Ursula Jakob
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Oxidative Stress and Redox Regulation

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I dedicate this book to my beloved partner and companion, Dori Reichmann, and to my remarkable parents, Ludmila and Semion Garber, for their unfailing love, endless support, and continuous enthusiasm to understand my research. I also dedicate this book to my amazing daughter, Noya, who keeps asking the question "Why?" which drives our life as researchers.

Dana Reichmann

I dedicate this book to my husband Jim and my children Alina and Adrian – every day I am amazed by their generosity, wisdom, humor, and love.

Ursula Jakob

Preface

Reactive oxygen species (ROS) have long been branded as undesired cytotoxic compounds to be avoided at all costs. This view has changed with the realization that ROS, which are constantly produced in all aerobic organisms, play essential roles in a plethora of signaling processes that act to control cell differentiation, proliferation, and apoptosis. It is now clear that organisms need to achieve a fine-tuned balance between oxidants and antioxidants (i.e., redox homeostasis) in order to function properly. At the heart of many cellular ROS-controlled processes are redox-sensitive proteins. These proteins use reversible posttranslational modifications, particularly on functionally and/or structurally important thiol groups, to regulate their activity and the activity of the pathway that they are part of. Based on extensive research particularly in the last decade, a picture is now emerging that cells harbor a highly complex network of redox-sensitive processes, which are constantly adjusting the cell's metabolism and physiology to the prevailing redox conditions of the environment. Alterations in a cell's redox homeostasis are involved in initiating stem cell differentiation, are associated with tumor development, and are considered to be a critical component in aging and age-related diseases.

The realization that redox-regulated processes influence almost every area of modern biology and will be inevitably encountered by many non-redox biologists provided a key motivation in the assembly of this book. We aim to provide a concise overview of the current state of the art in redox biology for a wide range of people from the novice to the expert. We start with a description of the chemical basis of thiol oxidation followed by a summary of the major enzymatic and non-enzymatic players involved in ROS detoxification and redox homeostasis in bacteria and eukaryotes. The second part of the book introduces state-of-the-art experimental and computational methodologies that are currently used in redox biology and discusses their advantages and limitations. We decided to focus on three major methodologies that nicely complement each other: (1) redox proteomics, which provides means to discover novel redox-sensitive proteins and pathways in the cell and is a useful approach to monitor changes in redox homeostasis during distinct physiological or pathological states; (2) fluorescence imaging of ROS in multicellular organisms using a variety of highly specific detection methods, which allow temporal and

spatial tracking of ROS in live organisms; and (3) redox bioinformatics to analyze, characterize, and eventually predict features of redox-sensitive and redox-regulated proteins in cells. Integration of these methods will eventually allow us to draw a global and system-wide picture about redox homeostasis and redox regulation in the living organism. The final, and the most applied, part of this book details biological examples of oxidative stress and redox regulation in a variety of different pro- and eukaryotic systems. Some chapters illustrate the importance of ROS in cell signaling, epigenetics, and stem cell differentiation, while others provide expert summaries and discussions about the role of redox regulation in infectious diseases, cancer, and aging. The closing chapter summarizes recent drug developments, which are aimed to modulate specific redox pathways in different human disease pathologies.

We are tremendously grateful to each of our contributors for their enthusiasm and willingness to provide the chapters for this book. Without them, this book would have not been possible. It was great fun putting this book together, and we hope that our book will infect the readers with the same enthusiasm about this fascinating research field that we experience every day in working with redox-regulated proteins.

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Ursula Jakob

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Chapter 1
The Chemistry of Thiol Oxidation and Detection

Mauro Lo Conte and Kate S. Carroll

Abstract The thiol functional group of the amino acid cysteine can undergo a wide array of oxidative modifications and perform a countless number of physiological functions. In addition to forming covalent cross-links that stabilize protein structure and functioning as a powerful nucleophile in many enzyme active sites, cysteine appears to be the principal actor in redox signaling, functioning as a regulatory reversible molecular switch. It is increasingly appreciated that the thiol group of cysteine in subset of proteins undergoes oxidative modification in response to changes in the intracellular redox environment. To understand these complex but critical biological phenomena, the chemistry of the thiol functionality and related oxidation products must also be taken into consideration. Selective methods to monitor and quantify discrete cysteine modifications will be central to understanding their regulatory and pathophysiologic function. Accordingly, this chapter focuses on the chemical feature of thiol oxidation and on selective methods for detecting oxidants and individual cysteine chemotypes.

Keywords Thiol oxidation • Cysteine • Reactive oxygen species • Reactive nitrogen species • Reactive sulfur species

1.1 Introduction

The thiol functional group of the amino acid cysteine can undergo a wide array of oxidative modifications and perform a countless number of physiological functions. In addition to forming covalent cross-links that stabilize protein structure and

This chapter is dedicated to the memory of Professor William S. Allison.

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functioning as a powerful nucleophile in many enzyme active sites, cysteine appears to be the principal actor in redox signaling, functioning as a regulatory reversible molecular switch. It is increasingly appreciated that the thiol group of cysteine in subset of proteins undergoes oxidative modification in response to changes in the intracellular redox environment. To understand these complex, but pivitol biological phenomena, the chemistry of the thiol functionality and related oxidation products must be taken into consideration. Along these lines, selective methods to monitor and quantify discrete cysteine modifications are key to understanding their regulatory and pathophysiologic function. This chapter focuses on the chemistry of thiol oxidation and detection.

1.2 The Biological Chemistry of Thiols

The cysteine side chain is generally considered the most potent nucleophile of all amino-acid side chains under physiological conditions. This notable level of reactivity is due to the presence of a thiol functional group. Thiol is a sulfur analogue of alcohol, but the smaller difference in electronegativity between the sulfur atom and the hydrogen atom makes the S–H bond less polarized than the O–H bond, leading to a diminished propensity to form hydrogen bonds. In contrast, thiols are much more acidic in comparison to alcohols, and this property can be explained by the weakness of the S–H bond and the greater likelihood that the negative charge will be distributed within sulfur 3d orbitals. Cysteine can be considered a triprotic acid in which the pK_a of thiol group has been determined to be 8.2 (Tajc et al. 2004). In glutathione, the most abundant low molecular weight thiol in the cytosol, cysteine residue has a pK_a of 9.1 (Tang and Chang 1996). In general, therefore, thiols are mild acids, but the protein microenvironment can dramatically influence the pKa value. The presence of a positively charged residue, such as lysine or arginine (Copley et al. 2004), as well as the formation of a hydrogen bond (Wang et al. 2001), may increase thiol acidity by 3–4 orders of magnitude. The reactivity of thiols is correlated with its pKa value (Szajewski and Whitesides 1980). In cysteines, a thiolate side chain becomes a stronger nucleophile and readily reacts with oxidants and electrophilic species, although interactions with specific residues or metals can also stabilize the thiolate form. With its remarkable reactivity, the cysteine thiol group can play a key biological role in catalysis and serve as an important site for many post-translational modifications. Considering the propensity of thiols to undergo oxidative reactions, the need for methods and chemical tools to monitor both reduced and oxidized cysteine residues has become clear. The key challenge for effective detection of bio-functional groups is summarized by the concept of chemoselectivity (Trost 1983) and, more specifically, in bioorthogonality (Bertozzi 2011). Indeed, chemists and biologists have sought to identify highly selective and facile reactions to detect biomolecules in living systems without interfering with native biochemical processes. Moreover, the relatively low abundance of cysteine, in comparison to other amino acids, combined with its remarkable nucleophilicity

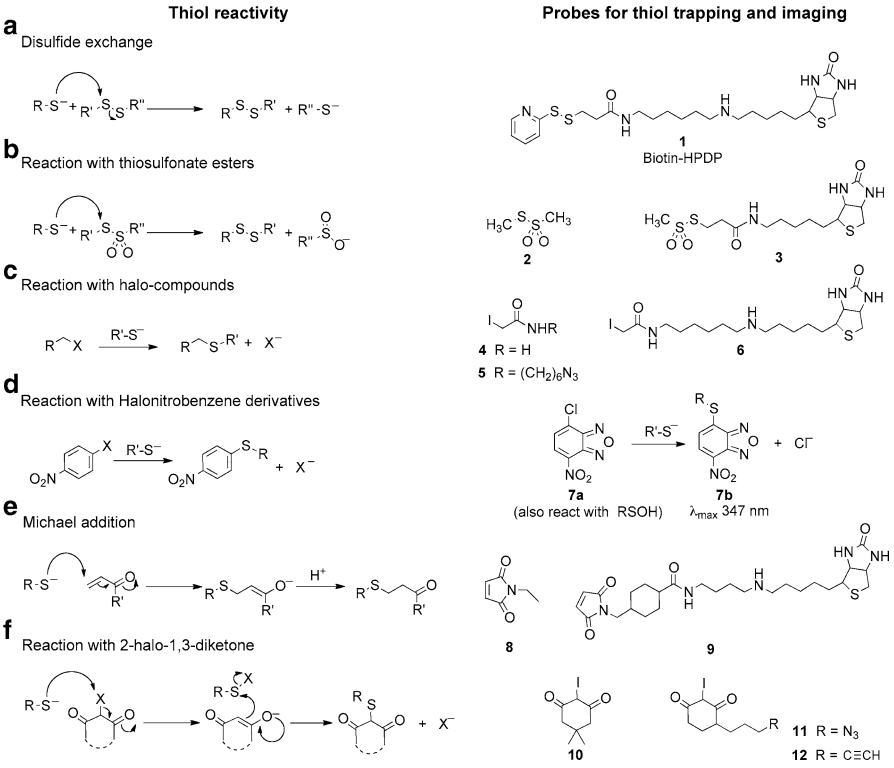

Fig. 1.1 Chemical reactivity and detection of thiols

has made cysteine the most common target for selective protein bio-conjugation (Chalker et al. 2009), creating fertile ground for the development of site-specific strategies for protein modification. In this context, the thiol reacts as a soft nucleophile with alkyl and aryl halides, carbonyl, phosphoryl, and sulfonyl groups as well as with unsaturated compounds (Fig. 1.1a–f). There are a large number of reagents that selective modify thiols, even in the presence of other strong nucleophiles such as lysine or histidine (Fig. 1.1, **1–12**). Such thiol conjugations can be further subdivided into two categories: reversible and irreversible.

To date, the most well studied example of cysteine modification is disulfide formation between two thiol groups and, thus it is no coincidence that one of the first strategies to detect thiols was inspired by the process of thiol-disulfide exchange (Fig. 1.1a). Bearing in mind that disulfide exchange is an equilibrium reaction (see Sect. 1.4.3), a series of specific disulfides were designed in order to favor one direction over the other. The well-known 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) or Ellman's reagent is perhaps most common disulfide-based reagent used to monitor and quantify protein free thiols (Ellman 1959). The notably low pKa of

the 5-mercapto-2-nitrobenzoate leaving group (4.4, Little and Brocklehurst 1972) ensures that this reaction occurs rapidly and, at the same time, does not perturb naturally occurring disulfides in the protein of interest.

Thiopyridyl disulfides are another class of common disulfide-exchange based detection reagents (Olsen et al. 2004). Thiopyridyl disulfides such as **1** (Fig. 1.1) react rapidly with thiols to afford an electro-stabilized compound, pyridine 2-thione, which can be followed by colorimetric analysis (i.e., absorption at 420 nm). Based on the same concept, a series of selenyl-sulfides (Gamblin et al. 2004) and thiosulfonates (Kenyon and Bruice 1997) have also been developed to selectively functionalize cysteine side chains. For example, methyl methane thiosulfonate (**2**) is the classic thiol-targeted reagent used to block free proteins thiols in the biotin switch assay (Jaffrey et al. 2001). All of these reagents (e.g., **1–3**) react with the thiol side chain to form a mixed disulfide that can subsequently be reduced to regenerate the free cysteine residue. The reversible nature of the disulfide linkage is acceptable in some circumstances, but in other situations this feature can be problematic. As a result, specific alkylating agents have been designed to irreversibly modify the cysteine side chain (**4–11**). Alkyl halides react with thiols to yield stable thioethers (Fig. 1.1c). Such reagents are usually not exclusive in their reaction with thiols; however, with careful control of pH, α-halo carbonyl compounds (**4–6**) can provide a satisfactory degree of bioorthogonality. Iodoacetamide (**4**) is employed in standard protein digestion protocols to block cysteine residues (Shevchenko et al. 1996). Despite the popularity of iodoacetamide, side-reactions with lysine, methionine, tyrosine, and histidine residues have been observed (Nielsen et al. 2008). In such cases, this issue can be resolved by using chloroacetamide (Weerapana et al. 2008).

Another unusual class of organo-halides is halo-nitrophenyl derivatives (Fig. 1.1d and 7). The strong electro-withdrawing nitro group makes haloarene extremely reactive toward thiols, although other sulfur species may also be modified in the presence of similar compounds (see Sect. 1.4). Thiolates are good Michael donors and the addition reaction to an α,β-unsaturated system is an alternative way to alkylate cysteine residues (Fig. 1.1e). Historically, maleimides have been the most widely used tools in biochemistry for the modification of thiols. The popularity of *N*-ethyl maleimide (**8**) is due to its good cell permeability and the ease with which the ethyl group can be elaborated by other substituents (e.g., **9**). However, what must be considered is that the reaction is only selective toward the thiol group at pH 7 (Crankshaw and Grant 1996). Vinyl sulfones are an alternative to the use of maleimide derivatives. Although they offer the advantage of yielding a single stereoisomer as a product (i.e., in theoretical models, maleimides can yield a mixture of two diastereoisomers), vinyl sulfones may cross react with the ε-NH_2 of lysine (Masri and Friedman 1988). A selective new reagent for thiol modification reported by Carroll's research group is based on the α-halo 1,3-diketone scaffold (Fig. 1.1f). In this reaction, 2-iodo-5, 5-dimethyl-1, 3-cyclohexandione (**10**, distinguished from the aforesaid α-halo carbonyl compounds by virtue of its secondary halogen) and related compounds (e.g., **11, 12**) react with a thiol to give the sulfenyl iodide species; this intermediate is rapidly attacked by the 1,3-diketone carbon nucleophile to afford a stable thioether (Seo and Carroll 2011).

1.3 Two-Electron Oxidants

Reactive oxygen species (ROS) are a family of molecules that are continuously generated in cells as consequence of aerobic life. Traditionally, their production has been strictly associated with oxidative stress, aging, and disease. However, growing evidence indicates that controlled generation of ROS contributes to physiological intracellular signaling events. ROS mediate redox modification of various biomolecules *via* two-electron oxidation or radical-based reactions, and they react in particular with cysteine residues. The term ROS encompasses a wide class of activated oxygen molecules which, can undergo further reaction with nitrogen or sulfur compounds, to produce reactive nitrogen species (RNS) and reactive sulfur species (RSS). Here, we focus on two-electron oxidants without mentioning the radical species, which will be described in subsequent chapters of this book.

1.3.1 Hydrogen Peroxide

A handful of enzymes generate H_2O_2 directly. For example, significant quantities of H_2O_2 are produced in the peroxisome by the action of enzymes, such as D-amino acid oxidase or uric acid oxidase. More generally, however, H_2O_2 is formed in cells *via* the disproportionation of superoxide ($O_2^{\bullet -}$). The major sources of $O_2^{\bullet -}$ production are the electron transport chain (ETC.) complex in the mitochondria (Murphy 2009) and the family of NADPH oxidases (NOXs) (Bedard and Krause 2007). Superoxide disproportionation may occur spontaneously (7.6×10^5 M^{-1} s^{-1}, Kutala et al. 2008) or may be catalyzed by superoxide dismutase (SOD) (Forman and Fridovich 1973; Fukai and Masuko 2011). Finally, several studies have showed the importance of monoamino oxidase (MAO) as an important source of H_2O_2 in neurons (Maker et al. 1981) and in relationship to neurodegenerative diseases (Andersen 2004). Hydrogen peroxide has the ability to move between different cellular compartments. Although H_2O_2 has long been thought to diffuse freely across membranes, its dipole moment of 2.2×10^{-18} C·m (Cohen and Pickett 1981) is only slightly higher than that of water (1.9×10^{-18} C·m), rendering its passive diffusion similarly limited. Recent studies have demonstrated the implication of aquaporin water channels as specific mediators of H_2O_2 passage across membranes (Bienert et al. 2007) and the involvement of these channels in intracellular signaling (Miller et al. 2010).

The unusual reactivity of H_2O_2 is generally attributed to relatively low O–O bond energy (47 kcal/mol) – hence, the ease with which it is homolytically or heterolytically cleaved (Bach et al. 1996). H_2O_2 is a strong oxidant and may behave as an electrophile as well a nucleophile depending on the nature of the reactant. In cells, the principal targets of H_2O_2-mediated oxidation are thiols by means of two electron nucleophilic substitution. A thiol with a low pK_a value reacts rapidly to yield sulfenic acid and water (Eq. 1.1).

$$R - SH + HO - OH \rightarrow R - S - OH + H_2O \tag{1.1}$$

Although glutathione (GSH) is the most abundant low molecular weight thiol, GSH contributes little to the reduction of H_2O_2 in cells. In fact, the total protein thiol concentration in human cells is at least two times higher than GSH (Hansen et al. 2009). At steady state, cellular H_2O_2 is kept in check by enzymes of the peroxiredoxin (Prx) family (Winterbourn and Hampton 2008). The rate of H_2O_2 reduction catalyzed by Prx is exceptionally fast (1×10^7 M^{-1} s^{-1} at pH 7), but this reactivity cannot be explained solely by the low pK_a of the peroxidative cysteine (~5.7, Rhee and Woo 2011). More generally, the reactivity of peroxides (RO–OH) shows an inverse relationship with the pKa of the leaving group RO (for this reason CH_3CO_3H is much more reactive than H_2O_2). The presence of specific proton-donating moieties within the protein microenvironment can serve to stabilize the poor hydroxide-leaving group and thus, accelerate the reaction rate. Along these lines, a recent series of experimental and computational studies by Nagy et al. suggests that two highly conserved arginine residues contribute to reactivity of Prx toward H_2O_2 *via* hydrogen bonding (Nagy et al. 2011).

Although the recombinant form of proteins may undergo H_2O_2-mediated thiol oxidation in the test tube, in many cases, it has been difficult to reconcile relatively low oxidation rates observed *in vitro* with intracellular regulation of enzymatic activity. For example, the rate constant measured for inactivation of recombinant protein tyrosine phosphatase 1B (PTB1B) is ~10 M^{-1} s^{-1} (Denu and Tanner 1998), which would appear to be incompatible with intracellular signaling events. With respect to this long-standing issue, recent work by Gates and co-workers has proposed that more reactive species, such as peroxymonocarbonate and peroxymonophosphate, oxidize PTPs in cells (Zhou et al. 2011). Peroxymonocarbonate, which may be spontaneously generated from bicarbonate anion in the presence of localized high concentrations of H_2O_2 (Eq. 1.3), can increase the rate of PTB1B oxidation by a factor of 20; the elevated reactivity is easily explained by the higher pK_a value of the hydrogen carbonate leaving group.

$$H_2O + CO_2 \rightleftharpoons H_3O^+ + HCO_3^- \tag{1.2}$$

$$HCO_3^- + H_2O_2 \rightarrow H_2O + HO_2COOH^- \tag{1.3}$$

Peroxymonophosphate is even more powerful and reacts with PTB1B more than 7,000 times faster than H_2O_2, although the generation and existence of such a species *in vivo* remains to be evaluated. One very speculative possibility is that peroxymonophosphate could be biologically accessible *via* direct enzymatic phosphorylation of H_2O_2 (LaButti et al. 2007). On the other hand, the proximity of proteins to the source of ROS/RNS can also have an obvious influence on target selectivity and rates of thiol oxidation within the cell (Chen et al. 2008; Paulsen et al. 2012).

Besides thiol oxidation, H_2O_2 can also easy react *via* one electron reduction with transition metals such as iron and copper. This reaction, known as the Fenton reaction, generates hydroxyl radical (Eq. 1.4; where L = ligand), a highly reactive

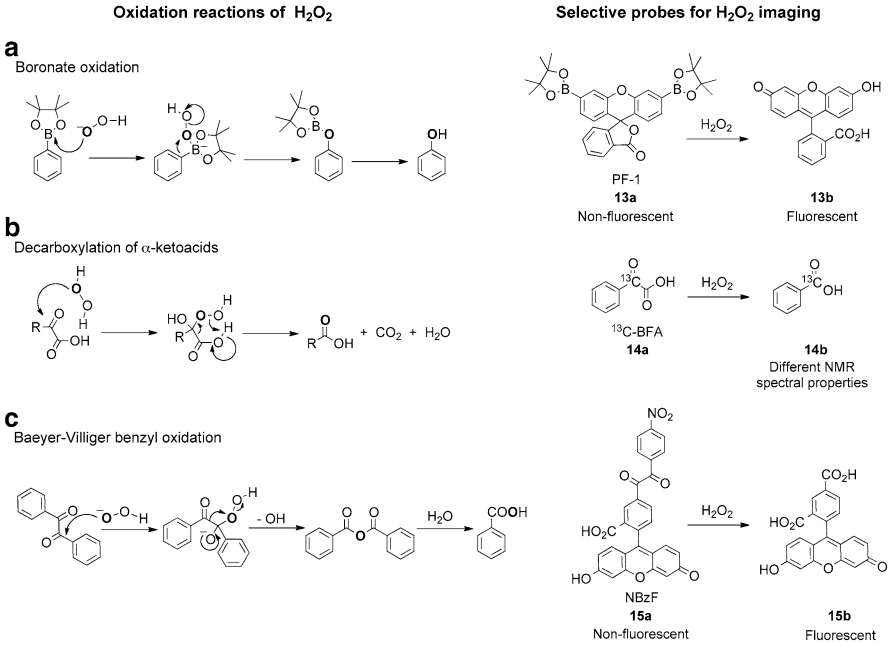

Fig. 1.2 Probes for the detection of hydrogen peroxide

species that can indiscriminately damage different biological target molecules such as DNA, proteins, or lipids *via* radical-mediated reactions and contributes significantly to the development of oxidative stress (Prousek 2007).

$$Fe^{2+} + H_2O_2 \rightarrow LFe^{3+} + HO^{\bullet} + HO^{-} \tag{1.4}$$

Although H_2O_2 has a relatively short half-life in cells ($t_{1/2} = 10^{-5}$ s), high variations in concentration can form locally and temporarily in cells (Giorgio et al. 2007). Molecular imaging is a powerful method for real-time monitoring of H_2O_2 in biological systems. Traditional tools for ROS detection, such 2′-7′-dichlorodihydroflurescein, are not specific for H_2O_2, but recent years has seen the development of many chemoselective probes, in particular by Chang's research group (Lippert et al. 2011). Because of its ambiphilic reactivity, H_2O_2 can be chemically differentiated from other ROS. With a pKa value of about 11, H_2O_2 is also a good nucleophile owing to the α-effect of adjacent nonbonding orbitals on its oxygen atoms (Jencks and Carriulo 1960), particularly in the deprotonated form. H_2O_2-mediated deprotection of aryl boronates to phenols (Kuivila and Armour 1957, Fig. 1.2a) has served as the starting point for the development of selective probes for H_2O_2 imaging. The installation of boronic esters on the 3′ and 6′ positions of the fluorescein core, for example, produces masked fluorescein (e.g., **13a**) in which the two boronates block the molecule in the closed lactone form, eliminating

its absorptive and emissive properties. The reaction with H_2O_2 transforms the boronates into phenols (e.g., **13b**) with a strong increase in fluorescence intensity (Chang et al. 2004). Although boronates should also react with alkyl peroxides (like lipid peroxides), the reaction with H_2O_2 should be faster because hydroxide anion is a better leaving group than alkoxides (RO^-). Obviously, the reaction is accelerated at higher pH levels, and local pH changes can alter the probe response. Based on the same approach, several other probes have been developed with enhanced sensitivity (Dickinson et al. 2011) or which are targeted to specific subcellular compartments (Dickinson and Chang 2008). Other bioorthogonal reactions have inspired alternative probes for selective H_2O_2 detection. Chang's group, for example, has developed a hyperpolarized ^{13}C probe based on oxidative decarboxylation of α-ketoacids (Fig. 1.2b) for ^{13}C MRI resonance imaging of H_2O_2 (**14a**, Lippert et al. 2011). Finally, Nagano's group has taken advantage of the less known Baeyer-Villiger benzil oxidations (Fig. 1.2c) to design a highly sensitive probe for H_2O_2 (**15a**, Abo et al. 2011). Together, these novel chemical tools have revealed fundamental new insights into the role of H_2O_2 in cells (Dickinson et al. 2011; Miller et al. 2010).

1.3.2 Hypo(Pseudo)halous Acid

The heme-containing enzymes myeloperoxidase (MPO) and lactoperoxidase (LPO) use H_2O_2 to oxidize halides (Cl^-, Br^- and I^-) and thiocyanate (SCN^-) into their respective hypohalous acids (HOXs). These species are more reactive than H_2O_2 and are used as antimicrobials by the immune system (Albrich et al. 1981). Although chloride is the most abundant negative electrolyte in living systems (1–60 mM in cell, 100 mM in extracellular fluids), the major substrate of MPO is SCN^-, whose affinity for the enzyme is a 1,000-fold greater than is that of Cl^- (Hawkins 2009). Generally SCN^- has a concentration of 20–250 μM (Van Dalen et al. 1997) but can reach a concentration of 1 mM in saliva, where LPO produces almost exclusively HSCN (Ashby 2008). Br^- is nearly 60-fold more specific for MPO but, with a concentration of 20–100 μM, the HOBr generated from neutrophils is estimated to be ~10% of HOCl production (Chapman et al. 2009). Finally, since the iodide concentration in cells is usually low (~1 μM), the production of hypoiodous acid *in vivo* can be considered negligible. Hypohalous acids are weak acids; their pKa values are 7.6 for HOCl, 8.7 for HOBr, 10.4 for HOI, and 5.3 for HOSCN (Davies et al. 2008). Except for HOSCN, which is completely deprotonated, these species exist as a mixture of their acid and anion forms at physiological pH. HOCl and, to a lesser extent HOBr, are extremely strong oxidants and indiscriminately react with a large variety of functional groups in proteins, nucleotides, and membrane lipids. HSCN is several times less reactive and selectively oxidizes thiols (Lloyd et al. 2008).

HOX species may also interconvert, and this reaction reflects the relative oxidizing strengths of these acids. As a result, if HOCl can oxidize Br^-, I^-, and SCN^-, HOBr reacts solely with I^- and SCN^- (Spalteholz et al. 2005), though

only the inter-conversion with SCN^- appears to be relevant *in vivo* (Nagy et al. 2006). HOXs are electrophilic oxidants and undergo two-electron nucleophilic substitution. Thiols react with HOCl and HOBr *via* formation of a very unstable sulfenyl halide (Eqs. 1.5 and 1.6), which readily rearranges to sulfenic acid (Nagy and Ashby 2007a). Otherwise, HOSCN generates a sulfonyl-thiocyanate (Eq. 1.8), which can react with another thiol to yield a disulfide and SCN^- (Eq. 1.9, Ashby and Aneetha 2004). Kinetic studies indicate that reaction proceeds *via* thiolate and hypothiocyanous acid reactants, indicating that this mild oxidant is very selective for proteins with low pK_a thiol residues (Nagy et al. 2009).

$$R - S^- + HO - Cl \rightarrow R - S - Cl + HO^- \tag{1.5}$$

$$R - S^- + HO - Br \rightarrow R - S - Br + HO^- \tag{1.6}$$

$$R - S - X + H_2O \rightarrow R - S - OH + X^- \tag{1.7}$$

$$R - S^- + HO - SCN \rightarrow R - S - SCN + HO^- \tag{1.8}$$

$$R_1 - S^- + R - S - SCN \rightarrow R - S - S - R_1 + SCN^- \tag{1.9}$$

It is generally accepted that the sulfur-containing residues (cysteine and methionine) react most rapidly with HOCl (with rate constants of $\sim 3\text{–}4 \times 10^{-8}$ M^{-1} s^{-1}), but strong nucleophilic amines (such as lysine and histidine) are also modified at high HOCl concentrations to yield chloramines (Pattison and Davies 2001).

$$R_1R_2NH + HO - Cl \rightarrow R_1R_2N - Cl + HO^- \tag{1.10}$$

$$R - S^- + R_1R_2N - Cl + H^+ \rightarrow R - S - Cl + R_1R_2NH \tag{1.11}$$

Small-molecule amines such as ammonia or glycine may react with HOCl to form membrane-permeable chloramines (Eq. 1.10), which selectively mediate thiol oxidations (Midwinter et al. 2006). Taurine-chloramine (TauCl) is another biologically relevant chlroamine. This cell-impermeable chloramine is generated especially in neutrophils, in which taurine is the most abundant free "amino acid" (10–30 mM) and also appears to play a scavenger role for chlorinated oxidants (Marcinkiewicz et al. 1995).

Although HOCl plays a fundamental role in destroying a wide range of pathogens, neutrophil-mediated HOCl production has also been implicated in several inflammation-associated diseases. A series of detection methods have been developed in order to shed light on its physiological and pathological roles (Fig. 1.3). These selective probes are based on the major oxidant strength of HOCl in comparison with other ROS. For example, *p*-methoxyphenol is selectively

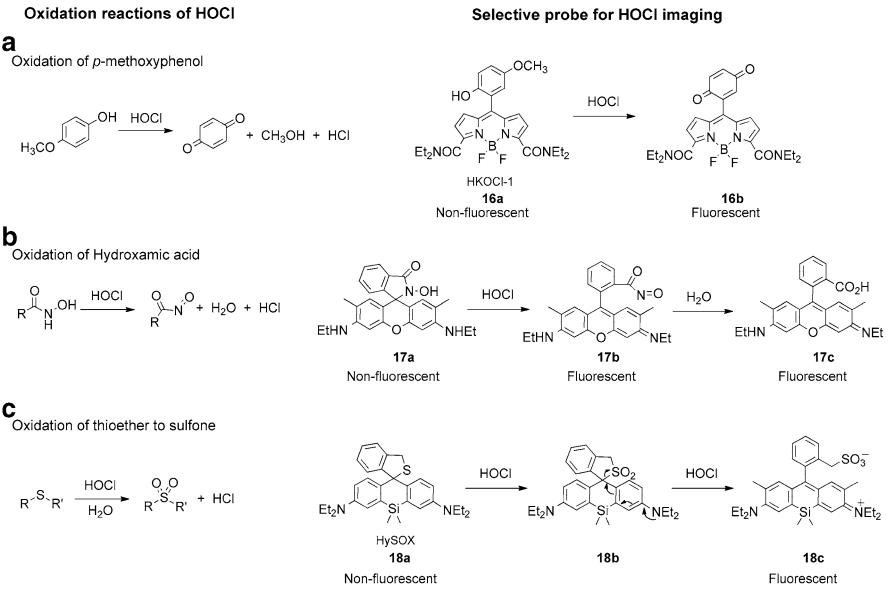

Fig. 1.3 Probes for the detection of hypochlorous acid

oxidized by HOCl (Fig. 1.3a) and shows good stability in the presence of other ROS and RNS. Yang's research group has taken advantage of this reaction to design a BODIPY-based probe (**16a**) that displays a significant increase of fluorescence after HOCl incubation (Sun et al. 2008). By analogy to chloramine formation, Tae's research group has instead developed a fluorescent probe based on HOCl-mediated oxidation of hydroxamic acid (Yang et al. 2009). Following generation of an unstable chlorinated intermediate, hydroxamic acid yields an acyl nitroso compound with elimination of HCl (Fig. 1.3b). Starting from a rhodamine scaffold, Tae's group designed a hydroxyamido derivative enclosed in a stable spirocyclic non-fluorescent form (**17a**), which demonstrates a rapid and selective fluorescent response in the presence of exogenous HOCl in cells as well as in mice. Recently, a thioether-rhodamine derivative was designed as a chemoselective probe for HOCl. In fact, thioethers and thiols are the major target of HOCl-mediated oxidation and, although thiols indiscriminately react with any ROS and RNS, thioethers show selective oxidation (Fig. 1.3c). The thioether group maintains the Si-rhodamine probe in the cyclic non-fluorescent form (**18a**), and the system recovers its highly conjugated structure through the generation of a sulfone (**18b**), followed by elimination of sulfinic acid (which then oxidizes to sulfonic acid) and a robust increase in fluorescence (Koide et al. 2011). Finally, although many efforts have been made, few methods (with the exception of probe **17a**) appear to have the appropriate characteristics for biological application *in vivo*.

1.3.3 Nitric Oxide and Peroxynitrite

NADPH-dependent nitric oxide synthase (NOS) catalyzes the synthesis of nitric oxide (NO) and L-citrulline *via* oxidation of the guanidine group of L-arginine (Alderton et al. 2001). NO, like carbon monoxide (CO) and hydrogen sulfide (H_2S), is a small gaseous signaling molecule implicated in numerous physiological processes (Cary et al. 2006). NO is a free radical species ($^{\bullet}N{=}O$) with a relative long half-life in comparison to other ROS/RNS ($t_{1/2} = 1$ s, Pacher et al. 2007) and can freely diffuse across membranes. Although an *S*-nitrosothiol could be directly generated by reaction with thiyl radicals (Jourd'heuil et al. 2003), most of its activity is mediated by development of intermediary nitrosating agents (Keszler et al. 2010). These nitrosating species are generated *via* a series of oxidation reactions. NO forms nitrous anhydride (N_2O_3) in the presence of oxygen (Eqs. 1.12a and 1.12b), and this unstable species can easy be hydrolyzed (Eq. 1.13) or undergo two-electron nucleophilic substitution by thiols (Goldstein and Czapski 1996) and amines (Caulfield et al. 1998). N_2O_3 is generally accepted as the major nitrosylating agent *in vivo*, although other pathways are proposed to explain *S*-nitrosothiol formation in cells (see Sect. 1.4.2).

$$NO + O_2 \rightarrow NO_2 \tag{1.12a}$$

$$NO + NO_2 \rightarrow O_2N - N = O \tag{1.12b}$$

$$O_2N - N = O + 2OH^- \rightarrow 2NO_2^- + H_2O \tag{1.13}$$

$$O_2N - N = O + R - S^- \rightarrow R - S - NO + NO_2^- \tag{1.14}$$

$$O_2N - N = O + R - NH_2 \rightarrow R - NH - NO + HNO_2 \tag{1.15}$$

$O_2^{\bullet -}$ transforms the relatively unreactive NO into peroxynitrous acid (ONOOH), a strong oxidant (Eq. 1.16). This diffusion-controlled reaction has the largest rate constant known for NO (1×10^{10} M^{-1} s^{-1}) and, *in vivo*, is spatially associated with the sources of superoxide (Radi et al. 2001). ONOOH is a weak acid with a pK_a value of 6.8 (Kissner et al. 1997) and is in equilibrium with its deprotonated form (peroxynitrite, $ONOO^-$) at physiological pH. Both species are strong oxidants and can participate in one- and two-electron oxidation reactions, although HONOOH shows a faster reactivity (Moro et al. 1994) and $ONOO^-$ appears to be a powerful nucleophile as well. Despite its short half-life, ONOOH appears able to diffuse across the membranes (Ferrer-Sueta and Radi 2009) where it can react with a wide group of substrates (Marla et al. 1997). Typically, thiols are oxidized to disulfides by ONOOH (Squadrito and Pryor 1998), but it is still uncertain which intermediate species are involved between the sulfenic acid (R-SOH) and *S*-nitrothiol ($R\text{-}SNO_2$).

Nevertheless, kinetic analysis clearly shows that this reaction involves the pairing of the thiolate/peroxynitrous acid. Other studies have shown that ONOOH can also mediate the *S*-nitrosylation of GSH with elimination of H_2O_2 (van der Vliet et al. 1998). In any case, considering that ONOOH is very unstable and can undergo hemolytic fission to generate the one-electron oxidant, hydroxyl ($^{\bullet}OH$), as well as $^{\bullet}NO_2$ radicals (Szabo et al. 2007), it is difficult to assess which reaction ONOOH participates in directly. ONOOH decomposition is also facilitated by bicarbonate (HCO_3^-). CO_2 and readily undergoes nucleophilic addition by $ONOO^-$ (with a constant rate of $5.8 \times 10^4\ M^{-1}\ s^{-1}$) to yield $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ (Eqs. 1.18 and 1.19). CO_2 also mediates the generation of other oxidant species (Eq. 1.20) and is considered to contribute to the nitration of tyrosine side chains (Squadrito and Pryor 1998).

$$NO + O_2^{\bullet -} \rightarrow ONOOH \tag{1.16}$$

$$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \tag{1.17}$$

$$ONOO^- + CO_2 \rightarrow ONO - OCO_2^- \tag{1.18}$$

$$ONO - OCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet -} \tag{1.19}$$

$$ONO - OCO_2^- \rightarrow O_2N - OCO_2^- \tag{1.20}$$

The development of powerful tools to detect NO signaling remains an active area of research. The first approach developed to detect NO was based on the *o*-phenylenediamine scaffold which, in the presence of NO and O_2 (Uppu and Pryor 1999), yields the corresponding aryl triazole (Fig. 1.4a). The electronic difference between the electron-rich diamine (**19a**) and the electron-poor triazole (**19b**) leads to a robust fluorescent switch (Kojima et al. 1998). Obviously, this is an indirect approach for NO detection. Specifically, the aryl diamine reacts with N_2O_3 to form *N*-nitrosylamine, which evolves to triazole *via* intramolecular nucleophilic displacement. Recently, Xu's research group has developed an elegant new probe based on the fluorescein scaffold; in this case, the diamine blocks the fluorophore in spirolactame form; reaction with NO leads to the formation of a triazole ring and concomitant fluorescence response (Zheng et al. 2008).

Parallel approaches for direct detection of NO have also been developed. It is well-known that NO has strong affinity towards transition metals, including the ability to bind the heme-group. Taking advantage of this property, several probes have been designed conjugating transition metals to a fluorescent core (Lim and Lippard 2007). This approach was based upon the release of a fluorophore, initially quenched by electron or energy transfer through coordination to a paramagnetic transition-metal center such as Fe(II), Co(II), or Cu(II). The presence of NO causes

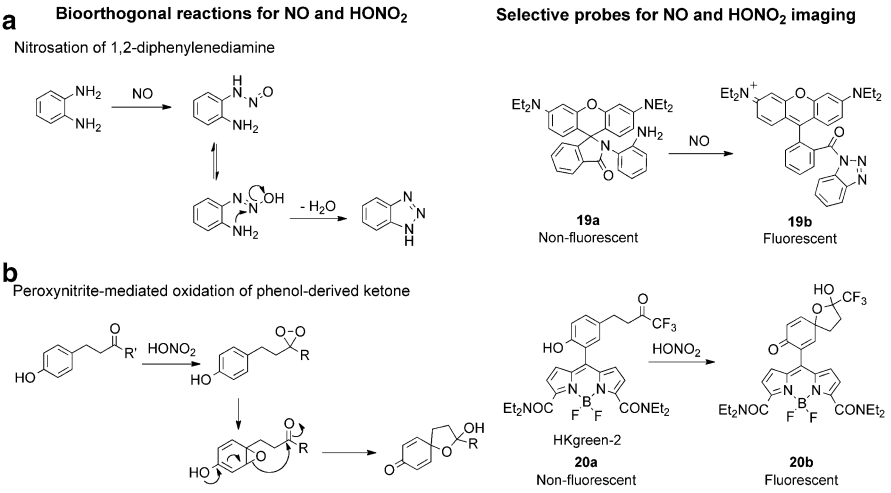

Fig. 1.4 Probes for the detection of nitric oxide and peroxynitrite

the displacement of the metal-complex with concomitant fluorescence activation. Taken together, the two approaches described are perfectly complementary for chemospecific NO detection in living systems.

Largely because of the ongoing difficulty of direct and unambiguous detection, the biological relevance of ONOOH remains controversial. Selective probes for peroxynitrous acid have been inspired based on the similar chemical behavior of $ONOO^-$ and peroxymonosulfate ($HOOSO_3^-$). In fact, $ONOO^-$ can react with ketones to yield dioxiranes (Yang et al. 1999). Furthermore, it is known that internal dioxiranes can intramolecularly oxidize phenol derivatives to quinones and that this reaction is facilitated when the dioxiranes are generated from ketones with electron-withdrawing groups (Yang et al. 2000). Yang's group has demonstrated that $ONOO^-$ can also oxidize anisole-derivative ketones *via* dioxirane *in situ* formation (Fig. 1.4b). Based upon this reaction, several fluorescent probes have been developed in which an anisole-derivative is conjugated with a fluorophore and the peroxynitrite-mediated oxidation selectively allows the release of the fluorescent molecule (e.g., **20a**; Yang et al. 2006; Peng and Yang 2010).

1.3.4 Hydrogen Sulfide

For many years, hydrogen sulfide (H_2S) was solely considered as a toxic gas, but in the last decade its involvement in mammalian cell signaling has become apparent (Li et al. 2011). H_2S is synthesized by two pyridoxal 5′-phosphate-dependent enzymes involved in cysteine metabolism (Chiku et al. 2009): cystathione β-synthase (CBS) and cystathione γ-lyase (CSE). H_2S can also be generated from L-methionine *via*

transsulfuration (Bearden et al. 2010) or release from a persulfide (RS–SH) by thiol-disulfide exchange. Like other gaseous signaling molecules, such as NO and CO, H_2S rapidly crosses cell membranes with no specific transporter (Mathai et al. 2009). In fact, although H_2S shows structural similarities with H_2O it does not form strong hydrogen bonds and, with a dipole moment of 0.9 D, H_2S can freely diffuse through the hydrophobic core of biological membranes.

H_2S is a diprotic acid with a pKa_1 of 6.9 and pKa_2 of 11.96; consequently, H_2S is in equilibrium with its deprotonated form (with a H_2S/HS^- of 1:3) at physiological pH (Liu et al. 2011). In many respects, H_2S exhibits a reactivity profile akin to cysteine. It is a powerful nucleophile and, like thiols, can react with electrophiles as well as oxidants. With two-electron oxidants (Eq. 1.21), H_2S forms hydrogen thioperoxide (HSOH – Carballal et al. 2011). HSOH is a very reactive sulfur species and can be oxidized rapidly to SO_4^{2-} or undergo reaction with thiols. *In vitro*, the reaction of H_2S with HSOH leads exclusively to the formation of a complex mixture of polysulfides (HS_X^- with x = 2–8) (Eq. 1.22), which have been proposed to mediate the *S*-sulfhydration of proteins (Nagy and Winterbourn 2010).

$$HS^- + H_2O_2 \rightarrow HS - OH + H_2O \tag{1.21}$$

$$HS - OH + n\,HS^- \rightarrow HS - S_n^- + H_2O \tag{1.22}$$

The biological functions of H_2S are still unclear, but growing evidence suggests a role for H_2S in the regulation of cardiovascular (Elsey et al. 2010) and gastrointestinal (Wang 2010) systems. Moreover, its biologically relevant levels are still debated, and a wide range of concentrations has been reported (Kabil and Banerjee 2010). To investigate such issues, a number of research groups have focused their efforts on the development of small-molecule probes for H_2S detection (Fig. 1.5). H_2S can be considered a strong reducing agent and this property has been widely exploited in organic synthesis to reduce aromatic nitro (Lin and Lang 1980) and azido (N_3) (Pang et al. 2009) groups to aniline. Chang's research group developed a selective probe for H_2S detection by installing an N_3 group in the 6′ position of a rhodamine core (**21a**), which locks the fluorophore in the spirocyclic non-fluorescent form. Reduction to the amine releases the fluorescence signal (**21b**) and such probes display high selectivity for H_2S over other biologically relevant reactive sulfur, oxygen, and nitrogen species (Lippert et al. 2011). Subsequently, Wang's research group used a similar approach to design a sulfonylazide dansyl derivative whereby the difference in electronegativity between the azide and amine group triggers a change in electronic and, thus, fluorescent properties (Liu et al. 2011). Both probes appear quite promising given that azido group is quite inert and has shown considerable compatibility for application in living systems.

As previously mentioned, H_2S exhibits a similar reactivity as compared to cysteine. Theoretically, H_2S can be considered a non-substituted thiol and can give nucleophilic attack twice, whereas other thiols, such as cysteine, can participate in just one nucleophilic attack. Exploring this property, Xian's research group

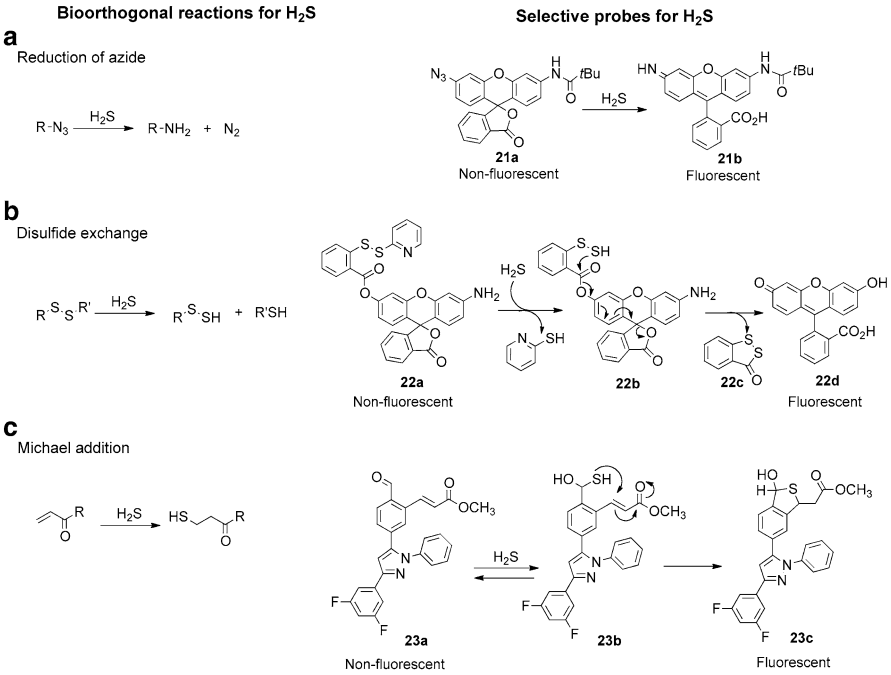

Fig. 1.5 Probes for the detection of hydrogen sulfide

hypothesized that a probe with two electrophilic centers could be selective for H_2S detection and developed a thiosalicylic acid ester of fluorescein in which the thiol group is functionalized as thiopyridyl disulfide (**22a**). Both thiols and H_2S readily yield disulfide exchange with activated disulfide, but only the disulfide intermediate from H_2S (**22b**) can rearrange, *via* nucleophilic attack on the intramolecular phenyl ester, leading to benzodithiolone (**22c**) and fluorescein (**22d**) products (Liu et al. 2011). Along these lines, Chuan's research group has developed a selective probe for H_2S using a Michael acceptor and an aldehyde group as two electrophilic centers (**23a**). H_2S forms a thioacetal with the aldehyde group (**23b**) followed by an intramolecular Michael addition with the internal α,β-unsaturated compound (**23c**). Such H_2S-specific rearrangement leads to a strong increase in fluorescence. Using this probe, Chuan's group has succeeded for the first time in imaging enzymatic H_2S production in living cells (Qian et al. 2011a).

1.4 Oxidative Modification of Protein Cysteine Residues

The cysteine side chain, with its high nucleophilic capacity, appears to be the principal target of ROS/RNS in cells. The sulfur atom of cysteine may assume a wide range of oxidation states (i.e., −2 to +4, Fig. 1.6) and each form exhibits a distinct

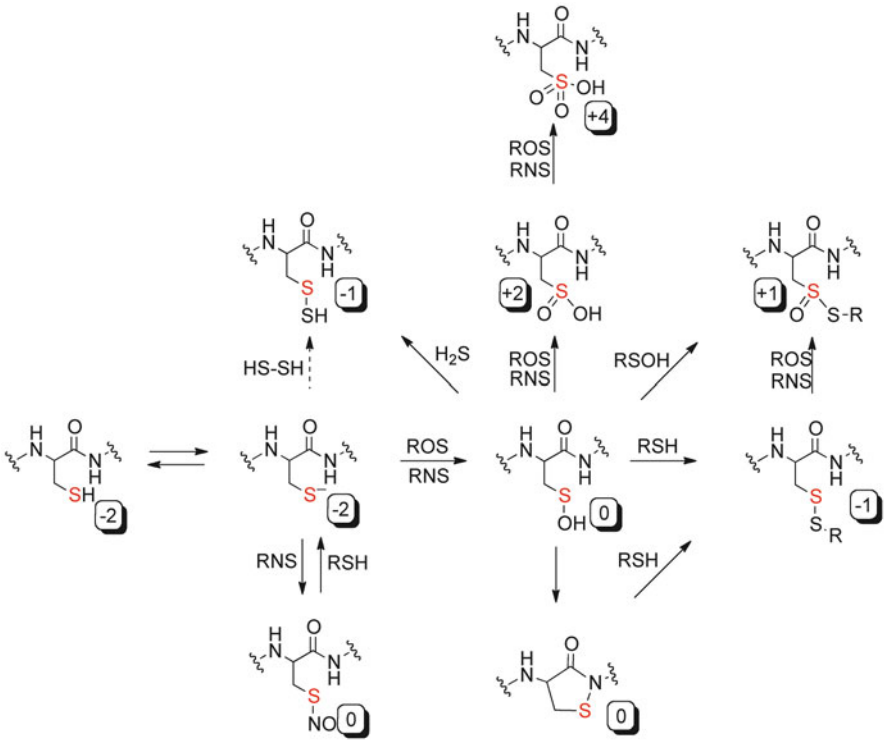

Fig. 1.6 Biologically relevant cysteine oxidation states. Oxidation number correspond to sulfur atom denoted in red

chemical reactivity. In the thiolate form, sulfur undergoes oxidation to generate a sulfenic acid, and this oxoform can be considered as a central species among thiol modifications. Sulfenic acid may be reduced to a disulfide by reaction with intra- and inter-molecular thiols or further oxidized to sulfinic acid at high ROS/RNS concentrations. In some cases, the sulfenic acid can lead to the formation of sulfenamide and thiosulfinate ester groups. The cysteine reactivity landscape becomes more complex given that the thiolate may react with RNS and RSS to form *S*-nitrosothiol and persulfide, respectively. Moreover, depending upon the nature of the protein microenvironment, many of these modifications are reactive and can interconvert with one another. In order to highlight the possible role and significance of each modification, their distinct physical and chemical properties are outlined below.

1.4.1 Sulfenic Acid

Sulfenic acids (RSOH) are directly formed *in vivo* by the oxidation of thiols with two-electron oxidants such as H_2O_2, ONOOH, or alkyl peroxides and many

enzymes form SOH intermediates during their catalytic cycles (Lim et al. 2011). In addition, RSOH can be generated from the hydrolysis of sulfonyl-halides (Nagy and Ashby 2007a), sulfonyl-thiocyanates (Ashby and Aneetha 2004), and thiosulfinates (Nagy and Ashby 2007b). Hydrolysis of *S*-nitrosothiols can also yield RSOH (Percival et al. 1999), but the reaction has a high activation barrier (Moran et al. 2011). The thiyl radical ($RS^{\bullet}$) can also yield sulfenic acid in the presence of hydroxyl radicals ($^{\bullet}OH$); however, this reaction is not likely to be widespread in a biological setting. Otherwise, sulfenic acids are usually obtained in organic synthesis from the thermolysis of sulfoxides (Sivaramakrishnan et al. 2005) or thiosulfinates (Block and O'Conner 1974).

The S–O bond length in RSOH is distinctly longer than that of sulfoxides, indicating that the tautomeric structure, R–S(O)H, can be excluded (Goto et al. 1997). RSOH is a weak acid but, because of their high reactivity, their pKa has been difficult to measure. In the case of some stable small-molecule sulfenic acids, an acidity of two to three orders of magnitude lower than the corresponding thiols has been found (Heckel and Pfleiderer 1983). This trend could quite realistically offer an estimation of pKa value for other sulfenic acids. For example, the pKa value of a high-hindered triptycenyl sulfenic species was recently reported to be 12.5 (McGrath et al. 2010), about three times higher than a typical thiol. Although they are usually defined as transient species, intramolecular hydrogen bonds (Heckel and Pfleiderer 1983) and steric hindrance (Nakamura 1983) can play major roles in stabilizing protein RSOH. More generally, hydrogen bonds in apolar protein microenvironments, and the absence of vicinal thiols can all stabilize sulfenic acids (Claiborne et al. 1993).

Sulfenic acids exhibit potent electrophilic (Fig. 1.7) and relative weak nucleophilic (Fig. 1.8) reactivity. In small-molecule RSOHs, this dual behavior can lead to self-condensation in which one sulfur atom functions as a nucleophile and the second as an electrophile to yield a thiosulfinate ester (Fig. 1.7a). In this reaction, intermolecular hydrogen bonding mediates the self-condensation of RSOH, thereby reducing the free energy of activation for thiosulfinate formation thermodynamically preferred over the acid (Davis et al. 1986). On the other hand, self-condensation of RSOH only competes with thiol-based reduction of sulfenic acid at high pH (Nagy and Ashby 2007a, b) and is otherwise considered negligible.

As indicated above, thiols can reduce sulfenic acid to form a disulfide (Fig. 1.8a) and this condensation represents an important biological reaction. Moreover, this reaction allows the recycling of sulfenic acid in as much as the disulfide can be reduced to thiol through the action of cellular-reducing agents such as glutathione (GSH), glutaredoxin (Grx) and thioredoxin (Trx). Dithiols (such as DTT) and Trx can also directly reduce RSOH back to the thiol form (Poole et al. 2004). Thiols are also used as trapping agents to demonstrate the formation of aleatory sulfenic acid in the thermolysis of sulfoxides (Kamiya et al. 1973). Several inorganic molecules, such as $NaAsO_2$ (Saurin et al. 2004), hydrazine, and NaN_3 (Allison 1976), have been reported to reduce RSOH to the thiol; however, their selectivity has not been sufficiently explored. Although sodium ascorbate (Fig. 1.9a) is generally considered a specific reducing agent for *S*-nitrosothiols (Turell et al. 2008), it can also reduce

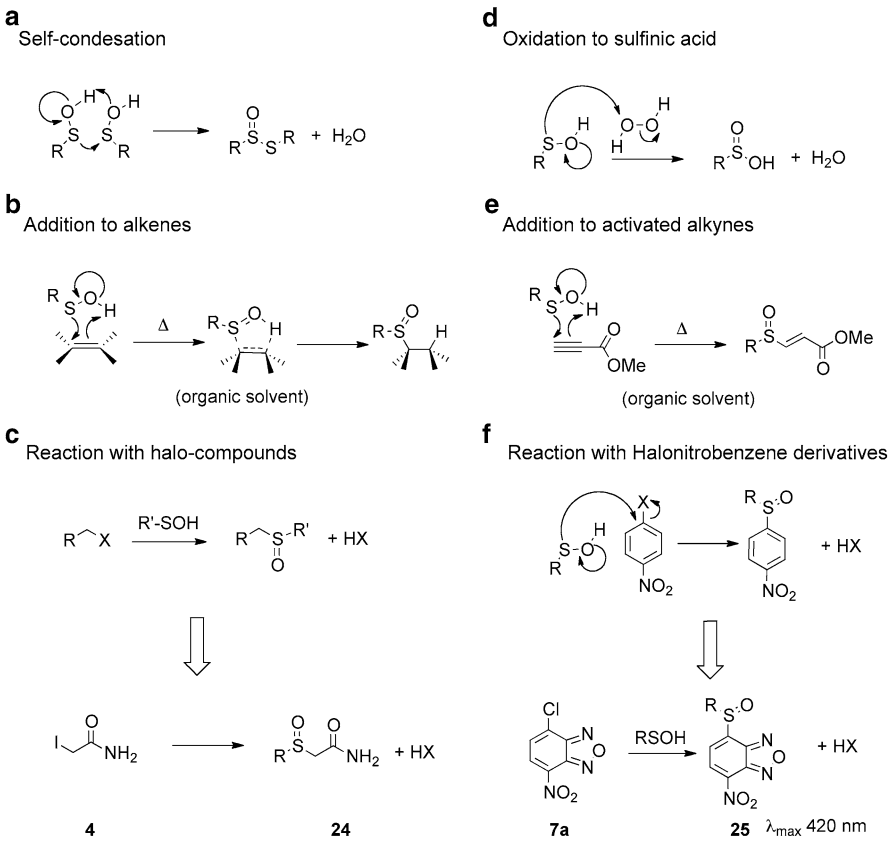

Fig. 1.7 Sulfenic acid reactivity: S atom as a nucleophile

RSOH through an addition/elimination mechanism (You et al. 1975; Fig. 8b); recently, the ability of sodium ascorbate to reduce sulfenic acid *in vivo* has also been proposed (Monteiro et al. 2007).

Sulfenic acids can also be oxidized by two-electron oxidants (Fig. 1.7b) or by O_2 in the presence of trace metal ions. The oxidation of non-hindered thiols leads only to the disulfide. Considering that sulfenic acids can be intermediates in disulfide formation, it is worth noting that oxidation of sulfenic acid is slower, compared to its condensation rate with thiols (Luo et al. 2004). Kinetic studies have showed that the rate constant for oxidation of cysteine sulfenic acid is approximately two to three orders of magnitude slower than cysteine (Hugo et al. 2009). Two pathways may be hypothesized for H_2O_2 oxidation of RSOH: first, a concerted mechanism mediated by formation of hydrogen bond and, second, the direct participation of a sulfenate anion (RSO^-). The pH profile of H_2O_2-mediated oxidation of RSOH indicates that the sulfenate is the reacting species and therefore, RSOH generated from a very low pKa cysteine should be more susceptible to irreversible oxidation to sulfinic acid.

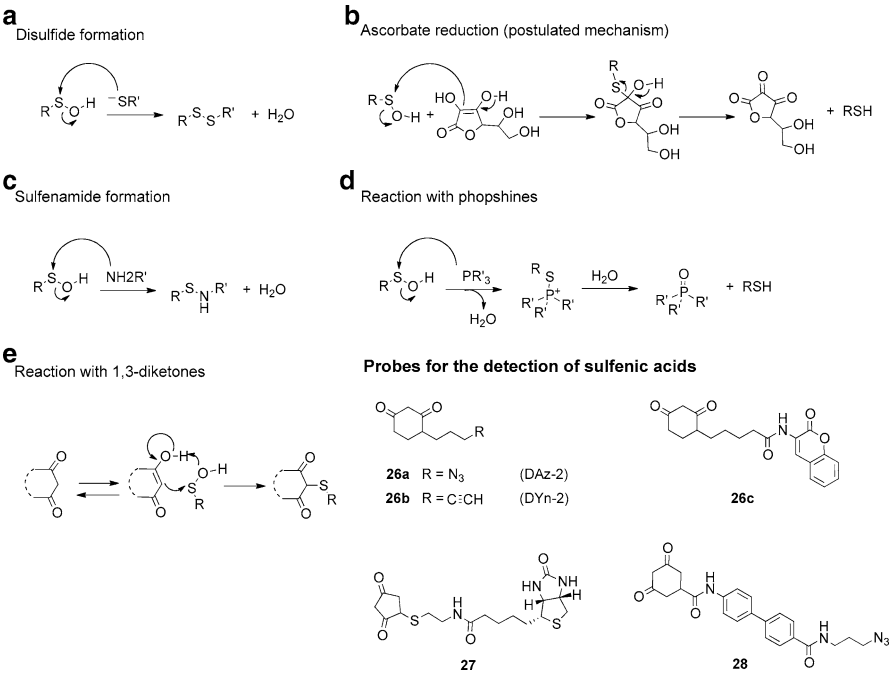

Fig. 1.8 Sulfenic acid reactivity: S atom as an electrophile

Reactive cysteine residues in specific proteins may readily oxidize to sulfenic acid. This reversible thiol modification plays a significant role in protein regulation and cell signaling (Paulsen and Carroll 2010). As the first (and sometimes only) oxidation product, RSOH is a key "marker" for ROS/RNS-sensitive cysteine residues and, as such, there is clear evidence of its role in cell signaling (Klomsiri et al. 2011; Roos and Messens 2011). As a result, interest in the RSOH detection has grown rapidly in the last decade and the reactivity of protein sulfenic acids continues to be explored. Sulfenic acids react as weak nucleophiles with alkyl or aryl halides, alkenes, and alkynes (Fig. 1.7c–e). Although sulfenate ions (RSO^-) should be an ambident nucleophile, alkylation with halides always yields the sulfoxide (Hogg and Robinson 1979). This reaction should be taken in account when blocking protein thiols with iodoacetamide since alkylation of sulfenic acid (Fig. 1.7e, **24**) becomes relevant at reagent concentrations typically employed in such procedures. The aromatic nucleophilic substitution of halonitroarene has been employed to detect the sulfenic acid group in proteins (Fig. 1.7f). For example, the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reacts with both sulfenic acids and thiols to yield covalent adducts (**25** and Fig. 1.7f) that are distinguishable from each other with respect to mass and their UV–vis absorption profiles (Ellis and Poole 1997). Conversely, alkenes (Kingsbury and Cram 1960) and electron-deficient alkynes (Goto et al. 1997) have long been used in organic synthesis in organic

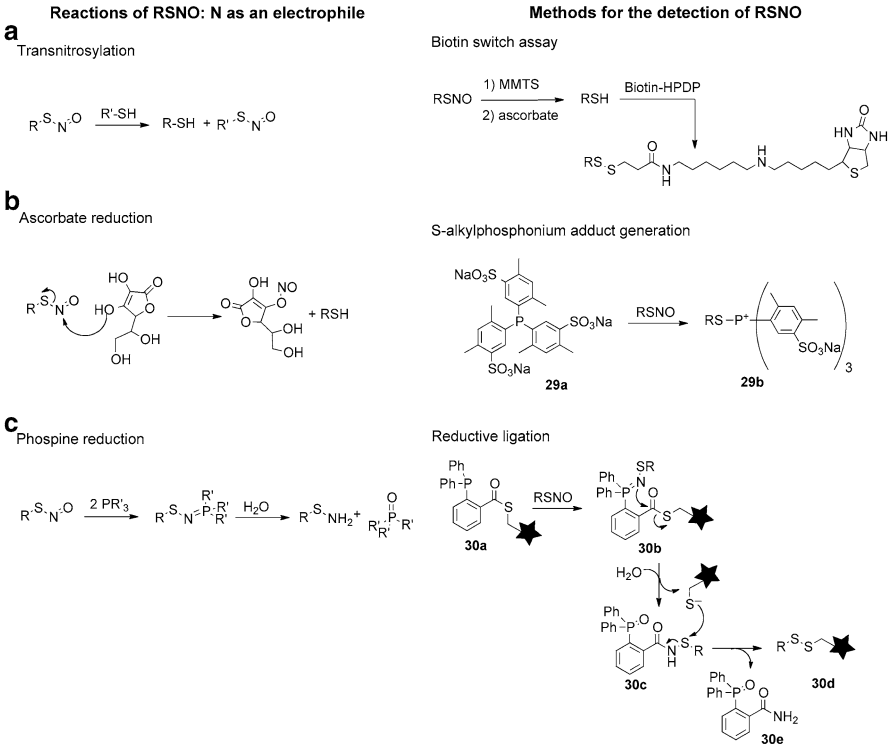

Fig. 1.9 Chemical reactivity and detection of *S*-nitrosothiols

solvent at high temperature for to trap transient sulfenic acid species (Fig. 1.7c–d). Such nucleophilic addition reactions occur through a concerted, cyclic process – the reverse of the elimination of sulfenic acids from sulfoxides – and should therefore proceed through the same transition state *via* regiospecific Markovnikov addition (Fig. 1.7c).

The sulfur atom in sulfenic acid also functions as an electrophile (Fig. 1.8a–e). Primary and secondary amines react with RSOH to yield sulfenamides (Fig. 1.8c, Allison et al. 1973). This reaction is quite slow and, in a biological context, only makes sense when this reaction takes place intramolecularly. In fact, in some proteins sulfenic acid can react with the nitrogen backbone of the neighboring residue to yield a five-member cyclic sulfenamide (see Sect. 1.4.4). Although RSOH could also react with intramolecular lysine residues, this species has been rarely detected (Raftery et al. 2001) and its formation *in vivo* remains unclear. Sulfenic acids also react with phosphorous III compounds to yield trialkylphosphine oxide and the corresponding thiol *via* hydrolysis of the intermediate phosphonium (Fig. 1.8d, Goto et al. 2003). To date, coupling of RSOH with cyclic 1,3-diketones is the only reaction that has demonstrated high selectivity for this cysteine oxoform.

Cyclic 1,3-diketones react with sulfenic acid in their enol-form, likely through hydrogen bond-mediated direct substitution (Fig. 1.8e).

5,5-dimethyl-1,3-cyclohexandione, commonly known as dimedone, is often used trapping agent for detecting protein sulfenic acid modifications (Benitez and Allison 1974). However, this reagent is not ideal for proteomic studies since it lacks an affinity and/or detection handle. In recent years, several advantageous derivatives of dimedone have been developed. The first approach was direct conjugation of 1,3-cyclohexandione with a biotin or a fluorophore (Fig. 1.8, **25**) moiety (Poole et al. 2005). More recently, Carroll's research group has functionalized the 1,3-cyclohexandione scaffold with an azide (**26a**) (Leonard et al. 2009) or alkyne (**26b**) (Paulsen et al. 2012) chemical reporter, known as DAz-2 and DYn-2, respectively. These small probes are membrane-permeable and enable detection and trapping of protein sulfenic acids directly in cells. After cellular tagging, lysates are generated and coupled to detection reagents *via* Staudinger ligation or Huisgen azide-alkyne 1,3-dipolar cycloaddition (also known as click chemistry). Carroll's group has also reported the development of a heavy-isotope coded dimedone analogue, which in combination with **10** (see Sect. 1.2) represents a powerful new approach to quantify the extent of sulfenic acid modification at individual protein cysteine residues (Seo and Carroll 2011). Furdui's research group has reported a new effective probe for sulfenic acids based on a related scaffold, 1,3-cyclopentadione (**27**, Qian et al. 2011b). Finally, Carroll's group has recently reported tri-functional probes consisting of a dimedone-like warhead, chemical reporter, and binding module (**28**) that enhances the detection of sulfenic acid in specific classes of signaling proteins (e.g., PTPs, Leonard et al. 2011). In parallel research, antibodies that recognize the protein-dimedone adduct have also been developed (Seo and Carroll 2009; Maller et al. 2011). Carroll and co-workers have applied such antibodies to visualize sulfenic acid modifications in tumor cells and profile growth factor-dependent changes in thiol oxidation among breast cancer cell lines (Seo and Carroll 2009).

1.4.2 Nitrosothiol

NO and its metabolites may react with the side chain of cysteine to yield *S*-nitrosothiols (Fig. 1.9, RSNO). This modification can lead to significant changes in the structure and function of proteins (Denninger and Marletta 1999). *S*-nitrosylation represents an important signaling mechanism and many proteins have been identified as *S*-nitrosylation targets (Jaffrey et al. 2001). S–NOs may be formed through the direct reaction of thiols with N_2O_3 and less commonly with ONOOH (see Sect. 1.3.3) or by trans-nitrosylation with an NO-donor (Fig. 1.9a). Trans-nitrosylation is also the most important reaction in which RS–NOs are involved in biology and appears to the principal mechanism upon which NO-signaling is based (Tsikas et al. 1999). RS–NOs are exceptionally labile (Kashiba-Iwatsuki et al. 1997) and easy react with other thiols by trans-nitrosylation or, in some cases, by disulfide formation. These two possible pathways are due to the concomitant presence of two electrophilic centers on the *S*-nitrosothiol: one is the

sulfur atom and the second is the nitrogen atom of the NO group. Sodium ascorbate (Fig. 1.9b) or Cu I (Dicks and Williams 1996) can also restore the thiol group from RS–NOs. Although the mechanism of ascorbate reduction is not entirely clear, the formation of *O*-nitrosoascorbic acid (Holmes and Williams 2000) appears quite accepted. The ascorbate-mediated reaction is used in the biotin switch assay. This technique is the most popular method for detecting *S*-nitrosothiols in proteins from cell extracts (Jaffrey et al. 2001) and involves three steps: (1) free thiols are blocked with a "thiol-specific reagent" (see Sect. 1.2) such as MMTS; (2) nitrosothiols are reduced by sodium ascorbate to thiols; and (3) nascent thiols are then conjugated to biotin with N-[6-(biotinamido)hexyl]-30-(20-pyridyldithio)-propionamide (biotin-HPDP, **1**) and analyzed by Western blot (Fig. 1.9).

Along with issues inherent to indirect or subtractive methods of detection, another challenge for this technique is the selective reduction of RSNOs with sodium ascorbate. It is well-known that sodium ascorbate can give false-positive signals for *S*-nitrosothiols when detected by the biotin switch assay (Huang and Chen 2006). In fact, ascorbate can also reduce sulfenic acids (see Sect. 1.4.1) and disulfides, albeit less efficiently. Furthermore, the ascorbate-mediated reduction of RS–NOs is quite slow and some nitrosothiols cannot be reduced during the incubation time. The reaction rate of reduction can be increased using catalytic amounts of Cu^{+} (Kirsch et al. 2009). Copper promotes the direct de-nitrosylation of RS–NOs while ascorbate maintains the minimum concentration of Cu(I) necessary to catalyze the reaction, reducing Cu^{2+} to Cu^{+} (Eqs. 1.23 and 1.24). However, it is critical to note that Cu^{+} may also lead to oxidation of protein thiols.

$$\mathrm{RS-NO} + \mathrm{Cu^{+}} \rightarrow \mathrm{R-S^{-}} + {}^{\bullet}\mathrm{NO} + \mathrm{Cu^{2+}} \tag{1.23}$$

$$\mathrm{Cu^{2+}} + \mathrm{Asc^{-}} \rightarrow \mathrm{Cu^{+}} + \mathrm{Asc^{\bullet -}} \tag{1.24}$$

In light these limitations the biotin switch assay has been superseded by direct and selective bioorthogonal reactions for RS–NOs in recent years. It is known that triaryl phosphines react with RS–NOs to yield an aza-ylide intermediate, which is hydrolyzed to phosphine oxide in water (Fig. 1.9c, Haake 1972). Xian's lab has employed such phosphine-mediated reactions to selectively modify *S*-nitrosothiols. In these studies, an aza-ylide nucleophile is proposed, and in the presence of an intramolecular electrophilic group (such as an ester), the *S*-nitrosothiols can be converted into a sulfenamide by intramolecular acyl transfer (Wang and Xian 2008). Although the resulting sulfenamide is more stable than the *S*-nitrosothiol, it is not suitable for RS–NO detection in proteins. In particular, the sulfenamide is reduced to the thiol in the presence of excess phosphine reagent. Consequently, Xian's research group has developed triarylphosphines (**30a**) with a thioester as a trap for the aza-ylide intermediate (**30b**). This kind of reagent reacts with RS–NOs to generate a sulfenamide (**30c**), which immediately undergoes nucleophilic attack of the thiol leaving-group (formed after the intramolecular acyl transfer) to yield a disulfide (**30d**), which is still prone to reduction, but is more stable than an S–N bond.

This approach appears promising and has been used to detect protein RS–NOs in cell extracts (Zhang et al. 2010). However, the relative instability of the disulfide link remains as a significant challenge for robust application of this approach *in vitro* and especially in cells.

Several other phosphine reagents have been developed to detect RS–NOs (see the review: Wang and Xian 2011). King's group reported the first water-soluble phosphine capable of direct labeling of *S*-nitrosothiols (Bechtold et al. 2010). Tris(4,6-dimethyl-3-sulfonatophenyl)-phosphine trisodium salt hydrate (TXPTS) appears to form a stable sterically hindered *S*-alkylphosphonium adduct, which can be detected by ^{31}P-NMR. However, the selectivity of all these reagents must be careful investigated. In fact, disulfides (see Sect. 1.4.3) and sulfenic acids (see Sect. 1.4.1) are also reduced by phosphines. On the other hand, triarylphosphines do not appear to be fast disulfide-reducing agents (Saxon and Bertozzi 2000) and the presence of electron-withdrawing substituents should further reduce their reactivity (Wang and Xian 2008). In contrast, Davis's group has shown that a disulfide formed between a short thio-peptide and methyl 4-thio-2-nitrobenzoate reacts very rapidly (∼15 min) with several phosphines, including triphenylphosphine (Chalker et al. 2011). This reaction can be explained by the low pKa of methyl 4-thio-2-nitrobenzoate, which works as an excellent leaving group during the phosphine attack. Along these lines, some disulfides could exhibit cross-reactivity with the phosphine reagents developed for RS–NOs detection (Bechtold et al. 2010). Finally, cross-reactivity with sulfenic acids remains poorly understood. Although the selectivity of triarylphospine probes for RS–NOs detection over sulfenic acids was recently suggested (Li et al. 2012), however, the use of PTB1B as a model appears contradictory. In fact, when PTB1B is exposed to H_2O_2 the catalytic cysteine residue forms a stable, cyclic sulfenamide (Salmeen et al. 2003), which is predicted to be less reactive toward phosphines.

1.4.3 Disulfides

The formation of a disulfide bond between two cysteine residues or between a cysteine residue and a low-molecular-weight thiol may also have a significant impact on the structure and function of the macromolecule (Fan et al. 2009; Wouters et al. 2010). Protein disulfide generation is generally mediated by sulfenic acid formation (Eq. 1.25, see Sect. 1.4.1) or by thiol-disulfide exchange (Eq. 1.26). Thiols can also react with sulfenyl halides (Eq. 1.27), sulfenyl thiocyanates (Eq. 1.28), and thiosulfinate esters (Eq. 1.29) to form disulfide. Moreover, the reaction of *S*-nitrosothiols (Eq. 1.30) with thiols can sometimes proceed with release of nitroxyl (HNO) and disulfide formation (Hogg 2002).

$$\mathrm{R-S-OH + R_1-S^- \rightarrow R-S-S-R_1 + OH^-} \tag{1.25}$$

$$\mathrm{R-S-SR_1 + R_2-S^- \rightarrow R-S-S-R_2 + R_1-S^-} \tag{1.26}$$

$$R-S-X+R_1-S^- \rightarrow R-S-S-R_1+X^- \quad (1.27)$$

$$R-S-SCN+R_1-S^- \rightarrow R-S-S-R_1+SCN^- \quad (1.28)$$

$$R-S-S(O)R_1+R_2-S^- \rightarrow R-S-S-R_2+R_1-S-O^- \quad (1.29)$$

$$R-S-NO+R_1-S^- \rightarrow R-S-S-R_1+NO^- \quad (1.30)$$

Thiol-disulfide exchange is one of the most important sulfur-based reactions in biology and can regulate the structure and activity of proteins that contain regulatory cysteines (Ilbert et al. 2007). Although this reaction is spontaneous, it can also be catalyzed by transition metals (Arisawa and Yamaguchi 2008). The driving force of the reaction is the relatively low activation energy required for thiols to break disulfide bonds (Fernandes and Ramos 2004). The thiol–disulfide exchange reaction is a bimolecular nucleophilic substitution reaction (SN2) in which the attacking nucleophile is the thiolate. The reaction proceeds with a linear transition state and this geometry has a significant impact on the reaction rate. Consequently, in proteins, cysteines that are situated in a position in which a linear transition state is difficult to achieve will be much less reactive.

The rate of protein thiol–disulfide exchange reactions is influenced by several additional factors. These include the pKa of the thiolate and the leaving-group thiol, nearby charged amino acid residues, and steric factors. When a thiolate anion attacks an asymmetrical disulfide (R_1S–SR_2), the best leaving group will be the thiol with lowest pKa (Jensen et al. 2009). Moreover, since thiol-disulfide exchange involves negatively charged species, the reaction rate can be influenced by electrostatic factors, such as negative charges adjacent to the reaction center (Bulaj et al. 1998). The formation of an intramolecular disulfide is typically more efficient than the formation of intermolecular disulfides. Consequently, the incubation of disulfide with dithiothreitol (DTT) always leads to the oxidation of DTT. In biology, thiol-disulfide exchange is the basis of the mechanism of action of many enzymes. Another thiol/disulfide exchange process that deserves special consideration is *S*-glutathionylation of cysteine in proteins. Although *S*-glutathionylation was thought to protect cysteine from irreversible oxidation (Thomas et al. 1995), it was later shown that this modification affects the catalytic activity for several enzymes, suggesting a regulatory role (Demasi et al. 2003).

Disulfides also react with strong oxidants to yield thiosulfinate and thiosulfinate esters. However, this reaction is significantly slower than oxidation of a thiol or sulfenic acid and thus, would take place only at extremely elevated, cytotoxic concentrations of ROS/RNS. Disulfides are reduced to thiols by a number of inorganic compounds, including sodium borohydride ($NaBH_4$) and sodium cyanoborohydride ($NaBH_3CN$). In biochemistry, dithiols and phosphite esters are the most common reducing agents. It has been demonstrated that sodium ascorbate can also reduce a disulfide (Holmes and Williams 2000), but this reaction appears to be catalyzed by Copper(II). Chemical approaches to the detection of disulfides

are largely indirect. The initial step consists of blocking free thiols with an irreversible alkylating agent (see Sect. 1.2), followed by a disulfide reduction (e.g., with tris(2-carboxyethyl)phosphine – TCEP). Finally, the nascent thiols are trapped with the usual reagents (see Sect. 1.2). Two important considerations are: (1) owing to a pKa of ∼8 for most thiols, the blocking step must be carried out at neutral pH or higher for this reaction to be efficient; (2) commonly used reducing agents also reduce other reversible cysteine oxoforms, such as sulfenic acids, nitrosothiols, sulfenamides, and thiosulfinates. Direct detection methods exist only for *S*-glutathionylation (RS–SG), which can be monitored through reduction of this bond by glutaredoxin (Lind et al. 2002), radiolabeled with ^{35}S-GSH (Fratelli et al. 2002) or biotinylated glutathione ethyl ester (BioGEE, Sullivan et al. 2000). Alternately, an immunochemical approach using specific antibodies against the protein-glutathione adduct may be employed (Dalle-Donne et al. 2003).

1.4.4 Sulfenamide

A sulfenamide is a compound containing a trivalent nitrogen atom bonded to divalent sulfur and is formally derived from the condensation of sulfenic acid with an amine (Allison et al. 1973). Although a sulfenic acid and sulfenyl halide may react with any nucleophilic amine to generate a sulfenamide, in general, this reaction is several orders of magnitude slower then the equivalent reaction with a thiol. Nonetheless, interest in such modifications has grown since the discovery of cyclic sulfenamide formation in the active site of protein tyrosine phosphatase 1B (PTP1B) (Salmeen et al. 2003). The sulfenic acid intermediate produced by oxidation of the PTP1B catalytic cysteine is rapidly converted into the sulfonamide form. The crystalline structure of oxidized PTP1B reveals that the sulfenamide is characterized by an isothiazolidinone ring formed by the binding of sulfur of the cysteine to the backbone nitrogen of the adjacent serine residue (van Montfort et al. 2003). This species appears to be generated by nucleophilic attack of the main-chain amide nitrogen on the electrophilic sulfur in sulfenic acid with elimination of water. Beyond PTP1B, few other proteins have been found to form cyclic sulfonamide modifications (Lee et al. 2007) and, as a result, it is difficult to say whether such modification has a wider role in biology.

Sulfenamides appear to have similar a reactivity profile akin to sulfenic acid and undergo attack by several nucleophilic species. For example they react with thiols to yield disulfides (Nti-Addae et al. 2011) and, consequently sulfenamide formation may mediate the *S*-glutathionylation of proteins such as PTP1B (den Hertog et al. 2005). Sulfenamides can also undergo trans-amination reactions (Craine and Raban 1989); considering that the only biologically relevant sulfenamide known to date is cyclic, however, this reaction is unlikely occur in cells because the equilibrium of the reaction is always shifted toward the cyclic form. Sulfenamides can be reduced to thiols by phosphite esters, which also react with disulfides, sulfenic acids, and *S*-nitrosothiols. The cyclic sulfonamide within PTP1B is often cited for its ability

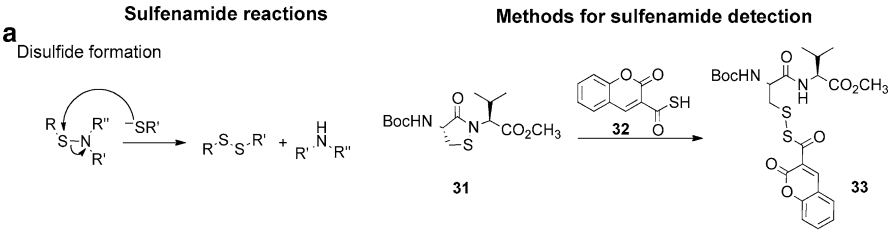

Fig. 1.10 Chemical reactivity and detection of sulfenamides

to protect the reactive thiol against irreversible oxidation to sulfinic and sulfonic acid. This statement can be rationalized by the lesser nucleophilic character of the sulfur atom in the cyclic sulfonamide as compared to the sulfenic acid. In aqueous basic solutions of H_2O_2, the sulfenamide is readily oxidized to sulfinamide and sulfonamide (Kharasch et al. 1946), but at physiological pH such oxidation is negligible. In fact, although sulfinamide formation has been reported in short peptides exposed to ROS (Shetty and Neubert 2009), no evidence of sulfinamide or sulfonamide formation has been observed in PTP1B. This is true even in the presence of high H_2O_2 concentrations, as PTP1B exclusively undergoes sulfinic and sulfonic acid modification, ascribed to the direct oxidation of the cysteine sulfenic acid intermediate (Held et al. 2010).

The development of specific probes for sulfenamides is made more difficult by the similarity with sulfenic acid reactivity (Fig. 1.10b). Shiau et al. have used a series of relatively weak nucleophilic thiols to identify compounds that react with sulfonamides, but not disulfides (Fig. 1.10). They identified a coumarin thioacid (**32**), which reacts with a model sulfenamide (**31**) to generate the fluorescent disulfide (**33**) and exhibits no cross-reactivity with disulfides (Shiau et al. 2006). Unfortunately, this study provided no data regarding reactivity of **32** with sulfenic acid; nonetheless, it is almost certain that the coumarin-derivative should also react with sulfenic acids and related thiosulfinates.

1.4.5 Thiosulfinate Ester

Thiosulfinate esters can be formed by the condensation of two sulfenic acids (see Sect. 4.1) or by oxidation of disulfides (Eq. 1.31). In a biological context, the auto-condensation of sulfenic acids appears to have poor relevance in proteins owing to steric hindrance, while it should be quite likely for small molecules such as cysteine or glutathione. The direct oxidation of disulfide to the thiosulfinate would require very high, localized concentrations of ROS/RNS and only biologically relevant for glutathione in its oxidized state (Giles et al. 2002). Interestingly, the thiosulfinate group forms as a reaction intermediate during sulfiredoxin (Srx)-mediated reduction

of cysteine sulfinic acid in Prxs; however, this modification is generated through a completely different mechanism (see Sect. 1.4.5).

The thiosulfinate ester is a highly reactive species and can readily undergo the nucleophilic attack by thiols to yield a disulfide and a sulfenic acid (Nagy et al. 2007). Glutathione disulfide *S*-monoxide has consequently been proposed as a mediator in *S*-glutathionylation (Huang et al. 2007). Thiosulfinates can also undergo hydrolysis (Eq. 1.32), a reaction that is in equilibrium with self-condensation of sulfenic acids (Nagy and Ashby 2007a, b). Formally, thiosulfinates can be oxidized to sulfonate esters (Eq. 1.33), but this should take place only in the presence of high concentrations of very strong oxidants such as those found in the phagosome. Finally, disproportionation of aromatic thiosulfinate esters has been reported to yield a disulfide and a thiosulfonate ester *via* a complex radical mechanism (Eq. 1.34, Poudrel and Cole 2001).

$$\mathrm{R-S-SR + H_2O_2 \rightleftarrows R-S-S(O)R + H_2O} \tag{1.31}$$

$$\mathrm{R-S-S(O)R + H_2O \rightleftarrows 2\,R-S-OH} \tag{1.32}$$

$$\mathrm{R-S-S(O)R + H_2O_2 \rightarrow R-S-S(O)_2\,R + H_2O} \tag{1.33}$$

$$\mathrm{2\,R-S-S(O)R \rightarrow RS-S-R + R-S-S(O)_2\,R} \tag{1.34}$$

Except for the Prx-thiosulfinate intermediate, no other thiosulfinates are known in proteins. This may be due, at least in part, to the difficulty of finding a selective strategy for detecting this thiol modification. The thiosulfinate ester, like a thiosulfonate ester (Schank et al. 2007) may react with dimedone (and its derivatives) to yield a dimedone-thioether and a sulfenic acid, which in turn reacts with a second molecule of dimedone. Rabinkov's group has developed a simple spectrophotometric assay to determine the presence of allicin (a natural thiosulfinate ester present in garlic) and of allinase activity by the reaction between 4-mercaptopyridine (4-MP) and allicin (Miron et al. 2002). The selectivity of this technique is based on the high reactivity of the thiosulfinate in comparison to the disulfide, but its application with proteins in complex biological samples appears since 4-MP should also react with the more abundant sulfenic acid.

1.4.6 Sulfinic Acid

Sulfenic acid can be further oxidized to sulfinic acid (RSO_2H) in the presence of excess ROS/RNS (see Sect. 1.4.1). RSO_2H is a relatively stable species as compared to RSOH, and with a pK_a value of ~2, is always deprotonated at physiological pH (Burkhard et al. 1959). Sulfinic acids can be also generated by disproportionation

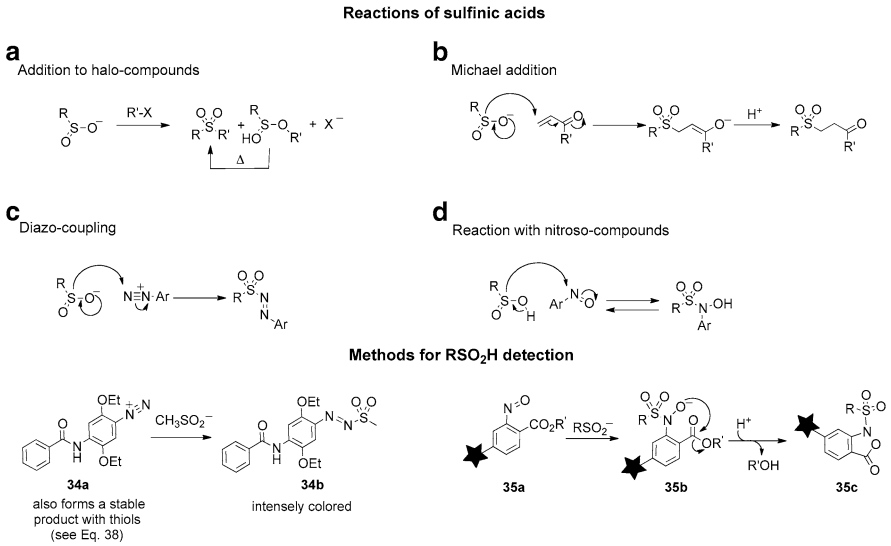

Fig. 1.11 Chemical reactivity and detection of sulfinic acids

of sulfenic acid (Eq. 1.35, Abraham et al. 1983) or by nucleophilic displacement of thiosulfonate esters (Eqs. 1.36 and 1.37, Harpp et al. 1979).

$$2\,R-S-OH \rightarrow R-SH + RS_2OH \tag{1.35}$$

$$R-S-S(O)_2\,R_1 + HO^- \rightarrow RSO_2^- + R_1-S-OH \tag{1.36}$$

$$R-S-S(O)_2\,R_1 + R_2-S^- \rightarrow RSO_2^- + R_1-S-S-R_1 \tag{1.37}$$

In the deprotonated form, the sulfinate group (RSO_2^-) is an ambident anion and the negative charge is delocalized between the sulfur and the two oxygen atoms. RSO_2^- behaves mainly as a soft nucleophile (Reddie and Carroll 2008) and can react with many electrophilic species such as halides (Fig. 1.11a) (Khamis et al. 2010) and α,β-unsaturated compounds (Fig. 1.11b) (Ogata et al. 1970). Sulfinic acids can therefore be alkylated by iodoacetamide as well as ethylmaleimide, although these reactions are slower in comparison to thiols. In all these reactions, *S*-attack is generally favored and leads to the thermodynamically more stable sulfone. In the presence of strong electrophiles, the sulfonyl ester can be kinetically generated (Baidya et al. 2010), but this unstable species slowly rearranges to a sulfone (Fig. 1.11a). Sulfinic acids can oxidize further to sulfonic acid, but neither of these oxoforms can be reduced directly by thiols, hence the term "irreversible". In recent years, a biological role for RSO_2H has emerged, for example, in both Parkinson's disease protein DJ-1 (Blackinton et al. 2009) and in matrilysin (MMP-7) activation (Fu et al. 2001), although the best-known cysteine sulfinyl modification is occurs

within the Prx family (Wood et al. 2003). Finally, the discovery of sulfiredoxin, an ATP-dependent protein that specifically reduces sulfinic acid in Prx, has opened the door to an additional layer of redox regulation (Jönsson et al. 2008) and increased interest in detecting this specific modification.

Antibodies against individual proteins with sulfinyl/sulfonyl modifications have been developed (see Sect. 1.4.7). At the moment, however, no selective chemical probes are available to detect sulfinic acid formation in proteins. On the basis of early physical-organic studies (Ritchie et al. 1961), the use of diazonium salts (Fig. 1.11c, **34**) as a trapping agent for protein sulfinic acids was recently proposed (Jacob and Ba 2011). Although this approach has proven to be effective for colorimetric detection of methane sulfinic acid (Babbs and Gale 1987), several complications are present in this system. Diazonium salts are highly unstable species that readily decompose under neutral aqueous conditions. Moreover, considering the potential cross-reactivity with tyrosine (Hooker et al. 2004), as well as with cysteine (Patt and Patt 2002), future application of this technique with proteins may be limited. Whereas avoiding basic conditions can minimize cross-reactivity with tyrosine, cysteine appears to react with diazonium salts under a wide range of pHs (Eq. 1.38) to yield a stable sulfenyldiazene species (Lo Conte and Carroll, unpublished data).

$$\mathrm{R-SH + Ar-N_2^+ \rightarrow R-S-N=N-Ar + H^+} \tag{1.38}$$

As an alternative, the Carroll research group has been working to developing a new selective ligation strategy using C-nitroso compounds, which condense with sulfinic acids to yield N-sulfonyl hydroxylamines (Fig. 1.11d). N-sulfonyl hydroxylamines are unstable at neutral or basic pH (Darchen and Moinet 1976). As a result, in order to convert the product into a stable adduct, we can take advantage of the nucleophilic behavior of hydroxylamine. In the presence of an electrophilic center (e.g., a carboxylic ester) on the C-nitroso compound (**35a**), N-sulfonyl hydroxylamine (**35b**) undergoes intramolecular cyclization to form a stable N-sulfonyl benzisoxazolone adduct (**35c**). Although thiols can also target the C-nitroso group, selectivity for sulfinic acid is ensured by the fact that reaction with the thiol leads to an unstable N-sulfenyl hydroxylamine linkage, which is readily cleaved in the presence of additional thiols; this chemical approach is currently under being adapted for detection of protein sulfinic acids *in vitro* (Lo Conte and Carroll 2012).

1.4.7 Sulfonic Acid

Sulfonic acid represents the highest oxidation state for the cysteine sulfur atom and no biological pathway is known to reduce this cysteine oxoform. Sulfinic acid can be further oxidized to sulfonic acid by strong oxidizing species such as peroxynitrous acid and hydrogen peroxide (Eq. 1.39, see Sect. 1.4.6). Sulfonic acid can also

be generated by the disproportionation of sulfinic acid (Eq. 1.40). As alluded to above, when an essential enzyme active site cysteine is oxidized to sulfonic acid the catalyst is irreversibly inhibited. However, it has also been proposed that sulfonic acid modification of Prx could enhance its proposed chaperone activity (Lim et al. 2008). This modification may also target proteins for degradation. For example, the oxidation of N-terminal cysteine residues to sulfonic acid can mediate arginylation and degradation of GTPase-activating proteins (Tasaki and Kwon 2007).

$$R - SO_2H + H_2O_2 \rightarrow R - SO_3H + H_2O \tag{1.39}$$

$$4\,R - SO_2H + H_2O \rightarrow RSO_3H + R - S(O)_2 - S - R \tag{1.40}$$

Sulfonic acid is one of the strongest organic acids. Cysteic acid, with a pKa of −3, is always present in the deprotonated form as sulfonate at physiological pH (Chang et al. 2010). Sulfonic acid is a very poor nucleophile and its derivatives are usually obtained by activation as the sulfonyl chloride. No facile chemical methods are available to trap and tag the sulfonic acid modification in proteins. Although base-mediated β-elimination of cysteine sulfonic acid to dehydroalanine and sulfite could provide a starting point for assay design (Dai et al. 2005), the concomitant β-elimination of phosphoserine and phosphothreonine (McLachlin and Chait 2003) represents a serious limitation of such as approach. To date, antibodies are the most common technique for detecting the sulfonic acid form of a specific protein (Woo et al. 2003). Unfortunately, this approach is not easily applied to all proteins, and the resulting antibodies typically exhibit the same affinity for sulfinic and sulfonic acid cysteine oxoforms. Recently, an innovative mass spectroscopy assay was developed to selective enrich and identify peptides containing cysteine sulfonic acid. This approach is based on ionic affinity capture using polyarginine-coated nanodiamonds that exhibit good specificity even in the presence of phosphopeptides (Chang et al. 2010). The technique was applied to selectively enriched sulfopeptides obtained from tryptic digests of over-oxidized BSA.

1.4.8 Sulfhydration

The process by which H_2S mediates *S*-sulfhydration of proteins is still unclear. For example, the direct reaction of cysteine side chains with H_2S (Mustafa et al. 2011) appears quite unlikely without any intermediary oxidant species. Persulfides can be generated by the direct reaction of sulfenic acids (Kabil and Banerjee 2010), sulfenyl halides, sulfenyl-thiocyanates, or thiosulfenyl esters with H_2S (Eqs. 1.41, 1.42, 1.43 and 1.44). Although less reactive, disulfides can also generate persulfides *via* disulfide exchange with H_2S (Eq. 1.45). Finally hydrogen persulfide (HSSH) has recently been proposed as the physiological sulfhydration agent in cells

(Nagy and Winterbourn 2010). HSSH is generated by reaction of H_2S with two-electron oxidants and can easy react with low pKa thiols (Eq. 1.46).

$$R - SOH + HS^- \rightarrow R - S - S^- + H_2O \tag{1.41}$$

$$R - S - X + HS^- \rightarrow R - S - S^- + HX \tag{1.42}$$

$$R - S - SCN + HS^- \rightarrow R - S - S^- + HSCN \tag{1.43}$$

$$R - S - S(O)R_1 + 2\,HS^- \rightarrow 2\,R - S - S^- + H_2O \tag{1.44}$$

$$R - S - S - R_1 + HS^- \rightarrow R - S - S^- + R_1S^- \tag{1.45}$$

$$R - S^- + HS - SH \rightarrow R - S - S^- + HS^- \tag{1.46}$$

Persulfides show intermediate behavior between thiol and disulfide reactivity. In fact, the terminal sulfur is ambiphilic and can behave as an electrophile or a nucleophile. For example, persulfides can undergo oxidation reactions to yield a wide range of products or may also undergo disulfide exchange (Eqs. 1.47 and 1.48), following the same rules described in Sect. 1.4.3. If the internal sulfur atom of a persulfide group has a pKa lower than 6.9 (corresponding to the pKa of hydrogen sulfide), the reaction may proceed *via* trans-sulfhydration; alternately, the result is disulfide formation and the release of hydrogen sulfide. The terminal sulfur, however, can also directly attack a disulfide or react with a sulfenic acid to generate a trisulfide (Eqs. 1.49 and 1.50). Trisulfides are widely distributed in the biological world, although they are not a common post-translational modification, and the number of proteins in which a trisulfide has been unambiguously identified is small (Nielsen et al. 2011).

$$R - S - SH + R_1S^- \rightarrow R - S - S - R_1 + HS^- \tag{1.47}$$

$$R - S - SH + R_1S^- \rightarrow R_1 - S - S^- + RS^- \tag{1.48}$$

$$R - S - S^- + R_1 - S - S - R_1 \rightarrow R - S - S - S - R_1 + R_1S^- \tag{1.49}$$

$$R - S - S^- + R_1 - S - OH \rightarrow R - S - S - S - R_1 + OH^- \tag{1.50}$$

In the presence of transition metals, persulfides can readily decompose to generate ROS, and such reactions could mediate the anti-microbial and anti-cancer properties of some natural products (Chatterji et al. 2005). The terminal sulfur is a strong nucleophile and can react with a huge range of electrophiles. The richness of its chemistry makes the persulfide group a versatile reagent for the incorporation of sulfur along many metabolic pathways (Mueller 2006).

In the last decade, interest in H_2S signaling has grown rapidly, but relatively few proteins have been found to be sulfhydrates (Krishnan et al. 2011). This is due

to the difficultly of monitoring such modifications uniquely. The Snyder research group has reported an assay for proteins persulfides, which is a modification of the classic biotin switch assay (Mustafa et al. 2009). In this method, MMTS is employed to block free thiols and any persulfides are reacted with biotin-HPDP (possibly forming a disulfide or trisulfide linkage) and analyzed by Western blot. Applying this approach to complex cell lysates, several proteins were reported as targets of H_2S signaling. A key feature of this approach requires that the MMTS reagent only modify thiols and not persulfides. Unfortunately, no rationale or data was provided by Mustafa et al. to validate this claim. In fact, our own research group has recently determined that MMTS does react with persulfides to yield a trisulfide linkage as expected from the nucleophilic nature of sulfane sulfur (Pan and Carroll, unpublished data). Therefore, considering that the chemical mechanism and selectivity remain unknown, results obtained using this approach should be interpreted with caution.

1.5 Conclusions

In the last decade, awareness of the number of proteins containing redox-sensitive cysteine has grown significantly. Cysteine can assume a wide range of oxidative states in response to dynamic changes in intracellular redox potentials. To understand the role of such protein modifications, methods have been developed to distinguish between different cysteine oxidation states. The role of the biologist is not simply to choose the most appropriate technique, but also to maintain a critical view of possible artifacts. Opting for an *in vitro* approach can prove unsatisfactory because many cysteine oxoforms are reactive and unstable outside of the cellular milieu. The use of indirect methods, by which all free thiols are trapped, must be preceded by the awareness that these thiol-trapping agents can react inefficiently or be incompletely selective, resulting in under- or over-estimation. The same considerations must be taken into account when using reducing agents to identify specific modifications. Indeed, the selectivity and efficiency of such reagents requires careful evaluation. Furthermore, in many cases, interconversion of cysteine redox states dramatically increases the challenges of studying an individual modification. Finally, given that the local microenvironment can exert a remarkable influence on protein thiolate reactivity, the exploration of probes and chemical methods exclusively in low-molecular-weight model systems may prove unsatisfactory. Although more complex, a recommended course for future research is the identification of selective reactions that can be employed to monitor cysteine oxidation *in situ*, directly in cells. Obviously, this presents a formidable challenge, but given the vast number of biological processes that thiol oxidation plays a significant regulatory role, it should be well worth the effort.

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Chapter 2
Radical Scavenging by Thiols and the Fate of Thiyl Radicals

Christine C. Winterbourn

Abstract Reduced glutathione reacts rapidly with the majority of biological free radicals and is regarded as a good radical scavenging antioxidant. However, the reaction generates thiyl radicals, which are strong, potentially damaging oxidants that need to be removed for the process to be effective. Sequential reactions of the radical with the thiolate anion and oxygen drive the scavenging reaction, producing the disulfide and superoxide radicals. Protein thiols are also good radical scavengers via a similar mechanism, and this is an efficient route for generating intramolecular disulfides, mixed disulfides with glutathione and nitrosothiols. These interactions are relevant not only to antioxidant defence but as part of a network of radical reactions that can regulate the oxidation state of glutathione and the extent of protein S-thiolation as well as acting as a source of superoxide and hydrogen peroxide. This chapter discusses the radical chemistry of glutathione and other thiols and how it could contribute to redox activity in the cell.

Keywords Free radical scavenging • Glutathione • Superoxide • Protein disulfide • Thiyl radical

2.1 Introduction

Thiols are prime biological targets for oxidation and their ability to undergo reversible oxidation and reduction enables them to contribute to many cell functions. Thiol groups are required for the activity of numerous enzymes and they have a major role in the antioxidant defenses of the cell. There are also a large

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number of regulatory proteins whose activity can be modified by oxidation of the sulfhydryl group. Thus, sulfhydryl oxidation can affect cell function not only through inactivating thiol enzymes, but also by altering the binding characteristics of molecules involved in signaling pathways (Winterbourn and Hampton 2008; Forman et al. 2004).

Thiol groups are generally reactive with all the oxidants that cells encounter, although reaction rates may differ by many orders of magnitude. The reaction may proceed either by a two electron mechanism to generate the sulfenic acid (or sufenyl halide) as the initial product, as is the case with peroxides, hypochlorous acid and peroxynitrite, or by a one electron free radical pathway to produce the thiyl radical. The ultimate products of the two pathways are not necessarily the same. As a consequence of this plus the difference in reactivity of individual reactive oxygen species (ROS), all ROS will not undergo the same reactions in the cell. Therefore, antioxidant strategies, as well as effects on cell metabolism or regulatory pathways will differ depending on the oxidant involved.

The focus of this chapter is on radical pathways, with non-radical pathways covered more extensively elsewhere in this book. However, it is useful to compare the two pathways. With a few exceptions (notably peroxiredoxins, thiol-containing glutathione peroxidases and the bacterial transcription factor oxyR) the direct reaction of peroxides with thiol groups is slow (Winterbourn and Metodiewa 1999; Winterbourn and Hampton 2008). Antioxidant defense against peroxides is not due to a direct reaction with reduced glutathione (GSH), but is provided by well characterized systems that involve either glutathione peroxidases plus GSH, or peroxiredoxins acting in combination with thioredoxin/thioredoxin reductase (Flohe et al. 2011; Rhee and Woo 2011). These enzymatic systems involve two electron steps that cycle between thiol and disulfide and are ultimately dependent on NADPH for reducing equivalents. Direct reactions between thiols and more reactive oxidants such as hypochlorous acid, chloramines or peroxynitrite are more favorable. These can result in oxidation beyond the sulfenic acid or disulfide, to the sulfinic ($-SO_2H$) or sulfonic acid ($-SO_3H$). Particularly in the case of HOCl, condensation with amino groups in GSH or proteins can give rise to sulfinamides or sulfonamides (Harwood et al. 2006; Raftery et al. 2001). Therefore a range of higher oxidation products is possible.

Thiols react with a wide range of radical species. These are generated by a variety of cellular mechanisms (Fig. 2.1) and include hydroxyl, phenoxyl, alkoxyl, arylamino, peroxyl, semiquinone and carbon centred radicals as exemplified in Table 2.1. Some of the parent compounds that give rise to these radicals occur physiologically, others are drugs or environmental chemicals. Some, such as flavonoids, are themselves radical scavengers and of interest for their potential health benefits as antioxidants. This article examines radical scavenging by GSH and by thiol proteins within the context of antioxidant defense and also as a potential mechanism for regulating redox-sensitive cell functions.

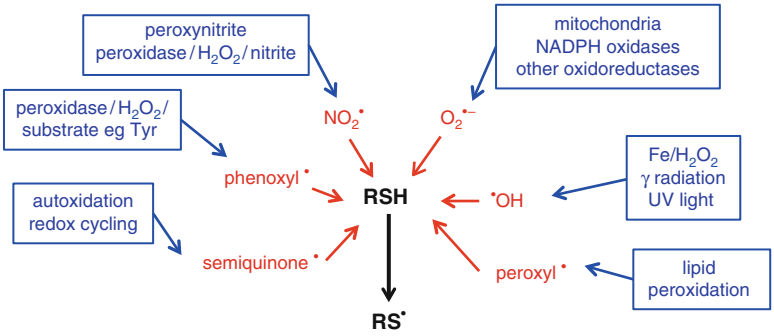

Fig. 2.1 Examples of physiological sources of thiyl radicals. As described in the text and Table 2.1, a majority of physiologically relevant free radicals can react with GSH and protein thiols (*shown as RSH in Figure*) to generate thiyl radicals. Major sources of such radicals are shown. Autoxidation and redox cycling mechanisms apply to many drugs and environmental chemicals including polyphenols, quinones and other aromatic compounds. Other oxidoreductases that generate superoxide radicals include xanthine oxidase, lipoxygenase and nitric oxide synthase. Superoxide reacts only slowly with thiols (*as designated by dashed line*) and these mechanisms are more relevant as a source of hydrogen peroxide for metal-catalyzed or peroxidase- mediated radical generation

Table 2.1 Examples of radical generating systems that cause oxidation of GSH to its thiyl radical

Class of compound	System	References
Tyrosine	Peroxidase	Nakamura et al. (1986), Pichorner et al. (1995)
Phenols	Peroxidase, radiolysis	Ross and Moldeus (1985), D'Arcy Doherty et al. (1986), Subrahmanyam and O'Brien (1985)
Sugars	Radiolysis	Baker et al. (1982)
DNA bases	Radiolysis	Willson (1983)
Nitrogen dioxide and peroxynitrite	Direct reaction	Quijano et al. (1997), Bonini and Augusto (2001)
Aromatic amines, phenothiazines	Peroxidase, autoxidation	Ross and Moldeus (1985), Subrahmanyam and O'Brien (1985), Subrahmanyam et al. (1987)
Ethanol (hydroxyethyl)	Thermal decomposition	Stoyanovsky et al. (1998)
Tocopherol	Radiolysis	Niki et al. (1982)
Hydroxpyrimidines	Autoxidation	Munday and Winterbourn (1989)

2.2 Radical Scavenging by Reduced Glutathione

Radical scavenging by GSH generates the glutathionyl radical (reaction 2.1). In most cases the reaction is fast; for example with tyrosine the rate constant, k, is 2×10^6 M^{-1} s^{-1} (Folkes et al. 2011) and with millimolar concentrations typically

present inside cells, GSH would be expected to be an effective physiological scavenger. However, the reduction potential of GSH is sufficiently high that scavenging of many radicals is not highly favored on thermodynamic grounds (Wardman and von Sonntag 1995). The reaction is reversible, and in many cases the reverse reaction is at least as fast and the equilibrium lies far to the left (e.g. for acetaminophen $K_1 = 3 \times 10^{-4}$ (Ramakrishna Rao et al. 1990)). For a scavenging reaction to provide antioxidant protection, the products of the reaction must be less reactive or damaging than the initial species. However, as discussed in Sect. 2.7, thiyl radicals are oxidizing species that can undergo a number of potentially damaging reactions with biological molecules. On these grounds, GSH would not appear to have the qualities of a good radical scavenging antioxidant. Yet in experimental systems where radicals are generated from substrates such as acetaminophen and tyrosine, they are efficiently scavenged by GSH (for example Ramakrishna Rao et al. 1990; Ross 1988; Pichorner et al. 1995).

$$R^{\bullet} + GSH \rightleftarrows RH + GS^{\bullet} \tag{2.1}$$

$$GS^{\bullet} + GS^{-} \rightleftarrows GSSG^{\bullet -} \tag{2.2}$$

$$GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -} \tag{2.3}$$

There are features of thiyl radical chemistry that enable GSH and other thiols to act as effective scavengers and antioxidants (Wardman and von Sonntag 1995; Winterbourn 1993; Wardman 1995). Most importantly, reaction (2.1) can be kinetically driven in the forward direction by removal of $GS^{\bullet}$ through a rapid reaction with the thiolate anion (GS^{-}) (reaction 2.2). Reaction (2.2) is also an equilibrium. It converts an oxidizing radical ($GS^{\bullet}$) into the disulfide radical anion ($GSSG^{\bullet -}$), which is probably the strongest reductant produced in biological systems (Buettner 1993). The position of equilibrium (2.2) is influenced by the thiolate ion concentration, which is dependent on the pH, the pK_a of the thiol (8.8 for GSH) and the GSH concentration. At pH 7.4 and 5 mM GSH, the ratio of $GS^{\bullet}$ to $GSSG^{\bullet -}$ is 2:1 (Wardman 1995). However, the equilibrium is established rapidly and reactions of $GSSG^{\bullet -}$ may dominate even if its concentration is relatively low. $GSSG^{\bullet -}$ reacts with oxygen irreversibly (reaction 2.3) at a near diffusion-controlled rate ($k = 2 \times 10^8\ M^{-1}\ s^{-1}$). This displaces equilibrium (2.2) to the right and provides the driving force for removing $GS^{\bullet}$. Thus reactions (2.1), (2.2), and (2.3) account for the good scavenging ability of GSH.

Physiologically, this is a major decay route for thiyl radicals. An alternative pathway is for the radical to react with oxygen to form a peroxyl radical (reaction 2.4). This reaction is fast, but it is also reversible and in most situations it contributes less to $GS^{\bullet}$ removal than reaction (2.3). However, it becomes more significant at lower pH or GSH concentration where less thiolate is present. Secondary reactions of the peroxyl radical are the most likely source of the higher oxidation states of glutathione (such as the sulfinic and sulfonic acid) that are minor products in some

radical systems (Wefers et al. 1985; Winterbourn and Metodiewa 1994). $GSSG^{\bullet-}$ may undergo alternative reactions to reaction (2.3) (e.g. reduce quinones and heme proteins) when oxygen is limiting. Dimerization of $GS^{\bullet}$ radicals to give the disulfide (GSSG) is of limited significance at the low steady state radical concentrations that are likely to be present physiologically and even under hypoxic conditions it should only be a minor route for radical decay. Indeed, radical transfer to a carbon centre on the peptide, as observed under anerobic conditions (Zhao et al. 1994, 1997) is likely to be more favourable than dimerization.

$$GS^{\bullet} + O_2 \rightleftarrows GSOO^{\bullet} \tag{2.4}$$

From the above argument, it is evident that oxygen plays an important role in enabling GSH to act as a good scavenger without the build up of potentially damaging thiyl radicals. As a corollary, in addition to producing GSSG as a final product, the reaction sequence consumes oxygen and generates superoxide, which will dismutate to hydrogen peroxide (reaction 2.5). Therefore, scavenging by GSH could be regarded as a generator of oxidative stress or a potential source of oxidants involved in redox regulation.

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2.5}$$

From the perspective of antioxidant protection, however, this mechanism enables GSH to act as an intermediary for removing radicals and channeling them to superoxide (Fig. 2.2). Superoxide acts as a radical sink and, with superoxide dismutase (SOD) present, the sequence provides a mechanism for a single enzyme to control the effects of radical generation (Munday and Winterbourn 1989; Winterbourn 1993). GSH acting in concert with SOD can therefore provide effective radical scavenging antioxidant activity, with the sum of reactions (2.1), (2.2), (2.3) and (2.5) for radical removal giving the net reaction (2.6). For full antioxidant protection, enzymatic removal of hydrogen peroxide is also required.

$$2R^{\bullet} + 4GSH + O_2 \rightarrow 2RH + 4GSSG + H_2O_2 \tag{2.6}$$

There is a plethora of evidence that superoxide and hydrogen peroxide are produced during radical scavenging by GSH (for example O'Brien 1988; Munday 1994; Pichorner et al. 1995; Stoyanovsky et al. 1995; Ross et al. 1985; Galati et al. 1999). These examples include systems where phenoxyl radicals are generated from tyrosine or dietary flavonoids by a peroxidase plus hydrogen peroxide (reaction 2.7), in which case the hydrogen peroxide generated in the scavenging reaction is re-used by the peroxidase. In this mechanism, referred to as thiol pumping (Ross et al. 1985), much more GSH is oxidized than the initial peroxide added.

$$2RH + H_2O_2 \xrightarrow{\text{peroxidase}} 2R^{\bullet} + 2H_2O \tag{2.7}$$

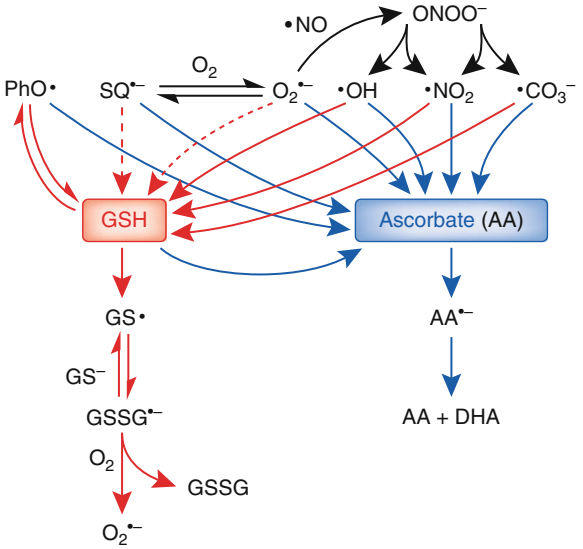

Fig. 2.2 Major aqueous phase radical scavenging pathways in the cell. Pathways show potential interactions with the two main aqueous scavengers, GSH and ascorbate. Note the reversibility and interconversion of many of the reactions. Reactions equivalent to those shown for GSH occur with thioredoxin and glutaredoxin and are also likely with other thiol proteins. A key difference between the glutathione (*red*) and ascorbate (*blue*) pathways is that the former is a radical chain, driven by the reaction of the disulfide radical anion ($GSSG^{\bullet-}$) with oxygen, and producing superoxide, whereas ascorbate generates ascorbyl radicals ($AA^{\bullet-}$). SOD removes superoxide radicals but the hydrogen peroxide generated could be pro-oxidant. *Dashed lines* signify less favourable reactions. *GS*$^{\bullet}$ glutathionyl radical, *PhO*$^{\bullet}$ phenoxyl radical, *SQ*$^{\bullet}$ semiquinone radical, *DHA* dehydroascorbate; reproduced from Winterbourn (2008) with permission

A similar mechanism operates when hydroquinones and hydroxypyrimidines such as dialuric acid autoxidize in the presence of GSH (Winterbourn and Munday 1989, 1990). These compounds undergo superoxide-dependent autoxidation via a semiquinone intermediate. GSH alone, by reducing the semiquinone and generating superoxide, enhances autoxidation and the resultant hydrogen peroxide production, and but with SOD also present, the whole process is inhibited. These are good examples of where both GSH and SOD are required for effective antioxidant protection.

2.3 Reaction of Superoxide with Thiols

The superoxide generated as a result of radical scavenging by GSH could potentially react with more GSH and set up a chain reaction. If this reaction were fast, then large amounts of GSH could be oxidized for each initial radical generated. Data

from a number of sources indicate that superoxide does react with GSH. Although a wide range of values for the rate constant is reported in the literature, there is now a consensus that the reaction is relatively slow and at physiological thiol concentrations there is only a short chain (Winterbourn and Metodiewa 1994, 1999). Rate constants of 200 M^{-1} s^{-1} for GSH (Jones et al. 2002) and 68 M^{-1} s^{-1} for N-acetylcysteine (Benrahmoune et al. 2000) have been measured. GSH reacts about 500 times more slowly than ascorbate with superoxide so even if SOD were limiting, this reaction should play a minimal role in superoxide removal or GSH oxidation in the cell. The same argument holds for N-acetylcysteine when it is added to experimental systems as an "antioxidant".

Experimental and theoretical studies with cysteine, GSH and dithiothreitol indicate that the reaction with superoxide is likely to proceed via a complex stabilized by a three electron sulfur – oxygen bond, which breaks down in reaction (2.8) to give a sulfinyl radical (Zhang et al. 1991; Winterbourn and Metodiewa 1994; Cardey and Enescu 2009). The thiyl radical is formed, but via a secondary reaction with the thiol (reaction 2.9) rather than direct electron transfer, and superoxide is regenerated via reactions (2.2) and (2.3). The short chain is probably due to superoxide reacting with the intermediate radicals. Direct electron transfer to give the thiyl radical should be more favorable for the hydroperoxyl radical ($HO_2^{\bullet}$) and may be more relevant at lower pH.

$$O_2^{\bullet-} + GSH \rightarrow [GS \bullet\bullet\bullet O_2H]^{\bullet-} \rightarrow RH + GSO^{\bullet-} \tag{2.8}$$

$$GSO^{\bullet-} + GSH \rightarrow GSOH + GS^{\bullet} \tag{2.9}$$

2.4 Radical Scavenging by Protein Thiols

Protein thiols should undergo similar radical scavenging reactions as GSH. The reactivity of a particular cysteine residue will depend on its pK_a (the thiolate anion is generally more reactive (Wardman and von Sonntag 1995)) and another thiol being accessible so the equivalent of reaction (2.3) can proceed. Cysteine thiyl radicals can be formed directly on proteins exposed to radical generating systems. In addition, cysteine can act as a sink for radicals generated at other sites such as tyrosine, tryptophan or carbon side chains (Schoneich 2008). For example, spin trapping studies with myoglobin have shown intramolecular transfer from an initial tyrosyl radical to cysteine (Witting and Mauk 2001). Radical transfer from tyrosine to cysteine is also seen in ribonucleotide reductase where it is an integral part of its enzymatic mechanism (Holmgren and Sengupta 2010). Generation of a protein thiyl radical has also been observed during the enzymatic activity of mitochondrial NADH dehydrogenase and endothelial nitric oxide synthase (Chen et al. 2005, 2011). With nitric oxide synthase this occurred in the absence of tetrahydrobiopterin cofactor when the enzyme switched from nitric oxide to superoxide production.

One consequence of intramolecular radical transfer is that nitration of tyrosine residues can be inhibited by the presence of neighbouring cysteine residues (Zhang et al. 2005). Tyrosine nitration proceeds by a mechanism that involves combination of a tyrosyl and nitrogen dioxide radical. When this reaction was studied with tyrosyl peptides containing cysteine residues, nitration was suppressed and formation of thiyl radicals due to electron transfer from the tyrosine radical was observed.

Some protein thiols could be much more effective than GSH as radical scavengers. As reaction (2.2) is critical in the scavenging pathway, the presence of a vicinal thiol group should facilitate formation of the disulfide bond and accelerate the reaction. The vicinal thiols in dihydrolipoic acid and thioredoxin have both been shown to scavenge phenoxyl radicals and generate superoxide in the process (Goldman et al. 1995). In contrast to GSH, it was not possible to trap the thiyl radical with these compounds. This implies a rapid reaction of the radical with the vicinal thiol group. We studied this mechanism using the stable tetramethylphenylenediamine (Wurster's blue) radical, following its disappearance by stopped flow spectrophotometry. In this system, dithiothreitol was 5,000 times more efficient than GSH at scavenging the radical (Winterbourn 2003). This observation raises the possibility that proteins containing vicinal thiol groups could be efficient radical scavengers and generators of superoxide. Radical mechanisms could then be a significant route to disulfide formation in these proteins.

If radicals are formed on single cysteine residues on proteins, they would be expected to react with GSH (reactions 2.2 and 2.3) to generate a mixed disulfide, and produce superoxide in the process. This route to glutathionylation of a specific cysteine residue has been observed for nitric oxide synthase (Chen et al. 2011). Glutathionylation by a different radical mechanism can also be facilitated by glutaredoxins. In addition to catalyzing disulfide interchange reactions with glutathione, these thiol proteins react rapidly with glutathionyl radicals, and accelerate oxygen-dependent formation of GSSG (Starke et al. 2003; Gallogly et al. 2008). The proposed mechanism involves an initial reaction between the glutathionyl radical and the active site thiolate of glutaredoxin (the equivalent of reaction 2.2), which is favored because of its low pK_a and because the enzyme has a high affinity for the glutathionyl moiety (Gallogly et al. 2009). An enzyme-$SSG^{\bullet -}$ radical anion would be formed and react rapidly with oxygen (equivalent of reaction 2.3) to give superoxide and glutaredoxin-SSG. This is the catalytic intermediate formed by glutaredoxin in thiol exchange reactions, and is turned over by GSH to regenerate the enzyme thiolate and GSSG. This reaction has the potential to be a significant radical scavenging pathway and source of superoxide in the cell. It may also be a significant route for S-glutathionylation of cell proteins. Glutaredoxins are efficient catalysts of this reaction and in studies with actin, glyceraldehyde-3-phosphate dehydrogenase and protein tyrosine phosphatase 1B, thiyl radical-mediated formation of glutathione adducts was found to be much more efficient than any of the other mechanisms tested (Starke et al. 2003).

Protein glutathionylation has been widely observed in cells under oxidative stress and is proposed as one of the major mechanisms for regulating redox sensitive signalling pathways (Fratelli et al. 2003; Gallogly et al. 2009; Dalle-Donne et al.

2003). The details of how this occurs are not fully understood, although two electron oxidation pathways (e.g. with hydrogen peroxide) or thiol exchange is usually invoked. However, with the addition of GSH to protein thiyl radicals and glutaredoxin-mediated addition of GSH radicals both being facile mechanisms, it is possible that radical mechanisms may play a more prominent role in redox signalling than is generally considered.

2.5 Reactive Nitrogen Species and Nitrosothiol Formation

Nitrosylation of cellular GSH and protein thiols has been observed widely and linked to the biological action of nitric oxide and regulation of cell signaling pathways (Foster et al. 2003). However, in spite the emphasis given to this process as a regulatory mechanism, there is surprisingly little understood about how nitrosothiols are formed from NO. Once one nitrosothiol has formed, it can readily exchange with others, as exemplified by the protein S-thiolation by GSNO. However, NO and GSH do not react directly. Either one or the other must be oxidized. The most commonly cited reaction is between GSH and N_2O_3, which can be formed from the reaction of NO with NO_2. However, calculations by Lancaster (2006, 2008) show that the NO concentrations required for this reaction to be significant (above 20 μM) are unrealistically high for it to be a physiological pathway. In contrast, the reaction between NO and the thiyl radical is fast (Madej et al. 2008), and it can be argued that virtually all *de novo* nitrosothiol formations should occur via this reaction. However, there are caveats here too, as competing reactions will decrease its efficiency. It has been argued that this reaction will compete poorly with hydrogen transfer to the thiyl radical from a carbon centre on the peptide (Hofstetter et al. 2007). In addition, other species that are likely to be present in the systems where thiyl radicals are generated, including the thiolate plus oxygen, are likely to react more efficiently than NO. Therefore, in such situations nitrosation may account for only a small fraction of the thiol modification. Lancaster (2008) has argued further that in the redox signaling field, nitrosothiol formation may be an indicator, but not necessarily the functionally important modification.

Peroxynitrite ($ONOO^-$) is produced in a very fast reaction from superoxide and NO (reaction 2.10). It oxidizes GSH and other thiols both by two electron and radical mechanisms (Ferrer-Sueta and Radi 2009). The direct (two electron) reaction occurs in competition with breakdown of the peroxynitrite, which in physiological media involves reaction with carbon dioxide. This forms an intermediate that decomposes to give carbonate and nitrogen dioxide radicals (reaction 2.11). Both of these react readily with thiols. At high millimolar GSH concentrations, some of the oxidation is likely to be direct, but under most conditions radical-mediated oxidation of GSH and protein thiols should predominate (Quijano et al. 1997). The outcome is typical of any thiyl radical pathway, with disulfides as the major product (reactions 2.12 and 2.13 followed by reactions 2.2 and 2.3). Even when peroxynitrite is generated from NO and superoxide, this is not an efficient mechanism

for generating nitrosothiols. However, the pathway does consume oxygen and at maximum efficiency more superoxide is generated than is used to form the initial peroxynitrite (net reaction 2.14). Therefore, in addition to being a route for the formation of GSSG or protein disulfides, this sequence is a physiological mechanism for generating superoxide at the expense of GSH and NO.

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \tag{2.10}$$

$$ONOO^- + CO_2 \rightarrow \text{intermediate} \rightarrow CO_3^{\bullet -} + NO_2^{\bullet} \tag{2.11}$$

$$CO_3^{\bullet -} + GSH \rightarrow HCO_3^- + GS^{\bullet} \tag{2.12}$$

$$NO_2^{\bullet} + GSH \rightarrow NO_2^- + GS^{\bullet} \tag{2.13}$$

$$NO^{\bullet} + 4GSH + 2O_2 \rightarrow NO_2^- + 2GSSG + H_2O + 2H^+ + O_2^{\bullet -} \tag{2.14}$$

2.6 Interactions Between Glutathione, Ascorbate and Other Radical Scavengers

Although GSH is an effective scavenger in many experimental systems, the question arises as to how efficient this process in relation to other scavenging pathways. Ascorbate is an obvious alternative. It is a better one electron reductant than GSH (Buettner 1993) and scavenges a wide range of radicals including thiyl radicals. Therefore, it could act either by scavenging other radicals directly by intercepting the glutathionyl radical, as shown in Fig. 2.2. The ascorbate radical is relatively stable, does not react with oxygen, and decays primarily by dismutation. Thus scavenging by ascorbate could bypass superoxide production from GSH. Wardman (1995) has considered radical reactions involving ascorbate and GSH from a thermodynamic perspective and calculated that with ascorbate and GSH concentrations in the physiological range, thiyl radicals would preferentially react with ascorbate, with a minor fraction giving rise to superoxide. Sturgeon and co-workers (1998) used a peroxidase system to generate tyrosyl radicals and showed that with 8 mM GSH, both oxygen uptake and thiyl radical formation were inhibited by ascorbate in the 25–100 μM range. It can be concluded from both approaches that ascorbate should dominate over GSH as a radical scavenger under typical intracellular conditions. If this mechanism prevails, there is still synergy between the two antioxidants, as the dehydroascorbate generated can be recycled intracellularly by GSH in a two-electron reduction (Cuddihy et al. 2008). The overall outcome is therefore oxidation of GSH to GSSG.

However, conditions will vary between cell types and within cell compartments, and over time of oxidant exposure as components are consumed, and there are a number of experimental studies in which thiyl radicals have been trapped in cells subjected to free radical stress (for example Kwak et al. 1995; Stoyanovsky

et al. 1996). Therefore, it is reasonable to assume that there will be conditions where scavenging by GSH is important, and that relative concentrations of GSH and ascorbate will influence the extent to which GSH undergoes radical-mediated oxidation with resultant superoxide generation. Furthermore, unless cultured cells are supplemented, they almost invariably contain no ascorbate. Most studies of oxidative stress responses and redox signalling have been carried out with cultured cells, in which scavenging by thiols should be more dominant and redox reactions could be different from normal tissues. How this affects signaling mechanisms needs to be addressed using ascorbate-replete cells.

Synergism is likely to be important for other antioxidant activities. Ascorbate, and to a lesser extent GSH, is able to scavenge vitamin E radicals, thereby transferring radicals generated by peroxidation in the lipid phase to the aqueous phase for disposal (Niki et al. 1982). Synergism may also be relevant to the action of dietary or pharmacological antioxidants such as polyphenolics and carotenoids. Even though these compounds are good radical scavengers, dietary intake is unable to increase tissue concentrations sufficiently to impact on total radical scavenging capacity (Halliwell 2007). One way in which they could act as radical scavenging antioxidants (and it should be noted that this mode of action is not unequivocally established), would be to facilitate radical transfer to more abundant species such as ascorbate or GSH.

2.7 Oxidative Reactions of Thiyl Radicals

$GS^{\bullet}$ is an oxidizing species that can react with hydrogen-donating molecules including NADH, polyunsaturated fatty acids, retinol, and ferricytochrome c and other proteins (Schöneich et al. 1992; Forni and Willson 1986a, b; Nauser et al. 2004; Chatgilialoglu and Ferreri 2005; Borisenko et al. 2004). Thus it can initiate lipid peroxidation, generate trans fatty acids through cis-trans isomerization and cause irreversible protein modification. Cysteine thiyl radicals generated on proteins can also undergo intramolecular hydrogen transfer to form carbon centred radicals that give rise to dehydroalanine (Mozziconacci et al. 2011) and other side chain modifications (as reviewed Schoneich 2008). Most of the experimental studies of these reactions have been studied under anaerobic conditions where the reaction of the radical with the thiolate and oxygen is not possible. However, they illustrate the point that without efficient removal of the thiyl radical by the latter mechanism, radical scavenging by thiols is more likely to be damaging rather than protective.

2.8 Detection of Thiyl Radicals

Detection of any short lived radical species in a biological system is difficult and thiyl radicals are no exception. However, they have been detected directly by electron paramagnetic resonance (EPR) in cell free systems and mechanistic studies also

provide strong evidence for thiyl radical generation. Recent technological advances have made it possible to detect thiyl radicals in cellular systems (Stoyanovsky et al. 2011). For the most part this involves the use of spin traps, such as DMPO (5,5′-dimethyl-1-pyrroline *N*-oxide), which form radical adducts that can be detected not only by EPR but also by chromatographic, mass spectrometric and immunological methods. Although the initial spin-trapped species is a metastable nitroxide radical, this is readily converted to the stable hydroxylamine or nitrone, which in the case of GSH can be quantified in extracts of cells exposed to a radical stress using HPLC (Stoyanovsky et al. 1996; Cuddihy et al. 2008). A major advance introduced by the Mason group was the introduction of immuno-spin trapping (Mason 2004). In this technique, DMPO is used in an experimental system to trap radicals, then an antibody raised against DMPO is applied to detect radical adducts. Western blotting has been used to detect radical formation on proteins such as myoglobin, and immunoprecipitation plus mass spectrometry to identify the protein modified and pinpoint the radical site that reacted with the DMPO (Bonini et al. 2007; Bhattacharjee et al. 2007). Formation of cysteine radicals on nitric oxide synthase and NADH dehydrogenase have been detected using this approach (Chen et al. 2005, 2011) and the method has wider potential for following thiol oxidation in cells.

2.9 Thiyl Radicals and Redox Regulation

The radical scavenging properties of GSH and other thiols are generally considered in relation to the biological antioxidant defense network. As described in this chapter, thiols perform this function efficiently, provided the oxygen/thiolate mechanism can operate to remove the thiyl radical. However, radical scavenging could have a physiological impact beyond antioxidant protection. The field of redox biology has in recent years evolved from having a sole focus on oxidative damage to encompass oxidative stress and redox regulation, and the current view is of a continuum with no distinct boundaries. The same concept can be applied to thiyl radical reactions, with their capacity both to be regulated by cellular conditions and to be regulators of the proteins with which they interact. They are a source of superoxide and hydrogen peroxide, generated as a result of radical scavenging and able to be regulated by factors such as thiol concentration, oxygen and ascorbate. Thiyl radical reactions are also routes to protein disulfides, glutathionylated proteins and nitrosothiols, which are major players in redox regulation. Currently, the main emphasis is on two electron oxidants as transmitters of redox signals. However, based on the above argument, and the knowledge that major enzymatic generators of reactive oxidants such as NADPH oxidases, nitric oxide synthases, peroxidases and mitochondrial oxidoreductases operate by one electron mechanisms, radical mechanisms warrant more attention.

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Chapter 3
Redox Homeostasis

Joris Messens, Nicolas Rouhier, and Jean-François Collet

Abstract Multiple factors, including small sulfur-containing molecules and oxidoreductases, are involved in the control of intracellular redox homeostasis. In this chapter, we first review properties and functions of the small sulfur-containing molecules glutathione, mycothiol, bacillithiol and trypanothione. These low molecular weight thiols, which cycle between a reduced and oxidized form, are present at high intracellular concentrations and function as redox buffers to protect cells against oxidative stress conditions. In the second part of this chapter, we focus on the two oxidoreductases, thioredoxin and glutaredoxin. These enzymes are key players in pathways aimed to reduce disulfide bonds in intracellular proteins and to maintain cellular redox homeostasis. We review the general properties of these enzymes and highlight their significant diversity. Finally, we discuss the recent discovery that monothiol glutaredoxins coordinate an iron sulfur cluster, which suggests a novel link between redox and iron homeostasis.

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3.1 Introduction

The redox environment of cellular compartments is tightly controlled by the complementary action of oxidoreductases and high concentrations of sulfur-containing molecules, such as glutathione, which play the role of redox buffers. We review in this chapter the role and function of the low molecular weight thiols glutathione, mycothiol, bacillithiol and trypanothione in maintaining the intracellular redox state under both physiological and oxidative stress conditions. We also focus on thioredoxins and glutaredoxins, the most important classes of oxidoreductases involved in controlling the cellular redox homeostasis in both prokaryotes and eukaryotes.

3.2 Low Molecular Weight Thiols

Most living organisms contain high concentrations of low molecular weight (LMW) thiols that serve as redox buffers to protect the cells against a variety of reactive chemical species, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive electrophilic species (RES), metalloids, and some antibiotics (Dalle-Donne et al. 2008; Roos and Messens 2011). Glutathione is the most ubiquitous of these LMW thiols but certain microorganisms contain high concentrations of other related compounds, such as bacillithiol, mycothiol and trypanothione.

3.2.1 Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a water-soluble, low molecular weight tripeptide (Fig. 3.1), which is present at millimolar concentrations in nearly all eukaryotic cells and in many bacteria, most of which are Gram-negative. Glutathione is synthesized by the consecutive action of two ATP-dependent enzymes. First, γ-glutamylcysteine synthetase catalyzes the rate-limiting formation of L-γ-glutamylcysteine from glutamic acid and cysteine. Then, glutathione synthetase catalyzes the ligation of L-γ-glutamylcysteine with glycine.

Glutathione cycles between two species, a reduced GSH-form and a disulfide bonded GSSG form. The standard redox potential of the GSH-GSSG couple is −240 mV at pH 7.0 (Meister and Anderson 1983). The reduced form is the most abundant form *in vivo*, and the overall cellular GSH/GSSG ratio ranges from 30:1 to 100:1 (Hwang et al. 1992), which corresponds to a redox potential of approximately −221 to −236 mV (Hwang et al. 1992). The GSH/GSSG ratio is maintained by glutathione reductase, a flavoenzyme that uses the reducing power of NADPH to reduce GSSG back to GSH (Fahey et al. 1978). Noteworthy, the ratio of GSH to GSSG is significantly lower in the secretory pathway, ranging from approximately

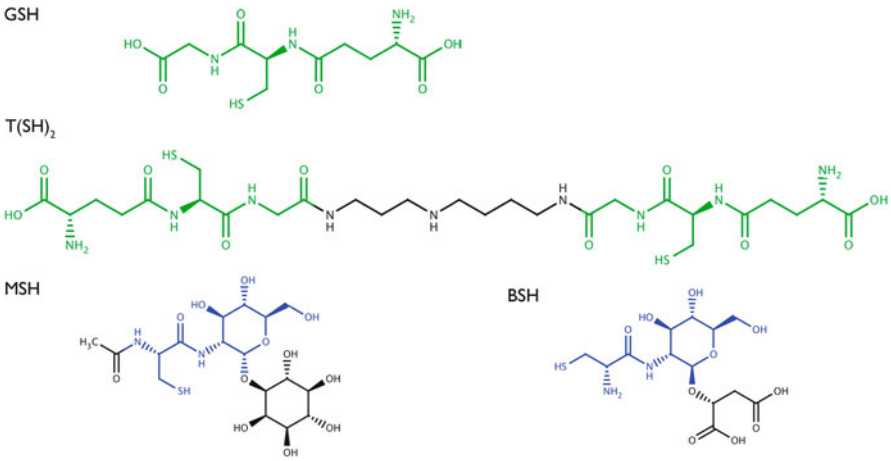

Fig. 3.1 The structure of low molecular weight thiol molecules in their reduced state. Glutathione (GSH): L-γ-glutamyl-L-cysteinylglycine; Dihydrotrypanothione [$T(SH)_2$]: N1,N8-bis(glutathionyl)spermidine; Mycothiol (MSH): N-acetylcysteine amide-linked to 1D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside [GlcN-α(1–1)-Ins] and Bacillithiol (BSH) are shown. Similar chemical motifs are shown in the *same color*. Different chemical motifs are in *black*. Structures were made with MarvinSketch 5.7.1 (ChemAxon)

1:1 to 3:1 (Hwang et al. 1992). This reflects a more oxidizing redox environment in the endoplasmic reticulum, the compartment where disulfide bond formation occurs in eukaryotes [for a review, see Depuydt et al. 2011].

Because of its high concentration and its low redox potential, glutathione, which is essential in eukaryotes but dispensable in bacteria such as *Escherichia coli*, is usually considered as a redox buffer that protects cells against oxidative damages. The thiol moiety of GSH enables this molecule to act as a scavenger reacting with various electrophilic compounds including ROS and RNS. Rate constants at pH 7.4 vary for each oxidant and range between 3×10^7 M^{-1} s^{-1} for NO_2 (Ford et al. 2002), 6.6×10^2 M^{-1} s^{-1} for peroxynitrite (Radi et al. 1991; Koppenol et al. 1992; Zhang et al. 1997; Quijano et al. 1997), 115 M^{-1} s^{-1} for taurine chloramines (Peskin and Winterbourn 2001), 3×10^7 M^{-1} s^{-1} for hypochlorous acid and 1×10^{10} M^{-1} s^{-1} for hydroxyl radicals (Winterbourn and Hampton 2008).

Glutathione is also indirectly involved in peroxide scavenging by participating in the recycling of ascorbate, an electron donor to ascorbate peroxidases (Rouhier et al. 2008), and by supplying some thiol peroxidases, like glutathione peroxidases and peroxiredoxins with reducing equivalents with or without the help of glutaredoxins (Grxs, see below). Glutathione peroxidases and peroxiredoxins are antioxidant enzymes that use a thiol-based chemistry to reduce H_2O_2 and lipid peroxides. However, among the so-called glutathione peroxidases, only the selenocysteine-containing enzymes are truly dependent on glutathione, whereas cysteine-containing enzymes are in fact dependent on thioredoxin (Trx) for their recycling (Navrot et al. 2006). In the peroxiredoxin family, several members have

been shown to be recycled by GSH alone or by the GSH/Grx couple, the first reported example being a poplar type II peroxiredoxin (Rouhier et al. 2001). Finally, some members of the glutathione S-transferase family, as well as some Grxs also exhibit a glutathione-dependent peroxidase activity (see the Chap. 8 by Jacquot et al., in this book).

In addition to its role in scavenging oxidizing molecules, glutathione can also covalently modify cysteine residues in a process called S-glutathionylation, although the cellular levels of glutathionylated proteins in non-stressed cells are low (<0.1% of the total protein cysteines) (Hansen et al. 2009). S-glutathionylation is reversible and is assumed either to protect cysteine residues from irreversible oxidation or to regulate proteins, whose cysteines are essential for activity or folding. In general, S-glutathionylation is reversed by the action of Grxs, which constitute a family of glutathione-dependent oxidoreductases that almost exclusively reduce cysteine glutathione adducts formed on proteins in the course of their catalytic cycle or under oxidative stress conditions (see Sect. 3.3.2 for more details). S-glutathionylation is mediated by mechanisms that can be schematically classified as ROS-dependent or ROS-independent (Ghezzi and Di Simplicio 2007). Although the final product is the same, the rates of the reactions are very different. The ROS-independent process occurs via thiol/disulfide exchange between GSSG and the proteins. However, under physiological conditions, the low concentration of GSSG in the cytosol is unlikely to favor the formation of mixed disulfides with protein thiols in this compartment. Moreover, the thermodynamic barrier limits the oxidation of proteins by GSSG, which occurs via a nucleophilic attack of a protein thiolate ($R\text{-}S^-$) on GSSG: the mixed disulfide formed must thus have a redox potential higher than that of the $[GSH]^2/[GSSG]$ couple, which is theoretically possible, but extremely unlikely (Forman et al. 2010). In contrast, in the endoplasmic reticulum, where the GSH/GSSG ratio is lower, the formation of protein-glutathione mixed disulfides is more likely to occur via the direct reaction of a thiolate with GSSG (Townsend 2007; Forman et al. 2010; Chakravarthi et al. 2006). In ROS-dependent processes, sensitive cysteine residues are first oxidized to a sulfenic acid upon ROS exposure. Sulfenic acids are extremely unstable modifications that can be irreversibly oxidized to sulfinic and sulfonic acids (Roos and Messens 2011). Glutathione reacts with sulfenic acid to form a glutathione-protein mixed disulfide, which effectively protects those cysteines against irreversible oxidation. For example, the α-glutamyl transpeptidase is protected against oxidative damage by S-glutathionylation (Dominici et al. 1999). S-glutathionylation is not only a protective mechanism but can also regulate protein function (Dalle-Donne et al. 2008). For instance, the enzyme α-ketoglutarate dehydrogenase is reversibly inactivated by S-glutathionylation in response to alterations in GSH levels in mitochondria (Nulton-Persson et al. 2003). For more examples, we refer to Mieyal et al. (2008). Altogether, the reversible S-glutathionylation of specific proteins has implications for the regulation of cellular homeostasis in health and disease. For instance, changes in the S-glutathionylation state of specific proteins play important roles in diabetes, cardiovascular, lung and neurodegenerative diseases (Dalle-Donne et al. 2008; Mieyal et al. 2008).

The data summarized above indicate that glutathione is a central player in redox homeostasis. However, depending on the organism, the essential function of glutathione might not always be due to its role in thiol-redox control but rather due to its role in iron homeostasis, as recently revealed by work done in the yeast *Saccharomyces cerevisiae* (Kumar et al. 2011).

3.2.2 Trypanothione

Since its discovery in 1985 (Fairlamb et al. 1985), trypanothione has enjoyed a lot of attention (Fig. 3.1). Trypanothione has not been found in prokaryotes but is present in kinetoplastida, which are primitive eukaryotes that parasitize animals and plants. In these organisms, most of the glutathione content is found in the form of a unique thiol, N1, N8-bis(glutathionyl)spermidine, also known as trypanothione. It is a conjugate of two glutathione molecules with one molecule of spermidine, and is synthesized by the ATP-dependent enzyme trypanothione synthetase (Oza et al. 2002).

Oxidized trypanothione (TS_2) is reduced to dihydrotrypanothione [$T(SH)_2$] by the FAD disulfide oxidoreductase trypanothione reductase [reviewed in Flohe et al. 1999]. Whereas the redox potential of the TS_2-$T(SH)_2$ couple (−242 mV) is similar to that of glutathione (Fairlamb and Cerami 1992), trypanothione is more reactive than glutathione in thiol-disulfide exchange reactions under physiological conditions, due to the lower pK_a value of its thiol group (7.4 compared to 8.7 in glutathione) (Krauth-Siegel et al. 2005).

$T(SH)_2$ functions as the donor of reducing equivalents to several enzymes of the parasite, including thioredoxin (Schmidt and Krauth-Siegel 2003), tryparedoxin (Nogoceke et al. 1997; Ludemann et al. 1998), monothiol glutaredoxin-1 (Filser et al. 2008) and ribonucleotide reductase (Dormeyer et al. 2001). $T(SH)_2$ can also spontaneously reduce protein sulfenic acids in the model protein glyceraldehyde-3-phosphate dehydrogenase (Filser et al. 2008).

Tryparedoxin is a distant relative of the thioredoxin superfamily (see below), which has a WCPPCR active site motif and is substantially larger (16 kDa) than most thioredoxins (Krauth-Siegel and Leroux 2012). As such, tryparedoxin is reduced by $T(SH)_2$ (Nogoceke et al. 1997; Gommel et al. 1997) and can reduce glutathione-protein mixed disulfides (Melchers et al. 2007). Tryparedoxin can also transfer reducing equivalents from $T(SH)_2$ to a variety of protein targets, making the tryparedoxin/$T(SH)_2$ couple the determining factor for the intracellular redox state of the parasite (Reckenfelderbaumer and Krauth-Siegel 2002).

In addition to providing reducing equivalents to oxidoreductases, $T(SH)_2$ can also efficiently scavenge hydrogen peroxide, peroxynitrite and radiation-induced radicals (Thomson et al. 2003; Awad et al. 1992; Carnieri et al. 1993). $T(SH)_2$ maintains the redox homeostasis by passing electrons to peroxidases via intermediate shuttle molecules, which can either be tryparedoxin, ascorbate, or even glutathione [reviewed in (Castro and Tomas 2008)].

3.2.3 Mycothiol

Glutathione was thought to be an essential molecule in all living cells until it was found that many Gram-positive bacteria do not produce this compound (Fahey et al. 1978). In the early 1990s, it was shown that glutathione-lacking bacteria *Mycobacterium bovis* and *Streptomyces clavuligerus* produce an alternative low molecular weight thiol that was given the common name "mycothiol" (Spies and Steenkamp 1994; Newton et al. 1995). Mycothiol is the major thiol in most Actinomycetes and is produced at millimolar levels in *Mycobacteria* and *Streptomycetes*. It is comprised of N-acetylcysteine amide-linked to a 1D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside [GlcN-α(1–1)-Ins] (Fig. 3.1). Mycothiol biosynthesis occurs by linkage of GlcNAc to inositol (Ins), deacetylation to GlcN-Ins, ligation of the latter to L-cysteine, and the transacetylation of the cysteinyl residue by acetyl Coenzyme A (CoASAc) (Newton et al. 2008).

Like glutathione, mycothiol is also present in a reduced (MSH) and a disulfide-bonded (MSSM) state. However, the redox potential of the MSH/MSSM couple is not known, probably due to the limited availability of mycothiol for research purposes. The chemical synthesis of mycothiol with the correct stereochemistry is a challenging task, making bacterial isolation of mycothiol still far more efficient (Ordóñez et al. 2009).

A key property of mycothiol is its high resistance to oxidation by molecular oxygen in the presence of redox metals. For instance, the copper-catalyzed autoxidation of mycothiol is about 30-fold slower than that of cysteine and 7-fold slower than that of glutathione (81), due to the acetyl and GlcN-Ins moieties blocking the amino and carboxyl groups of the cysteine, respectively. In Actinobacteria, mycothiol-disulfide-selective reductase (MTR), a NADPH-dependent flavoenzyme, reduces MSSM back to MSH in order to maintain the intracellular redox homeostasis required for the proper functioning of a variety of biological processes (Rawat and Av-Gay 2007). Interestingly, we showed in *Corynebacterium glutamicum* that mycothiol cannot be replaced by glutathione, which indicates that mycothiol plays a specific role in that organism (Ordóñez et al. 2009). Accordingly, mycoredoxin-1 (Mrx1), the glutaredoxin analog of Actinomycetes, does not function with glutathione but has a strict specificity for mycothiol in a reaction coupled to MTR and NADPH (Ordóñez et al. 2009). These results suggest that glutaredoxins and mycoredoxins have specific binding sites for glutathione and mycothiol, respectively.

Mycothiol serves as a storage form of cysteine in *Mycobacterium smegmatis* (Bzymek et al. 2007) and plays a role in the detoxification of thiol-reactive substances, including formaldehyde, various electrophiles and antibiotics (Newton et al. 2000; Rawat and Av-Gay 2007). Mycothiol S-conjugates derived from electrophiles and antibiotics are then cleaved by mycothiol S-conjugate amidase to release GlcN-Ins, which is used to resynthesize mycothiol, and a mercapturic acid, which is excreted from the cell. Some enzymes depend also on mycothiol for proper functioning, such as formaldehyde dehydrogenase MscR (Misset-Smits et al. 1997), later identified as nitrosomycothiol reductase with a role in the protection

against oxidative stress (Vogt et al. 2003), and maleylpyruvate isomerase (Feng et al. 2006; Wang et al. 2007). Moreover, we have recently found that the activity of *C. glutamicum* arsenate reductases 1 and 2 depends on reducing equivalents transferred from mycothiol by mycoredoxin (Ordóñez et al. 2009). Very recently, the identification of a mycothiol S-transferase has been reported in *Mycobacterium smegmatis* and *M. tuberculosis* (Newton et al. 2011). This enzyme is postulated to help protect the cells against antibiotics, as evidenced by the appearance of mercapturic acids in the fermentation broth of producing cultures (Rawat and Av-Gay 2007).

3.2.4 Bacillithiol

Bacillithiol (BshC) was discovered in *Bacillus subtilis* as a molecule disulfide-linked with OhrR. OhrR is a peroxide sensitive transcription factor, which contains a single cysteine residue required for redox sensing (Lee et al. 2007; Fuangthong and Helmann 2002). A thiol compound with the same mass was independently discovered in extracts of *Bacillus anthracis* after labeling with monobromobimane (Nicely et al. 2007).

Bacillithiol is widely found among low-GC Gram-positive bacteria (Firmicutes) and is also sporadically present in more distantly related bacteria, including *Deinococcus radiodurans* (Helmann 2011). The structure of bacillithiol was determined after purification from this latter bacterium (Nicely et al. 2007). Bacillithiol is structurally similar to mycothiol. The inositol group is replaced by L-malate, which makes bacillithiol an α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid (Fig. 3.1). Bacillithiol biosynthesis requires three enzymes that sequentially couple GlcNAc to malic acid (BshA), deacetylate the GlcNAc-Mal intermediate to generate GlcN-Mal (BshB), and finally couple this latter product to a cysteine to generate bacillithiol. Like mycothiol, bacillithiol is also not available in sufficient amounts to allow biochemical studies; therefore the redox potential of bacillithiol is not known and information on the rate constants of the reaction with ROS and RNS is not available.

By analogy with glutathione and mycothiol, bacillithiol is likely to be a central player in thiol-disulfide homeostasis. It can therefore be anticipated that bacillithiol-containing organisms encode a bacillithiol reductase to mediate the recycling of the oxidized form of bacillithiol as well as oxidoreductases for the reduction of protein-bacillithiol mixed disulfides. The name ‘bacilliredoxin’ (Brx) has been proposed for such proteins (Helmann 2011). A bacillithiol S-transferase responsible for the conjugation of bacillithiol with various substrates has been recently described in *B. subtilis* (Newton et al. 2011). Moreover, mutants lacking bacillithiol are highly sensitive to fosfomycin (Gaballa et al. 2010), probably due to the inability of FosB, a presumed bacillithiol-S-transferase, to detoxify this antibiotic.

As explained above, protein S-thiolation is thought to protect active site cysteine residues of essential enzymes against irreversible oxidation to sulfinic or sulfonic acids. In *B. subtilis* OhrR, it has been shown that S-bacillithiolation of a single cysteine functions as a redox-switch (Lee et al. 2007; Newton et al. 2009). It leads to the inactivation of the OhrR repressor and derepression of *ohrA* transcription. OhrA is a thiol-dependent peroxiredoxin that converts organic peroxides into organic alcohols and protects the cell against NaOCl. In a recent proteomic study of NaOCl-treated cells using shotgun-LC-MS/MS analysis, several other proteins, including two methionine synthase paralogs (MetE and YxjG), the inorganic pyrophosphatase PpaC, the 3-D-phosphoglycerate dehydrogenase SerA and the thiol-disulfide oxidoreductase YphP were found to be S-bacillithionylated (Chi et al. 2011). The latter enzyme YphP might function as a putative bacilliredoxin that reduces the mixed disulfide between bacillithiol and essential cysteines in proteins.

3.3 A Diversity of Oxidoreductases

Although cells have millimolar concentrations of glutathione and alternatives that serve as redox buffers to prevent the formation of unwanted disulfides, they need more efficient reducing systems to catalyze the reduction of disulfide bonds and maintain the cellular redox homeostasis. The major cellular reducing pathways involve thioredoxins and glutaredoxins.

3.3.1 Thioredoxins

Thioredoxins (Trxs) are ubiquitous antioxidant enzymes that were originally discovered in 1964 in the bacterium *Escherichia coli* as an electron donor for ribonucleotide reductase, an enzyme that provides deoxyribonucleotides required for DNA synthesis and repair (Laurent et al. 1964; Moore et al. 1964). Since their discovery, Trxs have been found to be at the heart of numerous fundamental processes in living organisms, ranging from Archaea to mammals. Trxs are present in various cellular compartments, including the cytosol (Arner and Holmgren 2000), nucleus (Hirota et al. 1997, 1999), mitochondria and plastids (Schürmann and Buchanan 2008), and are found to be attached to the cell membrane (Martin and Dean 1991) or secreted to the extracellular environment (Arner 1999; Xu et al. 2008).

Trxs are best known for their catalytic role in reducing disulfide bonds that form in proteins, either in the course of their catalytic cycle or upon exposure to oxidative stress conditions. Therefore, Trxs are part of the antioxidant defense against peroxides and other ROS. However, Trxs also play other roles in the cell. For instance, Trx proteins have been shown to regulate programmed cell death via denitrosylation (Benhar et al. 2008), to act as growth factor (Powis et al. 2000), to

modulate the inflammatory response (Nakamura et al. 2005), to play important roles in the lifecycle of viruses and phages (Holmgren 1989) or to activate antimicrobial peptides (Schroeder et al. 2011).

3.3.1.1 General Properties

Thioredoxins are characterized by a highly conserved structural fold consisting of two motifs (Fig. 3.2a), as described first by Holmgren et al. (1975). The structure of Trx consists of five β-strands surrounded by four α-helices (Holmgren et al. 1975) (Fig. 3.2b). The β-sheets and α-helices can be divided in a N-terminal $\beta_1\alpha_1\beta_2\alpha_2\beta_3$ and a C-terminal $\beta_4\beta_5\alpha_4$ motif connected by the α_3-helix. The β-strands of the N-terminal motif run parallel, and the β-strands of the C-terminal motif run anti-parallel. The α_2 and α_4 helices are located on one side of the central β-sheet while the α_3-helix is located on the opposite side (Fig. 3.2b). The α_3-helix is oriented perpendicularly to helices α_2 and α_4. The catalytic site of Trxs consists of a canonical WCGPC motif that is located on the surface of the protein in a short segment at the N-terminus of the α_2-helix. Many proteins, including glutaredoxins (see below) (Xia et al. 2001) (Fig. 3.2c), protein disulfide isomerases (McCarthy et al. 2000; Tian et al. 2006; Heras et al. 2004), glutathione S-transferases (Reinemer et al. 1991), some thiol-peroxidases (Ren et al. 1997), and the recently characterized protein DsbG (Depuydt et al. 2009; Heras et al. 2004) present a minimal version of the Trx fold. This version, known as the "Trx-fold" (Martin 1995), lacks the ß$_1$-strand and the α_1-helix of Trx.

The WCGPC motif is a landmark feature of Trx proteins. The cysteine residues of this motif are found predominantly reduced *in vivo*, allowing Trxs to break disulfide bonds in oxidized substrate proteins. The reaction can be seen as a transfer of the disulfide bond from the substrate protein to Trx, or as a transfer of electrons from the reduced cysteines of Trx to the substrate protein. The molecular details of the reaction catalyzed by Trx have been the subject of extensive research [see Collet and Messens 2010 for a review]. The reaction starts with a nucleophilic attack of the N-terminal thiol of the WCGPC motif on the disulfide of the target protein, leading to the formation of a mixed disulfide complex between Trx and the target protein. The N-terminal cysteine of the WCGPC motif has a pK_a value of about ∼7 (Dyson et al. 1991; Dillet et al. 1998), which is lower than the pK_a value of free cysteines in solution (∼9). Therefore, a large fraction of this cysteine is present as a thiolate under physiological conditions, enabling this residue to act as a nucleophile. The low pK_a value of the first cysteine residue results from the stabilization of the negative charge of the thiolate anion through the formation of hydrogen bonds between the sulfur atom and neighboring residues. Once a mixed-disulfide has formed between Trx and its substrate, the C-terminal thiol is activated as a thiolate and attacks the mixed-disulfide (Roos et al. 2009). This results in the release of the reduced substrate protein and oxidized Trx. The recycling of Trx to the reduced form is catalyzed by thioredoxin reductase (TrxR) at the expense of NADPH (Lennon et al. 2000), enabling Trx to start another reaction cycle.

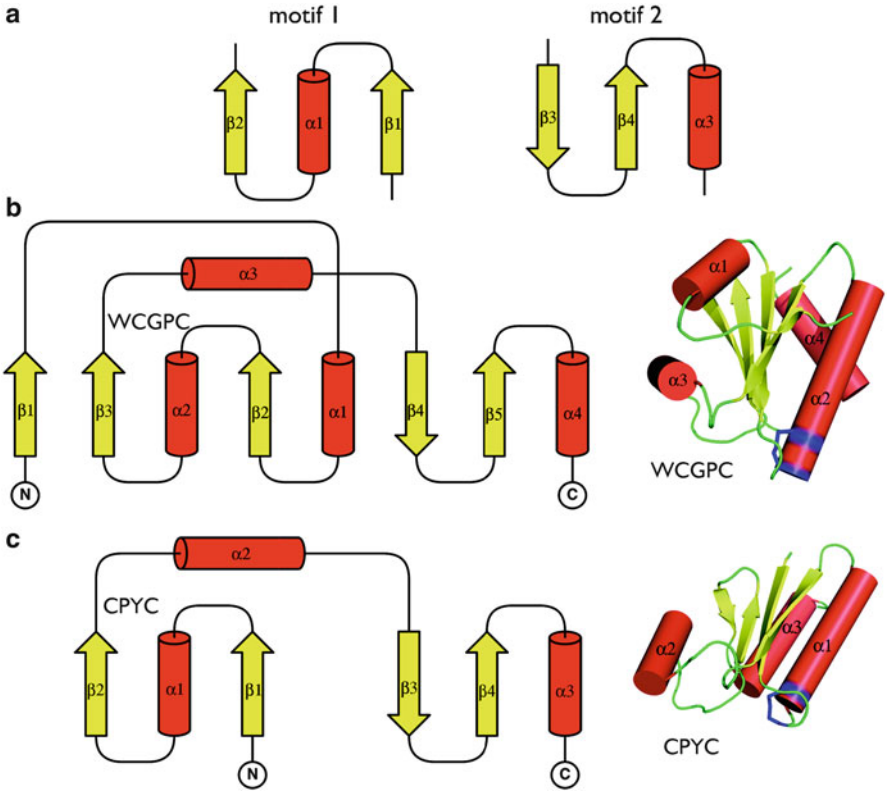

Fig. 3.2 The structures of the Trx-fold proteins, thioredoxin (Trx) and glutaredoxin (Grx). (**a**) The topology of the two motifs that constitute the Trx-fold. (**b**) The secondary and three-dimensional structures (PDB code: 2O7K) (Roos et al. 2007) of *Staphylococcus aureus* Trx are shown. The structure consists of 4 α-helices and a central 5-stranded β-sheet. The conserved WCGPC motif is located at the N-terminus of the α_2-helix. The α-helices are in *red*, the β-strands in *yellow* and the disulfide bond is shown in *blue*. (**c**) The secondary and three-dimensional structures of *E. coli* glutaredoxin are shown (PDB code: 1EGO) (Xia et al. 1992). Glutaredoxins have a Trx-fold consisting of two motifs connected by the α_2-helix. The conserved active site CXXC motif is always located at the same position, at the N-terminal site of an α-helix. The figure was generated using TopDraw from CCP4 suite and MacPyMol (Delano Scientific LLC 2006). An adapted version of the figure was reprinted with the permission of (Collet and Messens 2010)

3.3.1.2 Diversity and Roles of Thioredoxins

Escherichia coli Trx1 (Ec_Trx1), the first identified Trx, is usually considered as the prototype of the thioredoxin superfamily of which members have been identified in most genomes. Here, we briefly review the most prominent Trx proteins to highlight the diversity within this ubiquitous family.

Bacterial Thioredoxins

Ec_Trx1 is the most reducing protein present in the cytoplasm of *E. coli*. Its redox potential value has been estimated to be −270 mV (Krause and Holmgren 1991) but a more recent study suggests that it might be even lower ($E^{o'} = -284$ mV) (Cheng et al. 2007). Ec_Trx1 catalyzes the reduction of several cytoplasmic enzymes that form a disulfide bond in their catalytic cycle, including ribonucleotide reductase, methionine sulfoxide reductases (MsrA, MsrB and fRMsr) and 3-phosphoadenosine 5′-phosphosulfate (PAPS) reductase (Holmgren and Bjornstedt 1995). Ec_Trx1 also provides reducing equivalents to DsbD, an inner membrane protein, which transfers electrons across the membrane and delivers them to periplasmic oxidoreductases (Rietsch et al. 1997). Moreover, Ec_Trx1 is required for the growth of several bacteriophages including T7, M13 and f1 (Russel 1991). For this latter function, which does not require the catalytic cysteine residues, Ec_Trx1 binds to viral DNA polymerase, to mediate the interaction between DNA polymerase, DNA and other replication proteins (Ghosh et al. 2008; Hamdan et al. 2005).

The *E. coli* cytoplasm contains a second thioredoxin, Ec_Trx2 (Miranda-Vizuete et al. 1997). Ec_Trx2, which shares only 28% sequence identity with Ec_Trx1, has a redox potential of −221 mV (El Hajjaji et al. 2009), and is therefore a significantly less reducing enzyme than Ec_Trx1. Like Ec_Trx1, Ec_Trx2 is able to reduce ribonucleotide reductase, DsbD and PAPS reductase. However, Ec_Trx2 has two striking characteristics that distinguish it from Ec_Trx1 and suggest that this protein may have a specific function in *E. coli*. First, the expression of Ec_Trx2 is controlled by OxyR, a transcription factor that controls the response to oxidative stress (Ritz et al. 2000). Second, Ec_Trx2 contains an additional N-terminal domain of 32 amino acids that harbors two CXXC motifs. We found that these additional cysteine residues bind Zn^{2+} with an extremely high affinity (10^{18} M^{-1}) (Collet et al. 2003). The zinc-binding CXXC motifs of Ec_Trx2 are conserved in several other bacterial Trxs, making Ec_Trx2 the prototype of a new zinc-binding Trx family. Noteworthy, we recently showed that the zinc center of Ec_Trx2 fine-tunes its redox and thermodynamic properties (El Hajjaji et al. 2009).

Yeast and Mammalian Thioredoxins

In the budding yeast *Saccharomyces cerevisiae,* there are two cytosolic (Sc_Trx1, Sc_Trx2) and one mitochondrial (Sc_Trx3) thioredoxin (Herrero et al. 2008). A double mutant lacking both *sc_trx1* and *sc_trx2* is viable but exhibits a more severe phenotype than the single mutants, indicating that Sc_Trx1 and Sc_Trx2 are only partially redundant. For instance, mutants lacking both Trxs have a longer S-phase in their cell cycle due to an inefficient reduction of ribonucleotide reductase, are auxotrophic for sulfur amino acids (Muller 1991) due to their inability to reduce PAPS reductase, and are unable to use methionine sulfoxide as a source of organic sulfur due to their inability to reduce methionine sulfoxide reductases.

The function of the mitochondrial Trx, Sc_Trx3, is less defined than that of its cytosolic counterparts. This protein seems to play a role in the defense mechanisms against ROS generated in mitochondria, as suggested by the fact that a mutant lacking Sc_Trx3 is hypersensitive to hydroperoxide (Pedrajas et al. 1999).

Mammalian cells possess two Trx isoforms, ma_Trx1 (~12 kDa) and ma_Trx2 (~18 kDa), which are present in the cytosol and mitochondria, respectively. The ma_Trx1 has also been detected in the nucleus of certain normal and tumor cells, even though it does not have a nuclear localization sequence (Maruyama et al. 1999; Hirota et al. 1997). In addition to the catalytic cysteines present in the CGPC motif, ma_Trx1 contains three extra cysteine residues (Qin et al. 1994). In the human protein, two of these cysteines (C62 and C69) flank the α_3-helix that links both motifs of the Trx-fold (Fig. 3.1a). The third additional cysteine (C73) is located in a turn close to the CGPC active site motif. Several reports have shown that these extra cysteines are involved in regulating the function of Trx via post-translational modifications, such as glutathionylation and S-nitrosylation (Casagrande et al. 2002; Kuster et al. 2006). For instance, C73 has been shown to be *S*-nitrosylated after treating the human protein with S-nitrosoglutathione (Mitchell et al. 2005). This S-nitrosothiol can be transferred from C73 to caspase 3 *in vitro* (Mitchell and Marletta 2005) and *in vivo* (Mitchell et al. 2007). Moreover, under oxidizing conditions, a disulfide can be formed between C69 and C72 (Watson et al. 2003). The formation of this disulfide is predicted to have a profound effect on the structure of Trx and to decrease the rate by which the active site is regenerated by TrxR.

Plant Thioredoxins

The Trx family is particularly important in plants. Plant Trxs have been primarily found to regulate enzymes involved in carbon metabolism but, as suggested by the identification of putative target proteins by proteomic studies, are likely also involved in many other cellular processes, such as photorespiration, lipid metabolism, membrane transport, hormone metabolism, and ATP synthesis (Balmer et al. 2003, 2004; Schürmann and Buchanan 2008). Moreover, plant Trxs also play an important role in sustaining early seedling growth of germinating cereal seeds (Wong et al. 2004).

Plants possess the largest group of Trxs found in all organisms. For instance, recent genomic analyses identified more than 40 Trx genes in higher plants, taking into account atypical variants with modified active sites and multidomain proteins with at least one Trx domain, such as nucleoredoxins (Chibani et al. 2009). The fact that only 28 out of the 46 Trxs found in *Populus trichocarpa* contain the typical WCGPC active site motif illustrates the diversity of the plant Trx family (Chibani et al. 2009).

Plant Trxs with CGPC active site are distributed into nine classes: Trx f, h, m, o, x, y, z, tetratricopeptide domain-containing thioredoxins (TDX) and nucleoredoxins. They are found in several subcellular compartments such as the cytoplasm, the mitochondria and the chloroplasts. Whereas TrxR reduces

mitochondrial and cytoplasmic Trxs, Trxs present in chloroplasts are recycled by a ferredoxin-thioredoxin reductase (FTR) [reviewed in Gelhaye et al. 2005]. FTR is almost exclusively found in photosynthetic organisms and uses the reducing power provided by light to reduce Trxs. Although plant Trxs adopt the Trx-fold, some of them present specific characteristics that differentiate them from classical Trxs. For instance, the presence of an additional cysteine residue at position four in poplar Trx *h4* prevents the recycling by TrxR but renders the enzyme glutathione- and Grx-dependent (Koh et al. 2008). In addition, some other Trxs harboring an unusual CXXS active site motif are also recycled by glutathione (Gelhaye et al. 2003).

3.3.2 Glutaredoxins

3.3.2.1 General Properties

Glutaredoxins were first discovered in *Escherichia coli,* when Holmgren identified *E. coli* Grx1 as an alternative electron donor for ribonucleotide reductase in cells lacking Trx1 (Holmgren 1976). Grxs are glutathione-dependent disulfide oxidoreductases that catalyze a variety of thiol-disulfide exchange reactions, including the reduction of protein disulfides, as well as protein glutathionylation and deglutathionylation (Lillig et al. 2008; Mieyal et al. 2008; Gallogly et al. 2009). Grxs are usually small, heat-stable proteins that adopt a typical Trx-fold despite a low sequence homology with Trxs (Eklund et al. 1984; Martin 1995) (Fig. 3.2c). In Grxs, the solvent exposed CXXC active site motif is often replaced by a CXXS motif where the C-terminal active site cysteine is substituted by a Ser [for an overview, see Gallogly et al. 2009; Couturier et al. 2009].

When dithiol Grxs function as general protein disulfide oxidoreductases, they become oxidized upon reducing the protein disulfide, in a way similar to Trx. They are then converted back to the reduced state by glutathione (GSH), which forms a mixed-disulfide with the first cysteine of the catalytic cycle, which is then resolved by a second GSH molecule, releasing reduced Grx and GSSG (Fig. 3.3). As explained above, the intracellular GSH/GSSG ratio is maintained by glutathione reductase at the expense of NADPH. Albeit some Grxs, such as *E. coli* Grx1, catalyze the reduction of protein disulfide bonds, Grxs seem to function mostly as monothiol oxidoreductases for protein deglutathionylation reactions (Fernandes and Holmgren 2004). Under physiological conditions, the thiolate of the active site nucleophilic cysteine attacks the disulfide bond of a S-glutathionylated protein, releasing the protein thiol in the reduced form while becoming itself glutathionylated (Fig. 3.3). Then, GSH attacks the glutathionylated sulfur as explained above, releasing reduced Grx and GSSG. Alternatively, some Grxs were found to be either uniquely regenerated by TrxR or by both, a glutathione- or TrxR-dependent system (Johansson et al. 2004; Zaffagnini et al. 2008).

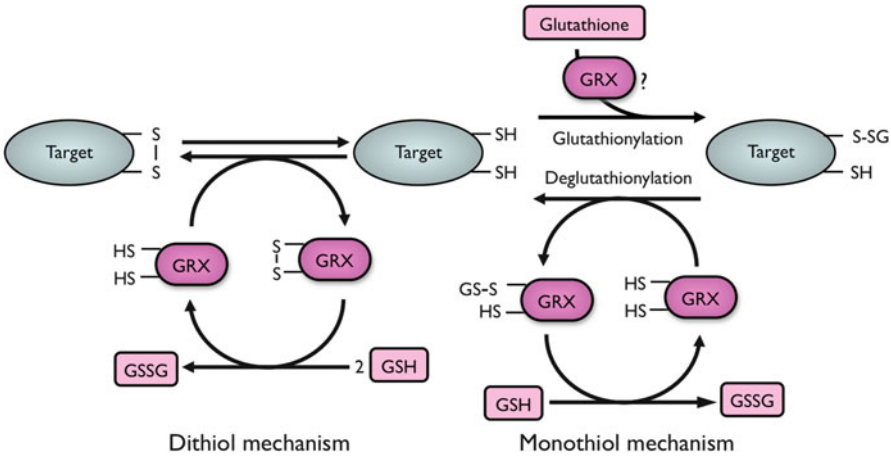

Fig. 3.3 Catalytic mechanisms employed by glutaredoxins. Grxs can reduce both disulfide bonds and protein-glutathione adducts by using either a dithiol or a monothiol mechanism. In the dithiol mechanism, the two cysteines of the active site are required and generally form an intramolecular disulfide bond, which is then reduced by GSH. This is similar to the reduction mechanism used by Trxs, except the latter are reduced by thioredoxin reductases instead of GSH. In the monothiol mechanism, only the catalytic cysteine (*first cysteine of the active site*) is required to reduce the protein-glutathione adducts. The Grx is glutathionylated in the course of the reaction, and is subsequently regenerated by a GSH molecule. Almost no information is available about the glutathionylation process *in vivo,* but it has been proposed that Grxs could also catalyze the glutathionylation of proteins

3.3.2.2 Diversity and Roles of Glutaredoxins

Grxs have been identified in most living organisms, including viruses, bacteria, plants and mammals. For instance, there are four Grxs in *E. coli* (Ec_Grx1 to 4), seven Grxs in *S. cerevisiae* (Sc_Grx1 to 7) and four in human (Hs_Grx1, Grx2, Grx3 and Grx5). Initially, Grxs were classified into a dithiol (with a CPY/FC catalytic motif) and a monothiol (with a CGFS catalytic motif) subgroup based on the active site sequence. However, this classification has now been further refined into six classes, considering the existence of either plant- or bacterial-specific isoforms or of multimodular isoforms containing additional domains (Couturier et al. 2009). The dithiol subgroup is now referred to as class I Grxs while the monothiol Grxs are included into class II. However, several monothiol Grxs with CPYS or CSYS active site motifs also belong to class I (Couturier et al. 2009).

Bacterial Glutaredoxins: The Example of *E. coli* Grxs

Four Grxs (Ec_Grx1, Ec_Grx2, Ec_Grx3 and Ec_Grx4) have been identified in *E. coli*. As stated previously, Ec_Grx1, which is about ten times less abundant than

Ec_Trx1, has been shown to catalyze the reduction of disulfide bonds in various cytoplasmic enzymes. For instance, Ec_Grx1 is an alternate electron donor for PAPS reductase and methionine sulfoxide reductases (Fernandes and Holmgren 2004). Moreover, Ec_Grx1 specifically reduces the disulfide bond that inactivates the transcription factor OxyR (see Sect. 3.2) under oxidative stress conditions (Aslund et al. 1999). As the gene that codes for Ec_Grx1 is induced by OxyR, the OxyR response to oxidative stress is therefore autoregulated [reviewed in Antelmann and Helmann 2011].

Much less is known about the other three *E. coli* glutaredoxins, particularly Ec_Grx3, which exhibits 5% of the activity of Ec_Grx1 with ribonucleotide reductase, and lacks activity with PAPS reductase. The *in vivo* substrates of this protein are unknown. With a molecular mass of about 24 kDa, Ec_Grx2 is unique among *E. coli* Grxs, which typically range in molecular weights from 9 to 14 kDa. Although Ec_Grx2 cannot reduce PAPS reductase or ribonucleotide reductase, this protein, which is up-regulated in stationary phase, is very active in catalyzing deglutathionylation. This is highlighted by the high catalytic activity of the enzyme towards mixed-disulfides between glutathione and artificial or physiological substrates (Vlamis-Gardikas 2008). Ec_Grx4 is the only *E. coli* Grx that belongs to the class of monothiol Grxs. Like other monothiol Grxs, Ec_Grx4 has been shown to bind an iron sulfur cluster (see Sect. 4.3) (Iwema et al. 2009). Its function remains obscure.

Eukaryotic Glutaredoxins: The Example of Plant Grxs

As for the Trx family, comparative genomic analyses revealed the presence of expanded Grx families in higher plants (from 27 to 35 genes), compared to non-photosynthetic organisms, which contain only a limited number of these genes (Couturier et al. 2009). Besides their roles associated with their capacity to bind and transfer Fe-S clusters, which is described in the next section, the established functions for plant Grxs are likely related to their capacity to reduce glutathionylated substrates. Several members from three out of the four Grx classes existing in land plants have been characterized so far. Grxs from class I have been divided into five subclasses (GrxC1, C2, C3, C4 and C5/S12), whereas class II is sub-divided into four subclasses (GrxS14, S15, S16, S17). Because the number of class III Grxs is variable between species (from 13 to 24 isoforms), a precise classification has not yet been achieved (Couturier et al. 2009). In general, if we exclude the targeting sequence, class I and III Grxs have a molecular weight comprised between 10 and 13 kDa. In contrast, the size of class II Grxs ranges from *ca* 12 kDa for GrxS14 and S15 to 53 kDa for GrxS17. The latter is composed of an N-terminal Trx-like domain fused to three Grx domains.

Owing to the large number of Grxs, deciphering the functions of plant Grxs is a daunting task. The present knowledge indicates that Grxs are important for the response of plants to oxidative stress. As already indicated, class I Grxs participate in the regeneration of specific subgroups of antioxidant enzymes, including type

II peroxiredoxins and methionine sulfoxide reductases of the B1 type (Rouhier et al. 2001; Tarrago et al. 2009). These two types of enzymes use a single redox active cysteine that is successively sulfenylated and glutathionylated during the catalytic cycle. Grxs thus contribute to the recycling of the active form by reducing the glutathione adduct. Among class I Grxs, knock-out mutants have only been described for GrxC1 and C2. Whereas the single mutants did not exhibit any phenotype, the double mutant was lethal at an early stage after pollinization. This probably indicates that GrxC1 and GrxC2 have redundant functions, at least for some early developmental stages (Riondet et al. 2011).

Among class II Grxs, the study of knock-out, knock-down or overexpressing lines indicated that seedlings of *A. thaliana* knock-out mutants for GrxS14 (otherwise named AtGrxcp) exhibited a higher sensitivity to oxidative stress treatment, whereas overexpression of the respective ortholog from the arsenic hyperaccumulator *Pteris vittata* increased arsenic resistance (Cheng et al. 2006; Sundaram et al. 2009). Quite similarly, silencing of tomato GrxS16 led to increased sensitivity to oxidative, salt and drought stresses while over-expression had opposite effects (Guo et al. 2010). Arabidopsis mutant plants, in which GrxS17 is disrupted, displayed several phenotypes under high temperature, including defects in proliferation and/or cell cycle control, accumulation of ROS, cellular membrane damages and altered auxin perception. These phenotypes highlight the important role of this protein for temperature-dependent postembryonic growth (Cheng et al. 2011). Last but not least, several studies have implied class III Grxs in developmental or stress response processes, via the regulation of transcription factors of the TGA family (La Camera et al. 2011; Laporte et al. 2011; Ndamukong et al. 2007; Xing and Zachgo 2008).

3.3.2.3 Glutaredoxins and Iron Homeostasis

While Grxs have been identified in the 1970s as an alternative reducing system to Trxs, it has only recently been recognized that several monothiol or dithiol Grxs are able to bind [2Fe-2S] cluster (Fig. 3.4) (Lillig et al. 2005; Rouhier et al. 2007; Bandyopadhyay et al. 2008). The [2Fe-2S] center, which is ligated by the catalytic cysteines of two Grx monomers and two glutathione molecules, can be efficiently transferred to acceptor proteins, as demonstrated for a plant plastidial Grx. Related to this observation, a role for *S. cerevisiae* Grx5, a protein with a CGFS active site, has been suggested as a carrier protein in Fe-S cluster biogenesis in yeast. In a *S. cerevisiae grx5* null mutant, deficient cluster assembly for the mitochondrial Fe-S enzymes aconitase and succinate dehydrogenase has been reported (Rodriguez-Manzaneque et al. 2002; Muhlenhoff et al. 2003). Furthermore, deletion of the zebrafish mitochondrial *grx5*, which also coordinates an iron sulfur cluster, causes hypochromic anemia. As such, the mitochondrial Fe-S cluster assembly machinery is required for heme biosynthesis (Wingert et al. 2005). Moreover, most prokaryotic or eukaryotic Grx orthologs were able to complement the zebrafish *grx5* mutant, which suggests that this function has been conserved throughout evolution (Molina-Navarro et al. 2006).

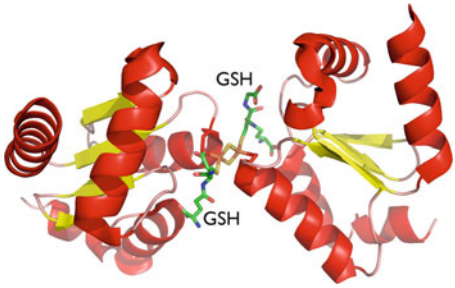

Fig. 3.4 Three-dimensional structure of the dimeric *A. thaliana* GrxC5 bridging a [2Fe-2S] cluster. The α-helices are shown in *red*, and β-strands are shown in *yellow*. The [2Fe-2S] center in *A. thaliana* GrxC5 is bridged by the catalytic cysteine of each monomer (PDB code 3RHC) (Couturier et al. 2011) and by two GSH molecules shown in stick representation. The figure was generated using MacPyMol

Some Fe-S containing Grxs function as redox sensors (Lillig et al. 2005; Rouhier et al. 2007; Couturier et al. 2011). *In vitro,* the [2Fe-2S] clusters on both poplar GrxC1 and human Grx2 are stabilized by GSH and destabilized by GSSG. Therefore, changes in the cellular GSH redox state could serve as a balance to promote or inhibit Grx activity. However, determining the exact ratio between the holo- and apo-protein *in vivo* is technically very challenging and has never been performed, which makes it difficult to differentiate the reductase activity from the capacity to bind Fe-S clusters.

Multidomain monothiol glutaredoxins formed by a Trx-like domain fused to one to three Grx domains have also a function in the nucleus where they are involved in intracellular iron trafficking. In *S. cerevisiae* and *Schizosaccharomyces pombe*, the nucleocytoplasmic class II Grxs (Grx3 and Grx4) regulate iron homeostasis by modifying the function of several transcription factors (Pujol-Carrion et al. 2006; Ojeda et al. 2006; Mercier and Labbe 2009; Jbel et al. 2011). Recently, Hoffman et al. showed that *S. cerevisiae* Grx4 functions as an iron sensor (Hoffmann et al. 2011). The C-terminal domain of this protein binds to the iron-sensing transcription factor Aft1p, whereas its N-terminal Trx domain was found to be essential *in vivo*. Furthermore, Mühlenhoff et al. found that Grx3 and Grx4 have an essential function in intracellular iron trafficking and sensing (Muhlenhoff et al. 2010). Depletion of *grx3* and *grx4* in *S. cerevisiae* specifically impaired all iron-requiring reactions in the cytosol, mitochondria, and nucleus, including the synthesis of Fe-S clusters, heme, and di-iron centers. From all these observations, we can conclude that these Grxs function in the intracellular iron trafficking and sensing through the bridging of a glutathione-containing iron sulfur center.

This class of Grxs forms also a complex with two other proteins, FRA1 and FRA2 (Fe repressor of activation-1 and 2), corresponding to aminopeptidase P- and BolA-like proteins, respectively (Kumanovics et al. 2008). The exact function of the two latter proteins in this complex is not yet clear, since BolA, in particular,

was initially described as a protein affecting the morphology and elongation of *E. coli* cells (Aldea et al. 1988). The whole complex is an intermediate that links the mitochondrial Fe-S cluster assembly machinery to the transcription of the iron regulon, which is under the control of the iron-sensing transcription factor Aft1p. The biochemical and spectroscopic characterization of the Grx-BolA heterodimeric complex allowed Li *et al*. to identify three out of the four iron binding sites, which are constituted by the active site cysteine of Grx3 or Grx4, glutathione and a histidine residue from BolA (Li et al. 2009, 2011). The fact that an Fe-S containing Grx homodimer is converted into a Grx-BolA heterodimer upon addition of BolA *in vitro* supports the proposal that BolA could act as an adaptor protein changing the function of Grxs from an Fe-S scaffold/delivery protein to an iron sensor. Based on the strong co-occurrence of *grx* and *bolA* genes and their frequent association in bacterial genomes, it is likely that this interaction is conserved between kingdoms (Couturier et al. 2009; Rouhier et al. 2010). However, the absence of Grx3/4 orthologues with an N-terminal Trx domain in bacteria together with the absence of Aft1p orthologues in non-yeast organisms suggest a diversity of iron sensing mechanisms and transcription factors that might be involved.

3.4 Conclusions

The redox biochemical and biological fields are emerging with the identification of several LMW thiols that function as redox buffers and a variety of oxidoreductases, mostly from the Trx superfamily. However, many cellular mechanisms are still not known. Moreover, further work is required to explore how redox regulation affects complex cellular processes, and to fully characterize the role and function of various Grxs and Trxs involved in redox pathways. How redox and iron homeostasis are interconnected also requires further investigation. To advance our knowledge in redox biochemistry, we need to monitor the redox state and to quantify cellular thiol/disulfide redox buffers *in vivo*. New methods need to be developed to individually quantify both symmetrical and mixed disulfide populations in the correct biological context. Furthermore, for the more recently discovered LMW sulfur-containing compounds, many new targets and mechanisms are waiting to be discovered. Unraveling the role of these sulfur molecules in thiol redox regulation pathways and in the interplay between cytosolic thiol redox status and intra/extracellular stimuli will be one of the challenges for the next decade.

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Chapter 4
Sulfenic Acids and Peroxiredoxins in Oxidant Defense and Signaling

Leslie B. Poole, Kimberly J. Nelson, and P. Andrew Karplus

Abstract In recent years, there has been tremendous growth in awareness of the importance of sulfenic acids to biological processes. This is in part related to reactive oxygen species in general and hydrogen peroxide in particular becoming widely recognized as intermediates or second messengers crucial to signaling processes that guide cell growth and development. Thus a picture is emerging of sulfenic acids being important both for defending against oxidative stress and for normal growth. Although the work is challenging due to the fleeting and localized nature of some of the intermediates involved, substantial progress has been made in identifying modified proteins and the roles they play. The landscape of modifications uncovered thus far suggests that we have barely scratched the surface of the many stress-induced and regulatory oxidation events taking place in cells on a regular basis. This chapter recounts the chemistry of sulfenic acids and their effects in modulating function in a few recently-studied proteins. We also discuss cysteine-based peroxidases in more depth, with a focus on the structural and biochemical features of the peroxiredoxin family.

Keywords Redox signaling • Peroxiredoxins • Sulfenic acids • Oxidative stress defenses • Redox regulation

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Abbreviations

ROS	reactive oxygen species
Prx	Peroxiredoxin
Orp1	oxidant resistance protein
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PTP	protein tyrosine phosphatase
SHP	Src homology 2 domain-containing phosphatase
DEP1	density-enhanced phosphatase-1
PTEN	phosphatase and tension homolog
HEK	human embryonic kidney
VEGF	vascular endothelial growth factor
EGF	epidermal growth factor
VEGFR	VEGF receptor
PDGF	platelet-derived growth factor
MEF	mouse embryonic fibroblasts
PKGIα	cGMP-dependent protein kinase
Ohr	organic hydroperoxide resistance protein
C_p	peroxidatic cysteine
C_r	resolving cysteine
Trx	thioredoxin
Grx	glutaredoxin
FF	fully folded
LU	locally unfolded
AhpC	alkyl hydroperoxide reductase C protein
TrxR	thioredoxin reductase
BCP	bacterioferritin comigratory protein
Yap1	yeast activator protein 1
Pap1	activator protein-1 from *Schizosaccharomyces pombe*
Tpx1	thiol peroxidase 1
Nrf-2	NF-E2-related factor
ARE	antioxidant response element
Keap1	Kelch like ECH-associated protein 1
CDK1	cyclin dependent kinase 1

4.1 Introduction and Scope

Reactive oxygen species (ROS) are produced in aerobic organisms by many avenues including as a result of incomplete oxygen reduction by the mitochondrial electron transport chain, as a result of exposure to certain environmental toxins and chemotherapeutics, and even purposefully as part of an immune response or of

signaling pathways. It has long been recognized that cellular proteins that remove these damaging species play a critical role in preventing or minimizing mutagenesis and in delaying carcinogenesis and aging (Greenberg and Demple 1988; Imlay 2008; Stadtman 2006). The peroxiredoxin (Prx) family of cysteine-based peroxidases is now thought to be the system responsible for removing the majority of intracellular hydrogen peroxide and other alkyl hydroperoxides (Winterbourn 2008; Adimora et al. 2010). The functions of these enzymes undoubtedly extend well beyond defense, however. It has more recently become apparent that ROS, and hydrogen peroxide in particular, are also produced during normal cell growth by NADPH oxidase enzymes as a part of receptor-mediated signaling pathways that lead to proliferation, differentiation and immune responses (Murphy et al. 2011). Activated cysteine thiol groups (SH) in proteins can be oxidized by peroxide to first form cysteine sulfenic acid (SOH) and, in many cases, then go on to form other reversible species (e.g., disulfides, sulfenamides) or irreversible species (e.g., sulfinic and sulfonic acids). Functionally, SOH has most clearly been established as a catalytic intermediate in cysteine-dependent antioxidant enzymes like Prxs and methionine sulfoxide reductases; however, it has also been shown to serve a role in regulating a range of other transcription factors and signaling proteins (Poole et al. 2004; Paulsen and Carroll 2010; Klomsiri et al. 2011). Here we describe the chemical attributes and summarize the biological roles for sulfenic acids as widespread posttranslational modifications in cellular proteins. We also describe in more detail structural and biochemical characteristics of the peroxiredoxin family as an elegant example of mechanisms by which cysteine oxidation can be used both catalytically and to regulate protein function.

4.2 Cysteine, a Special Amino Acid

A major site of oxidant reactivity that underlies cellular defenses and oxidant-sensitive switches in proteins is the thiol, or sulfhydryl group, of cysteine. This residue has very special physicochemical properties that are responsible for its high prevalence within protein functional sites in spite of its overall low abundance within proteins compared with most other amino acids (Marino and Gladyshev 2010a). These properties include a nominal pK_a value around 8.5 that allows cysteine to function as a nucleophile or an acid/base catalyst, redox properties that bring additional catalytic versatility to enzymes as well as the possibility of forming disulfide bonds that can stabilize proteins or lock in a specific conformation of a protein. Moreover, bioinformatic evidence suggests that the high tendency of this residue to be functional has led to an evolutionary selection against its presence on protein surfaces, leading to the overly simplistic interpretation that, because of its high propensity to be buried in protein structures, this amino acid residue is considerably hydrophobic in nature (Marino and Gladyshev 2010a). Only the very low abundance “21st” amino acid selenocysteine can be functionally interchanged

with cysteine in proteins; this feature has been the basis for a bioinformatic approach to identify putative redox-active cysteine residues within the sequences of divergent proteins (Fomenko et al. 2007).

An important chemical product of the oxidation of cysteine thiols is the sulfenic acid, formed when the nucleophilic thiolate anion attacks the terminal oxygen atom of hydroperoxides like hydrogen peroxide, lipid hydroperoxides and peroxynitrite, releasing the alcohol (or water) product. Compared to the rate of reaction with an unactivated thiol or thiolate-containing molecule like glutathione, this chemistry is highly accelerated in the active sites of cysteine-based peroxidases like peroxiredoxins (Parsonage et al. 2005) and NADH peroxidases (Parsonage et al. 1993), and in the oxidant-sensor sites of transcriptional regulators like OxyR (Åslund et al. 1999) and OhrR (Lee et al. 2007). The enhanced reactivity of these proteins toward peroxides reveals the large influence that protein microenvironment can have on the reactivity of particular cysteine residues, supporting second order reaction rates as high as 10^7–10^8 M^{-1} s^{-1}, far higher than the 10–20 M^{-1} s^{-1} expected for small molecule thiolates (Parsonage et al. 2005; Winterbourn 2008; Manta et al. 2009; Hall et al. 2010). The nucleophilic nature of activated cysteine residues also renders them susceptible to alkylation by electrophiles, S-nitrosation, and other types of modifications. A series of bioinformatic analyses have been developed with the goal of identifying features which activate or fine tune the oxidant or electrophile reactivity of given cysteinyl residues; however, this task has been difficult due to the limited number of known redox-regulated proteins and the range of structures and functions exhibited by the proteins reportedly exhibiting sensitivity *in vivo* or *in vitro* (Dennehy et al. 2006; Greco et al. 2006; Salsbury et al. 2008; Sanchez et al. 2008; Codreanu et al. 2009; Marino and Gladyshev 2010b). Early indications based on a functional site profiling and electrostatic analysis of ~50 sites known to be modified to sulfenic acid are that (1) the solvent exposure of modifiable cysteines is not different from the average cysteine, (2) charged residues are underrepresented in the structure near these modifiable sites, and (3) threonine and other polar residues appear to exert a large influence on the cysteine pK_a, thus playing a role in promoting the reactivity of these cysteinyl residues toward oxidants (Salsbury et al. 2008). In the case of the highly peroxide-reactive active site of the widespread and diverse Prx family of proteins, structural and computational analyses have been more informative regarding the active site features which promote peroxide reactivity of these proteins, described in more detail below.

4.3 The Fate of Sulfenic Acids in Proteins

Sulfenic acids are reactive species in solution and in many proteins. In some proteins, however, they are quite stable (e.g., in the cysteine-based peroxidases NADH peroxidase and human PrxVI), and this must be ascribed to features of the protein microenvironment including the presence of stabilizing hydrogen-bonding partners and the lack of nearby thiol groups (Choi et al. 1998; Claiborne

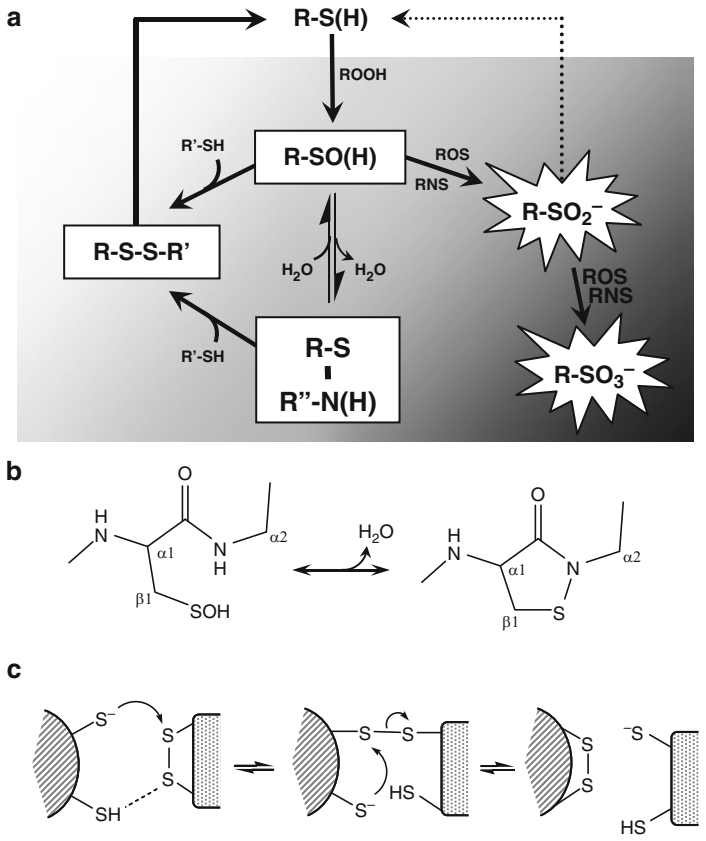

Fig. 4.1 Major redox forms of oxidized cysteinyl residues. (**a**) Sulfenic acids are formed by reactions of cysteine thiolates with oxidants, and particularly hydroperoxides (ROOH). In proteins, sulfenic acids can condense with thiol groups to form intramolecular, intermolecular or mixed disulfides (*left*), undergo a reversible condensation with a proximal amine or amide nitrogen to form a sulfenamide (*middle*), or become further oxidized to sulfinic and perhaps sulfonic acids (*right*). Species in the *middle* and *left* are readily reversible by cellular reductants back to the thiol state. Sulfonic acids are biologically irreversible, as are sulfinic acids, except in the case of the unique repair pathway catalyzed by sulfiredoxin that exists to recover hyperoxidized peroxiredoxins (*dotted line*). (**b**) A cyclic sulfenamide can be formed within protein tyrosine phosphatases like PTP1B through condensation of the cysteine sulfenic acid (*showing* α_1 *and* β_1 *carbons*) with the neighboring main-chain nitrogen of the next residue, serine in PTP1B (side chain attached to the α_2 carbon not shown). (**c**) Thiol-disulfide interchange reactions transfer electrons between dithiol/disulfide centers, effectively transferring the disulfide bond; this mechanism is used by common physiological reductases like thioredoxin and glutaredoxin

et al. 2001). Proximity of a protein sulfenic acid to thiol groups from small molecules, from other proteins, or from other parts of the same protein leads to disulfide bond formation (with loss of one water molecule). This may be the most common fate of cellular protein sulfenic acids, at least when exposed to relatively low levels of ROS where much of the oxidation is reversible (Fig. 4.1). Recent

recognition that a sulfenamide (also known as sulfenyl-amide) can form between an amine or amide nitrogen through a condensation reaction with the sulfenic acid in some proteins has highlighted this reversibly oxidized species as another way in which reversible, signaling-related protein oxidation may be sustained (Fig. 4.1a, b). Sulfenamides have been observed in *in vitro* experiments in several proteins (Lee et al. 2007; Salmeen et al. 2003; van Montfort et al. 2003; Yang et al. 2007). The cyclic sulfenamide formed at the active site of protein tyrosine phosphatase 1B (PTP1B) (Fig. 4.1b) has been observed crystallographically and proof of its occurrence and importance *in vivo* has recently been demonstrated using endogenously produced, conformation-specific, single chain antibodies (known as intrabodies) (Haque et al. 2011).

Disulfide or sulfenamide linkages (or those of related, further oxidized forms) have the potential to serve as cross-links between proteins within a complex, and/or to cause or stabilize large conformational changes within the oxidized proteins. These changes may enhance protein stability or make the downstream product(s) of the initial sulfenic acid more accessible to reductants. Disulfide bonds may also readily migrate to other sites containing free thiols through thiol-disulfide interchange reactions, the same type of thiol-disulfide reactivity by which dithiol-containing reductants reductively recycle oxidized proteins (Fig. 4.1c). Thus, any subsequently-generated disulfide bond(s), as downstream products of sulfenic acid formation, may be quite distant from the initial site where the sulfenic acid is formed, including being located on a different protein. This type of disulfide exchange occurs in *Saccharomyces cerevisiae*, for example, during the transfer of oxidizing equivalents from peroxides to Yap1, a stress-activated transcriptional regulator, which occurs via a sulfenic acid initially generated on a reactive cysteine within the Gpx3 glutathione peroxidase homologue Orp1 (i.e., oxidant resistance protein 1) (Delaunay et al. 2002).

Further oxidation of a sulfenic acid as is promoted by high levels of oxidants first generates the sulfinic acid ($R\text{-}SO_2^-$), and then subsequently the sulfonic acid ($R\text{-}SO_3^-$). Nearly all such "hyperoxidized" cysteine residues in proteins are irreversibly damaged in that the thiol group cannot be regenerated in biological systems. The exception to this is in the sulfinic acid-containing Prxs, where a specialized repair protein known as sulfiredoxin can catalyze the ATP-dependent regeneration of the Prx active site thiol (discussed further below) (Biteau et al. 2003; Jönsson et al. 2008b).

In vivo, the fate of sulfenic acids generated in cellular proteins will be highly dependent on the nature and levels of oxidants and reductants within the environment of the protein, as well as the proximity of the sulfenic acid to potential reactive groups. For many proteins that are rapidly oxidized by peroxide, the lifetime of the sulfenic acid that is formed is governed by how fast it forms a disulfide or becomes reduced by a relevant protein (e.g., thioredoxin or glutaredoxin) or small molecule (e.g., glutathione or cysteine) reductant. This allows for the RSOH lifetime to be tuned for functional purposes, with for example, oxidized forms of certain transcriptional regulators purposefully experiencing rather slow turnover so

as to optimize the oxidation signal that gives rise to transcriptional activation or derepression (Åslund et al. 1999; Lee et al. 2007; Parsonage et al. 2005; Horta et al. 2010; Reeves et al. 2011).

4.4 Approaches to Assess Cysteine Oxidation Within Proteins

The most straightforward assessment of protein oxidation products can be accomplished when the modification is not susceptible to reductant treatment and has little or no propensity to migrate to other locations during the analysis. Thus, the irreversibly oxidized sulfinic and sulfonic acids, as well as sulfinamide (R-S(O)-NH-R′) and sulfonamide (R-S(O_2)-NH-R′) products that are oxidized beyond the sulfenamide state, can be identified by mass spectrometry and in some cases by peptide mapping techniques (Griffiths et al. 2002; Raftery et al. 2001; Shetty and Neubert 2009; Shetty et al. 2007). Sulfinic and sulfonic acid products at the active sites of Prx and PTP1B proteins have also been characterized crystallographically (Sarma et al. 2005; Schröder et al. 2000; van Montfort et al. 2003), and hyperoxidized forms of mammalian Prxs can be recognized by redox state-sensitive antibodies that do not cross-react with the thiol-containing protein (Woo et al. 2003). Hyperoxidation of Prxs is also often detected by a change in the protein's isoelectric point (due to the more acidic pK_a of the hyperoxidized forms) that causes a shifted mobility in two-dimensional gel electrophoresis (Mitsumoto et al. 2001; Rabilloud et al. 2002; Yang et al. 2002). For human Prx I, the hyperoxidized form is separable from the thiol or sulfenic acid containing forms by reverse-phase HPLC, as was used to quantitatively assess sulfiredoxin activity (Jönsson et al. 2008a). As a point of caution, however, these hyperoxidized protein sites may in some cases be generated from sulfenic acids that become exposed and air oxidized during sample workup and/or analysis (Ellis and Poole 1997a; Fuangthong and Helmann 2002; Shetty et al. 2007). For this reason, chemical trapping of sulfenic acids as early as possible in the analytical protocol used is recommended to minimize such artifacts (Ellis and Poole 1997a; Klomsiri et al. 2010).

In pure proteins amenable to NMR analyses, sulfinic and sulfonic acids can in principle be distinguished from thiols and sulfenic acids using ^{13}C-NMR, although the sulfinic and sulfonic acids are expected to be quite similar to one another in $^{13}C^{\beta}$ chemical shift (Crane et al. 1997). Such analyses were applied to assess the redox states of cysteine within various forms of NADH peroxidase from *Enterococcus faecalis*.

In pure proteins of interest, disulfide-bonded cysteine residues may be accurately assessed as long as great caution is taken to rapidly alkylate free thiols prior to and during denaturation to prevent disulfide "scrambling," which is more of a problem in alkaline pH conditions (Hansen and Winther 2009; Chouchani et al. 2011). Traditional peptide mapping with Edman degradation or MS analyses to

sequence peptides, particularly in conjunction with comparisons between oxidized and reduced forms, can often be used to pinpoint the location of the disulfide bond of interest (Baker and Poole 2003; Poole 1996). Methods for detecting sulfenic acids in pure proteins have been more of a challenge to develop, particularly for proteins where this modification is not verifiable by crystallographic or NMR approaches. Because of the transient nature of this species, particularly when proteins are denatured, chemical trapping approaches have been developed that rely on either the nucleophilic or electrophilic properties of this species. The electrophilic compound NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) was found to be highly useful in this regard. While this reagent reacts with both thiols and sulfenic acids, the products of these two are readily distinguished by their spectral signatures (with thiol- and sulfenic acid-generated adducts giving absorbance maxima of 420 and 347 nm, respectively), and by the higher mass (by 16 atomic mass units) of the sulfenic acid-derived product (Ellis and Poole 1997a; Poole and Ellis 2002). While this approach has been useful for identifying sulfenic acids in a number of systems (Kettenhofen and Wood 2010; Poole 2008), the nucleophilic compound dimedone (5,5-dimethyl-1,3-cyclohexanedione), which forms a stable thioether bond with cysteine residues containing sulfenic acids, has found wider application. This reagent was used in early experiments with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), taking advantage of the ^{14}C-labeled dimedone that was available at the time; incorporation of the radiolabel was observed with the oxidized, but not reduced, form of the enzyme (Benitez and Allison 1974). As radiolabeled dimedone is no longer commercially available, most subsequent studies using dimedone have relied on the increase in mass (by 140 Da) observed upon covalent attachment of dimedone to proteins (Ellis and Poole 1997a; Kettenhofen and Wood 2010; Willett and Copley 1996). In the past several years, anti-dimedone antibodies have also become available, allowing for yet another way to detect incorporation of this compound into proteins (Seo and Carroll 2009).

For the most part, the methods described above are quite useful when assessing cysteine oxidation within pure proteins, but not for more complex mixtures of proteins as are typically encountered in proteomic experiments. Most strategies for detecting thiol modifications across large proteomes are designed to detect reversible thiol oxidation in general, not distinguishing between disulfides, sulfenic acids and S-nitrosothiols, for example (Leichert and Jakob 2006; Ying et al. 2007; Chouchani et al. 2011; Victor et al. 2012). Recent approaches have, however, been developed to specifically label and detect sulfenic acid formation across proteomes using conjugated derivatives based on dimedone that possess fluorescent or affinity labels (Poole et al. 2005, 2007; Charles et al. 2007; Leonard and Carroll 2011). A major advantage of specifically targeting sulfenic acid is that it is the direct protein product of cysteine modification by peroxides so that one can be confident that the site where the oxidation chemistry is initiated is revealed; this question is more difficult to resolve when subsequent products like disulfide bonds are analyzed. These approaches are beginning to reveal a large and growing list of oxidizable proteins that are important to redox regulation and oxidative stress across a wide range of biological systems.

4.5 Cysteine Sulfenic Acids as Catalytic and Regulatory Components of Enzymes and Transcriptional Regulators

4.5.1 Regulatory Sulfenic Acids in Cellular Proteins

The clearest examples of signaling-related sulfenic acid generation are in the above-mentioned bacterial transcriptional regulators OhrR and OxyR, which sense the presence of peroxides through their reactive cysteine residues, transducing the signal directly to modulate transcriptional activity. Both *Escherichia coli* OxyR and *Xanthomonas campestris* OhrR form intramolecular disulfide bonds upon oxidation; in OxyR, the "locking in" of the significant, oxidation-induced conformational change by disulfide bond formation has been referred to as "fold editing," altering the protein's structural and functional properties (Choi et al. 2001; Panmanee et al. 2006). Interestingly, *Bacillus subtilis* OhrR only has the one cysteine residue that forms sulfenic acid upon exposure to organic peroxides. Without a disulfide-bonding partner within the protein, this sensor site forms either a sulfenamide intermediate or a mixed disulfide with a low molecular weight thiol such as cysteine or the recently-discovered bacillithiol, promoting derepression and suppressing hyperoxidation, which would interfere with reductive recycling of this transcription factor (Lee et al. 2007; Soonsanga et al. 2008; Newton et al. 2009).

Protein sulfenic acids as regulatory features of thiol-dependent enzymes have been well established for many years, particularly through the work of Allison and co-workers, which demonstrated the oxidant sensitivity of the protease papain and the metabolic enzyme GAPDH (Allison 1976). Like papain, another related cysteine protease, cathepsin K, showed evidence of sulfenic acid formation after treatment with S-nitrosoglutathione, suggesting a role for this inhibitory modification in NO-mediated bone resorption (Percival et al. 1999). Cathepsins B and L were also shown to lose 50–75% of their activity after treatment with low levels (10 μM) of H_2O_2 (Headlam et al. 2006). As mentioned above, protein tyrosine phosphatases like PTP1B and PTPα, possess redox-sensitive cysteinyl residues at their active sites (Yang et al. 2007; Tonks 2005); *in vivo* sulfenic acid formation at the active sites of other cysteine-dependent phosphatases like SHP1 and SHP2, DEP1 and PTEN has also been observed after treatment of T-cells with antigen (Michalek et al. 2007), endothelial cells with vascular endothelial growth factor (VEGF) (Oshikawa et al. 2010), and HEK 293 cells with tumor necrosis factor α, respectively (Michalek et al. 2007; Oshikawa et al. 2010; Nelson et al. 2010). Some of these same phosphatases were also shown to be oxidized *in vivo* to sulfenic acid by EGF treatment of A431 cells (Paulsen et al. 2011). Enzymes which use cysteine at the active site to defend against cellular oxidants are also prone to oxidative regulation, and these are discussed in the next section.

Reactive cysteine residues adjacent to or even remote from the active site or binding site have also been shown to modulate the function of a wide variety of signaling-relevant proteins through sulfenic acid formation. Like with

phosphorylation, whether oxidation is activating or inhibitory varies with the protein, the site of the modification, and potentially the nature of the downstream oxidation product(s). In the absence of a localized PrxII to remove nearby H_2O_2, the murine VEGF receptor 2 (VEGFR2) is subject to oxidative inhibition of its tyrosine kinase activity through the oxidation of Cys1206 near the C-terminus which forms an intramolecular disulfide with Cys1199 (Kang et al. 2011). In contrast, direct oxidation of epidermal growth factor receptor (EGFR) at Cys797 by H_2O_2 elicited by EGF treatment of A431 cells enhances its tyrosine kinase activity (Paulsen et al. 2011). Another important sulfenic acid modification target of VEGF-induced ROS also appears to be the scaffolding protein IQGAP, which is oxidized preferentially at the leading edge of actively migrating cells (Kaplan et al. 2011). As IQGAP plays a pivotal role in regulating cytoskeletal elements and facilitating localized ROS production, this localized modification potentially serves to promote directional endothelial cell migration. Increasingly, non-receptor kinases are also being identified which undergo oxidative regulation *in vivo* in ways that modulate their activities (reviewed in Cross and Templeton 2006; Klomsiri et al. 2011; Poole and Nelson 2008). A recent study demonstrated that sulfenic acid and disulfide bond generation through platelet derived growth factor (PDGF)-induced ROS occurs in and inhibits serine-threonine protein kinase Akt2 but not Akt1, leading to changes in glucose uptake (Wani et al. 2011b). In a separate study, Akt2 knockout MEFs expressing recombinant Akt2 proteins (wild type and an oxidation insensitive mutant) were also used to demonstrate a role for Akt2 oxidation in promoting cell migration (Wani et al. 2011a). As an example of oxidative activation, disulfide bond formation through initial sulfenic acid formation in cGMP-dependent protein kinase (PKGIα) upon perfusion of rat hearts with H_2O_2 led to intersubunit disulfide bond formation, enzyme relocalization, and an increase in kinase activity of this protein (Burgoyne et al. 2007). Src is another very important nonreceptor, membrane-associated tyrosine kinase which is activated by oxidation at two critical cysteine residues which may form an intrasubunit disulfide bond, although direct evidence for the latter is still lacking; interestingly, *in vitro* oxidation at a different site in this and other Src-family kinases is inhibitory, suggesting that the redox regulation of this class of enzymes may be quite complex (Giannoni et al. 2010). As more methods become available to reveal sites of SOH formation, the list of proteins susceptible to sulfenic acid formation will continue to grow and more specific roles for oxidation to SOH in cellular processes will undoubtedly be established.

4.5.2 Sulfenic Acids in Catalysis and Regulation of Oxidative Defense Enzymes, Focusing on Prxs

As examples of sulfenic acid-generating proteins, cysteine-based peroxidases have been much studied, in the 1980s and 1990s with the NADH peroxidase from lactic acid bacteria (Crane et al. 1997; Poole and Claiborne 1989; Yeh et al. 1996)

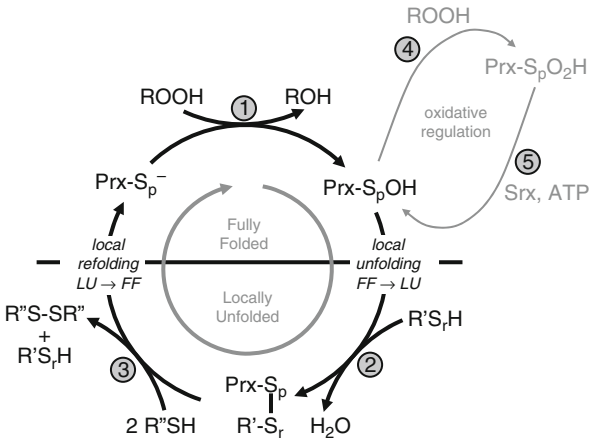

Fig. 4.2 Prx catalytic cycle. The first step (1) of the Prx catalytic cycle is conserved across all Prxs and involves the thiolate form of the C_p (S_p^-) reacting with peroxide to form cysteine sulfenic acid (S_pOH). In step 2, the sulfenic acid is resolved through the formation of a disulfide with a resolving thiol (S_rH) from the same subunit, from another Prx subunit, or from an exogenous molecule. In step 3, the Prx is recycled back to the reduced form through a disulfide exchange reaction with Trx or a similar protein or domain. Local unfolding occurs in the C_p-loop (and in 2-Cys Prxs in the region of the protein including C_r) during step 2 so that the disulfide bond can form. Overoxidation of the C_p (step 4) and reduction of the Cys-S_pO_2H by sulfiredoxin (Srx; step 5) depict redox regulation and repair occurring in eukaryotic sensitive 2-Cys Prxs (Adapted from Hall et al. 2009a, b)

and more recently with the widespread Prx proteins (Ellis and Poole 1997a, b; Baker and Poole 2003; Hugo et al. 2009). In these proteins, as well as Ohr (a bacterial organic hydroperoxide resistance enzyme with no structural relationship to Prxs) and non-selenium glutathione peroxidase homologues (Lesniak et al. 2002; Flohé et al. 2011), the peroxidatic cysteine (the C_p) is oxidized to the sulfenic acid by the hydroperoxide substrate, then forms a disulfide bond through condensation with a "resolving" cysteine (the C_r) that is either also present in the peroxidase or provided by another protein or small molecule (Fig. 4.2). The return of the oxidized protein and C_p to the activated thiol state typically involves a thiol-containing reductant like thioredoxin (Trx). Prxs are good models for cysteine chemistry because, with the possible exception of sulfenamides, all of the examples described above of cysteine chemical states (including S-glutathionylated and S-nitrosylated species) are observed in these proteins (Fang et al. 2007; Chae et al. 2012). It may be no coincidence that one group of Prxs (the Prx1/AhpC group) would not be able to form the type of cyclic sulfenamide observed in PTP1B because the residue following the C_p is almost always a proline (99.5% of the members identified from the 2008 release of GenBank) (Nelson et al. 2011).

Any sulfenic acid generated in an active site during catalysis may also impart oxidant sensitivity to that protein through the potential to form the typically

irreversible sulfinic and sulfonic acids through further reactions with oxidants. Findings alluded to above showed that some eukaryotic Prxs are indeed sensitive to this type of inactivation, whereas other eukaryotic Prxs and most bacterial Prxs are much less sensitive (Hall et al. 2009a; Wood et al. 2003). The fact that an ATP-dependent repair system (the sulfiredoxins) has evolved to reduce and restore the activity of these easily hyperoxidized Prxs highlights the importance of this redox switch in biology. The nature of when and where Prx hyperoxidation plays an important role is less clear, but it may be critical to the ability of H_2O_2 to serve as a transiently-generated chemical signal or second messenger within cells. One proposal for how this might work is the "floodgate hypothesis" (Hall et al. 2009a; Wood et al. 2003). It suggests that Prxs are generally reduced and abundantly present to rapidly detoxify peroxides when the cell is in a "resting" state, but that during an oxidative signaling event, H_2O_2, superoxide and/or lipid hydroperoxide is locally generated, and the high concentrations at the site of generation lead to inactivation of the nearby Prx molecules before reductants are able to reduce them. This would turn off flux through these enzymes and, in a positive feedback loop, contribute to heightened oxidant concentrations. The built-up oxidants would in turn have the effect of promoting the oxidation of other proteins in the vicinity that would normally not be able to compete with reactive peroxidases. In this way the oxidation signal could influence susceptible sites in a wide range of signaling-relevant proteins. An alternative, but not mutually exclusive, model has also been suggested by the propensity of hyperoxidized Prx proteins in yeast to form large aggregates with chaperone activity; in these organisms, they act like heat shock proteins in helping to ameliorate protein damage due to heat stress (Jang et al. 2004). Such chaperone activity may carry over to higher organisms as well, although this has been a difficult question to address (Moon et al. 2005; Kim et al. 2009; Park et al. 2011). Oxidized Prxs may also behave similarly to the Orp1 protein described above, transmitting the original oxidation signal from the peroxide to other interacting proteins through a series of thiol-disulfide exchange interactions (D'Autreaux and Toledano 2007; Hall et al. 2009a).

The cellular conditions under which Prx hyperoxidation has a significant impact on defense and/or signaling are still being investigated. Direct exposure of cells to low, chronic levels of H_2O_2 can be quite informative in this regard (Phalen et al. 2006; Yang et al. 2002). Recent evidence shows that in mammalian cells, sensitive Prxs are hyperoxidized at even very low levels of H_2O_2 (<10 μM) (Baek et al. 2012). These low H_2O_2 levels were not toxic to wild-type cells, but were toxic to cells that were missing sulfiredoxin, verifying that the sulfiredoxin-catalyzed repair process is of critical importance to maintaining the redox homeostasis under these conditions. In a similar vein, studies in yeast showed that normal aging results in an accumulation of the hyperoxidized form of the Prx Tsa1, but that sulfiredoxin expression was upregulated by caloric restriction and blocked that accumulation so as to maintain an effective oxidant defense system (Molin et al. 2011). Strikingly, the addition of an extra copy of the sulfiredoxin gene mimicked the life-extending effects of caloric restriction.

4.6 Active Site Features and Oligomeric Forms Impacting Catalytic Function and Oxidant Hypersensitivity in Prxs

4.6.1 Roles for Conserved Residues in the Specificity and High Catalytic Efficiency of Prxs Toward Hydroperoxide Substrates

All Prxs share a highly conserved active site structure with a nearly universal PXXXTXXC_p sequence and a conserved Arg that is distant in sequence but nearby in the three-dimensional fold. These peroxidases have diverged from a common ancestor of the Trx-fold superfamily that includes Trxs and glutaredoxins (Grxs); comparisons of sequences and structures indicate that the TXXC active site in Prxs directly corresponds to the CXXC active sites in Trxs and Grxs (Fomenko and Gladyshev 2003; Copley et al. 2004). Interestingly, the conserved Arg of the Prxs corresponds in position to a conserved *cis*-Pro of the Trxs and Grxs which, in these proteins, is particularly important for accommodating large protein substrates in the active sites (Copley et al. 2004).

Using a standardized nomenclature for the secondary structural elements (α-helices and β-strands) of Prxs (Hall et al. 2011), the central 5-stranded beta sheet with an initial beta hairpin has two helices on the "back" side (α_1 and α_4) and three on the "front" where the active site is situated (at the N-terminus of helix α_2) (Fig. 4.3). This arrangement of secondary structural elements has been described as a cradle, where the C_p-containing central α_2 helix (the "baby") rests upon the β-sheet mattress, flanked by α_3 and α_5 helices that make up the sides of the cradle (Hall et al. 2009c, 2011). This structural arrangement provides stability to the α_2 helix and accommodates the large, though localized, conformational changes which take place during the course of catalysis – conformational changes which vary between Prxs from different subfamilies (see below). These conformational changes are necessary to accomplish the multi-step catalytic cycle of Prxs. At the beginning of the catalytic cycle, the enzyme is in its "fully folded" (FF) state with the deprotonated C_p (thiolate) prepared to encounter the peroxide substrate in the specialized environment of the FF Prx active site (Fig. 4.2). After the oxidation of C_p, the sulfenic acid must flip out of the protected pocket of the active site to reach and react with the thiol of the C_r, from wherever it comes. This requires an unfolding of the C_p-containing loop and helix to generate the "locally unfolded" (LU) state. While details vary somewhat with the different Prxs (Hall et al. 2009c, 2011; Perkins et al. 2012), approximately one turn of the α_2 helix is unraveled, and a disulfide bond is formed between C_p and C_r, locking in the LU state. Reductants can then, in principle, attack either sulfur of the disulfide bond to finish the recycling process. However, several studies focused on the Prx1/AhpC group that includes four of the six human Prxs have demonstrated that the physiological reductants attack at the C_r sulfur in these proteins (Budde et al. 2003; Jönsson et al. 2007). The enzyme is reactivated with the refolding of the segment with the regenerated C_p thiol to form the active site in the FF state (Fig. 4.2).

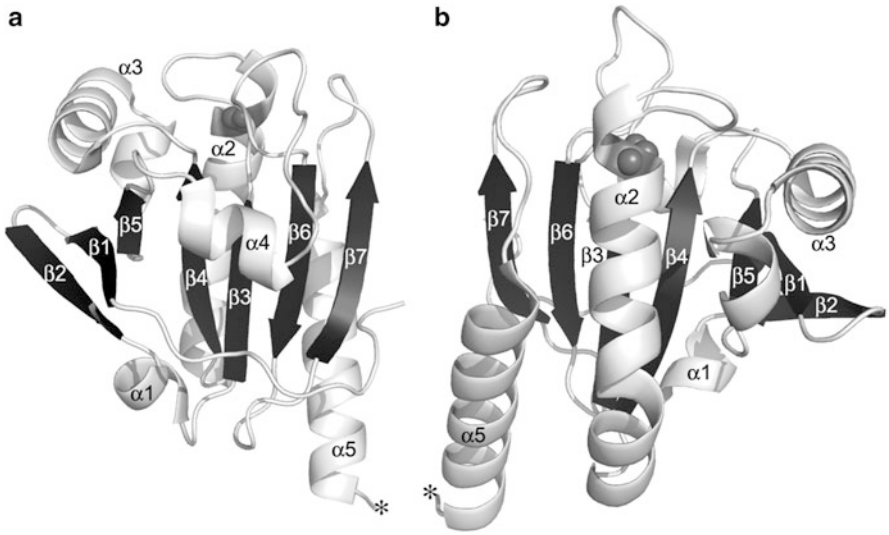

Fig. 4.3 Core secondary structure of Prx proteins. (**a**) Shown is the back view of a representative monomer of a fully folded (FF) Prx (*Salmonella typhimurium* AhpC; PDB entry 1N8J) with the conserved structural elements labeled and β-sheets shown in *black*. The C_p is located in the first turn of helix α_2 and is shown as *spheres*. An *asterisk* represents the position of attachment of the rest of the C-terminal tail that has been hidden in this view (normally absent in other subfamilies); the C-terminus of Prx1 and Prx6 subfamily members extends from here to make extensive interactions with an adjacent monomer. (**b**) The same monomer viewed from the front side of the cradle (related to A by a ∼180° rotation around a vertical axis). This and other molecule model figures were prepared using Pymol (DeLano 2002)

Because the thiolate is a much stronger nucleophile than the protonated thiol group, one of the widely recognized features of reactive cysteine residues, including those at the active site of Prxs, is their lowered pK_a relative to that of an unperturbed cysteine ($pK_a \sim 8.5$). For Prxs, pK_a values have been measured by several approaches. A simple one is to take absorbance measurements at 240 nm over a pH range, with the deprotonation event being measureable due to the higher extinction coefficient of the thiolate (Benesch et al. 1955; Kortemme et al. 1996; Roberts et al. 2005). An alternative approach is to measure the pH dependence of catalysis in competition with heme-containing peroxidases; this has the advantage of yielding a function-relevant pK_a that likely specifically reflects the C_p protonation in the FF state (Nelson et al. 2008; Ogusucu et al. 2007). Direct determination of the pH dependence of alkylation rates was not particularly useful, at least for bacterial AhpC, because the C_p in the FF active site is apparently unable to react with iodoacetamide or its derivatives; this means that the pK_a value measured would reflect the C_p in the LU form of the protein (Nelson et al. 2008). Published pK_a values for the C_p residue in the FF state of Prxs have ranged from 5.2 to 6.3 (Ogusucu et al. 2007; Trujillo et al. 2007; Nelson et al. 2008; Hugo et al. 2009; Manta et al. 2009; Horta et al. 2010; Reeves et al. 2011). Using the average pK_a

value of 5.75, about 94% of the C_p residues would be deprotonated at pH 7 as compared with only ~3% if the thiol pK_a were unperturbed. It should be noted, however, that the nucleophilicity of the thiolate decreases as the pK_a is lowered, so it would be a disadvantage to decrease the C_p's pK_a much below the 5.75 value.

During catalysis by Prxs, the two oxygen atoms of the hydroperoxide substrate move apart, with the bond between them ultimately breaking to form the hydroxide/alkoxide and the C_p-sulfenic acid products. The reaction is a classic in-line S_N2 attack by the thiolate nucleophile on the terminal oxygen of ROOH (called O_A here) that weakens the peroxyl -O-O- bond and generates a planar intermediate around O_A, followed by inversion of stereochemistry, bond breakage, and departure of the leaving group. Many of the various ligands found bound to Prxs in crystal structures in fact possess one or two oxygen atoms bound in a way which could mimic the peroxyl oxygens of the substrates or products (Hall et al. 2010). Analysis of these structures, as well as one Michaelis complex with H_2O_2 (PDB identifier 3a2v) and structures with water molecules bound in the active site, together paint a picture of the catalytic process. Interestingly, with all of the structures taken together, the positions of the oxygen atoms from the various structures are all found within the active site distributed along a narrow track (Fig. 4.4a). We interpret these positions as representing a thermodynamic well that stabilizes the positions occupied by substrate oxygens during the bond breaking process. In this way, catalysis can simplistically be viewed as the sliding of the terminal oxygen (O_A) along the S-O_A-O_B track, away from O_B and toward the attacking C_p sulfur, ultimately transferring O_A to the sulfur and releasing the alkoxide (or hydroxide) product (Fig. 4.4b). Close inspection of the hydrogen bonding interactions around the active site indicates that some of the interaction geometries will be stronger with the O_A located partway between O_B and the C_p sulfur, as would be consistent with preferential binding of the enzyme to the transition state (Hall et al. 2010). Such evidence for transition state stabilization fits well with the concept that enzymes accelerate chemical reactions by lowering the activation energy through such preferential interactions with the high-energy transition state, as originally proposed many years ago by Linus Pauling (1946).

Dissection of the hydrogen-bonding and electrostatic interactions around the active site also clarifies specific roles for the active site side chains and backbone interactions, as well as the α_2 helix, not only in activating the C_p sulfur, but also in positioning and activating the incoming peroxide substrate (Fig. 4.4b; Hall et al. 2010). An independent mechanistic analysis of substrate recognition and thiolate activation within the Prx active site offered similar conclusions in this regard, although some aspects of the proposed interactions were different (Ferrer-Sueta et al. 2011). Together, these analyses support the following specific roles for the conserved Prx residues in promoting catalysis: (1) the threonine hydroxyl (on rare occasion substituted by a serine hydroxyl) positions and activates the protonated O_A atom of the incoming peroxide substrate; (2) the arginine positions and interacts with both the active site thiolate and the peroxide substrate through both charge and hydrogen-bonding interactions; (3) two backbone NH groups from the first turn of the α_2 helix help position and activate the peroxide O_A and O_B atoms; and (4) the proline shields the active site and positions the backbone of the next two residues

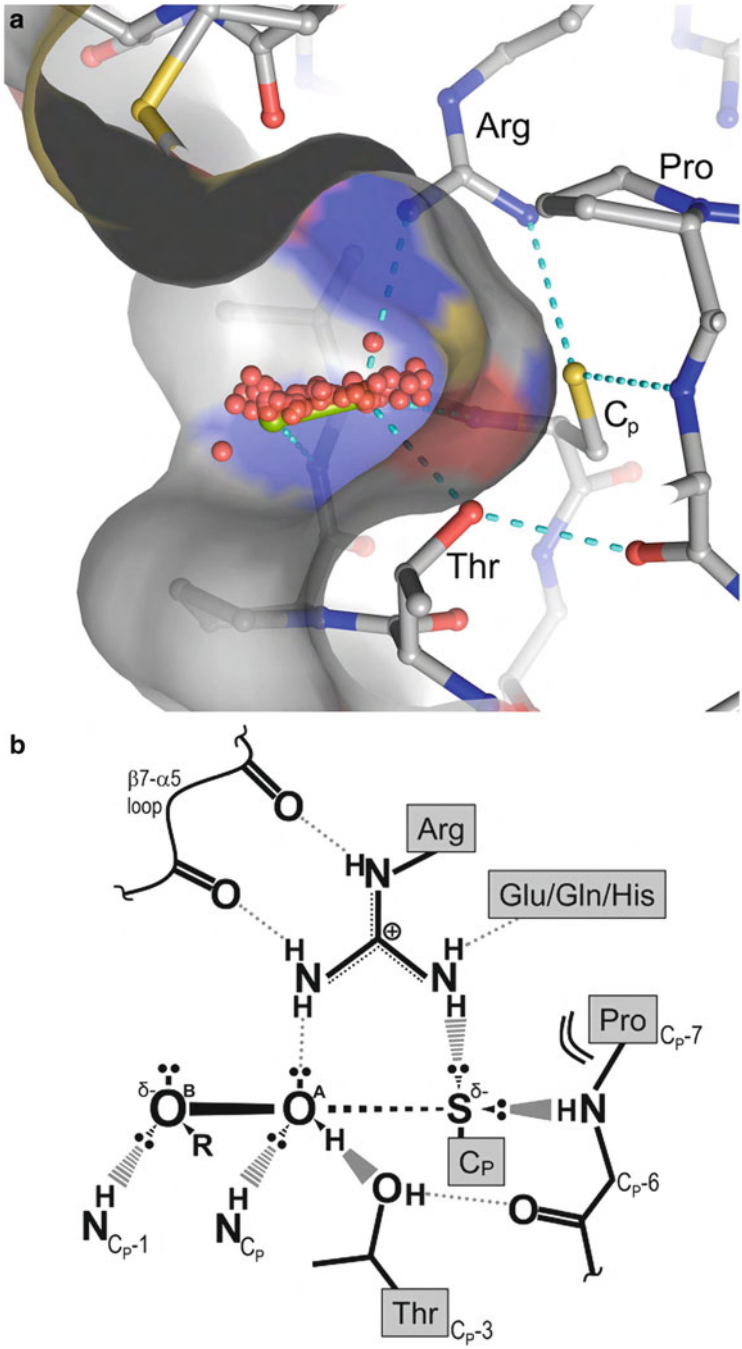

Fig. 4.4 (continued)

to provide additional hydrogen bonding interactions with C_p, and indirectly (via the threonine hydroxyl) with the peroxide O_A (Hall et al. 2010). These analyses have brought considerable insight into the origins of the specificity of Prxs – in other words, how the extremely high catalytic power of the Prx active site toward hydroperoxides is generated without a parallel enhancement of reactivity toward alkylating agents and other oxidants such as chloramines (Peskin et al. 2007; Nelson et al. 2008).

4.6.2 The Structural Basis for Oxidation Sensitivity

As alluded to above, the most abundant eukaryotic Prxs are sensitive to inactivation resulting from hyperoxidation of the C_p sulfenic acid when a second molecule of peroxide outcompetes the resolution reaction and generates the sulfinic acid at C_p. Some bacterial and cyanobacterial Prxs have also exhibited a relatively high degree of oxidant sensitivity (Baker and Poole 2003; Pascual et al. 2010). It has also been seen that a third molecule of peroxide can, in some cases, further oxidize this site to the sulfonic acid form, a redox form which cannot be repaired by sulfiredoxin (Sarma et al. 2005; Seo et al. 2009). The structural basis for this sensitivity has been explored, in particular, within the "typical 2-Cys" (Prx1/AhpC) group of Prxs (see below), where C_r resides near the C-terminus of another monomer within the dimeric (or oligomeric) protein. Comparisons of the structures of representative sensitive versus robust Prxs revealed specific structural features, especially a conserved YF motif within the C-terminal α-helix, correlated with sensitivity, and as described below, provided a rationale for the sensitivity (Wood et al. 2003).

Because the Prx catalytic cycle requires local unfolding of the active site loop and helix to allow C_p-C_r disulfide bond formation, excessive stabilization of the FF form will hinder the resolution reaction and promote hyperoxidation of the longer-lived sulfenic acid within the Prx active site. What was noted by Wood et al. (2003) is that the C-terminal α-helix present in sensitive Prxs packs against the active site C_p-loop to stabilize of the FF form (Fig. 4.5, upper left). In contrast, the architecture and mobility of the protein in the vicinity of the active site in the bacterial AhpC protein

Fig. 4.4 (continued) Interactions in the Prx active site. (**a**) Cutaway view of the active site pocket of a peroxiredoxin (PDB structure 3A2V) with bound H_2O_2 (*green*) overlaid with water molecules (*red spheres*) taken from many other peroxiredoxin structures. Surrounding atoms including the C_p-loop and conserved arginine are shown as *sticks* and *colored* by atom type and select hydrogen bonds are shown as *dashed cyan lines*. (**b**) Schematic drawing of the peroxide-bound active site emphasizing the hydrogen-bonding interactions present. The in-line geometry for the C_p thiolate to attack O_A for the peroxidatic S_N2 reaction (*bold broken line*) and key hydrogen-bonding interactions (*pale broken and dotted lines*) are indicated. The view is as in (**a**). Backbone atoms in the C_p-loop are identified by their residue position relative to C_p. The negative charge on the C_p-thiolate is indicated. Panel **b** is reproduced with permission from the Journal of Molecular Biology (Hall et al. 2010)

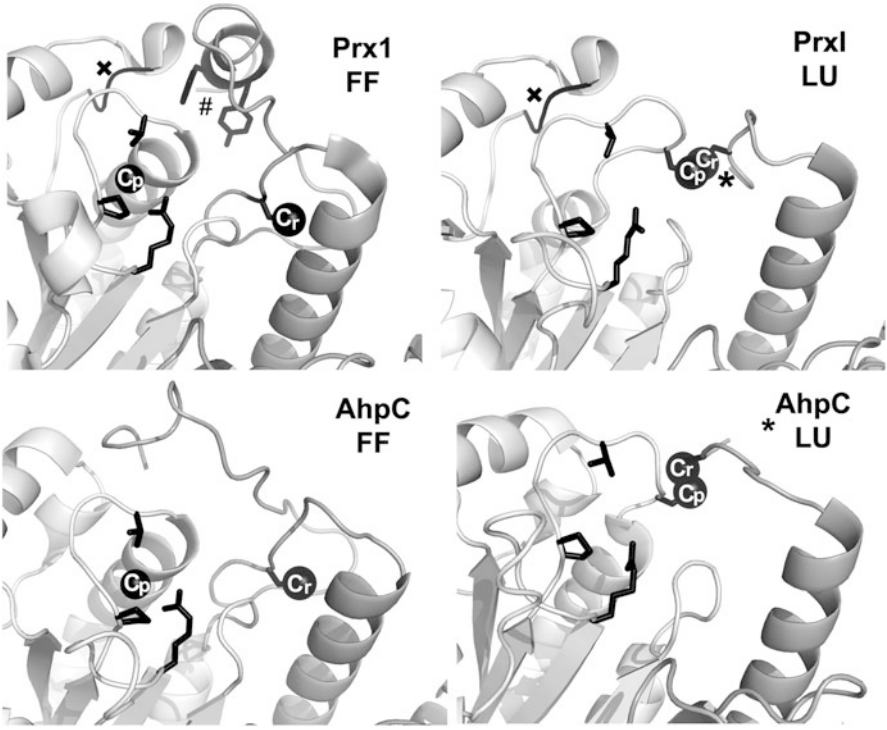

Fig. 4.5 Structural differences between robust and sensitive Prxs identified by Wood et al. (2003). *Ribbon* diagrams of the peroxidatic active site region of sensitive (*top*, *Rattus norvegicus* PrxI) and robust (*bottom*, *S. typhimurium* AhpC) structures in the fully folded (FF) and the locally unfolded (LU) conformations. Side chains for C_p and C_r cysteines (with balls emphasizing the sulfur positions), the four universally conserved active site residues (*black*), the YF motif of PrxI (*# in upper left panel*), and the main-chain segment containing the conserved GGLG motif of PrxI (*top panels in black and marked with an "x"*) are shown. The partner subunit contributing the C_r is depicted in a *darker shade of gray*. Structures shown are: *top left*, FF, C52S form of sensitive PrxI (PDB entry 2Z9S); *top right*, LU, disulfide form of sensitive PrxI (1QQ2); *lower left*, FF, C46S form of *St*AhpC (1N8J); and *lower right*, LU, disulfide form of *St*AhpC (1YEP). *Asterisks* marking the ends of the visible C-termini of the LU conformations indicate that many additional C-terminal residues are disordered. Comparison of the upper and lower FF panels (*left*) provides the most dramatic image of how the two sequence features common to sensitive enzymes result in a major difference in the burial of the C_p-containing helix

is noticeably different (Fig. 4.5, lower left), with a shorter C-terminus and greater mobility in all regions near the C_p (as suggested by the higher crystallographic B-factors in these regions). Local unfolding to allow for disulfide bond formation between C_p and C_r is therefore less hindered by the active site microenvironment in AhpC. This explanation of the origins of sensitivity also emphasizes the important role played by C_r in protecting the Prx C_p from overoxidation (Ellis and Poole 1997b; Trujillo et al. 2006).

Validation of this structural explanation for the heightened "redox sensitivity" of certain Prxs has come from mutational and biochemical experiments conducted with several eukaryotic enzymes. Koo et al. demonstrated that a sensitive Prx from *Schizosaccharomyces pombe* became "robust" toward high peroxide levels upon experiencing either single residue substitutions or small deletions at the C-terminus that disrupted the wild type packing interactions (Koo et al. 2002; Wood et al. 2003). Furthermore, simple swapping, through genetic manipulations, of the C-terminal tails of sensitive and robust Prxs from a single organism, *Schistosoma mansoni*, showed that the sensitivity of the enzymes toward oxidative inactivation tracked with the distinct C-terminal sequences (Sayed and Williams 2004). This has been taken as strong evidence that a "tuning" of the sensitivity of Prxs to hyperoxidation has taken place during evolution to match the properties of the specific enzymes to their biological function(s) (Poole et al. 2004).

4.6.3 Quaternary Structures of Prxs

Most Prxs form dimers or in some cases higher order oligomers that are associations of dimers. Only *E. coli* BCP and related proteins in the same subfamily have been shown to exist as stable monomers (Hall et al. 2011; Reeves et al. 2011). Interestingly, Prxs form two types of dimers (Sarma et al. 2005), and these are characteristic of the subfamily to which they belong (see below). A-type dimers, which arguably represent a more ancestral mode of interaction, are formed through a tip-to-tip association at strands β_1 and β_2 (the β-hairpin) and the loops preceding helices α_2, α_3 and α_4 (Fig. 4.6a). B-type dimers are quite distinct, with head-to-tail interactions at the edges of the β-sheets (through the β_7 strands) that generate an extended, 10-stranded β-sheet (Fig. 4.6b). B-type dimers, which form only in proteins in the Prx1 and Prx6 subfamilies (see below) can also form higher order doughnut-shaped structures (typically decamers, but also dodecamers or octamers) through association through the A-interface (Fig. 4.6c). These dimer-dimer interactions were found to be redox-sensitive in proteins like bacterial AhpC and plant and human Prx1-like proteins, and were seen to promote catalysis through stabilization of the FF active site and through a limitation of the dynamic sampling of side chain conformations that strongly affect the C_p pK_a (Barranco-Medina et al. 2008; Parsonage et al. 2005; Wood et al. 2002; Yuan et al. 2010). Larger aggregates of Prx proteins have also been observed and associated with chaperone activity, as mentioned above (Jang et al. 2004); studies of human PrxII using transmission electron microscopy and 3D reconstruction were consistent with the formation (on a holey carbon support film) of dodecamers of the decameric forms that were large, spherical particles with $M_r \sim 2.62$ MDa and an external diameter of ~20 nm (Meissner et al. 2007).

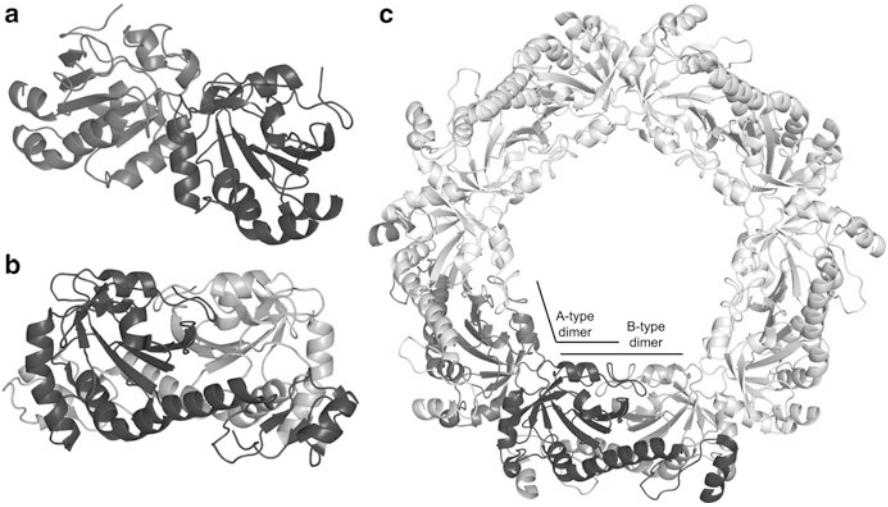

Fig. 4.6 Quaternary structures of Prxs. Many Prxs form dimers either through A-type dimers (**a**) that interact near helix α3, or through B-type dimers (**b**) that interact edge to edge to form an extended 10-strand β-sheet. (**c**) Members of the Prx1 and Prx6 subfamily form B-type dimers and many of them form higher order doughnut-like oligomers through the A-interface that can be comprised of 8, 10, or 12 subunits, with the majority being decameric. Structures depicted are for human PrxV (1HD2) (**a**), *Arenicola marina* Prx6 (2V2G) (**b**), and human PrxII (1QMV) (**c**)

4.7 Prx Subfamilies

Prxs are a diverse group of proteins, which can be classified into 6 subfamilies (Prx1, Prx6, Prx5, Tpx, PrxQ, and AhpE) based on sequence similarities, structural homology, and residue conservation in the vicinity of the C_p (Copley et al. 2004; Hall et al. 2011; Nelson et al. 2011). Most species contain multiple Prx proteins from multiple subfamilies; for example, humans have 6 Prxs from 3 subfamilies, *E. coli* has 3 Prxs from 3 subfamilies, *Saccharomyces cerevisiae* has 5 Prxs from 4 subfamilies, and *Arabidopsis thaliana* has 10 Prxs from 4 subfamilies. While the mechanisms for sulfenic acid formation and peroxide reduction, as well as the PXXX(T/S)XXC_p motif, are conserved across all Prxs, other features, including oligomeric state, susceptibility to hyperoxidation, substrate specificity, and the presence and location of the C_r can vary between and within the subfamilies (Hall et al. 2011; Nelson et al. 2011). While the C_r is now known to reside in at least five different locations within the Prx structure (Fig. 4.7a and Lian et al. 2012), in the early 1990s, only a single C_r location was recognized (Chae et al. 1994a). This C_r, near the C-terminus of the Prx, forms a disulfide bond between two chains across the B-type interface (Chae et al. 1994b; Poole 1996) and at that time, the terms "2-Cys" and "1-Cys" were used to distinguish the only two known patterns for resolution of the C_p (Chae et al. 1994a). Later, with the discovery that other Prx proteins formed intrasubunit disulfide bonds using C_r residues in other

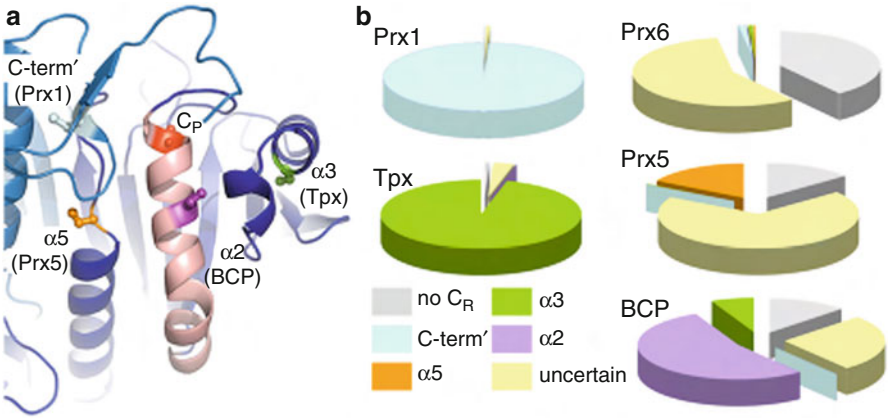

Fig. 4.7 Variability in locations of C_r within and between Prx subfamilies. (**a**) The four prototypical locations for the C_r (labeled by location and the subfamily it is commonly associated with) are mapped onto a composite structure based on *St*AhpC (1YF1). The conserved C_p (*red*) is also shown. The two chains of the B-type dimer are colored in *dark* and *light blue* and helix α2 is colored *pink*. (**b**) Pie charts based on ~3,500 Prx sequences showing the frequency at which the C_r is in a given location for each subfamily (Nelson et al. 2011). Wedges are colored by C_r position consistent with panel A using the same notation: no C_r (*gray*), C-term' (*cyan*), α5 (*orange*), α3 (*green*), α2 (*purple*) and uncertain (*pale yellow*). The exact positions are defined as in Hall et al. (2011). Sequences marked as "uncertain" have additional Cys residues present, but none align exactly with one of the known locations. We suspect most of them are 1-Cys Prxs. This figure is reproduced with permission from the journal Antioxidants and Redox Signaling (Hall et al. 2011)

locations, the term "typical 2-Cys" was used to distinguish the earlier-recognized Prxs containing a C_r in the C-terminus of the protein from "atypical 2-Cys Prxs" with a C_r in any other location. The terms 1-Cys, typical 2-Cys, and atypical 2-Cys are still sometimes used to describe the mechanism by which the protein resolves the sulfenic acid, but they are not particularly useful in distinguishing Prx subfamilies because all subfamilies likely include both 1-Cys and 2-Cys members and because the "atypical 2-Cys" category is not homogeneous (Fig. 4.7b). In all cases, disulfide bond formation requires the localized unfolding of the region surrounding C_p (and often C_r), as described above, promoting the next step of reduction by a thiol-containing disulfide reductase system. The preferred reductant can also vary among and within subfamilies. For many Prxs, Trx serves as the direct reductant, but Grx, AhpF, and small molecule thiols like glutathione have also been shown to reduce particular Prxs.

4.7.1 Prx1 Subfamily

The Prx1 subfamily, also called the Prx1/AhpC subfamily (Nelson et al. 2011), includes highly expressed and widely distributed proteins such as the bacterial alkyl hydroperoxide reductase C protein (AhpC), the tryparedoxin peroxidases, the yeast

TSA1 and 2, and the human PrxI-IV proteins. While members of this subfamily have been shown to react very rapidly with H_2O_2 (catalytic efficiencies of $\sim 10^7$ M^{-1} s^{-1} with the best substrates), turnover in *in vitro* assays is typically limited by the rate of reduction ($\sim 10^5$ M^{-1} s^{-1}) (Baker and Poole 2003; Horta et al. 2010; Manta et al. 2009; Parsonage et al. 2005). Prx1 subfamily members contain a 40–50 residue extension at the C-terminus compared to the common core structure. All known members form stable B-type dimers and, in the FF form, the resolving cysteine in >99% of the subfamily members is buried within the C-terminal extension approximately 14 Å away from the C_p in the adjacent monomer. Upon oxidation, the region surrounding both the C_p and the C_r become locally unfolded, allowing for the formation of a disulfide bridge across the B-type interface (Fig. 4.5). As described above, Prx1 subfamily members can further associate through the A-interface to form oligomeric, doughnut-like structures of 4–6 dimers. The redox sensitivity of the oligomeric state was first explored in detail for AhpC from *S. typhimurium*. Using analytical ultracentrifugation, reduced AhpC was shown to exist as a stable decamer whereas disulfide bond formation destabilized the decamer, promoting dissociation into dimers at low concentration (Wood et al. 2002). The importance of the decamer interface was further highlighted by studies in which disruption of the decamer interface led to a 100-fold decrease in catalytic efficiency (Parsonage et al. 2005). While eukaryotic Prx1 subfamily members are typically reduced by Trx, many of the bacterial AhpC-like subfamily members have a specific partner flavoprotein reductase, AhpF, encoded within the same operon (Poole 2005). AhpF enzymes have a specially evolved double Trx-fold domain (Roberts et al. 2005; Hall et al. 2009b) that is fused to a C-terminal thioredoxin reductase (TrxR)-like region which, unlike the thioredoxin system, is NADH- rather than NADPH-dependent (Poole and Ellis 1996; Poole et al. 2000).

4.7.2 Prx6 Subfamily

The first Prx to be structurally characterized was human PrxVI from which this subfamily takes its name. Prx6 sequences are widely distributed and have sometimes been grouped with Prx1 subfamily members due to their somewhat similar sequences, the formation of B-type dimers, and the presence of a C-terminal extension (Copley et al. 2004). However, in contrast to the Prx1 subfamily, only a subset of Prx6 members forms decamers using the A-type interface. Also, members of this subfamily contain a longer C-terminal extension than observed in the Prx1 subfamily and the large majority appear to be 1-Cys Prxs (i.e., lacking a C_r in the protein) (Nelson et al. 2011). One member of this subfamily, *Aeropyrum pernix* "Tpx", has a cysteine (C213) in the extended C-terminal extension that serves as a C_r (Jeon and Ishikawa 2003), and ~16% of the Prx6 sequences identified in a large-scale bioinformatic analysis of Genbank(nr) contain a cysteine residue in the same location (Nelson et al. 2011). The identity of reductant(s) of Prx6 subfamily

members is also largely unknown; some subfamily members are reduced by Trx while others are not. At least some subfamily members may be regenerated by GSTπ and glutathione, or by ascorbate (Hall et al. 2011).

4.7.3 Prx5 Subfamily

The best characterized member of this subfamily is human PrxV, which is less reactive with H_2O_2 (second order rate constant $\sim 10^5$ M^{-1} s^{-1}) than human PrxI or PrxII, but is more reactive with both organic hydroperoxides ($\sim 10^6$ M^{-1} s^{-1}) and peroxynitrite ($\sim 10^7$ M^{-1} s^{-1}) (Trujillo et al. 2007; Manta et al. 2009). Prx5 subfamily members are found in archea, bacteria and higher eukaryotes and are localized to various locations within eukaryotic cells including mitochondria, peroxisomes, cytoplasm, and plant chloroplasts. This family includes a group of bacterial proteins that contain a Grx domain fused to the C-terminus of the Prx5 domain. Although the canonical location of the C_r has been considered to be in helix α_5 as observed in human Prx5, recent analysis shows that a cysteine is found in this location only 17% of the time in this subfamily (Nelson et al. 2011), implying that most Prx5 subfamily members are 1-Cys Prxs.

4.7.4 Tpx Subfamily

Whereas the Tpx subfamily takes its name from the first family member to be biologically characterized, *Escherichia coli* thiol peroxidase, a major confusion is that this same moniker (Tpx) has been used to abbreviate "thioredoxin peroxidase" and is the common name for many Prxs not belonging to this subfamily. Tpx subfamily members are found almost exclusively in bacteria (Nelson et al. 2011) and are comprised of A-type dimers that stay associated in both oxidized and reduced states (Baker and Poole 2003; Hall et al. 2009c). The K_m for cumene hydroperoxide (a bulkier and more hydrophobic substrate than H_2O_2) is more than two orders of magnitude lower than the K_m for H_2O_2 in *E. coli* Tpx (Baker and Poole 2003). For more than 99% of Tpx subfamily members the C_r is located in helix α_3, with the remainder apparently being 1-Cys proteins (Nelson et al. 2011).

4.7.5 PrxQ Subfamily

This subfamily contains the plant PrxQ chloroplast proteins as well as the bacterial BCP proteins and, based on recent bioinformatic analysis, is distributed widely across archea, bacteria, plants, and fungi (Nelson et al. 2011). While the PrxQ subfamily is not as well characterized as other Prx subfamilies, recent structural

and kinetic studies have shown that members of this subfamily are rather diverse in terms of oligomeric state, presence and location of the C_r, and kinetic activity, supporting the hypothesis that this subfamily is most similar to the ancestral Prx and that C_r-positions have arisen independently multiple times in the course of evolution (Copley et al. 2004; Hall et al. 2011; Horta et al. 2010; Reeves et al. 2011). PrxQ subfamily members have been shown to exist as either monomers or A-type dimers, and mechanistically can be either 1-Cys or 2-Cys Prxs with the C_r existing in one of two locations: 54% of the subfamily members contain a C_r five residues after the C_p in helix α_2 (Fig. 4.7) while another 13% exhibit a C_r in helix α_3 (the position commonly associated with members of the Tpx subfamily) (Nelson et al. 2011). The catalytic activity of this family also varies widely. *E. coli* BCP exhibits a similar, slow rate of reaction with H_2O_2 and other alkyl hydroperoxides (second order rate constants $\sim 10^4$ M^{-1} s^{-1}) and can be reduced efficiently by a range of reductants including Trx1, Trx2, Grx1, and Grx3 (Reeves et al. 2011). In contrast, a plant PrxQ from *Xylella fastidiosa* has been shown to react much more rapidly with H_2O_2 ($\sim 10^7$ M^{-1} s^{-1}) (Horta et al. 2010). Members of the PrxQ subfamily have also been shown to exhibit both the highest (at −146 mV, *E. coli* BCP) and lowest (at −325 mV, poplar PrxQ) redox potentials reported to date for any Prx (Reeves et al. 2011; Rouhier et al. 2004).

4.7.6 AhpE Subfamily

The AhpE subfamily is comprised of a small number of proteins that have to date only been found in a limited number of gram positive bacterial species closely related to *Mycobacterium tuberculosis* (Nelson et al. 2011). AhpE does not group clearly with any other Prx subfamily, however, it shares ~30% amino acid sequence identity with Prx1 subfamily members and ~25% sequence identity with members of the PrxQ subfamily. *M. tuberculosis* AhpE has been shown to react faster with peroxynitrite ($\sim 10^7$ M^{-1} s^{-1}) than with H_2O_2 ($\sim 10^5$ M^{-1} s^{-1}) and is the only structurally characterized subfamily member to date (Hugo et al. 2009). Although *M. tuberculosis* AhpE is a 1-Cys Prx, other members of this subfamily contain a potential C_r in helix α_2 similar to many PrxQ subfamily members (Nelson et al. 2011).

4.8 Regulation of Prx Activity

4.8.1 Transcriptional Regulation of Prx Expression

Peroxiredoxin expression is frequently up-regulated in response to oxidative stress. In *E. coli*, H_2O_2 activates OxyR through reaction with its sensor site, Cys199, to form a sulfenic acid; subsequent disulfide bond formation with Cys208 leads to

activation of the transcription factor, inducing expression of a variety of antioxidant proteins including AhpC and AhpF, KatG catalase, glutathione reductase, and Grx1 (Åslund et al. 1999). Transcriptional activity of OxyR is subsequently modulated by these antioxidants which directly or indirectly decrease H_2O_2 levels in the cell; in addition, Grx1 directly reduces and inactivates OxyR, providing multiple feedback mechanisms to regulate OxyR activity (Åslund et al. 1999). Although OxyR is present in many bacteria, a distinct transcriptional regulator known as PerR regulates Prx expression in *Bacillus subtilis* in response to specific peroxide or general oxidative stress (Mongkolsuk and Helmann 2002). In *Saccharomyces cerevisiae*, the Gpx homolog Orp1 serves as a peroxide sensor by forming a sulfenic acid on Cys36 in the presence of H_2O_2 (Delaunay et al. 2002). Although this protein has its own C_r for intramolecular disulfide bond formation, a kinetic pause at this step allows formation of an intermolecular disulfide bond with yeast activator protein 1 (Yap1), a transcription factor that controls antioxidant gene expression. A second cysteine in Yap1 then attacks the intermolecular disulfide to form an intramolecular disulfide bond within Yap1 and release Orp1, activating transcription and upregulating Prx expression (D'Autreaux and Toledano 2007). In *Schizosaccharomyces pombe*, the Yap1 homologue, activator protein-1 (Pap1), is activated by "low" peroxide levels (below 0.2 mM) in concert with thiol peroxidase 1 (Tpx1), a member of the Prx1 subfamily, and both are inactivated at H_2O_2 levels greater than 1 mM. At this high peroxide level, the Sty1 transcription factor is independently activated through a mechanism involving a bacterial-like His phospho-relay system and potentially promoted by disulfide bond formation either intermolecularly with Tpx1 or intramolecularly through direct oxidation of a cysteine on Sty1 (Day et al. 2012; Karplus and Poole 2012). In *Homo sapiens*, the transcription factor, NF-E2-related factor-2 (Nrf-2) has been shown to regulate the expression of Prx1 and Prx4 as well as the Prx reductants, Trx, TrxR1, and Srx (Bell and Hardingham 2011). Transcriptional regulation of these genes involves a *cis*-acting DNA sequence known as the antioxidant response element (ARE), which recruits the transcription factor Nrf2. Under non-stress conditions, Nrf2 is ubiquitinated and targeted for degradation by Kelch like ECH-associated protein (Keap 1); during oxidative stress, oxidation of one or more cysteine(s) on Keap1 releases Nrf2, allowing for Nrf2 accumulation in the nucleus and subsequent activation of ARE-containing genes (Brigelius-Flohé and Flohé 2011). In parasites, Prxs are very important factors for defending against the immune system, and their expression tends to be regulated by developmental stage and environment (Gretes et al. 2012).

4.8.2 Regulation of Prx Activity by Phosphorylation and Other Posttranslational Modifications

Recent studies have identified a group of post-translational modifications (in addition to redox modifications described above) that regulate Prx activity, including phosphorylation, acetylation, and glutathionylation (Parmigiani et al. 2008; Woo

et al. 2010; Chae et al. 2012). Most of these studies have focused on human PrxI and PrxII, and it is still too early to completely understand the role some of these modifications play in the cell. In particular, PrxI has been shown to be phosphorylated by cyclin dependent kinase 1 (CDK1) at Thr90 during the mitotic phase in HeLa cells (Chang et al. 2002) and at Tyr194 in response to stimulation by growth factors (e.g. EGF and PDGF), or by antibodies to IgM and CD3 in the case of B-cells and T-cells, respectively (Woo et al. 2010). Both phosphorylation events appear to exhibit specificity for the PrxI isoform over PrxII, and decrease the peroxidase activity of the phosphorylated Prxs, thus allowing for a localized increase in H_2O_2 levels in response to growth factor signaling (Rhee and Woo 2011). Acetylation at the C-terminus of PrxI and PrxII by HDAC6 was reported to increase Prx activity (Parmigiani et al. 2008), perhaps a result of disrupting the packing of the C-terminal helix to promote disulfide bond formation during catalysis and avoid oxidative inactivation. Glutathionylation of the non-catalytic Cys83 has recently been shown to shift the oligomeric state of human PrxI from predominantly decameric to predominantly dimeric structures, eliminating its ability to serve as a chaperone; additional effects of glutathione in recycling or regulating Prx activity likely varies with the specific protein, and adds to the complexity of glutathionylation as a regulatory mechanism for Prxs (Parmigiani et al. 2008; Woo et al. 2010; Chae et al. 2012).

4.9 Outlook

The last two decades have seen a tremendous growth in the recognition of the importance in biology of sulfenic acid derivatives of cysteine. Although much research involving sulfenic acid derivatives does not include the word “sulfenic” in the abstract, Pubmed searches using the word “sulfenic” yield 20, 41, 102 and 199 hits for the 5 year periods of 1990–1994, 1995–1999, 2000–2004, and 2005–2009, respectively. Related to this, hydrogen peroxide has since early in this century transitioned from being simply numbered among the many dangerous oxidative stressors to being in its own right seen as an important and widespread second messenger crucial to fundamental signaling processes that guide normal growth and development. These research areas were initially connected by the recognition of the evolutionary significance of sensitive Prxs (Wood et al. 2003) and their reactivation by sulfiredoxin (Woo et al. 2003; Biteau et al. 2003), along with the discovery of the sulfenamide-inhibited form of protein tyrosine phosphatases (Salmeen et al. 2003; van Montfort et al. 2003), and the identification of widely expressed isoforms of NADPH oxidases that function in growth factor signaling (Oakley et al. 2009). Our understanding of these processes is still rather primitive but the results in hand are enough to predict confidently that discoveries in this area – including the challenging processes of mapping of the temporal and spatial dynamics of the occurrence of sulfenic acids *in vivo* – will play a prominent role in advancing our

understanding of basic eukaryotic cell biology and our ability to combat major health problems such as obesity, cancer, and aging.

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Chapter 5
Fluorescent Imaging of Redox Species in Multicellular Organisms

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Abstract Redox processes play a crucial role in many aspects of physiology and changes in cellular redox species are increasingly being linked to a wide range of pathological conditions. Redox species can change dynamically at the subcellular compartment-, cell- and tissue-level and different redox species likely convey different biological information. The investigation of redox biology in a living multicellular organism is particularly difficult and is hampered by the lack of tools which offer redox species specificity and the necessary spatial and temporal resolution. In recent years there has been intense development of small organic chemical and genetically encoded fluorescent probes which have vastly improved our ability to investigate cellular redox processes. In this chapter we describe the currently available fluorescent probes, focusing in particular on those which have already been applied to multicellular organisms or those which we believe have the potential for *in vivo* use in the future. We discuss advantages and disadvantages of the different kinds of probes and highlight their major problems and limitations.

Keywords Reactive oxygen species • Redox-sensitive dyes • Redox-sensitive fluorescent proteins

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5.1 Introduction

5.1.1 Why Study Redox Changes In Vivo?

It is becoming increasingly clear that changes in biological redox species are associated with many aspects of human pathology. However, obtaining a detailed understanding of organism redox biology is incredibly difficult. Redox species are regulated at the subcellular compartment-, cell-, and tissue-specific level. Furthermore, many cellular redox species are often independently regulated and change dynamically over time.

Contrary to the classically assumed status of reactive oxygen species (ROS) as unwanted and damaging agents, certain ROS are now known to fulfill crucial roles in many cellular contexts. Hydrogen peroxide (H_2O_2) is specifically produced in response to a range of stimuli, including growth factors and cytokines (Veal et al. 2007). In this context H_2O_2 acts as a second messenger, leading to the post-translational modification of a range of proteins involved in cellular signaling pathways. Thereby, H_2O_2 can influence cellular decisions including proliferation, differentiation, migration and apoptosis (Veal et al. 2007).

Both H_2O_2 and nitric oxide (NO·) are produced in phagocytic immune cells where they contribute to both the killing of invading pathogens and to the regulation of immune signaling pathways, including the pro-inflammatory response (Wink et al. 2011; Grivennikov et al. 2010; Harrison et al. 2011). Inflammation in this context is a crucial part of the immune response; however, prolonged high levels of ROS and reactive nitrogen species (RNS) are associated with chronic inflammation which plays a major role in the initiation and progression of a wide range of pathologies, including atherosclerosis, diabetes and cancer (Drummond et al. 2011; Pashkow 2011; Harrison et al. 2011; Grivennikov et al. 2010). Perturbations in other cellular redox species have also been linked to a range of diseases, for example, sustained alterations of the glutathione system have been associated with malignant, cardiovascular and neurodegenerative disorders (Mieyal et al. 2008; Mieyal and Chock 2012). It goes without saying that redox processes are clearly of huge biomedical interest.

In reality, the actual biomedical significance of redox processes, e.g. protein redox regulation, remains to be demonstrated in the truly *in vivo* context of model animals. It also remains unclear if pharmacological intervention can target cellular redox processes accurately and specifically enough in order to facilitate a beneficial medical outcome. For example, it is becoming clear that even commonly used redox agents such as *N*-acetyl cysteine (NAC) can elicit unexpected outcomes in the context of a multicellular organism (Albrecht et al. 2011).

To date, our understanding of redox processes *in vivo* has been constrained by the lack of appropriate investigative tools and methodology. In particular there are several key difficulties associated with measuring redox processes in the context of multicellular organisms. These include (i) obtaining redox species-specificity, (ii) obtaining subcellular compartment-specificity, (iii) obtaining cell- and tissue-type

resolution (iv) obtaining temporal resolution and (v) dealing with the high optical opacity of many model organisms, which impedes standard microscopic investigation.

Redox studies of multicellular organisms have typically relied on disruptive whole organism techniques, but such methods are prone to *post mortem* artifacts and offer little in the way of spatial resolution as evidenced for example by whole organism reduced glutathione (GSH)/oxidized glutathione (GSSG) assays. *In situ* staining (for example of tissue sections) has also been widely used, however, problems include the frequent use of non-specific probes and the observation of late-stage markers of oxidative damage which are indirect and offer little information relating to the initial redox species involved. Cell or tissue culture is more amenable to investigation with redox probes, but at best cell culture only crudely approximates the true *in vivo* environment and must be viewed in light of the typically non-physiological molecular oxygen concentration (de Souza 2007; Atkuri et al. 2007).

In this chapter we focus on the challenge of investigating redox processes taking place inside multicellular organisms. In particular we focus on the organic chemical and genetically-encoded fluorescent probes which are currently available, and discuss their applicability for use in observing biologically relevant redox species in the context of multicellular organisms.

We believe the ultimate goal should be the development of methodologies that allow the non-invasive, real-time observation of defined redox species, with subcellular compartment resolution in living multicellular organisms, even when not optically transparent. At present, in most situations, this goal is far from being realized. The best approximation that is currently available is the imaging of genetically encoded redox probes in transparent model organisms such as *C. elegans, Drosophila* larvae and *Danio rerio* (zebrafish) (Niethammer et al. 2009; Back et al. 2011; Albrecht et al. 2011). For non-transparent organisms including mice, the next best choice is a minimally invasive technique such as intravital imaging, i.e. observation through a window preparation. However, to reach deep tissues, non-transparent organisms must be sacrificed and measurements performed on organ or tissue preparations or tissue sections. While such procedures in principle allow *in situ* redox measurements, there is a high risk that the disruption of organism integrity changes the redox processes of interest. The chemical conservation of the biosensor redox state during dissection is one possible solution to the problem (Albrecht et al. 2011).

5.1.2 Which Redox Species Are of Particular Interest for In Vivo Measurements?

We consider there to be two classes of redox species that are particularly worthy of investigation. The first class consists of those oxidants that are primarily produced and involved in signaling and regulation. Most prominently, these are superoxide

($O_2^{\cdot-}$), H_2O_2 and NO·. $O_2^{\cdot-}$ is of interest because in most situations it is the primary ROS produced and a precursor of H_2O_2. Although $O_2^{\cdot-}$ reactivity with thiols is very low, it can react with 4Fe-4S clusters, as seen in the $O_2^{\cdot-}$-responsive bacterial transcription factor SoxR (Winterbourn and Hampton 2008). Thus $O_2^{\cdot-}$ may in principle also play a role in regulating protein function. In terms of redox signaling and redox regulation, H_2O_2 is arguably the most important endogenous oxidant. It is the most stable of the ROS, clearly involved in regulating protein function, and under certain circumstances it is also a precursor of the hydroxyl radical (HO·) and hypochlorous acid (HOCl) (Dickinson and Chang 2011), which along with peroxynitrite ($ONOO^-$) constitute the highly reactive oxygen species (hROS). These species are also of interest as they are very short-lived and indiscriminately react with biological molecules, thus they are likely to be the actual source of ROS-induced cellular damage. NO· is specifically produced by nitric oxide synthases (NOS) and serves as both an autocrine and paracrine signaling molecule, for example in vasodilation and neurotransmission. NO· is known to regulate protein function via post-translational modification. Indeed, S-nitrosylation is reported to affect as many as 3,000 different proteins (Hess and Stamler 2012).

The second class of redox species of fundamental importance comprises redox couples that are central to metabolism and redox homeostasis. Here we focus primarily on the redox couples for which fluorescent probes are currently available, GSH/GSSG and NAD^+/NADH. The glutathione redox couple provides an abundant source of reducing equivalents that protect cells from oxidative stress and are crucial for detoxification of xenobiotics (Grant et al. 1996; Meister 1988). S-glutathionylation is a mechanism for regulating redox-sensitive signaling pathways (Dalle-Donne et al. 2009) and alterations of glutathione homeostasis are known to accompany cell death, senescence, aging and a number of pathophysiological conditions. Likewise, the NAD^+/NADH redox pair has many important roles in the cell. Disrupted NAD^+/NADH homeostasis has been found to be associated with several pathological conditions, including cancer (Zhang et al. 2006).

5.1.3 What Kinds of Redox Probes Exist?

Generally we can distinguish between optical and non-optical redox probes. The most prominent non-optical redox probes are those based on electron paramagnetic resonance (EPR) spectroscopy (Vikram et al. 2010). The main advantage of EPR is the ability to non-invasively image redox species in whole living animals, e.g. mice (Vikram et al. 2010; Shulaev and Oliver 2006). However, the disadvantages of EPR include (i) the low spatial resolution of images (cellular or subcellular compartment imaging is not possible), (ii) the necessity to load organisms with high concentrations of spin-trap probes, which can be toxic and suffer from many of the pharmacokinetic complications described below for chemical fluorescent probes (Shulaev and Oliver 2006) and (iii) the lack of chemical specificity of those EPR probes amenable to *in vivo* application, with the notable exception of NO· specific probes (Yoshimura et al. 1996).

Most optical probes are either based on fluorescence or luminescence. Only relatively few examples of luminescence-based redox probes exist compared to fluorescent probes. In this review we focus primarily on the fluorescent probes, because they have been most widely used for imaging in multicellular organisms and show promise for further improvements.

The vast majority of fluorescent redox probes are either organic chemical or genetically-encoded, and these types are the main focus of this review. However, it should be mentioned that there are now fluorescence-based redox probes, which are neither small organic chemicals nor proteins, namely nanotube- and nanoparticle-based probes designed to react to signaling concentrations of H_2O_2 or NO· (Jin et al. 2010; Casanova et al. 2009; Boghossian et al. 2011).

5.2 Chemical Fluorescent Probes and Their Applications

Chemical fluorescent probes have two key advantages over genetically encoded probes. The first and major advantage is that chemical probes can be applied to almost any sample without the prior need for lengthy genetic manipulation steps. Consequently, chemical probes can be directly applied, for example to human clinical samples, which may ultimately be useful in the context of disease diagnosis.

Chemical fluorescent probes are also especially flexible as their properties can be readily modified via the addition of chemical moieties. This strategy has been used to create novel probe variants with increased specificity towards target molecules and increased membrane permeability. The latter can be achieved by protecting hydroxyl and carboxyl groups with acetyl or acetoxymethyl (AM) groups, thus allowing cells to be more efficiently loaded with fluorescent probes. Chemical modifications can also be used to manipulate spectroscopic properties such as the fluorescence emission wavelength. This can be particularly useful for *in vivo* imaging; near-infrared (NIR) fluorescence, in the range of 650–900 nm enables deeper imaging of animal tissue than visible wavelengths because of low background fluorescence and minimal absorption by hemoglobin and water at these longer wavelengths (Weissleder and Ntziachristos 2003). The exact tissue penetration efficiency of NIR fluorescence is dependent on the particular tissue type examined, but is usually in the order of 1 cm (Lee et al. 2007). In particular, the cyanine fluorophore exhibits strong fluorescence in the NIR range and has been widely used as the basis of fluorescent probes or labeling agents for *in vivo* imaging applications (Klohs et al. 2008).

Chemical fluorescent probes can be broadly categorized into two main classes:

1. Hydro-type fluorescent probes, which are molecules delivered in the non-fluorescent reduced state and which become fluorescent upon oxidation. These are the ‘conventional’ probes that can react non-specifically and indirectly with a broad range of oxidants.

2. Fluorescent probes selective to particular oxidants. These probes have been designed from the outset to respond specifically to defined oxidant species.

Representative probes and their key properties are summarized in Tables 5.1 and 5.2.

5.2.1 Hydro-Type Fluorescent Probes

In the past, hydro-type chemical fluorescent probes, in particular dichlorodihydrofluorescein, dihydrorhodamine 123 and hydroethidium, have been widely used to report changes in 'cellular redox status' or 'ROS production'. However, fluorescent changes observed with this class of probes can be the result of a wide range of cellular processes and thus great care must be taken when designing and interpreting experiments. Hydro-type fluorescent probes are non-specific, highly susceptible to oxidation by undefined enzymatic processes (Halliwell and Whiteman 2004) and easily excluded from the cell by membrane transporters, including multidrug resistance protein (MDR) and multidrug resistance-associated protein (MRP) transporters (Huai-Yun et al. 1998; Ludescher et al. 1992). Due to the low specificity of these probes and the complexity of the cellular environment, the biological basis of fluorescence changes remains poorly understood in most cases. In fact, it may be difficult to determine if observed fluorescence changes are the result of changes in ROS concentration at all (Wardman 2007). In this section we focus on the three most commonly used hydro-type fluorescent probes, as well as the recently introduced hydrocyanines.

5.2.1.1 2′,7′-Dichlorodihydrofluorescein (DCFH)

DCFH is one of the most commonly used chemical fluorescent redox probes. An acetylated derivative, DCFH-DA, is typically used for intracellular measurements. Intracellular esterases catalyze the deacetylation of DCFH-DA to DCFH, which can then be oxidized to yield the highly fluorescent product 2′,7′-dichlorofluorescein (DCF) (Fig. 5.1a). However, the intracellular process of DCFH oxidation is complex and ill-defined. *In vitro*, DCFH appears to be unreactive toward H_2O_2 and $O_2^{\cdot -}$. DCFH oxidation by H_2O_2 can be facilitated by peroxidase activity (LeBel et al. 1992). DCFH oxidation can also be mediated by ferric iron and cytochrome *c* via the generation of HO· or peroxidase compound I-type oxoferryl species, respectively (LeBel et al. 1992; Lawrence et al. 2003). 5-lipoxygenase (LOX) was also reported as a potent catalyst of DCFH oxidation (Hempel et al. 1999). DCFH oxidation may generally be influenced by the intracellular concentration of heme and heme proteins (Ohashi et al. 2002). Additionally, DCFH is prone to photo-oxidation, which may pose problems when the probe is exposed to high-intensity laser irradiation. In general, DCFH oxidation proceeds via a radical intermediate ($DCF^{\cdot -}$) which is

Table 5.1 Hydro-type fluorescent probes

Name	Target species	Fluorophore	Features	Sensing mechanism	Physiological application	Ref.
DCFH[a]	Nonspecific	Fluorescein	Susceptible to photo-oxidation Non-specific oxidation	Oxidation	✓	1
DHR123[a]	Nonspecific	Rhodamine	Susceptible to photo-oxidation Non-specific oxidation	Oxidation	✓	1
DHE[a]	$O_2^{\cdot -}$ (partially nonspecific)	Ethidium	Highly susceptible to photo-oxidation Non-specific oxidation	Oxidation	✓	2
MitoSOX™ Red[a]	$O_2^{\cdot -}$ (partially nonspecific)	Ethidium	Highly susceptible to photo-oxidation Non-specific oxidation Targeted to mitochondria	Oxidation	✓	2
Hydrocyanines	HO·, $O_2^{\cdot -}$	Cyanine	NIR fluorescence Susceptible to photo-oxidation	Oxidation	✓	3

1. Halliwell and Whiteman (2004), 2. Robinson et al. (2006), 3. Kundu et al. (2009)
[a]Commercially available

Table 5.2 Fluorescent probes selective to oxidants

Name	Target species	Fluorophore(s)	Features	Sensing mechanism	Physiological application	Ref.
BESSo[a]	$O_2^{\cdot -}$	Fluorescein	AM derivative available	Deprotection of BES	✓	1
PG1[a], PC1[a]	H_2O_2	Fluorescein, Resorufin	Slow reaction rate (1 M^{-1} s^{-1})	Deprotection of boronate	✓	2
PF2[a], PF3[a], PE1[a], PY1[a], PO1[a]	H_2O_2	Fluorescein, Rhodol	Different fluorescence wavelength applicable to multicolor imaging	Deprotection of boronate	✓	3
NPF1[a]	H_2O_2	Naphtho-fluorescein	NIR fluorescence	Deprotection of boronate		4
RPF1[a]	H_2O_2	Coumarin and Fluorescein	Ratiometric measurement	Deprotection of boronate		5
PL1[a]	H_2O_2	Naphthalimide	Ratiometric measurement	Deprotection of boronate	✓	6
Mito-PY1[a]	H_2O_2	Rhodol	Targeted to mitochondria	Deprotection of boronate		7
SPGs[a]	H_2O_2	Fluorescein	Controllable localization by SNAP tag expression	Deprotection of boronate		8
PCL-1	H_2O_2	Luciferin	Bioluminescence detection	Deprotection of boronate	✓	9
PF6[a]	H_2O_2	Fluorescein	High intracellular retention	Deprotection of boronate	✓	10
NucPE-1	H_2O_2	Rhodol	Localization to nucleus High background fluorescence	Deprotection of boronate	✓	11
QCy7 based	H_2O_2	Cyanine	NIR fluorescence	Deprotection of boronate	✓	12
NBzF	H_2O_2	Fluorescein	High fluorescence activation upon reaction (~150 fold)	Baeyer-Villiger reaction with H_2O_2	✓	13
HPF[a]	HO·, $ONOO^-$	Fluorescein	Resistance to photo-oxidation	*O*-dearylation	✓	14
APF[a]	HO·, $ONOO^-$, HOCl	Fluorescein	Resistance to photo-oxidation	*O*-dearylation	✓	14
APC	HO·, $ONOO^-$, HOCl	Calcein	High intracellular retention	*O*-dearylation	✓	15

MitoHR	HO·, $ONOO^-$	Rhodamine	Targeted to mitochondria Resistance to photo-bleaching	*O*-dearylation		16
MitoAR	HO·, $ONOO^-$, HOCl	Rhodamine	Targeted to mitochondria Resistance to photo-bleaching	*O*-dearylation	✓	16
SNAPF	HOCl	Naphtho-fluorescein	NIR fluorescence	*O*-dearylation	✓	17
HySOx	HOCl	Rhodamine	Colorless Resistance to photo-bleaching	Sulfur atom oxidation	✓	18
MMSiR, ws-MMSiR	HOCl	Si-rhodamine	Colorless NIR fluorescence Resistance to photo-bleaching	Sulfur atom oxidation	✓	19
NiSPY1[a]	$ONOO^-$	BODIPY	High fluorescence activation	Nitration		20
HKGreen1	$ONOO^-$	Fluorescein	Low conversion efficiency to fluorescent product after reaction	Reaction with trifluoromethyl ketone		21
HKGreen2	$ONOO^-$	BODIPY	pH insensitivity	Reaction with trifluoromethyl ketone	✓	22
Cy-PSe	$ONOO^-$	Cyanine	NIR fluorescence Reducible by GSH	Selenium atom oxidation	✓	23
FOSCY	HO·, $ONOO^-$, HOCl, $O_2^{\cdot -}$	Cyanine	Static quenching of fluorescence between Cy5 and Cy7	Oxidative decomposition of Cy7 moiety	✓	24

(continued)

Table 5.2 (continued)

Name	Target species	Fluorophore(s)	Features	Sensing mechanism	Physiological application	Ref.
			NIR fluorescence			
DAF2[a]	NO	Fluorescein	Reactive to NO^+	Triazole formation	✓	25
			DA derivative available			
DAF-FM[a]	NO	Fluorescein	Reactive to NO^+	Triazole formation	✓	26
			Less pH sensitivity than DAF2			
DAR[a]	NO	Rhodamine	Reactive to NO^+	Triazole formation	✓	27
			Resistance to photo-bleaching			
DCl-DA Cal	NO	Calcein	Reactive to NO^+	Triazole formation	✓	28
			High intracellular retention			
DAC-P, DAC-S	NO	Cyanine	Reactive to NO^+	Triazole formation		29
			NIR fluorescence			
CuFL[a]	NO	Fluorescein	Reactive to NO·	Reduction and release of Cu^{2+}	✓	30
Cu_2(FL2A)	NO	Fluorescein	Reactive to NO·	Reduction and release of Cu^{2+}	✓	31
			High intracellular retention			
MNIP-Cu	NO	MNIP	Reactive to NO·	Reduction and release of Cu^{2+}	✓	32
mBCl[a]	GSH	Bimane	Applicable to two photon excitation	Glutathionylation	✓	33

1. Maeda et al. (2007), 2. Miller et al. (2007), 3. Dickinson et al. (2010), 4. Albers et al. (2008), 5. Srikun et al. (2008), 6. Albers et al. (2006), 7. Dickinson and Chang (2008), 8. Srikun et al. (2010), 9. Van de Bittner et al. (2010), 10. Dickinson et al. (2011a), 11. Dickinson et al. (2011b), 12. Karton-Lifshin et al. (2011), 13. Abo et al. (2011), 14. Setsukinai et al. (2003), 15. Izumi et al. (2009), 16. Koide et al. (2007), 17. Shepherd et al. (2007), 18. Kenmoku et al. (2007), 19. Koide et al. (2011), 20. Ueno et al. (2006), 21. Yang et al. (2006), 22. Sun et al. (2009), 23. Yu et al. (2011), 24. Oushiki et al. (2010), 25. Kojima et al. (1998a, c), 26. Kojima et al. (1999), 27. Kojima et al. (2001), 28. Izumi et al. (2009), 29. Sasaki et al. (2005), 30. Lim et al. (2006), 31. McQuade et al. (2010), 32. Ouyang et al. (2008), 33. Rice et al. (1986)

[a]Commercially available

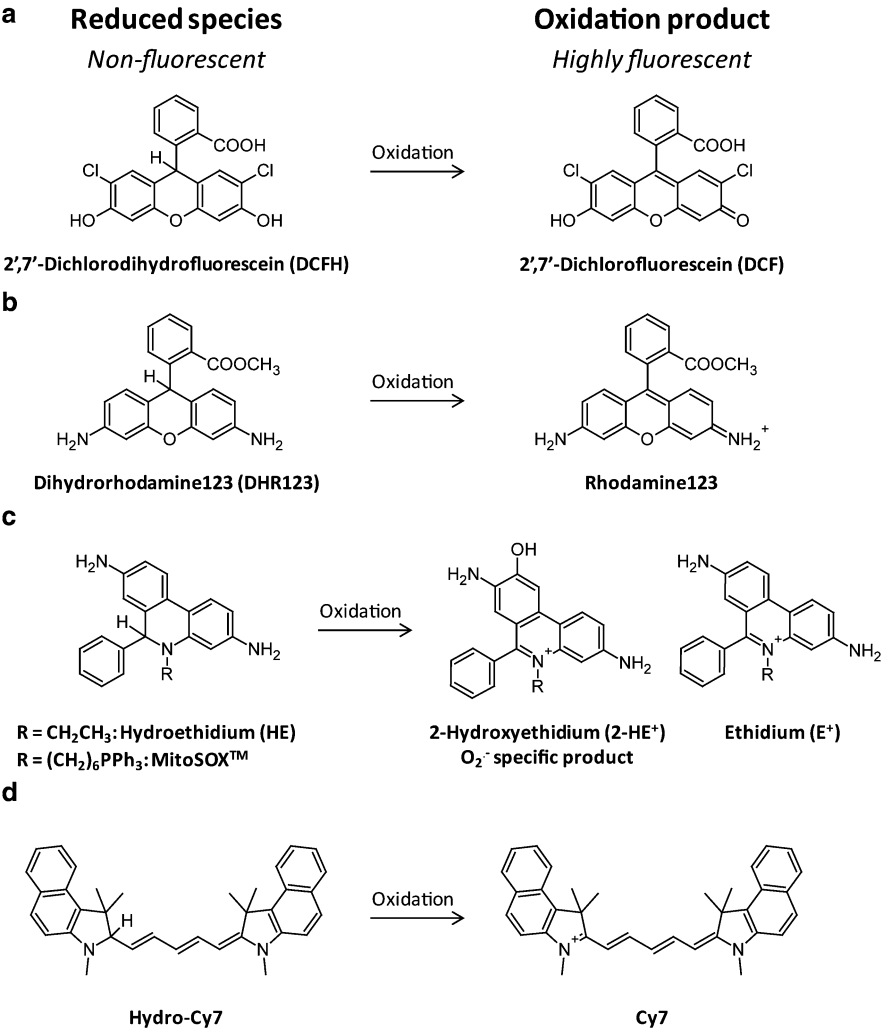

Fig. 5.1 Hydro-type fluorescent probes DCFH (**a**), DHR123 (**b**), DHE and MitoSOX™ Red (**c**), and hydroCy7 (**d**)

further oxidized to DCF with the concomitant generation of $O_2^{\cdot -}$. Thus, DCFH-induced $O_2^{\cdot -}$ formation (and subsequent formation of H_2O_2) likely fuels further DCFH oxidation, potentially leading to an overestimation of ROS production (Wrona et al. 2005). In addition to these considerations, DCFH may also diffuse spontaneously out of cells or be actively removed by membrane transporters. Thus it is often not clear to what extent differences or changes in fluorescence actually reflect intracellular ROS production (Halliwell and Whiteman 2004; Wardman 2007). Despite these limitations, DCFH-DA has been applied widely to the study

of redox changes in multicellular organisms, including *C. elegans* and zebrafish (Schulz et al. 2007; Morgan et al. 2010; Kishi et al. 2008; Harding et al. 2003).

5.2.1.2 Dihydrorhodamine123 (DHR123)

DHR123 is based on the rhodamine fluorophore and is frequently used as a ROS probe (Fig. 5.1b). DHR123 readily crosses cellular plasma membranes and thus will likely distribute in the cytosol. DHR123 appears to suffer from many of the problems already described for DCFH and the intracellular reactivity of DHR123 is complex and poorly understood. Like DCFH, DHR123 has been shown to have very low intrinsic reactivity towards H_2O_2 and $O_2^{\cdot -}$ (Hempel et al. 1999). Instead, the reaction of DHR123 with H_2O_2 is dependent on catalysis and was shown to be mediated by peroxidases and oxidases, including xanthine oxidase (Hempel et al. 1999). Consequently, differences or changes in the expression level of peroxidases and oxidases may affect intracellular DHR123 oxidation. Like DCFH, DHR123 is susceptible to photo-oxidation. Following oxidation, the positively charged and hydrophobic product, rhodamine123, accumulates in the mitochondrial matrix due to the negative potential of the mitochondrial inner membrane (Johnson et al. 1981). Therefore, the extent of matrix accumulation may be affected by changes in mitochondrial membrane potential (Johnson et al. 1981). Finally, it should be noted that the level of MRP expression is known to influence the intracellular concentration of rhodamine123 (Ludescher et al. 1992).

5.2.1.3 Hydroethidium (HE) and Mito-HE

Hydroethidium (HE) and Mito-HE (known as MitoSOX™ Red) are commercialized as probes specific for $O_2^{\cdot -}$ (Fig. 5.1c). MitoSOX™ Red is an HE derivative with a triphenylphosphonium (TPP) moiety for mitochondrial targeting. Photo-oxidation of HE is ten times more rapid than that of DCFH and DHR123, leading to high background fluorescence (Buxser et al. 1999). It has been shown that HE can react directly with ferricytochrome c (Benov et al. 1998) and similar to DCFH and DHR123, HE may be oxidized by peroxidases (Wardman 2007). However, recent work suggests that $O_2^{\cdot -}$-mediated HE oxidation can be measured more specifically. It is believed that HE initially interacts with heme proteins to form an ethidium radical ($HE^{\cdot +}$) intermediate, independent of the presence of $O_2^{\cdot -}$ (Robinson et al. 2006). By radical coupling with $O_2^{\cdot -}$ the intermediate can further oxidize to yield 2-hydroxy-ethidium ($HO\text{-}E^+$). In contrast, one electron oxidation by other electron acceptors generates ethidium (E^+), in a reaction possibly mediated by heme proteins (Robinson et al. 2006). Both oxidation products rely on intercalation into DNA to become highly fluorescent. The much faster rate of radical coupling compared to one electron oxidation likely ensures that $HO\text{-}E^+$, rather than E^+, is the dominant product that results from the interaction with $O_2^{\cdot -}$ (Robinson et al. 2006). Thus, for specific detection of $O_2^{\cdot -}$ it is crucial to differentiate between the two different

oxidation products. The different excitation wavelengths (385–405 and 480–520 nm for HO-E^+ and E^+, respectively) may allow to discriminate between the two species (Robinson et al. 2006, 2008). However, in nearly all previous studies using HE or MitoSOX™ Red, fluorescence excitation was in the 480–520 nm range, therefore E^+ rather than HO-E^+ was measured, and it remains unclear if $O_2^{\cdot -}$ production was specifically detected. Astrocytes expressing a superoxide dismutase mutant associated with amyotrophic lateral sclerosis were shown to exhibit increased HO-E^+ generation (Robinson et al. 2008).

5.2.1.4 Hydrocyanine-Based Probes

Reduced cyanine-based probes known as hydrocyanines, e.g. Hydro-Cy3 and Hydro-Cy7, fluorescent in the NIR range, were recently employed as ROS probes (Kundu et al. 2009). Hydrocyanines can be obtained by $NaBH_4$-mediated reduction of commercially available cyanine dyes. Similar to other hydro-type probes, reduction disrupts π-conjugation of the cyanine fluorophores, making them non-fluorescent. *In vitro,* direct oxidation by $O_2^{\cdot -}$ or HO· recovered the original cyanine fluorophore (Fig. 5.1d). Hydro-Cy3 was found to be oxidized in live cells and tissue explants in response to angiotensin II and lipopolysaccharide (LPS) stimulation, respectively (Kundu et al. 2009). Hydro-Cy7 oxidation was observed *in vivo* during acute inflammation in LPS-treated mice (Kundu et al. 2009). However, in each case, it remains unclear which ROS are driving probe oxidation. Further probe characterization is required to understand the mechanism of oxidation and to assess specificity and pharmacokinetics.

5.2.2 Fluorescent Probes Selective to Oxidants

Recently, chemical fluorescent probes which promise much greater ROS specificity have been developed (Chen et al. 2011). The deliberate design of chemical moieties which react directly and selectively with defined redox species forms the basis for this group of probes. Although these probes should be preferable over hydro-type probes, most of them are not yet commercially available. Several of these probes have so far only been described in one publication and thus remain incompletely characterized, especially in terms of their *in vivo* applicability.

5.2.2.1 $O_2^{\cdot -}$-Selective Fluorescent Probes

The probe 4, 5-dimethoxy-2-nitro-benzenesulfonyl tetrafluorofluorescein (BESSo) becomes fluorescent in the presence of $O_2^{\cdot -}$ (Maeda et al. 2007). Fluorescence is initially quenched because the fluorescein hydroxyl group is protected as a 4, 5-dimethoxy-2-nitro-benzenesulfonyl (BES) ester. Nucleophilic substitution of

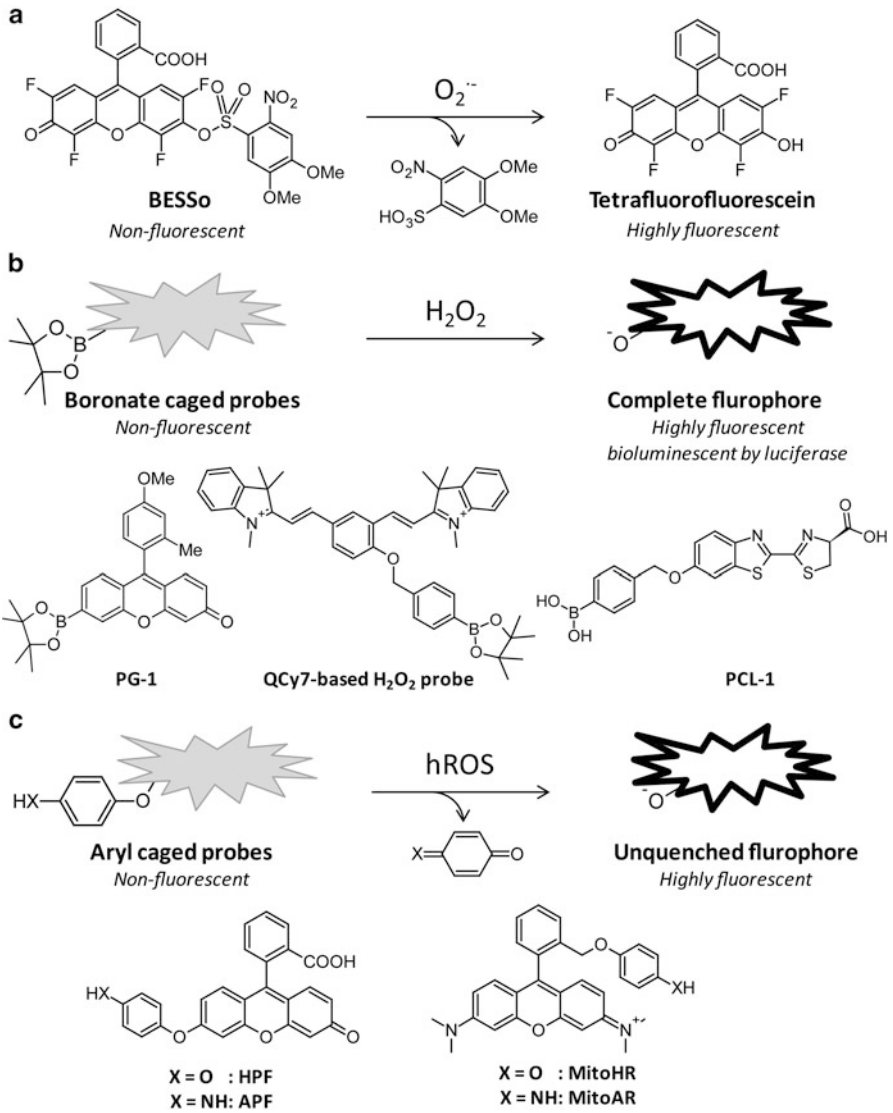

Fig. 5.2 Fluorescent probes selective for superoxide (**a**), hydrogen peroxide (**b**) and hROS (**c**)

the sulfur atom in the BES ester by $O_2^{\cdot -}$ releases the highly fluorescent tetrafluorofluorescein (Fig. 5.2a). *In vitro* experiments suggest that BESSo has little reactivity with a wide range of other redox species, including the dominant cellular nucleophile GSH. An acetoxymethyl derivative of BESSo, which allows for more efficient cellular loading, was used to detect $O_2^{\cdot -}$ generation in activated human neutrophils and following butyric acid treatment of Jurkat T cells (Maeda et al.

2007). The reaction rate of BESSo oxidation by $O_2^{\cdot -}$ has not yet been determined. Thus, the ability of the probe to compete with endogenous $O_2^{\cdot -}$ scavenging systems remains unclear. This aspect may be especially important when studying processes generating low levels of $O_2^{\cdot -}$.

5.2.2.2 H_2O_2-Selective Fluorescent Probes

The selective detection of H_2O_2 has been accomplished by the use of three different chemical strategies. The first strategy utilizes aryl boronate cages as protecting groups. H_2O_2 selectively opens the boronate cage, thus deprotecting the fluorescent dye (Fig. 5.2b). This principle forms the basis for a series of H_2O_2 probes with various spectral and chemical properties, as described below. Probes with a range of different fluorescence emission wavelengths have been created, offering much greater flexibility for making simultaneous measurements with other probes, or potentially for parallel H_2O_2 measurement in more than one subcellular compartment (Albers et al. 2008; Dickinson et al. 2010). Aryl boronate cage-based probes based on fluorescence resonance energy transfer (FRET) (e.g. Ratio Peroxyfluor-1) or internal charge transfer (ICT) (e.g. Peroxy Lucifer-1) have also been generated (Albers et al. 2006; Srikun et al. 2008). These probes enable ratiometric measurement which reduces concerns relating to differential cellular probe accumulation. Further, targeting of these probes to the mitochondrial matrix and nucleus has been demonstrated, thus enabling a certain degree of compartment-specific H_2O_2 detection. (Dickinson and Chang 2008; Srikun et al. 2010; Dickinson et al. 2011a, b). NucPE1, which localizes to the nucleus, has been used for H_2O_2 detection in *C. elegans* (Dickinson et al. 2011b).

A new type of cyanine, QCy7, which fluoresces in the near-infrared region, has also been developed to utilize the boronate-cage protection mechanism for H_2O_2 detection (Karton-Lifshin et al. 2011). π–conjugation in the fluorophore is disturbed by caging with a boronic acid benzyl ether, resulting in fluorescence quenching. The reaction with H_2O_2 drives the release of the caging moiety to generate highly fluorescent, deprotected QCy7. A QCy7-based H_2O_2 probe has already been applied to a mouse model of inflammation, and a fluorescent signal was observed in inflamed tissues (Karton-Lifshin et al. 2011).

The boronate cage protection strategy has also been extended to a bioluminescence-based probe (Van de Bittner et al. 2010). PeroxyCaged Lucifecin-1 (PCL-1) is a firefly luciferin derivative that is unreactive towards luciferase. Its reaction with H_2O_2 leads to the selective removal of the boronate cage, releasing luciferin which then acts normally as a substrate for luciferase, thus producing a bioluminescent readout. PCL-1 was used to show H_2O_2 production in an LNCaP-luc tumor xenograft model following testosterone stimulation (Van de Bittner et al. 2010).

Some boronate-based probes have the disadvantage that they react with H_2O_2 rather slowly, with rate constants of $\sim$1 M^{-1} s^{-1} *in vitro*, as shown for Peroxy Green-1 (PG1) and Peroxy Crimson (PC1). It is not clear what drives the observed fluorescence increase of these probes *in cellulo* given that the highly abundant and

efficient H_2O_2 scavenging enzymes (rate constants up to 10^7–10^8 M^{-1} s^{-1}) should almost completely outcompete the probes' reaction with H_2O_2 (Rhee et al. 2010). Further, it should be noted that phenyl boronates were recently shown to react much more efficiently with $ONOO^-$ and HOCl than with H_2O_2 (Sikora et al. 2009), which may be an important consideration when applying boronate-based probes to inflammation models.

The second chemical strategy for the detection of H_2O_2 is the use of a benzil (dibenzoyl) moiety, which selectively reacts with H_2O_2 to form benzoic anhydride via a Baeyer-Villiger reaction and subsequent hydrolysis. The only probe currently based on this mechanism, NBzF, has a much larger fluorescence enhancement ratio than boronate-based probes due to stronger fluorescence quenching prior to the reaction (Abo et al. 2011). NBzF is able to detect physiologically relevant signaling concentrations of H_2O_2 as demonstrated in epidermal growth factor (EGF)-stimulated A431 cells (Abo et al. 2011).

A third strategy for the detection of H_2O_2 involves peroxalate nanoparticles, which carry a peroxylate ester and a fluorescent dye (Lee et al. 2007). One advantage of this probe is light-source independence. The peroxalate ester is thought to react with H_2O_2 specifically, giving rise to a high energy dioxetanedione intermediate which excites the dye to emit in the NIR range. Another advantage of this probe is that the fluorescence emission wavelength can be tuned by the choice of different fluorescent dyes. Fluorescence emission from peroxalate nanoparticles was observed in a mouse inflammation model (Lee et al. 2007).

5.2.2.3 hROS-Selective Fluorescent Probes

2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) have been introduced as probes for highly reactive oxygen species (hROS) (Setsukinai et al. 2003). The intrinsic fluorescence of HPF and APF is quenched by the protection of the phenolic hydroxyl group at the 6′-position of fluorescein by electron-rich 4-hydroxyphenoxyl and 4-aminophenoxyl groups, respectively. Upon reaction with hROS, an O-dearylation reaction releases fluorescein (Fig. 5.2c) (Setsukinai et al. 2003). HPF is known to react selectively with HO· and $ONOO^-$ to become highly fluorescent (Setsukinai et al. 2003). In addition to HO· and $ONOO^-$, APF also reacts with HOCl, thus HOCl production can be infered by comparison between APF and HPF. Both probes are highly resistant to photo-oxidation. Although HPF and APF have clear advantages for detecting hROS, the fluorescent product fluorescein can be transported out of cells. Intracellular retention of APF was improved by the introduction of two imino-diacetic acid groups, yielding APC (Izumi et al. 2009). This simple concept of increasing cellular retention by introducing negative charge to yield a calcein–based structure may serve as a general strategy to increase the sensitivity of fluorescent probes in living cells (Izumi et al. 2009).

MitoHR and MitoAR are based on the rhodamine fluorophore. The positive charge of rhodamine drives localization to the mitochondrial matrix. One advantage of rhodamine-based probes is a general resistance to laser irradiation-induced photo-bleaching, making them especially suitable for fluorescence microscopy (Koide et al. 2007). The intrinsic probe fluorescence is quenched by the electron rich 4-hydroxyl- and 4-aminophenyl ether moieties. Reaction with hROS leads to cleavage of the ether moiety, giving rise to 2-hydroxymethyl tetramethylrhodamine (HMTMR), a highly fluorescent product (Fig. 5.2c) (Koide et al. 2007). MitoHR mainly reacts with HO·, and to a lesser extent with $ONOO^-$ and HOCl, whereas MitoAR reacts with HO· and HOCl, and to a lesser extent with $ONOO^-$. MitoAR was shown to detect myeloperoxidase (MPO)-derived HOCl in HL60 cells (a human promyelocytic leukemia cell line) stimulated with H_2O_2, but not in HeLa cells (which do not express MPO) (Koide et al. 2007).

There are several fluorescent probes considered to detect HOCl specifically, including sulfonaphthoaminophenyl fluorescein (SNAPF), which emits in the NIR region (Shepherd et al. 2007). Similar to the protection mechanism employed in APF, the fluorescence of the unreacted probe is quenched by a 4-aminophenoxyl moiety. In SNAPF, but apparently not APF, this moiety is selectively removed by reaction with HOCl. SNAPF has been used *in vivo* to demonstrate HOCl production in a mouse peritonitis model (Shepherd et al. 2007).

HySox is also designed to specifically detect HOCl and is based on a ring-opening mechanism (Kenmoku et al. 2007). It is initially colorless and non-fluorescent because of a closed ring structure that disrupts π–conjugation in the fluorophore. Upon oxidation of the sulfur atom in the five-membered ring structure, the rhodamine fluorophore is formed. An advantage of this probe is the complete absence of fluorescence in the unreacted molecule. Thus, any increase in fluorescence can be ascribed with a high degree of certainty to HOCl rather than other factors such as unreacted probe accumulation. This property of HySox leads to excellent signal-to-background ratios in contrast to probes that contain the mature fluorophore prior to activation and thus can generate high background signals by simple accumulation (Kenmoku et al. 2007). HySox has been used to detect HOCl production in activated neutrophils (Kenmoku et al. 2007). The same principle has been extended to the NIR fluorophore MMSiR and its water-soluble analogue, wsMMSiR. These are based on the Si-rhodamine NIR fluorophore, and have enabled HOCl detection *in vivo* in a mouse peritonitis model (Koide et al. 2011).

Several fluorescent probes are considered to specifically detect $ONOO^-$. Mechanistically, these probes are based on either nitration (NiSPY-1) (Ueno et al. 2006), formation of a dienone from a trifluoromethyl ketone (HKGreen-1 and HKGreen-2) (Yang et al. 2006; Sun et al. 2009) or selenium oxidation (CyP-Se) (Yu et al. 2011). Of these, HKGreen-2 and CyP-Se, based on BODIPY and cyanine fluorophores, respectively, were applied to demonstrate $ONOO^-$ generation in stimulated macrophages (Sun et al. 2009; Yu et al. 2011). As CyP-Se fluoresces in the NIR region, it may have potential for future applications *in vivo*.

The cyanine-based probe FOSCY-1 consists of two fluorophores, $Cy5SO_3H$ and the Cy7-based IR786S (Oushiki et al. 2010). Fluorescence is quenched by self-stacking between the two hydrophobic fluorophores. Activation of fluorescence is based on the different reactivities of Cy5 and Cy7 to a range of ROS. Upon oxidation of the IR786S moiety by HO·, $ONOO^-$ or HOCl, and to a lesser extent by $O_2^{\cdot -}$, the Cy5 moiety becomes fluorescent due to the loss of intramolecular stacking. FOSCY-1 has been successfully used to detect ROS production in an *in vivo* peritonitis model (Oushiki et al. 2010). Although reactive to a broad range of ROS, the oxidation mechanism of this probe suggests that one advantage may be a very slow rate of photo-oxidation.

5.2.2.4 NO· Selective Fluorescent Probes

NO·-selective fluorescent probes are based either on an *o*-phenylenediamine moiety or a metal complex. The former are indirect NO· probes because they actually react with nitrosonium equivalents (NO^+/N_2O_3) to generate a triazole (Fig. 5.3a). It is assumed that in the presence of molecular oxygen, nitrosonium equivalents are continuously generated from NO· (Ignarro et al. 1993) and can be taken as a proxy for NO·. The diaminofluorescein (DAF) probes are based on this principle (Kojima et al. 1998a, b, 1999). DAF2-DA has been used to detect NO· production in the CA1 region of hippocampus brain slices following stimulation with *N*-methyl-D-aspartic acid (NMDA) (Kojima et al. 1998b). However, DAF2-DA suffers from pH sensitivity and susceptibility to photo-bleaching. DAF-FM was developed to be less sensitive to pH and photo-bleaching than DAF2. Using a di-acetate derivative of DAF-FM, changes in NO· production were observed in the notochord and caudal fin of developing zebrafish (Lepiller et al. 2007). However, it remains to be unambiguously demonstrated that the changes observed with DAF-FM-DA are solely a result of NO· production rather than a differential accumulation of the unreacted probe or fluorescent product. Diaminorhodamine (DAR)-4M, based on the rhodamine fluorophore, is less sensitive to photo-bleaching and pH changes than any of the DAF probes (Kojima et al. 2001). DAR-4M has been used to demonstrate cell layer-specific NO· production in freshly prepared mouse cortical slices upon NMDA stimulation (Imura et al. 2005).

Although the DAF and DAR probes are promising tools for detecting intracellular NO· in cell culture or transparent model organisms, they have the disadvantage of limited cellular retention, thus decreasing their sensitivity for NO· detection. This problem has recently been overcome by the introduction of imino-diacetic acid groups into DAF4, producing dichloro-diamino-calcein (DCl-DA-Cal). DCl-DA-Cal was found to be more sensitive than DAF2 in detecting NOS-generated NO· in bovine aortic endothelial cells upon bradykinin stimulation (Izumi et al. 2009). Cyanine-based diaminocyanine (DAC)-P and DAC-S have also been created to enable NO· detection in the NIR region. DAC-P detected NO· release in isolated rat kidneys perfused with the NO·-generating compound NOC13 (Sasaki et al. 2005).

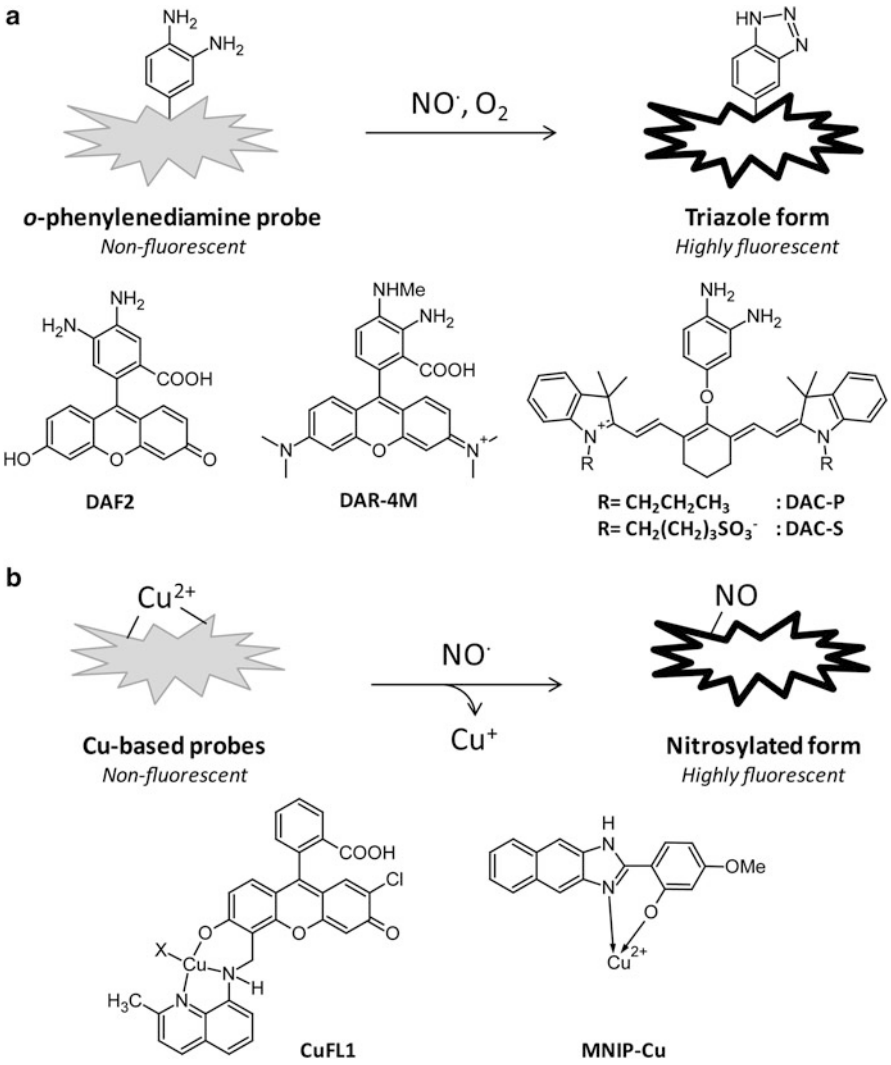

Fig. 5.3 Fluorescent probes selective for nitric oxide based on *o*-phenylenediamine (**a**) or metal complex (**b**)

In contrast to *o*-phenylenediamine-based probes, metal complex-based probes detect the NO· radical directly (Fig. 5.3b). To date, all probes developed in this class consist of a fluorophore ligand coordinating a paramagnetic Cu^{2+} ion, which quenches the fluorescence of the fluorophore. Reduction of Cu^{2+} to Cu^{+} by NO· releases a nitrosylated and highly fluorescent fluorophore ligand. CuFL, a fluorescein-based probe based on this mechanism has been reported to detect NO· generated by constitutive and inducible NOS in SK-N-SH human neuroblastoma

cells and Raw264.8 murine macrophages, respectively (Lim et al. 2006). In a direct comparison with DAF2-DA, CuFL generated a brighter signal in response to cNOS activation. The intracellular retention of Cu-based NO· probes has been improved with the development of Cu_2(FL2A), in which two additional negative charges have been introduced (McQuade et al. 2010). Cu_2(FL2E) contains two carboxylic acids protected as ethyl esters which can be cleaved by intracellular esterases. Despite the fact that this probe needs to react with two NO· molecules in order to become fluorescent, its improved cellular retention renders it more sensitive to NO· than CuFL. Application of Cu_2(FL2E) to murine olfactory bulb brain slices successfully detected cell layer-specific NO· generation (McQuade et al. 2010). Another probe utilizing a Cu-based quenching mechanism, MNIP-Cu, is based on the 4-methoxy-2-(1H-naphtho [2,3-d] imidazol-2-yl) phenol (MNIP) fluorophore (Ouyang et al. 2008). Reaction with NO· generates *N*-nitroso MNIP as an NO· reaction-specific fluorescent product. MNIP-Cu does not seem to exhibit acute *in vivo* toxicity. MNIP-Cu injection has been applied to a mouse liver injury model, where a NO·-dependent fluorescent increase was detected in cryosections *in situ* (Ouyang et al. 2008).

5.2.3 Fluorescent Probes Reactive to Thiols

The compartment-specific concentration and redox state of intracellular glutathione is one of the key elements of cellular redox homeostasis. Chemical probes for glutathione are only able to measure relative differences in the concentration of free GSH. Such probes must be membrane-permeable and should be able to discriminate GSH from other thiol-containing molecules, in particular protein thiols and cysteine. Only monochlorobimane (mBCl), i.e. syn-(1-chloroethyl)-1,5-diazabicycla[3.30]acta-3,6,-diene-2,8-dione, is commercially available at this time, although several other fluorescent probes for the detection of intracellular GSH have been reported (Chen et al. 2010). mBCl is essentially non-fluorescent until it forms a strongly fluorescent glutathione-bimane adduct. Adduct formation is catalyzed by intracellular glutathione S-transferases (GSTs) and it is typically assumed that mBCl reacts mainly with cytosolic GSH, due to the abundance of GST activity in this compartment. mBCl is frequently applied to measure relative differences in cytosolic GSH concentration between cell types. However, when making such comparisons it is necessary to ensure that the fluorescent signal has reached a 'plateau' level for all samples. Otherwise, the measurements can be affected by cell-type specific differences in GST activity, which influence the rate of fluorescence accumulation but should not alter the final fluorescence intensity. When mBCl was applied to measure the distribution of GSH concentrations in cancer cells from a primary tumor, substantial variability in mBCl fluorescence was observed (Rice et al. 1986; Cook et al. 1991). Further, cell-type specific mBCl fluorescence was observed by microscopy in both plant tissues and brain slices (Keelan et al. 2001; Meyer and Fricker 2000; Meyer et al. 2001; Sun et al. 2006). As a note of

caution, mBCl is routinely used to measure the activity of glutathione conjugate transporters (Ishikawa et al. 1994), whose expression varies between cell types and situations. Thus the process of glutathione-bimane adduct efflux likely influences the intracellular fluorescence to different extents in different cell types. Further, the general specificity of mBCl for glutathione has been questioned (van der Ven et al. 1994).

5.3 Genetically Encoded Redox Probes and Their Applications

Genetically encoded probes have two major advantages over currently available chemical probes. These are (i) precise targeting and (ii) redox species specificity. In principle, genetically encoded probes can be accurately targeted to almost any subcellular location. This is achieved either by the addition of appropriate targeting sequences or via translational fusion to a protein which localizes to the cellular location of interest. Redox species specificity is not an inherent property of all genetically encoded probes but is typically realized by the incorporation of an oxidant-specific protein domain within the probe structure, which serves to couple the probe response to a defined redox species. Current genetically encoded redox probes can be categorized by their mode of operation, into the following four classes:

1. Fluorescent proteins (FPs) that have been engineered to be redox sensitive by placement of a pair of cysteines onto the surface of the beta barrel. There are two archetypes in this class: redox-sensitive yellow fluorescent protein (rxYFP) and reduction-oxidation-sensitive green fluorescent protein (roGFP).
2. Fusion proteins based on the redox-sensitive FPs in class 1. They are engineered redox relays in which a redox enzyme is translationally fused to a redox-sensitive FP in order to catalyze its equilibration with a defined redox pair. This class includes the E_{GSH} probe Grx1-roGFP2 and the H_2O_2 probe roGFP2-Orp1.
3. Probes based on a FP that is not redox sensitive in itself but is responsive to conformational changes in a redox-sensitive protein domain to which it is fused. The best known example being the H_2O_2 probe HyPer.
4. FRET probes which either make use of thiol-containing peptide linkers or redox-sensitive protein domains to modulate the spatial arrangement of two FPs which make up a donor-acceptor FRET pair.

Representatives of the four classes are listed in Table 5.3 and those of particular interest will be discussed briefly in the following subsections. Aspects that have been covered in recent reviews (Meyer and Dick 2010; Morgan et al. 2011; Bjornberg et al. 2006a), in particular molecular mechanisms, thermodynamic considerations, and principles of ratiometric fluorescence measurements, will not be discussed here.

Table 5.3 Genetically encoded redox probes

Type	Name	Ratiometric (pH sensitivity)	FP	Additional sensing domain	Specificity	*In vivo* applications reported	Ref.
I	rxYFP		YFP	none	GSSG/GSH (dependent on endogenous Grx)		1
I	roGFP1	✓ (−)	GFP	none	GSSG/GSH (dependent on endogenous Grx)	✓	2
I	roGFP2	✓ (−)	EGFP	none	GSSG/GSH (dependent on endogenous Grx)	✓	2
II	rxYFP-yGrx1		YFP	Grx1 (*S. cerevisiae*)	GSSG/GSH (E_{GSH})		3
II	Grx1-roGFP2	✓ (−)	EGFP	Grx1 (*H. sapiens*)	GSSG/GSH (E_{GSH})	✓	4
II	roGFP2-Grx1	✓ (−)	EGFP	Grx1 (*H. sapiens*)	GSSG/GSH (E_{GSH})	✓	5
II	roGFP2-Orp1	✓ (−)	EGFP	Orp1 (*S. cerevisiae*)	H_2O_2	✓	6
III	HyPer	✓ (!)	cpYFP	OxyR (*E. coli*)	H_2O_2	✓	7
III	OHSer		cpVenus	OhrR (*X. campestris*)	ROOH (organic hydroperoxides)		8
III	Peredox	✓ (−)	cpGFP	Rex (*Thermus aquaticus*)	$NADH/NAD^+$		9
III	Frex	✓ (!)	cpYFP	Rex (*B. subtilis*)	NADH		10
IV	HSP-FRET	✓ (?)	CFP/YFP	Hsp-33 (*E. coli*)	? (incl. H_2O_2, NO·, HOCl)		11
IV	CY-RL5 & CY-RL7	✓ (?)	ECFP/EYFP	Peptide linker with 4 Cys	? (incl. GSH/GSSG)		12
IV	Redoxfluor	✓ (?)	Cerulean/Citrine	Peptide linker with 4 Cys (C-terminus of Yap1)	? (incl. H_2O_2, GSH/GSSG, Trx)		13
IV	FRET-MT	✓ (?)	ECFP/EYFP	Metallothionein (MT)	? (incl. NO·)	✓	14

1. Ostergaard et al. (2004), 2. Dooley et al. (2004), Hanson et al. (2004), 3. Bjornberg et al. (2006b), 4. Gutscher et al. (2008), 5. Albrecht et al. (unpublished data), 6. Gutscher et al. (2009), 7. Belousov et al. (2006), 8. Simen Zhao et al. (2010), 9. Hung et al. (2011), 10. Zhao et al. (2011), 11. Waypa et al. (2006), 12. Kolossov et al. (2008, 2011), 13. Yano et al. (2010), 14. Pearce et al. (2000)

In the following discussion of genetically encoded probes we have not included circularly permuted YFP (cpYFP), which has been suggested to be responsive to the presence of $O_2^{\cdot -}$ (Wang et al. 2008). The validity of this suggestion remains controversial (Muller 2009; Schwarzlander et al. 2011; Huang et al. 2011), with the main issue being that cpYFP is inherently highly pH sensitive. Therefore, it is conceivable that a pH response has been misinterpreted as a $O_2^{\cdot -}$ response (Schwarzlander et al. 2011). pH sensitivity must always be taken into account when considering the use of cpYFP or cpYFP-based probes as discussed for HyPer below.

5.3.1 Fluorescent Proteins with an Engineered Dithiol-Disulfide Pair

The founding members of this class are rxYFP (Ostergaard et al. 2004) and the roGFPs 1 and 2 (Hanson et al. 2004; Dooley et al. 2004). Both types of probe are engineered to contain a pair of cysteine residues, C149/C202 in the case of rxYFP and C147/C204 in the case of the two roGFPs. In both instances, the formation of a disulfide bridge between the engineered cysteine residues changes the fluorescent properties of the protein, thus allowing the determination of the redox state of the engineered dithiol-disulfide redox pair. When expressed inside cells, both probes predominantly, if not exclusively, equilibrate with the glutathione redox couple. Importantly, this equilibration is not a spontaneous process, but rather depends on the catalytic activity of endogenous glutaredoxins (Grx) (Ostergaard et al. 2004; Meyer et al. 2007). However, this implies that differences and changes in endogenous Grx expression or activity may affect the state of the probe in a way that does not properly reflect the actual E_{GSH}. This possibility must be kept in mind when comparing different cell types or the same cell type under different conditions.

Applications in multicellular organisms have focused on roGFPs (roGFP1 and roGFP2), rather than rxYFP, for two main reasons: (i) they allow ratiometric measurements and (ii) the measured ratio is resistant to perturbation by changes in pH and halide ion concentration. RoGFP1 is based on wild type GFP and exhibits a redox potential of about −290 mV, whilst roGFP2 is based on the brighter EGFP and has a slightly less negative redox potential of about −280 mV. These low midpoint potentials make both probes suitable for measurements in the cytosol, nucleus, mitochondria, and peroxisomes. However, their utility is limited in the more oxidizing environment of the endoplasmic reticulum (ER). For this reason, roGFP variants with higher midpoint potentials (roGFP1-iX probes) have been developed recently (Lohman and Remington 2008). Attempts to establish the use of these probes in the ER are ongoing (Delic et al. 2010; van Lith et al. 2011).

The first application of roGFPs in a multicellular organism was in the plant *Arabidopsis thaliana*. One early study asked if different cell types in the root display recognizable redox differences; transgenic expression of cytosolic and mitochondrially-targeted roGFP1 under a viral promoter apparently identified

increased probe oxidation in the elongation zone of the root tip relative to the other parts of the root (Jiang et al. 2006). Several studies have since applied roGFPs to investigate the impact of stress conditions. Increased oxidation of roGFP2 was observed in response to mechanical wounding of leaves (Meyer et al. 2007). Oxidation of mitochondrial-targeted roGFP2 (mt-roGFP2) was visualized in seedlings exposed to heat, salt and heavy metals (Schwarzlander et al. 2009). Apparent oxidation of cytosolic roGFP1 was observed in response to water stress (Jubany-Mari et al. 2010) and dark-induced senescence in leaves (Rosenwasser et al. 2011). Finally, two *Arabidopsis* studies combined redox imaging with genetic manipulations. Depletion of mitochondrial manganese superoxide dismutase (MnSOD) led to increased oxidation of mitochondrially-targeted roGFP1, but had no significant effect on cytosolic roGFP1 in leaf epidermis cells (Morgan et al. 2008). Further, deletion of all three *Arabidopsis* homologues of the *Plasmodium falciparum* chloroquine-resistance transporter family led to increased cytosolic roGFP1 oxidation, but had no effect on plastid-localized roGFP1. This study showed that the transporters have a role in regulating cytosolic GSH levels, probably by mediating glutathione or γ-glutamylcysteine transport to the cytosol (Maughan et al. 2010).

The first application in mice was the expression of roGFP2 in the liver by adenoviral gene transfer. Hepatic ischemia was found to induce roGFP2 reduction, whilst subsequent reperfusion led to roGFP2 oxidation, the degree of which was dependent on the length of the preceding ischemia and which correlated with subsequent liver damage as assessed by caspase-3 activity and serum levels of damage markers (Haga et al. 2009). So far, two kinds of roGFP-transgenic mice have been reported. First, mice expressing mitochondrially-targeted roGFP2 in dopaminergic neurons under the control of the tyrosine hydroxylase promoter have been used for two photon imaging of midbrain slices. The results indicated that roGFP2 in the substantia nigra is more oxidized than in the ventral tegmental area (Guzman et al. 2010). Second, mice have been created to express roGFP2 under the control of a β-globin mini-promoter, which mediates expression in red blood cells (Xu et al. 2011). The authors concluded from their data that roGFP2 in red blood cells becomes more oxidized with increasing cellular age.

Finally, roGFP1 has been expressed in the mitochondria of body wall muscle cells in *C. elegans* with the aim of observing redox changes linked to the mitochondrial fusion associated proteins EAT-3 and FZO-1. Increased roGFP1 oxidation was observed in strains containing mutant *eat*-3 alleles, but not in *fzo-1* mutants (Johnson and Nehrke 2010).

5.3.2 Fusion Proteins Based on roGFP

The observation that rxYFP and roGFPs require the activity of endogenous Grx to equilibrate with the glutathione redox couple, led to the concept of genetically fusing a Grx directly to the redox-sensitive FP. The advantage is twofold: first, the resulting fusion protein is now a complete glutathione-specific biosensor,

independent of endogenous enzymes. Second, the translational fusion increases the effective concentration of Grx by 2–3 orders of magnitude relative to unfused Grx, thus the rate of equilibration with the glutathione redox couple is strongly enhanced. The first example in this class was the fusion of rxYFP to yeast Grx1p, which was used to gain mechanistic insight into the reaction between Grx1p and rxYFP *in vitro* (Bjornberg et al. 2006b). This study found that the fusion increased the rate of GSSG-mediated rxYFP oxidation by a factor of 3,300 while the rate of rxYFP oxidation mediated by other oxidants including cystine remained unchanged. Thus, the Grx1p fusion also dramatically increased the specificity of rxYFP towards glutathione. Based on this principle the ratiometric roGFP2 was fused to human glutaredoxin-1; this construct was used to provide the first real-time measurements of the glutathione redox potential *in cellulo* (Gutscher et al. 2008).

Subsequently the concept of roGFP-based bipartite redox relays was extended to create a probe for H_2O_2 (Gutscher et al. 2009). To this end, the GSSG-sensing Grx domain was replaced with the H_2O_2-sensing oxidant receptor peroxidase 1 (Orp1) protein. Orp1 is well characterized as a sensitive and specific H_2O_2 sensor protein by which yeast cells regulate their transcriptional response to H_2O_2. Orp1 appears to contribute to H_2O_2 tolerance only as a sensor and not as a conventional H_2O_2-scavenging peroxidase (Delaunay et al. 2002). The physiological function of Orp1 is to convert H_2O_2 into a disulfide bond and to relay that disulfide bond to a recipient protein, the transcription factor Yap1, which is activated by oxidation. An adapter protein, Ybp1, is required to bring Orp1 and Yap1 into proximity and position for disulfide exchange. The key idea is to mimic the natural H_2O_2-Orp1-Yap1 relay by creating a H_2O_2-Orp1-roGFP2 relay. A peptide linker between Orp1 and roGFP2 provides proximity and thus abrogates the need for an adaptor protein. Furthermore, roGFP2 replaces Yap1, hence yielding a fluorescent instead of a transcriptional response.

Both Grx1-roGFP2 and roGFP2-Orp1 have been applied as *in vivo* probes in multicellular organisms. First, transgenic expression of cytosolic Grx1-roGFP2 in *A. thaliana* was applied to demonstrate the relative contributions of glutathione reductase and the thioredoxin system to glutathione redox homeostasis (Marty et al. 2009). Second, four different lines of transgenic *Drosophila* have been created to express either Grx1-roGFP2 in the cytosol or in mitochondria or roGFP2-Orp1 in the cytosol or in mitochondria. Of note, the domain order is critical for proper mitochondrial expression in plants and insects. While Grx1-roGFP2 and roGFP2-Grx1 are equivalent probes in terms of specificity, kinetics and dynamic range, in plants and insects only the latter one can be properly targeted to mitochondria (Albrecht et al., unpublished data). Living larvae from each of the four redox probe lines were used in combination with reverse genetics and feeding experiments (Albrecht et al. 2011). Interestingly, E_{GSH} and H_2O_2 levels were found to respond independently of each other, in a subcellular compartment- and tissue-specific manner. The pattern of changes that occurred varied according to the specific chemical treatment or genetic modification studied. In addition a chemical redox conservation method has been developed to prevent artificial roGFP disulfide-bond

formation during tissue dissection and paraformaldehyde (PFA) fixation, thereby allowing the imaging of optically-inaccessible tissue from non-transparent adult flies (Albrecht et al. 2011).

5.3.3 *Fluorescent Proteins Coupled to Redox-Responsive Proteins Domains*

Probes in this class include the H_2O_2 probe HyPer (Belousov et al. 2006), and the very recently introduced NAD^+/NADH probes Peredox (Hung et al. 2011) and Frex (Zhao et al. 2011). All three probes allow ratiometric measurements.

HyPer is based on the bacterial H_2O_2 sensor OxyR, which has been fused to cpYFP. The OxyR domain is selectively oxidized by H_2O_2 to form an intramolecular disulfide bond. The resulting conformational change subsequently changes the fluorescent properties of cpYFP. An improved variant, HyPer2, exhibits a wider dynamic range and a faster response (Markvicheva et al. 2011). Although there is no doubt that HyPer responds to H_2O_2 in a sensitive and specific manner, the significant pH sensitivity, which is inherent to cpYFP, should be given careful attention, especially in the context of *in vivo* studies. The pH sensitivity of the HyPer fluorescence ratio was already reported in the original publication (Supplementary Figure 1 of Belousov et al. 2006), but only recently it has been documented that HyPer measurements can be confounded by pH effects *in cellulo* (Roma et al. 2012). A HyPer mutant with an inactivated H_2O_2-sensing domain (C199S) can be utilized as a ratiometric pH probe (named 'synthetic pH sensor' = SypHer) (Poburko et al. 2011). In conclusion, if HyPer and SypHer are used in parallel experiments it should be possible to discriminate between H_2O_2 and pH mediated ratio changes.

To date, HyPer has been expressed in zebrafish, plants and *C. elegans*. General cytosolic HyPer expression in zebrafish was achieved by injection of HyPer mRNA into oocytes. Interestingly, following tail fin wounding, increased H_2O_2 production was observed at the wound edge, which extended up to 200 μm into the surrounding tissue, creating a gradient of decreasing H_2O_2 away from the wound margin (Niethammer et al. 2009). Very recently a Src family kinase (SFK) was proposed to act as a redox sensor in neutrophils which responds to the wounding-induced H_2O_2 gradient and mediates neutrophil recruitment to the wound site (Yoo et al. 2011). HyPer was also expressed in *A. thaliana*; cytosolic and peroxisomally-targeted HyPer was observed in epidermal leaf cells and stomatal guard cells (Costa et al. 2010) and used to suggest Ca^{2+}-dependent peroxisomal H_2O_2 scavenging.

Finally, HyPer was introduced into *C. elegans*. A plate-reader based method was used to measure HyPer fluorescence. A slight increase in the HyPer fluorescence ratio was observed in a longer lived SOD1 overexpressing strain which was interpreted as an increased steady-state H_2O_2 level. This interpretation was supported by an Amplex red assay performed on worm lysates (Cabreiro et al. 2011). Also HyPer fluorescence was measured in aging *C. elegans* (Back et al. 2011). Differences in HyPer fluorescence ratio were observed dependent upon the region of worm imaged. Further an age-dependent increase in HyPer fluorescence ratio was observed, which was delayed in calorically-restricted worms.

Most recently, two genetically encoded probes for the NAD^+/NADH redox couple have been developed. Both of them are based on the bacterial NADH-binding protein Rex. The Peredox probe (Hung et al. 2011) uses two copies of the *Thermus aquaticus* Rex protein, and interposed between them, the non-ratiometric cpGFP variant T-Sapphire. A tandemly attached mCherry provides the ratiometric reference for signal normalization. Peredox seems to report the NAD^+/NADH ratio. With a pKa of about 5, T-Sapphire should be pH insensitive in the physiological range of most cellular compartments. The Frex probe (Zhao et al. 2011) uses a tandem dimer of the Rex protein from *Bacillus subtilis* in combination with the ratiometric cpYFP. Thus, like other cpYFP-based probes, Frex is pH sensitive. Frex appears to measure changes in NADH concentration, rather than the NAD^+/NADH ratio. As these probes have just been reported in the literature, their *in vivo*/*in situ* application in model animals is a task for the future.

5.3.4 FRET-Based Redox Probes

In comparison to the probes mentioned above, FRET-based redox probes are the least investigated and their overall potential for *in vivo* studies is still to be established. FRET probes are ratiometric by design, yet their dynamic range appears to be quite limited. At least four FRET-based redox probes have been described in the literature. All of them place a redox-sensitive peptide or protein domain between the two FPs that make up the FRET pair.

In the family of RL-FRET probes, an α-helical linker peptide with four cysteine residues separates ECFP from EYFP (Kolossov et al. 2008). Formation of disulfide bonds within the α-helical linker increases FRET efficiency. The mid-point potential of the variant RL7-FRET was determined as −143 mV suggesting that this probe may find applications for measurements in the endoplasmic reticulum (Kolossov et al. 2011). In the 'Redoxfluor' probe Cerulean and Citrine are separated by a tandem repeat of the cysteine-containing 50-amino acid C-terminal peptide of the yeast transcription factor Yap1. Here, disulfide bond formation decreases FRET efficiency (Yano et al. 2010). In HSP-FRET the redox-sensitive heat shock protein Hsp33 links CFP and YFP (Waypa et al. 2006). Oxidation of Zn^{2+}-coordinating thiols in Hsp33 leads to a conformational change (Jakob et al. 1999) which is responsible for a change in FRET efficiency. Finally, FRET-MT places human metallothionein (hMTIIa) between ECFP and EYFP (Pearce et al. 2000). The probe primarily monitors binding and release of metal ions by MT. It was shown to respond to NO· which triggers release of metal ions from MT. FRET-MT seems to the only FRET-based redox probe that has been employed in a transgenic multicellular context. Expression in the mouse lung was achieved by adenoviral somatic gene transfer. The isolated lung was freshly imaged by confocal microscopy and appeared to show hypoxia-induced NO· production (Bernal et al. 2008).

All FRET probes have in common the problem of undefined specificity. Metal ion binding and release by MT is likely influenced by a number of redox- and non-redox processes. The Zn^{2+}-coordinating thiols of Hsp33 are known to react with various oxidants, including H_2O_2, NO· and HOCl. The cysteine-containing peptides of RL-FRET and Redoxfluor seem to equilibrate with GSH/GSSG, but it is unclear which additional redox reactions may occur *in vivo*. It would seem important to define which redox catalysts actually interact with the cysteines in the peptide linkers. Redoxfluor is suggested to communicate with both the glutathione and thioredoxin systems (Oku and Sakai 2012). It remains to be seen how mixed measurements of various redox couples can contribute to the study of physiological processes.

5.4 Conclusions and Outlook

5.4.1 Problems and Considerations

In recent years there has been an explosion of interest in the use of chemical and genetic probes to visualize redox species *in vivo*. There have been many publications using many different redox probes in many different systems. However, in general, very little comparison or cross-checking has been done and many examples of contradictory results exist. In addition, very few examples of established probe application methodology exist. This raises the questions: Which observations and interpretations can be trusted? And what kind of errors can lead to false conclusions? Obviously there are two major sources of human error. First, measurement and/or data processing errors (e.g. the failure to recognize measurement artifacts) and second, interpretation errors, typically based on the belief that the probe is measuring what it is supposed to measure compounded by the lack of appropriate control experiments. Here we give a few examples.

For most fluorescent probes, especially when applied on the level of tissues and organisms, fluctuating signal-to-noise-ratios and auto-fluorescence are serious issues. For example, when analyzing microscopic images based on ratiometric genetic probes, it is critical to calculate the fluorescence emission ratios only for those pixels that show above-background signals in both channels and which are not affected by autofluorescence. Inappropriate image processing procedures can lead to incorrect ratio images that indicate probe oxidation where there is in fact none. Great care should be taken to obtain control images, to identify auto-fluorescence signals and to avoid auto-fluorescent areas altogether.

In many situations and with many of the probes, interpretation of the measurements is difficult. The interpretation of hydro-type probe oxidation is most difficult because it can be influenced by factors unrelated to ROS, and the process of probe oxidation may generate further oxidants. The *in vivo* distribution of small molecule chemical fluorescent probes is usually unpredictable and uncontrolled. Systemic administration may give rise to accumulation in certain locations, leading to areas

of increased fluorescence that are unrelated to ROS production. Differential uptake and efflux can lead to the possibility that probe fluorescence is observed at a site different from the site of probe oxidation.

The response of genetically encoded probes should also be interpreted with caution. In the case of the cpYFP-based probes it may be difficult to determine if fluorescence changes are due to changes in pH rather than the redox species of interest. A general concern that applies to probes which utilize two different fluorescent proteins (e.g. FRET probes) is that differences in pH sensitivity, maturation rate and photo-stability can lead to measurement artifacts (Miyawaki 2011).

Interpretation issues can also arise when genetically-encoded probes are adapted for use in other subcellular compartments. For example, roGFPs predominantly equilibrate with the glutathione redox couple, but this requires the presence of Grxs. Nonetheless, roGFP1-iX probes are increasingly being applied with the aim of measuring E_{GSH} in the ER, which is not known to harbor any Grxs, thus it is unclear what is actually measured by these probes. Instead, the ER harbors a protein thiol oxidizing machinery and a host of protein disulfide isomerases (PDIs) involved in thiol oxidation, disulfide reduction and isomerization. At present it not known to what extent roGFP1-iX probes interact with any of these factors. Therefore, the assumption that roGFP1-iX probes report E_{GSH} in the ER is at best premature.

5.4.2 Future Developments

Evidently, more detailed probe characterization and especially cross-checking would be of great interest and utility to the redox biology field. It should be possible to compare a panel of different probes (chemical and genetic, e.g. all probes considered to respond to H_2O_2) in the same experimental system and compare the responses under various conditions and treatments. Such experiments should initially be performed in a cell culture setting, but ultimately could be extended to *in vivo* applications.

We are still a long way from obtaining the goal highlighted in Sect. 4.1.1 of being able to image any redox species of interest, in a defined subcellular compartment, with high temporal resolution in a multicellular organism. Below we describe a wish-list of developments which we believe are reasonable to expect in the near future and which are likely to make a substantial contribution to the study of redox biology.

5.4.2.1 Further Development of Chemical Probes

- *Targeting*: very few possibilities exist to target chemical probes to defined subcellular compartments of interest. The recently developed SNAP-tag technology offers the possibility to target chemical probes to specific locations,

with the limitation that this approach relies on prior genetic manipulation. A protein residing in the location of interest is genetically fused to a mutant O6-alkylguanine-DNA alkyltransferase, which reacts specifically and rapidly with benzylguanine (BG) derivatives. Fluorescent probes containing a BG moiety will be retained in the specific location of the SNAP-tag (Srikun et al. 2010). The SNAP-tag principle may be applicable to a range of chemical ROS probes.

- *Reaction rate*: in order to react with oxidants, chemical probes must compete with endogenous oxidant-scavenging systems. At present, ROS-specific chemical probes have very low reaction rates, and fluorescent signals accumulate slowly. Ideally, novel probes would combine ROS specificity with greatly enhanced reaction rates.

5.4.2.2 Further Development of Genetic Probes

- *Multi-color redox imaging*: the development of a redox-sensitive red fluorescent protein (RFP) would greatly enhance the possibilities for making simultaneous measurements of different redox species or of the same redox species in different subcellular compartments.
- *Novel probe specificities*: genetically encoded probes for several key biological redox species are still lacking. Most prominently, probes for the oxidants NO· and $O_2^{\cdot -}$, and for the redox couples NADPH/$NADP^+$, thioredoxin (ox/red) and ascorbate/dehydroascorbate, do not currently exist.

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Chapter 6
Redox Proteomics

Alexandra Müller and Lars I. Leichert

Abstract In the presence of oxidative and nitrosative stress, proteins can undergo oxidative modification. A large variety of such modifications have been identified to date, including carbonylation, oxidation of aromatic amino acids, methionine sulfoxidation, and cysteine oxidation. While unintended oxidative modifications most often lead to the damage of the affected proteins, oxidative modifications also play important roles in cellular redox sensing. Classical redox sensor proteins use reversible oxidative modifications to change their activity in response to a changing redox environment. These redox sensors are the focus of oxidative stress research and have been identified in all three kingdoms of life. They are involved in a wide variety of cellular processes ranging from central energy metabolism over protein quality control to the regulation of the oxidative stress response. Proteomic methods have been used to globally monitor the oxidation state of these redox sensors and to identify novel redox sensitive proteins. These methods can help us in understanding redox regulation and the role of protein oxidation under physiological and pathological conditions. In this chapter, we will provide an overview of the different oxidation products of amino acid side chains in proteins, discuss examples of their physiological relevance and present a selection of global methods to identify them. We put an emphasis on quantitative proteomic methods that are able to identify targets of oxidative modifications down to the amino acid.

Keywords Amino acid oxidation • Cysteine modification • Post-translational modification • Reactive oxygen species • Redox signaling

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6.1 Introduction: Reactive Oxygen Species, Oxidative Stress, and Cellular Defense Strategies

6.1.1 Reactive Oxygen Species and Redox Homeostasis

The oxygen-rich atmosphere of present-day earth has emerged some 2.4 billion years ago, when cyanobacteria first started to use water as electron source during photosynthesis (Buick 2008). Thus, molecular oxygen became available as an electron acceptor for metabolic processes such as respiration, which is energetically highly favorable compared to anaerobic fermentation and therefore regarded as a prerequisite for the evolution of multicellular life-forms (Buick 2008; Donoghue and Antcliffe 2010). However, oxygen utilization is a two-edged sword as molecular oxygen is a highly reactive element and its utilization in the respiratory chain and in oxidizing enzymes is accompanied by the generation of reactive oxygen species (ROS) (see Chambers et al. 1985; Turrens 2003 for a comprehensive review). ROS have the potential to damage virtually all cellular macromolecules including DNA, proteins, lipids, and carbohydrates. Three well-studied and common ROS in cellular systems are superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$). They correspond to the reduction of molecular oxygen by one, two, or three electrons and differ with respect to reactivity, half life, and abundance. Superoxide ($O_2^{-\cdot}$) reacts quite specifically with iron-sulfur clusters and possesses a half life of 0.05 s at 0.1 M concentration (Fridovich 1983). The probably most stable ROS is hydrogen peroxide (H_2O_2) (Pryor 1986). Like superoxide, H_2O_2 is mainly generated as a result of oxygen reduction in the respiratory chain but may also be produced by oxygenases and in the endoplasmic reticulum during disulfide bond formation (Gross et al. 2006; Kuthan and Ullrich 1982; Loschen et al. 1971). $HO^{\cdot}$, which may be generated through biologically relevant Fenton chemistry, i.e. the reduction of hydrogen peroxide catalyzed by redox-cycling metal ions, occurs at very low concentrations and possesses a half life as low as 10^{-9} s (Halliwell and Gutteridge 1989; Sies 1993). Nevertheless, $HO^{\cdot}$ is regarded as the most harmful of these ROS because it reacts instantly with any cellular macromolecules in its vicinity. It is thought that in non-photosynthetic organisms, ROS are predominantly generated in the respiratory chain, whereas in photosynthesizing cells the photosystems are by far the main contributors of partially reduced oxygen (Halliwell and Gutteridge 1989; Ivanov and Khorobrykh 2003).

Because proteins are the most abundant targets of ROS in the cell, organisms have evolved sophisticated defense strategies to protect them against the consequences of oxidative damage (Rinalducci et al. 2008). These cellular defense strategies basically act on three different levels: prevention of ROS formation, interception, and repair or removal of damaged proteins (Fig. 6.1). Prevention of ROS formation includes the chelation of metals that could otherwise drive radical-forming reactions, protection from radiation by specialized pigments, and structural hindrance of radical release by enzymes that are generally prone to radical

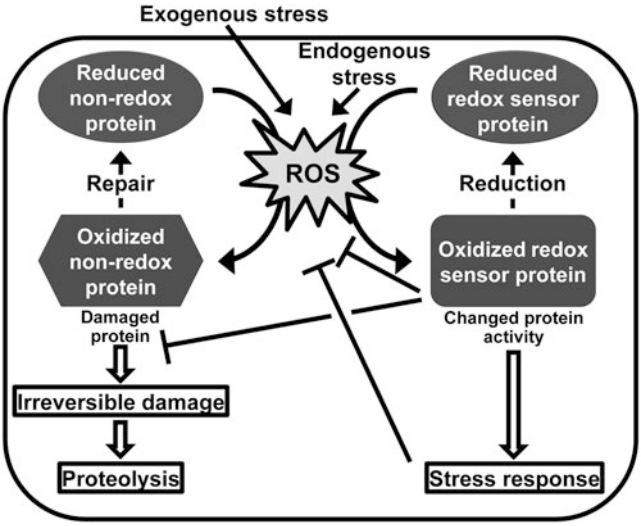

Fig. 6.1 Protein damage and redox regulation in cells exposed to oxidative stress. Endogenous or exogenous oxidative stress leads to the accumulation of reactive oxygen species (ROS) within the cell. These ROS lead to protein oxidation. While typical (non-redox) proteins are damaged by ROS and need to be repaired or degraded, redox sensor proteins alter their activity upon oxidation and often protect cells from oxidative stress. One example would be the redox-regulated chaperone Hsp33, which can prevent protein aggregation under severe oxidative stress conditions. If the redox-sensing protein is a regulator, it can induce different cellular pathways. A prominent example of this mechanism is the induction of the oxidative stress response in *E. coli* following oxidation of the regulator OxyR

formation as for example ribonucleotide reductase (Reichard and Ehrenberg 1983; Sies 1993). When generation inside the cell cannot be prevented or when organisms are exposed to exogenous oxidative stress, cells can remove ROS through enzyme-based decomposition or by the help of small-molecule antioxidants (Halliwell and Gutteridge 1989). Superoxide dismutase converts superoxide to oxygen and hydrogen peroxide. The latter is further dismutated to oxygen and water by catalase or reduced by peroxidase (Chance et al. 1979). These systems are supported by non-enzymatic antioxidants such as glutathione, ascorbic acid, α-tocopherol, flavonoids, and carotenoids. They work in concert with the thioredoxin and the glutaredoxin systems (Holmgren 1989). The thioredoxin/thioredoxin reductase system has been identified in all kingdoms of live. Thioredoxins are small proteins with a highly conserved Cys-Gly-Pro-Cys motif, which is exposed at the surface. They exhibit a highly reducing redox-potential, which allows rapid reduction of oxidized cysteine thiols under conditions of increased oxidative stress. This reduction is accompanied by the formation of a disulfide bond within the Cys-Gly-Pro-Cys motif, which is then re-reduced by thioredoxin reductase in an NADPH-dependent manner. Glutaredoxins are related small redox proteins with a similar active site consisting of two cysteines. In contrast to thioredoxins, however, glutaredoxins are reduced

non-enzymatically by the tripeptide glutathione (Holmgren 1989). As long as ROS production does not exceed the capacity of these defense systems, the thiol-disulfide ratio remains fairly stable (Ghezzi et al. 2005). If the exposure to ROS lasts, detoxifying and repairing systems become overwhelmed and irreversibly modified proteins may accumulate under these conditions. These are then subjected to the cellular degradation machinery, which represents the third level of the oxidative stress defense strategies (Grune and Davies 1997; Sies 1993).

6.1.2 Oxidative Stress

However, under certain circumstances, for example under nutrient deficiency, disease, and infection by pathogens, the above mentioned systems may be overwhelmed by ROS, a condition termed oxidative stress (Halliwell and Gutteridge 1989; Sies and Cadenas 1985). This imbalance between ROS generation and proper disposal by cellular defense strategies has been proposed to be one of the underlying mechanisms of aging (Harman 1956). Additionally, a variety of diseases has been linked to oxidative stress for a long time, among them neurological diseases, such as Alzheimer's and Parkinson's, as well as cardiovascular diseases, including heart failure and stroke (Alexandrova and Bochev 2005; Dai et al. 2011; Ogawa et al. 2002). However, in many cases it is still not clear, if oxidative stress is one of the causes or a symptom of these diseases, spawning the development of global and targeted methods to determine the mechanism of cell damage and to study the oxidative damage to biomolecules.

6.1.3 Redox Sensing

Over the past decades it has increasingly been realized that reactive oxygen species not only produce oxidative stress and cause damage of cellular components but also serve as potent signaling molecules involved in important cellular processes, including cell proliferation, differentiation, and apoptosis (D'Autréaux and Toledano 2007; Janssen-Heininger et al. 2008). Therefore the concept of 'redox signaling', which describes entire cascades from sensing of reactive oxygen species down to cellular responses was introduced (Bochner et al. 1984; Proctor and McGinness 1970). This concept is similar to the signaling by nitric oxide ($NO^{\cdot}$), a radical molecule that was also initially thought to be too unstable and harmful for signaling. While the canonical $NO^{\cdot}$-sensing is accomplished by a heme center in guanylate cyclase (Craven and DeRubertis 1978; Katsuki et al. 1977), more recently it has been discovered that $NO^{\cdot}$-signals can be transduced by amino acid modifications, including tyrosine nitration and S-nitrosylation (Ischiropoulos et al. 1992; Stamler et al. 1992; van der Vliet et al. 1994). Similar to oxidative stress, a disturbance of the physiological $NO^{\cdot}$-levels causes nitrosative stress and uncontrolled modifications, which led to protein damage (Calabrese et al. 2009).

Since the early 1990s a variety of so-called redox sensors has been identified, among them transcription factors like the bacterial OxyR and the eukaryotic NFκB, metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and carbonic anhydrase, as well as molecular chaperones like Hsp33 and peroxiredoxins (Chae et al. 1994; Chai et al. 1991; Jakob et al. 1999; Ravichandran et al. 1994; Staal et al. 1990; Storz et al. 1990; Zheng et al. 1998). What all these proteins have in common is the possession of redox-reactive cysteines, which are specifically oxidized, leading to an alteration of the protein's activity. Once the cause of the oxidation is removed, the cysteines can be reduced by the cellular antioxidant systems and the protein returns to its original redox state and activity (Fig. 6.1). The identification and characterization of these redox sensors is probably just a starting point on our way to understand the full impact of ROS on cellular metabolism, physiology, and signaling. While cysteine, due to the reversibility of thiol modifications in low oxidation states, is a particularly well-suited amino acid for redox sensing, oxidative modifications of other amino acids, such as methionine sulfoxidation have been demonstrated to have functions in redox signaling as well (Vogt 1995; Wong et al. 2008). Hence it is not surprising that in the 'omics' era, a variety of techniques have been developed to globally detect and identify oxidative changes within cellular proteins. The term 'redox proteomics' has emerged to set a boundary to classical proteomic approaches. Redox proteomics "aims to detect and analyze redox-based changes within the proteome both in redox signaling scenarios and in oxidative stress" (Sheehan et al. 2010). This chapter aims to give a comprehensive overview about the most recent proteomic approaches for the large-scale identification of different oxidative protein modifications. In the context of signaling it is important to know which fraction of a redox-sensitive protein is modified and which amino acids are targeted by ROS. Therefore, we will focus on quantitative techniques that allow for the identification of redox-sensitive sites down to the amino acid.

6.2 ROS-Mediated Protein Modifications and Methods for Their Detection

Although virtually all amino acids are vulnerable to oxidation and more than 35 different oxidation products of amino acids have been identified so far, only a few of them have been investigated with respect to their physiological relevance (Madian and Regnier 2010). The observed oxidative modifications can be grouped into two categories: *in vivo* reversible and irreversible modifications. It could be argued that an effective regulative oxidative modification should be reversible *in vivo*, as only reversibility allows for a back-and-forth change and adjustment of the activity of a redox-regulated protein to the changing redox environment. Of the known oxidative amino acid modifications, only the lower oxidation states of cysteine and methionine sulfoxidation fit these criteria and

these two amino acids can therefore serve in their corresponding proteins as true 'redox switches'. Most of the regulative oxidative modifications described so far are cysteine modifications, such as disulfide bond formation or sulfenic acid formation, although recent publications suggest a regulative role for methionine sulfoxidation as well (Abulimiti et al. 2003; Ciorba et al. 1997; Sun et al. 1999). In contrast, amino acid carbonylation and the oxidation of aromatic amino acids is irreversible *in vivo*, and proteins modified in this way typically are rapidly degraded to circumvent harmful aggregation (Nyström 2005). However, recent studies suggest that these irreversible modifications may have a regulatory impact as well (Wong et al. 2008). In the next sections, we will discuss these oxidative protein modifications and proteomic methods, which have been successfully used to study them.

6.2.1 Protein Carbonylation

Protein carbonylation is a post-translational modification resulting from at least four different reactions. The most disruptive event is the oxidation of the protein backbone, leading to the formation of protein fragments with an N-terminal α-ketoacyl amino acid and hence total loss of protein integrity (Stadtman 1990). Additionally, the side-chains of lysine, proline, arginine, and threonine may undergo metal ion-catalyzed oxidation leading to the corresponding ketone or aldehyde derivatives (Fig. 6.2). Carbonylation can also occur by 4-hydroxy-2-nonenal (HNE), which is a product of lipid peroxidation (Dalle-Donne et al. 2003a, b). Finally, lysine, cysteine, and histidine can be glycated or glycoxidated through the conjugation with reducing sugars and their oxidation products, respectively. The latter two reactions are referred to as 'secondary reactions' (Dalle-Donne et al. 2006).

Carbonylation is irreversible *in vivo*, and the fate of such modified proteins is degradation by the proteasome in higher organisms or the Lon protease in *E. coli* (Grune et al. 2003; Grune and Davies 1997; Levine 2002). Mildly carbonylated proteins are much more susceptible to degradation than non-modified proteins and it has been suggested that the carbonyl group can act as a recognition tag for the proteasome (Grune et al. 2003; Grune and Davies 1997). Age-related disorders like Parkinson's disease, Alzheimer's disease, cancer, and diabetes are associated with an accumulation of carbonylated proteins, which escape the cellular degradation machinery, causing the accumulation of cytotoxic protein aggregates (Dalle-Donne et al. 2003a, b; Levine 2002). Carbonylated proteins therefore often serve as biomarkers for the detection of oxidative stress conditions and several proteomic approaches have been developed to gain insights into the 'carbonylated proteome' (Aksenov et al. 2000; Dalle-Donne et al. 2003a, b; Reinheckel et al. 2000). As evidence for a regulatory function of carbonylation emerges (Lee and Helmann 2006; Wong et al. 2008), these methods are constantly improved to enable the site-specific identification of amino acids undergoing carbonylation.

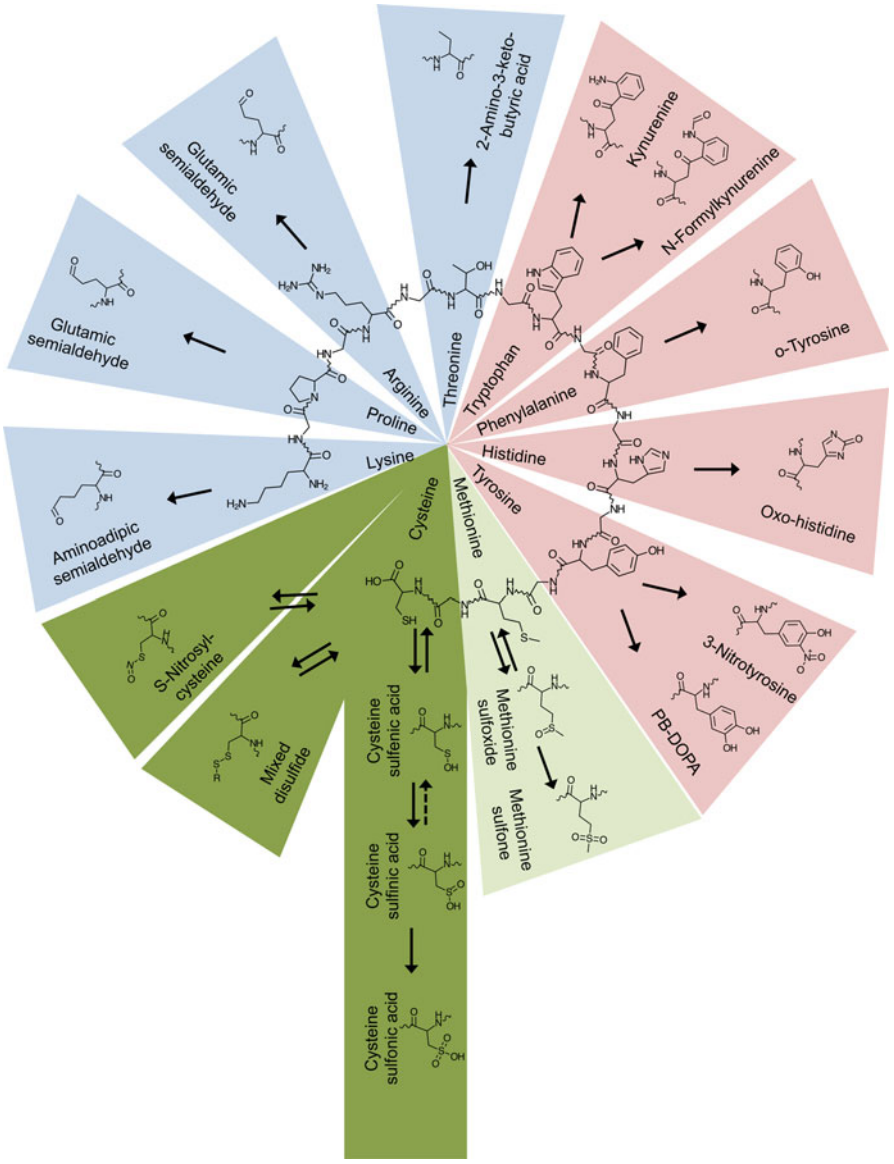

Fig. 6.2 Biologically relevant amino acid side chain modifications caused by reactive oxygen and reactive nitrogen species. *Bidirectional arrows* indicate modifications that are potentially reversible *in vivo*

6.2.1.1 Identification of Carbonylated Proteins

One of the first methods developed to detect carbonylated proteins was the so-called OxyBlot technique. The carbonyl group readily reacts with hydrazine and

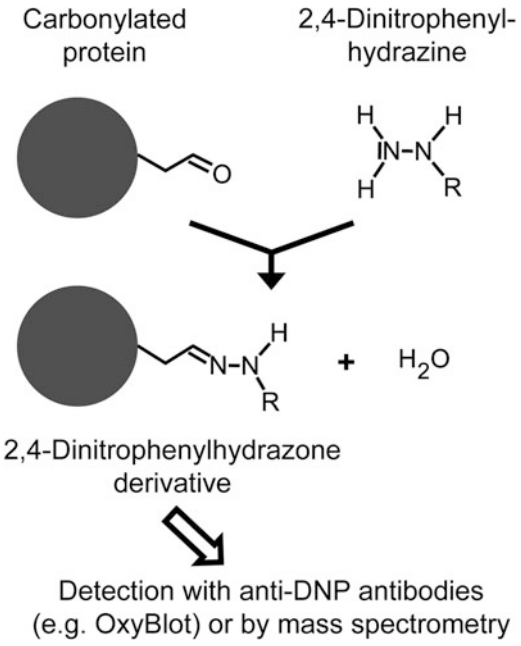

Fig. 6.3 Detection of protein carbonylation by derivatization with 2,4-dinitrophenyl hydrazine ("OxyBlot"). The carbonylated amino acid side chain of a protein is derivatized through a nucleophilic addition of the hydrazin group to the carbonyl group and the formation of a 2,4-dinitrophenylhydrazone. The hydrazone can be detected with antibodies in a western blot or analyzed by mass spectrometry

its derivatives, such as dinitrophenyl hydrazine (Fig. 6.3). This condensation of dinitrophenyl hydrazine with a carbonyl leads to the formation of a stable, intensely colored dinitrophenyl hydrazone product in a reaction well-known from carbohydrate analysis. In 1995, Nakamuro and Goto developed an antibody against dinitrophenyl hydrazine-treated carbonylated BSA, which proved to be specific for the dinitrophenyl hydrazone moiety. Western blots with this antibody allowed them for the first time to identify carbonylated proteins on a 2D gel from rat tissue (Nakamura and Goto 1996). Reinheckel et al. improved this procedure by adding the dinitrophenyl hydrazine in gel, after the separation of the proteins in the first dimension, which made it possible to directly correlate spots from the blot with the stained protein pattern, because there is no isoelectric shift caused when the hydrazone condensation is done after isoelectric focusing (Reinheckel et al. 2000).

Still, the site-specific identification of carbonylated side chains poses a challenge, as the carbonylated form of a given protein is often in low abundance when compared to the unmodified protein. However, Bollineni et al. recently reported a mass spectrometric method, in which dinitrophenyl hydrazine-modified peptides are separated by 2-dimensional liquid chromatography and which makes use of the fact that dinitrophenyl hydrazine can directly act as the matrix for MALDI-based mass spectrometry. In a bottom-up approach they could identify carbonylation-sensitive sites in BSA and β-lactoglobulin (Bollineni et al. 2011).

To further enrich carbonyl-containing proteins it is possible to chemically couple affinity tags to hydrazine. The use of biotin-hydrazine and subsequent affinity-purification with avidin columns to reduce sample complexity made it possible to

identify carbonylation sites of rat liver proteins by LC-MS/MS (Mirzaei and Regnier 2005). The same group successfully identified oxidation sites in transferrin by using Girard P reagent, which has the hydrazine linked to a quaternary amine that allowed for enrichment of carbonylated peptides by strong cation exchange chromatography (Mirzaei and Regnier 2006).

Even more sophisticated carbonyl-reactive probes, which combine affinity tags with a so-called element code have been developed. O-ECAT (oxidation-dependent carbonyl-specific element-coded affinity mass tag) specifically labels aldehyde and keto groups of oxidized amino acid side chains by forming an oxime and adds a chelating site for metal ions to the peptide. Differential insertion of metals such as terbium or holmium into the chelating site prior to liquid chromatography allows quantification of the relative oxidation of different samples based on the mass difference of the two metals. With this method oxidation sites in recombinant human serum albumin and *E. coli* RNA polymerase could be identified (Cheal et al. 2009; Lee et al. 2006).

To date these novel mass spectrometry-based methods have been used mainly for model proteins, while experiments on a global level in a physiological context are still missing and the quantification of carbonylations in more complex biological samples seems still challenging.

6.2.2 Oxidation of Protein-Bound Aromatic Amino Acids

The aromatic amino acid residues tryptophan, phenylalanine, histidine, and tyrosine are highly prone to oxidation, yielding very different oxidation products (Fig. 6.2) (Guan and Chance 2005). Furthermore, tyrosine is readily nitrated to 3-nitrotyrosine by reactive nitrogen species (Ischiropoulos et al. 1992), which we will discuss here as well. Because of the wide variety of possible modifications and the issue of physiological relevance, site-specific identification of aromatic amino acid modifications in complex samples is still challenging and is therefore, with few exceptions, a relatively unexploited research area to this day.

6.2.2.1 Tryptophan Oxidation

Oxidation of tryptophan may occur by singlet oxygen, derived from UV radiation of molecular oxygen, a so-called type 2 photo-oxidation, or by the reactive nitrogen species peroxynitrite (Ischiropoulos and al-Mehdi 1995; Nakagawa et al. 1977). Although in total 11 different oxidation products of tryptophan have been identified in an *in vitro* system using synthetic peptides, only a few of them have been found *in vivo* (Grosvenor et al. 2010). Among the products found *in vivo* are N-formylkynurenine and kynurenine (Anderson et al. 2002; Taylor et al. 2003b). As antibodies against tryptophan oxidation products are lacking, mass spectrometry is the method of choice for the detection of tryptophan modifications. Oxidation

of tryptophan results in mass shifts ranging from +4 Da (kynurenine) to +64 Da (dihydroxy-N-formylkynurenine), which can be used for the identification of modified peptides from MS/MS spectra.

Site-specific identification of oxidized tryptophan was mostly carried out for isolated proteins or protein complexes. In these experiments, the protein of interest is exposed to oxidative stress *in vivo* and then isolated from the complex protein mixture before digestion and LC-MS/MS. This experimental set up was successfully used to identify oxidized tryptophans in histidyl-tRNA synthetase and rat muscle actin (Fedorova et al. 2010; van Dooren et al. 2011). Anderson et al. studied isolated photosystem II from spinach chloroplasts and identified a single tryptophan residue (Trp-352) of CP43 core antenna complex in three distinct oxidation states, namely as kynurenine, oxindolylalanine, and a hydroxylated indole derivative, by tandem mass spectrometry. A role of this tryptophan oxidation as signal for the turnover of photosystem II was proposed (Anderson et al. 2002).

Because of the tell-tale mass shift, the identification of tryptophan oxidation sites in complex samples is possible. Two independent research groups investigated oxidative tryptophan modifications in mitochondrial proteins. Taylor et al. re-evaluated a previously compiled proteomic MS/MS data set of cardiac mitochondria for the occurrence of N-formylkynurenine, making use of the 32 Da mass increase (Taylor et al. 2003a). In total, 51 different N-formylkynurenine-containing peptides from 39 proteins of all mitochondrial respiratory complexes except complex II were discovered upon re-inspection of this data set (Taylor et al. 2003a). The observed tryptophan oxidation of mitochondrial proteins was proposed to be the consequence of exposure to ROS generated during respiration in the mitochondria. Møller and Kristensen studied rice leaf and potato tuber mitochondria and also searched specifically for N-formylkynurenine-containing proteins. 29 peptides containing oxidatively modified tryptophan from 17 different proteins where identified (Møller and Kristensen 2006). Both of these studies identified largely proteins directly involved in cellular respiration, demonstrating that these proteins, often the source of reactive oxygen species themselves, are particularly prone to oxidative damage. However, according to Perdivara et al., some kynurenine and N-formylkynurenine formation can potentially be attributed to artifactual modification during sample processing. A comparison of the extent of kynurenine and N-formylkynurenine occurrence in several antibodies, based on sample handling showed that oxidative tryptophan modifications can be readily identified after separation of the proteins on SDS-PAGE and tryptic in-gel digest but were not detectable after in-solution digest (Perdivara et al. 2010). These results demonstrate the challenges of redox-proteomics, and the need for suitable sample preparation procedures to avoid artifactual oxidative protein modifications.

6.2.2.2 Phenylalanine Oxidation

Oxidation of phenylalanine by $HO^{\cdot}$ or peroxynitrite leads to the formation of ortho-, meta-, and para-tyrosine (Fig. 6.2) (Maskos et al. 1992). The atypical amino

acid isomers ortho- and meta-tyrosine are used as biomarkers for the detection of oxidative protein damage associated with diseases such as β-thalassemia and atherosclerosis (Leeuwenburgh et al. 1997; Matayatsuk et al. 2007). Site-specific information about phenylalanine oxidation may be obtained by mass spectrometry, taking advantage of the 16 Da mass increase of tyrosine over phenylalanine. However, a global screening for phenylalanine oxidation has not been reported to date.

6.2.2.3 Histidine Oxidation

Oxidative histidine modification is used as biomarker for oxidative stress (Uchida and Kawakishi 1993). Histidine is particularly prone to metal-catalyzed oxidation leading to 2-oxo-histidine (Fig. 6.2). Histidine oxidation has deleterious effects on proteins: oxidation of histidine in Cu,Zn superoxide dismutase leads to a significant decrease in enzyme activity in aging rats (Maria et al. 1995). But regulatory effects of histidine oxidation have been described as well. In the *Bacillus subtilis* H_2O_2-sensing transcription factor PerR, three histidine and two aspartic acid residues typically coordinate an Fe^{2+}-ion in the regulatory site. Hydroxyl radicals, generated *in situ* through H_2O_2 reduction by the coordinated Fe^{2+}-ion oxidize histidine residues 37 or 91 of PerR to 2-oxo-histidine. The direct consequence is the loss of the metal ion and dissociation of PerR from the DNA followed by the transcription of target genes. This irreversible mechanism allows PerR to sense low levels of hydrogen peroxide *in vivo* (Herbig and Helmann 2001; Lee and Helmann 2006).

Despite its physiological importance, global proteomic screenings for 2-oxo-histidine have not yet been reported. Identification of histidine oxidation sites is complicated by changes in the MS/MS dissociation pattern of peptides containing oxidatively modified histidine due to the lower proton affinity and weaker nucleophilicity of 2-oxo-histidine. This can be particularly challenging in highly oxidized proteins, when several isomers differing in their site of oxidation are present (Bridgewater et al. 2007).

6.2.2.4 Tyrosine Oxidation

Tyrosine residues can be converted to protein-bound 3,4-dihydroxyphenylalanine (PB-DOPA; Fig. 6.2), or if two tyrosines are present in the immediate vicinity, they can be cross-linked to bi-tyrosine (Gross and Sizer 1959; Simpson et al. 1992). Formation of PB-DOPA occurs enzymatically, via metal-catalyzed oxidation, gamma irradiation, or UV light (Ito et al. 1984; Simpson et al. 1993). Similar to phenylalanine and histidine oxidation, oxidative tyrosine modifications can serve as biomarkers for the detection of oxidative stress-associated diseases: PB-DOPA levels were shown to be increased in a number of pathologies including atherosclerosis and lens cataract (Fu et al. 1998; Woods et al. 2003).

A global screening for PB-DOPA was recently published by Lee et al., demonstrating growing interest in this research field (Lee et al. 2010). Lee and co-workers

specifically searched for the PB-DOPA modification in LC-MS/MS spectra and identified as many as 67 PB-DOPA-bearing sites in *E. coli* and nine sites in HeLa mitochondria using this approach. These included proteins typically prone to oxidative modification, such as GAPDH and superoxide dismutase.

Sharov and co-workers have developed a derivatization technique transforming PB-DOPA to 6-amino-substituted benzoxazoles using benzylamine derivatives (Sharov et al. 2010). The resulting modified proteins display characteristic fluorescence properties making this method suitable for gel-based identification of PB-DOPA. In addition, the use of substituted benzylamines carrying functional groups to enable enrichment will conceivably facilitate MS-based identification of target proteins.

6.2.2.5 Tyrosine Nitration

In addition to hydroxylation, nitration of tyrosine to 3-nitrotyrosine (3-NT; Fig. 6.2) has been extensively studied. Accumulation of 3-NT is considered a typical marker of peroxynitrite stress (Beal et al. 1997). A variety of techniques for site-specific identification of 3-NT are available, including immunoprecipitation and immunoaffinity chromatography using anti-3-NT antibodies, gel-based techniques with subsequent immunoblotting and mass spectrometry, as well as gel-free mass spectrometry-based techniques (see Abello et al. 2009 for a comprehensive review).

The concentration of 3-NT in human plasma is in the pM to nM range and has been shown to remain fairly constant, even under conditions of disease, physical exercise, and pharmacological treatment. This low abundance in highly complex biological samples complicates its detection, making selection of a suitable methodology most critical for the generation of reliable data (Tsikas 2010). This challenge is further complicated by artifactual tyrosine-nitration, which occurs by acid-catalyzed reactions in the presence of nitrate or nitrite in solution. To avoid this problem, 3-NT can be converted chemically into stable derivatives, preferably during early steps of sample preparation (Tsikas 2010).

A variety of such derivatization techniques, including fluorogenic tagging with ABS (4-(aminomethyl)benzenesulfonic acid), APPD ((3R,4S)-1-(4-(aminomethyl)phenyl-sulfonyl)pyrrolidine-3,4-diol), or benzylamine as well as isotope-coded tagging via iTRAQ after protection of free amino groups and chemical modification was proven to be suitable for identification of 3-NT (Chiappetta et al. 2009; Dremina et al. 2011; Sharov et al. 2008, 2010). APPD derivatization of 3-NT-containing proteins from peroxynitrite-exposed cell lysates was developed recently and makes it now possible to quantify the amount of 3-NT through fluorescence spectrometry or boronate affinity chromatography followed by MS-based proteomics. In addition, localization and quantification of 3-NT has been performed by a clever extension of the iTRAQ chemistry. Specific iTRAQ labeling of 3-NT-bearing sites is facilitated by protection of lysine residues and N-termini of trypsin-digested peptides prior to reduction of the nitro group in 3-NT to an amino group, which can then be iTRAQ modified (Chiappetta et al. 2009).

These methods allowed for site-specific identification of 3-NT even at its typically very low abundance in complex protein samples, such as human uremic plasma and cell lysates (Chiappetta et al. 2009; Dremina et al. 2011).

6.2.3 Methionine Oxidation

Oxidation of protein-bound methionine residues to methionine sulfoxide is one of the few protein oxidation events that are reversible *in vivo* (Fig. 6.2). Methionines that undergo sulfoxidation can thus serve as on/off sensors of oxidative stress in certain proteins. A number of such proteins has been identified, among them calmodulin and glutamine synthetase (Levine et al. 1996; Wolff et al. 1980; Yao et al. 1996). Surface-exposed methionines are especially prone to oxidation, and once oxidized to methionine sulfoxide can be further oxidized to methionine sulfone, a modification that is irreversible *in vivo* (Fig. 6.2) (Levine et al. 2000). The modified amino acid methionine sulfoxide has different physico-chemical characteristics from reduced methionine, which in turn can lead to conformational changes in the affected protein, causing loss of function or altered susceptibility to degradation (Bigelow and Squier 2005). Therefore almost all organisms, from bacteria to human, possess methionine sulfoxide reductases (Msr), which reduce methionine sulfoxide back to methionine in a thioredoxin-dependent manner. Two different classes of methionine sulfoxide reductases, MsrA and MsrB, can be distinguished, based on their stereospecificity towards S- and R-enantiomers of methionine sulfoxide, respectively (see Weissbach et al. 2005 for a comprehensive review).

Methionine sulfoxide reduction activity is required under oxidative stress conditions. Consequently, deletion of *msrA* causes severe phenotypes related to oxidative stress both in prokaryotes and in eukaryotes. Increased sensitivity towards oxidative stress has been observed in *Escherichia coli* and *Saccharomyces cerevisiae msrA* deletion strains (Moskovitz et al. 1995, 1997). Knock-out of *msrA* in mice resulted in a 40% reduced life span, abnormal behavior and significantly increased brain dopamine levels, demonstrating the often observed connection between oxidative stress, aging and neurodegenerative diseases (Oien et al. 2008).

In contrast, *msrA* overexpression in *Drosophila melanogaster* and *S. cerevisiae* extended the life span and increased resistance against oxidative stress, respectively (Moskovitz et al. 1998; Ruan et al. 2002). The observed positive effects of *msrA* overexpression are attributed to an antioxidant effect of methionine oxidation. While reducing oxidized methionines, methionine sulfoxide reductase becomes oxidized, which is then reversed through thioredoxin-based reduction. Hence, methionine oxidation and the subsequent reduction of methionine sulfoxide is thought to be able to act as a sink for reactive oxygen species, protecting amino acids in close vicinity from oxidation (Levine et al. 2000).

In addition to this protective function of methionine oxidation, several studies found a role of methionine sulfoxidation in regulation of enzyme and peptide hormone activity. One of the earliest examples for regulation of protein activity

by reversible methionine oxidation was the discovery of the oxidation of Met3 in the voltage-gated potassium channel ShC/B from *Drosophila melanogaster*. Sulfoxidation of this specific methionine residue causes a significant slowdown of channel inactivation, which can be restored by co-expression of *msrA*. The authors proposed that membrane depolarization itself could induce oxidation and that the associated modulation may play a role in learning and memory (Ciorba et al. 1997).

In the case of IκBα (inhibitor of kappa B alpha), which is involved in the immune response, it was shown that oxidation of a methionine residue increased its resistance against proteolytic degradation. This stabilization leads to a decreased activation of the immune response (Agbas and Moskovitz 2009).

However, it is still not clear if the reduction by methionine sulfoxide reductases is a non-specific process on all cellular proteins with exposed methionines or if there are preferred targets of this antioxidant system. To trap potential target proteins, affinity chromatography with immobilized plastidial MSRB1 was carried out in *Arabidopsis thaliana*. Interestingly, 13 out of the 24 identified MRSB1-interacting proteins directly participate in photosynthesis, including the ATP synthase α- and β-subunits, the large RubisCO subunit and GAPDH (Tarrago et al. 2011). These results indicate that methionine sulfoxide reductase activity is of special importance in compartments that are generally exposed to oxidative stress, such as plastids. The authors noted that homologs of several identified MSRB1-interacting proteins were also shown to be methionine sulfoxide reductase substrates in other organisms. These include catalase and GAPDH from *Helicobacter pylori* and *Synechocystis*, respectively (Alamuri and Maier 2006; Sato et al. 2007), indicating that methionine oxidation itself could be targeted to some highly susceptible substrate proteins.

In a recent study, a combination of SILAC (stable isotope labeling with amino acids in cell culture) and COFRADIC (combined fractional diagonal chromatography) was used to identify methionine sulfoxide-containing peptides from complex samples and to quantify their degree of oxidation (Ghesquière et al. 2011). Human Jurkat cells were cultured in medium containing $^{13}C_5$-methionine and treated with hydrogen peroxide prior to cell lysis and tryptic digest. This hydrogen peroxide-treated sample was mixed with a completely oxidized 'reference sample' labeled with $^{12}C_5$ methionine. In contrast to the hydrogen peroxide-treated sample, the 'reference sample' was oxidized *in vitro* after trypsin digest to ensure complete oxidation of all methionines. The subsequently performed COFRADIC method relies on the fact that the retention time of a modified peptide in an LC run is different from the retention time of the unmodified peptide. Thus, the removal of the methionine sulfoxide modifications from a fraction of a first LC run and a subsequent second LC run of this fraction under the same conditions will lead to characteristic peak shifts, which make it possible to identify modified peptides (Fig. 6.4). Subsequent tandem mass spectrometry could identify the oxidation-sensitive methionine of these peptides. Furthermore, by comparison of the peak areas of the light and heavy peptides, which correspond to the fully oxidized reference and the hydrogen peroxide-treated sample, respectively, the degree of sulfoxidation in the cells could be determined. As many as 2,000 oxidation-sensitive methionines from more than 1,600 proteins were identified in this way. As these proteins were not associated with particular pathways or protein classes,

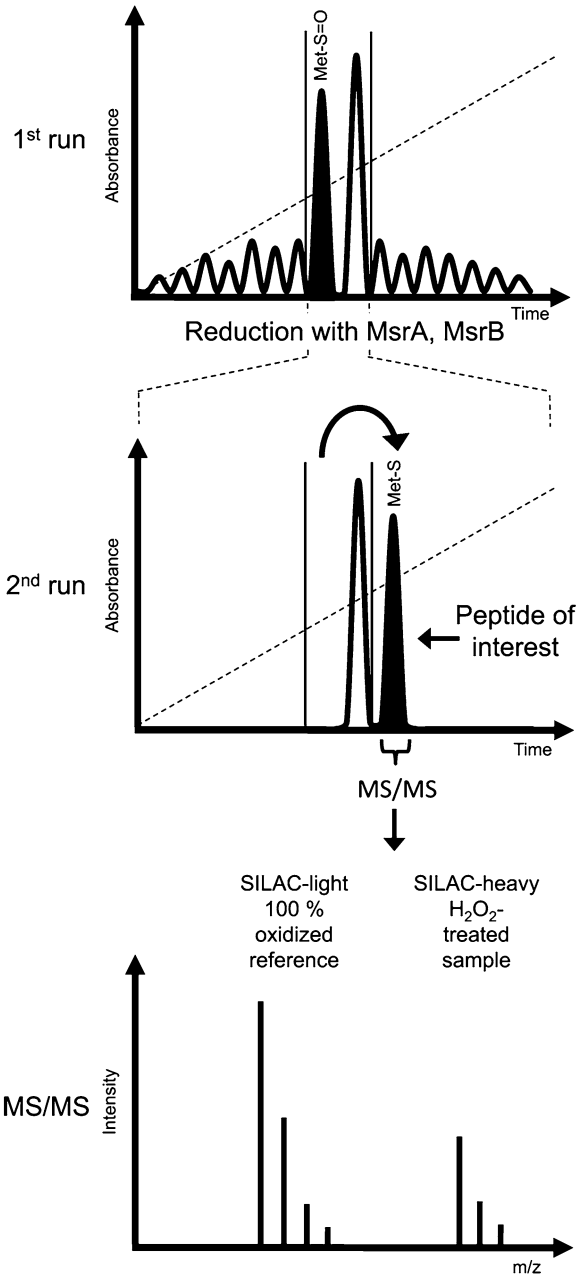

Fig. 6.4 Application of COFRADIC (Combined Fractional Diagonal Chromatography) and SILAC for the identification and quantification of peptides containing methionine sulfoxides. In the first HPLC run, a complex peptide mixture including $^{12}C_5$ methionine and $^{13}C_5$ methionine labeled peptides from a fully oxidized reference and an H_2O_2-treated sample, respectively, is separated. The peptides are collected in fractions and then reduced with methionine sulfoxide reductase A and B, which removes all sulfoxides. A second HPLC run is performed using the same chromatographic conditions. Peptides that were sulfoxidized in the original sample and were reduced by MsrA or MsrB, elute now at a later time from the column and are identified by tandem mass spectrometry. The ratio of the intensities of the $^{12}C_5$ and the $^{13}C_5$ signal in the MS allows for a quantification of the oxidized fraction of the peptide in the H_2O_2-treated sample

it was assumed that hydrogen peroxide-mediated oxidation of methionine is a non-targeted event (Ghesquière et al. 2011). This non-specificity and the susceptibility of methionine-containing peptides to form methionine sulfoxide through air oxidation during sample handling is a particular challenge when studying this modification.

The SILAC/COFRADIC method could also be used by the same group to detect and quantify methionine oxidation in a mouse sepsis model. In mice, the intravenous injection of *Salmonella* leads to a massive oxidative burst event due to the septic shock. Under these conditions 35 oxidatively modified methionines from 27 different proteins were identified in the serum. Most of these proteins, including myoglobin and NADP-dependent malic enzyme are not typical serum proteins and were thought to be released by the onset of necrosis (Ghesquière et al. 2011).

6.2.4 Cysteine Oxidation

Cysteine is one of the least used proteinogenic amino acids, with its fraction ranging between 0.7% in archaea and 2.3% in mammals. One of the reasons seems to be the high reactivity of its terminal thiol group, which brings a high risk of damaging oxidation to proteins that carry this amino acid (Giles et al. 2003). However, this high reactivity is critical for some proteins and can facilitate their structural stabilization by disulfide bond formation, mediate their binding of catalytic metal ions, ensure catalytic activity, and regulate their protein function (Miseta and Csutora 2000).

Depending on the cellular location of a protein, cysteine oxidation can occur under physiological conditions or under pathological stress conditions. In highly oxidizing compartments, such as the bacterial periplasm or the eukaryotic endoplasmic reticulum, protein cysteines are oxidized to form disulfide bonds under physiological conditions. This stabilizes the structure of extra-cellular proteins and helps them to maintain proper function in an environment devoid of active protein-folding systems (Darby and Creighton 1995). In these compartments, oxidoreductases such as protein disulfide isomerases in eukaryotes or the Dsb-system in Gram-negative bacteria introduce disulfide bonds during protein maturation (Depuydt et al. 2011).

In contrast, the cytoplasm is a highly reducing environment and most cysteines are required to be in their free thiol state in this compartment for proper protein function. Here, the thioredoxin and glutaredoxin systems keep cysteines in their reduced state. However, upon oxidative stress, cysteine thiol groups may become oxidatively modified. On the one hand, this protein oxidation often results in protein damage, e.g. through non-native disulfide bond formation, misfolding, or direct blockage of active sites. On the other hand, in so-called redox-regulated proteins, the oxidation can have regulative effects and change the protein's function. Cysteines are well-suited to function in this way as redox-sensing nano-switches, because oxidative thiol modifications in low oxidation states are fully reversible *in vivo* through the thioredoxin and glutaredoxin systems. These reversible modifications include cysteine sulfenic acid (Fig. 6.2), inter- and intramolecular disulfide bonds, and mixed disulfides with free cysteine, glutathione, or other low molecular weight thiols (Dickinson and Forman 2002).

The reactivity of a cysteine residue towards oxidants and other chemicals depends on its solvent exposure and its pK_a. The latter is strongly influenced by the local amino acid environment. The average pK_a of cysteine is 8.5. Because the

negatively charged thiolate is the redox-active form, typical cysteines are somewhat protected from oxidative modifications under physiological pH-conditions. However, in specific protein domains or in special local environments, the pK_a may be lowered significantly and the thiol group may exist mainly in its thiolate anion form even at physiological pH. These cysteines are highly prone to oxidation (Lindahl et al. 2011; Salsbury et al. 2008).

6.2.4.1 Formation of Disulfide Bonds

Disulfide bond formation occurs under physiological conditions during oxidative folding in specialized compartments, such as the bacterial periplasm or the eukaryotic endoplasmic reticulum. Disulfide bonds can be formed intramolecularly by two cysteines in the same peptide chain or intermolecularly between two different proteins (Fig. 6.2). Introduction of structural disulfides is achieved by specific oxidoreductases such as the Dsb-system in bacteria and the protein disulfide isomerases in eukaryotes (Depuydt et al. 2011).

Disulfide bond formation can also be observed within the cytoplasm. These disulfide bonds can be formed as part of a catalytic cycle, but under oxidative stress conditions, non-native disulfides may form. Some cytoplasmic proteins are especially prone to the formation of non-native disulfide bonds upon oxidative stress and such disulfides typically need to be removed to restore the protein's activity. This reduction can be carried out by thioredoxin, which leads to the formation of a catalytic intramolecular disulfide bond in thioredoxin. This disulfide bond is subsequently reduced by thioredoxin reductase. Electrons for the reduction of thioredoxin reductase are provided by NAD(P)H (Bindoli et al. 2008).

A well-studied example of a protein prone to form a disulfide bond in its active site is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate during glycolysis. During this reaction, NAD^+ is reduced, yielding NADH. GAPDH possesses two cysteines in its active site, one of which is essential to overcome the high activation energy of the reaction (Harris et al. 1963). Interestingly, these cysteines are not only important for catalysis but are also able to sense oxidative and nitrosative stress through reversible thiol oxidation. The inactivation of GAPDH under these stress conditions is thought to divert glucose to the pentose phosphate pathway, resulting in increased NADPH levels, an important cofactor of the thioredoxin and glutaredoxin systems (Godon et al. 1998; Ralser et al. 2007).

6.2.4.2 Sulfenic Acid Formation

Formation of disulfide bonds within proteins is thought to be preceded by the formation of sulfenic acid when the reaction is initiated by certain oxidants, such as hydrogen peroxide (Fig. 6.2). Generally, cysteine sulfenic acids are considered to be unstable and easily react with other free thiol groups to form disulfides. However,

a cysteine sulfenic acid may be stabilized and can have regulatory function, as has been shown in protein tyrosine phosphatase, which catalyzes the dephosphorylation of tyrosine. A reactive cysteine in the active center of this enzyme is reversibly oxidized to sulfenic acid by hydrogen peroxide resulting in inactivation of the enzyme (Meng et al. 2002).

6.2.4.3 Sulfinic and Sulfonic Acid Formation

Persistent oxidative stress may result in the formation of sulfinic acid in proteins (Fig. 6.2). This modification has long been viewed as an irreversible overoxidation product but this assessment dramatically changed with the identification and characterization of sulfiredoxin. This oxidoreductase has been identified in yeast, mammals, and cyanobacteria and catalyzes the regeneration of cysteine sulfinic acid of overoxidized peroxiredoxins (Biteau et al. 2003; Boileau et al. 2011; Chang et al. 2004). Peroxiredoxins are ubiquitous in all kingdoms and belong to the antioxidant enzyme systems, as they catalyze the removal of hydroperoxides and hydrogen peroxide (Hofmann et al. 2002; Wood et al. 2003).

In typical dimeric 2-Cys peroxiredoxins, a sulfenic acid intermediate is formed during the reaction with hydrogen peroxide at an N-terminally located cysteine residue during the reaction with hydroperoxides. The thiol group of a C-terminal cysteine residue from the other subunit reacts with the sulfenic acid to form an intermolecular disulfide bond, which is then reduced by thioredoxin. However, the sulfenic acid intermediate may also be further oxidized to sulfinic acid by excess hydrogen peroxide, which blocks disulfide bond formation and results in the inactivation of the peroxiredoxin (see Wood et al. 2003 for a comprehensive review). Sulfiredoxin can reduce the overoxidized cysteine and thus restore peroxiredoxin activity (Biteau et al. 2003). So far, sulfinic acid reduction by sulfiredoxin has only been demonstrated for peroxiredoxins and a growing body of evidence suggests that sulfiredoxins are highly specific for peroxiredoxin reduction (Woo et al. 2005).

Sulfinic acids can be further oxidized to sulfonic acid (Fig. 6.2), an oxidation event that is thought to be irreversible even in peroxiredoxins and typically leads to the inactivation of the corresponding protein. Sulfonic acid is hence used as a marker of cumulative damage due to excessive oxidative stress. Selective detection of proteins bearing cysteine sulfonic acid can be achieved by nano-diamonds coated with poly-arginine, which specifically bind to the sulfonate groups of over-oxidized proteins (Chang et al. 2010).

6.2.4.4 Formation of Mixed Disulfides by S-Thiolation

Cysteine overoxidation often goes along with protein damage. Glutathione and other low molecular weight thiols can play an important role in preventing this overoxidation both during conditions of oxidative stress and under physiological conditions. In many organisms, the tripeptide glutathione is present at millimolar concentrations in the cytoplasm. Under normal physiological conditions, it has

been shown that up to 20% of glutathione exists in a protein-bound state as mixed disulfide (Ghezzi et al. 2005). A protein sulfenic acid that might form when a protein encounters oxidative stressors can react with the free thiol group of glutathione, releasing a water molecule, and forming a glutathione adduct. The mixed disulfide is then protected from overoxidation.

In addition, S-glutathionylation has been shown to modulate the activity of several proteins that are involved in different cellular processes, including signal transduction and metabolism (Rinna et al. 2006; Zaffagnini et al. 2007). Cobalamine-independent methionine synthase (MetE) in *E. coli* is a prominent example of a protein, whose activity is regulated by reversible S-glutathionylation. MetE catalyzes the formation of methionine from homocysteine by the use of methyl-tetrahydrofolate as a methyl group donor. Under oxidative stress conditions, MetE was shown to become S-glutathionylated at cysteine 645 at the entrance of the active site, resulting in a conformational change and thereby the loss of enzyme activity. As a consequence, cells treated with oxidizing agents become methionine auxotroph (Hondorp and Matthews 2004). It has been proposed that the inactivation of MetE by S-glutathionylation protects the enzyme against irreversible oxidation damage with the possibility to restore the activity when the redox-environment is returned to pre-stress conditions.

In eukaryotes, regulation of protein activity by reversible S-glutathionylation seems to be a far more common event than in prokaryotes. Examples of proteins regulated by S-glutathionylation include metabolic enzymes, such as carbonic anhydrase III and α-ketoglutarate dehydrogenase, as well as transcription factors, such as NF-κB and c-Jun. The list of newly identified proteins that become S-glutathionylated is constantly growing (Dalle-Donne et al. 2007). In most organisms, reversibility of S-glutathionylation is achieved by the glutaredoxin system. Not surprisingly, the disturbance of this system is associated with the onset or the progression of neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease (Sabens et al. 2012).

Not all organisms use glutathione as a cellular low molecular weight thiol (Zaffagnini et al. 2007). Most Gram-positive bacteria, as for example the soil bacterium *Bacillus subtilis*, do not possess glutathione and although S-thiolation was readily observed, the nature of such modifications remained unclear (Hochgräfe et al. 2005). Interestingly, cysteine biosynthesis is greatly increased upon oxidative stress in *B. subtilis*, suggesting a role of high cysteine levels for oxidative stress protection. Hochgräfe et al. demonstrated, that proteins in *B. subtilis* undergo reversible S-cysteinylation (Hochgräfe et al. 2007). Using a mass spectrometry-based approach, they identified six different proteins that become S-cysteinylated by oxidative stress treatment. One of these proteins is methionine synthase MetE that is regulated in *E. coli* by glutathionylation. This reversible inactivation of MetE in *B. subtilis* is thought to increase cysteine levels by redirecting of synthesis capacity from methionine towards cysteine biosynthesis (Hochgräfe et al. 2007). Hence, though the nature of cysteine modification may differ in particular organisms, it seems that similar metabolic pathways are targeted through the protective inactivation of functionally related proteins. Another common low molecular weight thiol in *B. subtilis* is the recently discovered bacillithiol. Bacillithiol is synthesized from

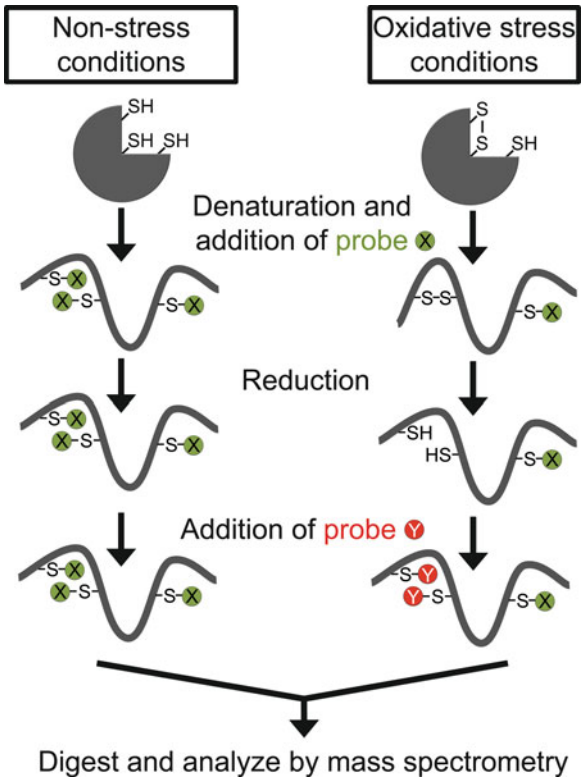

Fig. 6.5 Detection of oxidative cysteine modifications by differential thiol labeling. A hypothetical protein in its reduced (*left*) and oxidized form (*right*) is denatured and a thiol reactive probe "X" is added. This probe blocks all free thiol groups while oxidized cysteines will not be modified. In a subsequent step, reducing agent is added, which reduces the disulfide bond in the oxidized protein. Addition of a second thiol reactive probe "Y" with different properties will then label thiol groups that were initially oxidized. Based on the probes properties, the oxidation state of the protein can be determined, e.g. by radioactivity, fluorescence, or mass difference

L-cysteinyl-D-glucosamine and L-malic acid and functions as a redox buffer similar to glutathione in this organism. It was shown to directly react with protein-bound cysteine residues in regulative events leading to S-bacillithiolation. The anorganic peroxide-sensing transcription factor OhrR in *B. subtilis* is S-bacillithiolated upon hydroperoxide stress, resulting in its release from the operator, which allows transcription of oxidative stress response genes (Lee et al. 2007).

6.2.4.5 Proteomic Identification of Thiol Modifications

The proteomic identification of thiol modifications is typically based on modification of the thiol group with specific probes. The reduced thiolate is highly nucleophilic and is therefore easily modified by nucleophilic substitution or addition reactions. Typical probes used for this purpose are derived from iodoacetamide or maleimide. Because oxidized cysteines typically do not undergo these reactions, this allows for differential thiol trapping: after modification of all free cysteines in a protein sample with one probe, the sample can then be reduced and the now accessible, previously oxidized thiols can be labeled with a different probe in a second labeling step (Fig. 6.5). Thus, reduced and oxidized cysteines can be clearly

distinguished and potentially quantified, based on the nature of the probe. When typical thiol reductants such as DTT or TCEP are used in the reduction step, all reversible thiol modifications will be reduced and thus labeled with the second probe.

One of the first approaches to quantify the thiol status by the use of differential thiol trapping was accomplished in *E. coli* using a gel-based approach with unlabeled and ^{14}C-labeled iodoacetamide (Leichert and Jakob 2004). A number of so far unknown redox-regulated proteins were identified by the comparison of control and oxidative stress conditions. The same technique also allowed for the identification of substrates of the disulfide bond forming DsbA-system and the antioxidant thioredoxin system.

The well-known DIGE (differential gel electrophoresis) technique has also been adapted to redox proteomics by the use of derivatized cyanine (Cy) fluorescent dyes, which specifically react with free thiol groups (Chan et al. 2005; Hurd et al. 2007). In a typical workflow, a control sample and an oxidative stress-treated sample are first treated with NEM (N-ethyl maleimide) to block all free thiol groups. DTT is then added, which unspecifically reduces all reversibly oxidized cysteines. Subsequently, two different DIGE dyes (e.g., Cy3 maleimide and Cy5 maleimide) are used to label the formerly oxidized cysteines in the control and the treated sample, respectively. Samples are then mixed and 2-dimensional gel electrophoresis is performed. Due to the use of two distinguishable fluorophores, differences in the thiol redox state of individual proteins can be detected with this so-called redox-DIGE method.

Nevertheless, several control steps are needed, as differences in the relative expression of proteins and their stability, as well as differences in the labeling efficiency of the two dyes might have an influence on spot intensities. Swapping of the two labels and analysis of several gels in parallel can avoid false-positives (Hurd et al. 2007).

Among gel-free methods for the identification of reversible cysteine modifications, the ICAT technique (isotope-coded affinity tag) is well established. This tagging system is composed of a biotin moiety for purification, a linker region that can be isotopically light (light ICAT) or heavy (heavy ICAT), and iodoacetamide. ICAT has initially been developed for quantitative proteomics based on the differential expression of proteins and is typically used with fully reduced samples to gain the highest labeling efficiency (Gygi et al. 1999). But as labeling is based on the reaction of the iodoacetamide moiety with free sulfhydryl groups of cysteines, it also proved useful to detect changes in the oxidation state of cysteines. Light and heavy versions of ICAT are added to sets of non-reduced samples, which are expected to have differences in the thiol state. Samples are subsequently mixed, digested, purified, and analyzed by mass spectrometry. Because the ICAT reagent only modifies reduced cysteines, a decrease in intensity in a given sample (light or heavy) indicates a decrease in available free cysteines and in reverse an increase in oxidation. Using this approach, Sethuraman et al. identified and quantified oxidized cysteines in a hydrogen peroxide-treated particulate membrane fraction of rabbit heart (Sethuraman et al. 2004). However, as two different samples are compared, differential protein expression and protein stability needs to be taken into account

as they will also change signal intensities. A modified technique, which uses a differential thiol trapping approach as it labels reduced cysteines with light ICAT and oxidized cysteines with heavy ICAT avoids this problem, and was termed OxICAT (Leichert et al. 2008). In this setup, the ratio of reduced and oxidized cysteines in a peptide sample can be quantified and these ratios can be compared across samples to identify redox-sensitive proteins. This technique has successfully been used in *E. coli*, *Caenorhabditis elegans* and *S. cerevisiae*, identifying a number of putative redox-regulated proteins affecting a variety of cellular pathways (Brandes et al. 2011; Kumsta et al. 2011; Leichert et al. 2008).

6.2.4.6 Global Approaches for the Identification of Specific Thiol Modifications

It is often desirable to distinguish between different reversible thiol modifications, such as disulfide bonds, glutathionylation, sulfenic acids, or nitrosothiols. This can be accomplished by the use of specific reductants, such as for example ascorbate, which selectively reduces nitrosothiols with some specificity. Another approach is the use of probes that react specifically with the modified cysteine, such as dimedone, which exclusively reacts with sulfenic acids. However, these approaches can be sometimes complicated by the instability of the modification in question or the lack of specificity of the reductant.

The biotin-switch technique, which detects S-nitrosylated proteins in a three step procedure is based on the use of ascorbate as a specific reductant (Jaffrey and Snyder 2001). Free thiol groups are blocked by a thiolating agent, such as MMTS (methyl methanethiosulfonate) or an alkylating agent, such as NEM. Proteins are then treated with ascorbic acid, which reduces S-nitrosylated cysteines. These cysteines are then biotinylated by biotin-HPDP. Biotinylated proteins can be detected either by electrophoresis and subsequent western blotting using streptavidin-conjugated fluorophores or anti-biotin antibodies or by mass spectrometry after tryptic digest and affinity purification (Huang et al. 2009). Instead of biotin-HPDP, probes such as the cysTMT sixplex reagent may be used for protein labeling. The cysTMT is a mass spectrometric probe that has six different isobars, which release different reporter ions when fragmented in an MS/MS experiment. These reporter ions can then be used to quantify up to six different samples in a single experiment (Murray et al. 2012).

Although ascorbic acid is considered to be a specific reductant of S-nitrosothiols, there is evidence that it may also reduce disulfide bonds in the colorimetric thiol probe 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), cystine, mixed disulfides, and even in biotin-HPDH in a pH and concentration-dependent manner (Giustarini et al. 2008; Landino et al. 2006). To circumvent this problem, triphenylphosphate ester derivatives such as PE PEG or PE SO3, which have been shown to be even more specific reductants of S-nitrosothiols, can be used (Li et al. 2011).

Sulfenic acid is a very unstable intermediate and often reacts with free thiols to disulfides or is further oxidized to sulfinic and sulfonic acid. This instability

complicates the specific detection of sulfenic acid. Different dimedone-based probes including DAz-1 and DCP-Bio1 have been developed that react specifically with sulfenic acid. These reagents contain affinity or fluorescent tags, which enable enrichment or visual detection, respectively (Klomsiri et al. 2010; Poole et al. 2005; Reddie et al. 2008). DAz-1 was successfully used to demonstrate that the thioredoxin-related protein DsbG protects single cysteine residues of periplasmic proteins from oxidation to sulfenic acid (Depuydt et al. 2009).

Specific detection of S-glutathionylation can be achieved by reduction of S-glutathionylated cysteines with glutaredoxin (Grx) and subsequent labeling with N-ethylmaleimide–biotin (Lind et al. 2002). Other methods that have been used for detection of S-glutathionylated proteins include the detection of proteins modified by ^{35}S-labeled or biotinylated glutathione (Brennan et al. 2006; Lind et al. 1998).

6.3 Summary

Redox-proteomics methods can provide us with global insights into protein damage caused by oxidation, as well as into the molecular mechanisms that underlie redox-regulation. But the identification of target proteins of oxidative stress is only a first step in understanding the connection between oxidative stress and its pathological consequences. The availability of precise mass spectrometers and highly specific derivatization techniques already facilitated the identification of numerous proteins that are prone to oxidation and the role of their oxidative modification in redox-regulation, oxidative protein folding, and protein damage. The ongoing development of probes that show high specificity for defined oxidative modifications has the potential to further expand our insights, especially when these efforts are targeted at unstable and transient oxidative modifications. Global data sets gathered from redox-proteomics experiments will continue to provide a useful starting point for concise analyses of the role of protein oxidation within global cellular networks and will help us understanding the role of oxidative stress and redox signaling in health and disease.

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Chapter 7
Computational Redox Biology: Methods and Applications

Stefano M. Marino, Goedele Roos, and Vadim N. Gladyshev

Abstract Cysteine (Cys) is the most important amino acid in redox biology: it is the premier residue used by proteins to maintain redox homeostasis, sense redox changes in the environment, and counteract oxidative stress. Cys is often used as a catalytic redox-active residue and plays a key role in protein structure stabilization via disulfides and metal binding. Cys is much different from other common amino acids in proteins: its unique chemical and physical properties provide high affinity for metal ions, support formation of covalent bonds with other Cys, and confer response to changes in the environment. These features are largely responsible for the broad variety of its biological functions. Thus, a better understanding of basic properties of Cys is essential for understanding the fundamental roles Cys plays in redox biology, as well as for prediction and classification of functional Cys residues in proteins. In this chapter, we provide an overview of theoretical and computational tools that have been developed in the area of thiol regulation and redox biology. In particular, we introduce and discuss methods to investigate basic properties of Cys, such as exposure and pKa, and a variety of algorithms for functional prediction of different types of Cys in proteins.

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7.1 Introduction

One of the major mechanisms of regulating protein function involves posttranslational modifications of specific amino acid residues. The modifications can occur at certain regulatable positions (residues not directly involved in protein function) or functional sites in proteins (e.g., catalytic residues). Through these modifications, enzymes are able to sense and respond to changes in the cellular environment. In the case of the response to redox perturbations, the major sites involved typically include the amino acid cysteine (Cys). This residue is the predominant target of redox control of protein function and is key to the redox regulation of cellular processes. This chapter provides a general overview of computational methods used in redox biology. Because of their importance for the subject of this chapter, we will initially focus on Cys properties and theoretical methods available to investigate these properties.

Among the 20 common amino acid in proteins, Cys is often an outlier with respect to descriptors used to classify and systematize properties and features of amino acid residues. For a start, it is one of the least abundant amino acids in organisms, but in spite of this, it is frequently observed in functional sites of proteins, where it serves catalytic, regulatory, structure stabilizing, cofactor binding, or other functions. Furthermore, Cys is thought to be a relatively recent addition to the genetic code (Trifonov 2004), but it appears to accumulate more than any other amino acid in present day organisms (Jordan et al. 2005). Other distinctive features of Cys, highlighting its importance in biology, include the following: (i) in humans, Cys mutations lead to genetic diseases more often than expected on the basis of its abundance (Wu et al. 2007); (ii) the content of this amino acid in mitochondrially encoded proteins is particularly low and inversely correlates with lifespan of animals (Moosmann and Behl 2008). The biological interpretation of this relationship is debated (Jobson et al. 2010; Moosmann 2011; Schindeldecker et al. 2011), but it highlights the fact that Cys residues in proteins appear to be under strict evolutionary control. This feature has to be associated with several unique biological functions of Cys, as detected by genome-wide analyses of its tendency to form functional clusters, such as structural disulfides and metal-binding sites (Beeby et al. 2005; Marino and Gladyshev 2010a). Indeed, in the protein realm, a very unique property of Cys is its ability to react with another Cys forming disulfide bonds. The only other naturally occurring amino acid, albeit much less frequent in proteins, capable of a similar reactivity is selenocysteine (Sec). Sec is a selenium-containing amino acid that differs from Cys by a single atom (i.e., Se in place of S). In all characterized selenoproteins, Sec is a redox active functional residue (e.g. directly involved in catalytic function), albeit recently an exception to this rule has been reported (Lee et al. 2011). Its function can be partially preserved when Cys replaces Sec, although the replacement often leads to a considerable decrease in catalytic efficiency (Bock et al. 1991; Kim and Gladyshev 2005). A recent study made the relation between Cys and Sec even more intriguing. It has been found that Cys can be inserted in proteins in place of Sec (Xu et al. 2010).

What are the relevant physico-chemical features of Cys? What makes this amino acid so special for protein structure, function and regulation? The main features appear to be the high reactivity and chemical capabilities of its sulfur-based functional group. As mentioned above, the nature of its side chain allows for a unique reactivity in the protein world, i.e. the covalent interactions with other thiols create intra- and intermolecular disulfide bonds. In addition, its functional group can coordinate a variety of metals and metalloids. Therefore, Cys residues hold a special role in terms of protein structure stabilization (e.g., through metal binding and covalent bonds that help hold the structure together).

As incorporated into proteins, the side chain of Cys contains a sulfhydryl group, representing the most reduced state of sulfur in proteins. This moiety is very reactive when it comes in contact with various oxidants and other electrophiles under both physiological and pathophysiological conditions. Reversible oxidation of Cys thiols is known to play a role in redox regulation of proteins via the formation of sulfenic acid intermediates (Cys-SOH), overoxidation to sulfinic acids (Cys-SO_2H) (Leonard et al. 2009; Poole et al. 2004; Shenton and Grant 2003; Wood et al. 2003), intra- and intermolecular disulfide bonds (Paget and Buttner 2003), and mixed disulfides with glutathione (Cabiscol and Levine 1996). Additionally, Cys is the main target of nitrosative stress, leading to the formation of reversible S-nitrosothiols (Hess et al. 2005). The susceptibility of Cys to these modifications is largely dependent on the reactivity of each specific thiol: Cys thiolates are good nucleophiles and more prone to oxidation than Cys thiols (Winterbourn and Metodiewa 1999). Cys residues are also very polarizable (i.e., the thiol dipole can be easily perturbed by interaction with other residues of the protein). Documented examples include the effects of N-terminal helix dipole (Iqbalsyah et al. 2006), proximity to other titrable residues (Marino and Gladyshev 2009; Salsbury et al. 2008) and hydrogen bond partners (Foloppe et al. 2001). Therefore, Cys reactivity ultimately depends on local environmental features (e.g., secondary structure composition, proximity with charged residues, H-bond donors, etc.). A relevant functional consequence is that its sensitivity to oxidation and over-oxidation greatly varies with changes in the environment: an exposed Cys residue can be turned into a very reactive residue and thus more likely to be either functional or dysfunctional.

7.2 Computational Methods to Investigate Thiol Reactivity

7.2.1 General Aspects of Cys Reactivity in Proteins

The general features of Cys in proteins are difficult to define. Their chemico-physical properties are greatly influenced by the environment: deeply buried Cys residues tend to behave as hydrophobic residues (due to hydrophobic packing inside the protein body) while solvent accessible Cys residues have an opportunity to interact with many H-bond partners and titratable groups of polar residues, which

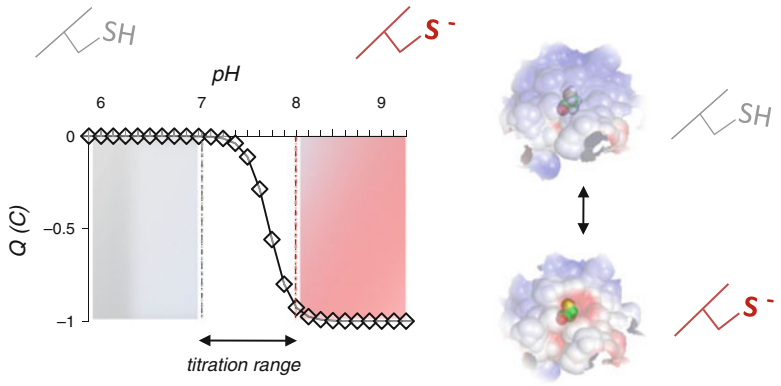

Fig. 7.1 Effect of pH perturbation on Cys located on molecular surfaces. For many exposed and polar Cys, pKa is close to the physiological pH (shown in *white*, between pH values of 7 and 8, in the figure). In this scenario, assuming Henderson-Hasselbach (HH) behavior for a monoprotic acid (in a physiological solution), sudden net charge switches can happen in response to even small local pH shifts, e.g. small pH increase, favoring deprotonation. Cys is in the anionic form, as shown above the *red shaded* portion on the graph. Conversely, relatively small pH changes may promote protonation and loss of the net negative charge. This can translate into sudden charge switches, which can affect considerably physico-chemical properties of molecular surfaces in proximity to the "switching" Cys. The latter event can affect protein-protein interaction patterns

are abundant on protein surfaces. These interactions can polarize considerably the exposed Cys. Indeed, Cys can be easily perturbed by closely positioned titratable residues (and also by interaction with the solvent), significantly affecting both its pKa and its titration range (Marino and Gladyshev 2010a).

According to computer-based evaluations, exposed Cys are the naturally occurring titratable residues with the closest pKa (i.e., average pKa in proteins) to the physiological pH (Marino and Gladyshev 2010a). This observation is significant *per se*: it implies that even for small variations of local pH (within the physiological range), exposed Cys residues may experience sudden charge shifts and significant electrostatic changes, which may extend to proximal portions of the molecular surface (e.g., Cys can act as a charge switch, potentially affecting local electrostatic properties and protein-protein interactions) (Fig. 7.1). For the same reason (i.e., pKa of many exposed Cys being close to physiological pH), Cys residues on molecular surface can experience sudden variation in their ability to serve as nucleophiles.

These observations highlight the intrinsically high responsiveness of exposed Cys to changes in physiological states and environmental conditions, with considerable implications in terms of protein function. Consequently, Cys aptitude may provide a biological explanation for why Cys residues are found much less frequently (than expected) on molecular surfaces. Unless employed for a specific function, exposed Cys residues tend to be removed from protein surfaces (Marino and Gladyshev 2010a).

7.2.2 *Exposure and pKa*

It follows from the discussion above that, in computational biology, two particularly important descriptors of Cys reactivity are its exposure to solvent and the protonation status of its functional group. In both cases, structural information is needed to evaluate Cys properties. To estimate protein exposure, a common approach consists of rolling a molecular probe (e.g., a sphere of 1.4 Å radius to mimic dimensions of the water molecule) over the protein body; the latter, usually, is treated as rigid body: the probe can just touch its residues, but cannot penetrate the surface. Commonly used (and free for download) programs include Naccess (v2.1.1, http://www.bioinf.manchester.ac.uk/naccess/) and Surface Racer© (http://apps.phar.umich.edu/tsodikovlab/index_files/Page756.htm). These methods exist in stand-alone versions and, therefore, are suitable for automated large-scale analysis. Relevant to exposure calculations are algorithms searching for residues, which, albeit accessible to the solvent, are fostered in small pockets of the protein body (Laurie and Jackson 2005). These methods are usually called pocket predictors (e.g. http://www.modelling.leeds.ac.uk/pocketfinder/) and can be useful for investigation of Cys modifications that occur in particularly controlled environments (e.g., some cases of S-nitrosylation) (Marino and Gladyshev 2010b). Additionally, some programs use simplified docking procedures to discover regions of proteins, which, alongside being exposed, are also suitable to accommodate larger substrates (Laurie and Jackson 2005). One such program is Q-site finder (http://www.modelling.leeds.ac.uk/qsitefinder/). It can be useful for detection of particularly reactive types of residues, such as catalytic Cys.

In the case of pKa predictions, no definitive computational protocols exist. As hinted before, thiolates are better nucleophiles than their protonated counterparts. Also, thiolates can react more rapidly with natural oxidants, such as peroxide (Winterbourn and Metodiewa 1999), even though this depends on the local environment (Winterbourn and Hampton 2008). For these reasons, the ability to correctly predict Cys pKa is a much sought after feature that would prove to be extremely valuable in redox biology.

Different approaches have been used to study reactive Cys. One method makes use of DFT (density functional theory) calculations to calculate pKa through Natural Population Analysis (NPA) charge on Cys sulfur atoms (Roos et al. 2009a, b). The method worked well when tested with some known cases (e.g. thioredoxins, and other thioredoxin fold thiol oxidoreductases). Considering the intrinsic complexity of this analysis, DFT methods for pKa prediction can be computationally demanding: usually, reduced protein models of the active site (i.e. the functional site under investigation) are studied instead. Another method, which has been applied to the investigation of reactive Cys is the empirical pKa predictor PROPKA (Sanchez et al. 2008). For a titratable residue, a pKa shift is evaluated as a function of the sum of energy contributions provided by surrounding residues. Although the approach's theory is relatively simple, it has been praised for its balance of speed and performance (Marino and Gladyshev 2011a, b).

A third category of methods, which found applications in the analysis of redox Cys (Tosatto et al. 2008; Foloppe et al. 2001), is based on the numerical solution of the Poisson-Boltzmann (PB) equation. The electrostatic calculations provide (free) energies of each of the protonation microstates in the system. This allows calculation of partition functions and, ultimately, for each titrable residue, the probability of protonation over a range of pH values. The latter is a bonus of PB-based pKa predictors: along with the pKa value, they can produce information about the titration curve for pH values close to the $pK_{1/2}$, a feature which can be very informative (Ondrechen et al. 2001; Marino and Gladyshev 2009). PB-based approaches for pKa prediction have been applied successfully to various redox related problems, such as glutathione peroxidases (Tosatto et al. 2008).

Each approach has its unique features, e.g. PROPKA is ultrafast while still providing acceptable results. If properly set-up, the quantum mechanics (QM)-based methods can be very accurate, and PB methods can compute, besides the pKa, the whole titration curve in proximity to the $pK_{1/2}$. So, rather than in competition, these methods can be considered complementary, as they can provide insights from different perspectives and ultimately help establish a more complete picture of Cys reactivity.

Related to Cys reactivity, a new proteomics approach, called isoTOP-ABPP (Weerapana et al. 2010), allows high-throughput identification of all reactive Cys in proteins, while at the same time quantifying their reactivity (through a reactivity score, R). As R-values reflect Cys nucleophilicity, and the latter depends (among other factors) on the acid dissociation constant of its functional group, methods like isoTOP-ABPP could be very useful for large-scale comparison between theoretical prediction of reactivity and experimental data. It would be interesting to compare theoretically based ranking of Cys reactivity such as pKa and exposure with R-values, experimentally obtained for all reactive Cys.

Besides pKa prediction, QM investigations can be useful in unraveling other aspects of Cys properties and reactivity, as detailed in the following section.

7.2.3 DFT in the Study of Redox Proteins

Cys reactivity can be assessed using reactivity descriptors founded in conceptual density functional theory, DFT (Parr and Yang 1995; Geerlings and De Proft 2008), describing the preferred reaction energetics and thus the kinetics in terms of the properties of the reagents in the ground state. Conceptual DFT looks at energy perturbation (Parr and Yang 1995; Geerlings and De Proft 2008) upon changing the number of electrons or the external potential, i.e. the potential felt by the electrons due to the nuclei. Sulfur is an electron rich element having d-electrons not tightly bound to the nucleus making it polarizable. Therefore, sulfur can be classified as a ‘soft’ element. The softness (S) is the reciprocal of the hardness, which is defined as the second derivative of the energy to the number of electrons. It measures the resistance of the system to changes in the number of electrons (Pearson

and Parr 1983; Geerlings and De Proft 2008). The *S* can be approximated via the ionization energy (IE) and the electron affinity (EA), i.e. $S \approx 1/(IE - EA)$. When the reactive part of a molecule (e.g., the functional group, the thiol/thiolate for Cys) is considered, the Fukui function $f(\underline{r})$ can be used as a local descriptor (Pearson and Parr 1983; Geerlings and De Proft 2008). The condensed Fukui functions on A can be approximated as:

1. $f_A^- = q_A(N_0 - 1) - q_A(N_0)$, for the nucleophilic attack (performed by atom A)
2. $f_A^+ = q_A(N_0) - q_A(N_0 + 1)$, for the electrophilic attack (received by atom A)

where $q_A(N_0)$, $q_A(N_0 + 1)$ and $q_A(N_0 - 1)$ are the atomic populations for atom A in the neutral molecule (N_0 electrons) and the corresponding anion ($N_0 + 1$) or cation ($N_0 - 1$), as evaluated at the geometry of the N_0 electron system. Both, the Fukui function and the softness have been successfully applied to reveal details of enzymatic reaction mechanisms, as well as in the interpretation of biochemical experimental observations (Roos et al. 2009a, b). For example, the detailed mechanism of the reaction for thioredoxin mediated reduction of target disulfides (a process involving formation and dissociation of mixed disulfides) was studied via these descriptors (Roos et al. 2009a, b). Using the Fukui function and the softness it was possible to identify and rationalize the succession of events involved in the reaction. In the catalytic C_1xxC_2 motif (where x denotes any amino acid, and C stands for Cys) of thioredoxin, the first Cys (C_1) is not only the attacking nucleophile (i.e., the one which initiates the reaction) but also the site receiving the final nucleophilic attack from the second Cys (C_2) of thioredoxin (i.e., C_1 attacks the substrate, then C_2 attacks C_1, releasing the reduced substrate). It was found that C_2 activation, after the mixed disulfide bond complex is formed, is due to hydrogen bonds (Roos et al. 2009a, b). This example shows that the Fukui function is able to identify the preferential sites for nucleophilic attack in disulfide bonds, a useful feature that can provide valuable insights in the investigation of reaction mechanisms involving this type of covalent modifications (e.g., the Dsb family).

As discussed above, sulfur is very prone to oxidation (Winterbourn and Metodiewa 1999; Winterbourn and Hampton 2008) and can adopt oxidation states ranging from −2 (the thiol group in Cys residues), to +4 (the sulfonic acid, R-SO_3H). In most biologically relevant forms, Cys oxidation states include −2 (thiol), −1 (disulfide), and 0 (sulfenic acid, R-SOH). Cys oxidation thermodynamics have not been experimentally studied, due to the high reaction rates between thiols and oxidants (Roos and Messens 2011). From a computational perspective, reduction potentials are real challenges: for small and medium sized systems, high level free energy calculations can be performed, albeit being computationally expensive, in a thermodynamic cycle linking the process in the gas phase with that in solvent (Baik and Friesner 2002; Schmidt Am Busch and Knapp 2005). For large protein systems, the free energies can be calculated in a quantum mechanics-molecular mechanics framework (Hu and Yang 2008; Mark et al. 2002; Kamerlin et al. 2009). However, to the best of our knowledge, these techniques have never been applied in redox biology, e.g. to study Cys oxidation in proteins. Recently, an alternative approach, called the *R*eduction potentials from *E*lectronic *E*nergies

(REE) method, was developed (Billiet et al. 2012). The REE approach is based on the correlation between reaction path, independent reaction energies and free energies. To assess its applicability in redox biology, the oxidation of thiol to sulfenic acid in human peroxiredoxin was studied (Billiet et al. 2012). It was found that a polar environments favor the sulfenylation of the deprotonated thiolate RS^-. Furthermore, when redox couples at different protonation states were compared, it was calculated that RSH/RSOH oxidation is thermodynamically favored over deprotonated RS^-/RSO^- oxidation. This thermodynamic preference for the protonation state can, however, be reversed in the active site of human Prx by a conserved arginine: the hydrogen bond between this arginine and the active site Cys favors the oxidation of the deprotonated couple (RS^-/RSO^-). With this interaction, in the active site of peroxiredoxin, not only kinetics but also thermodynamics favor the oxidation of thiolates (i.e., the reaction between oxidants and RS^- groups is favored over the reaction with RSH groups, both energetically and kinetically).

The REE method is not limited to the study of sulfenic acid/thiol redox couple and can also apply to other oxidized Cys couples, such as sulfinic acid or sulfonic acid derivatives. Furthermore, methionine oxidation and Sec oxidation can, in principle, be studied this way too. Therefore REE is one of few existing tools for detailed mechanistic studies of redox control. Once available to the community, it would offer a significant addition to the current arsenal of computational tools in the redox biology area (e.g., to provide *in silico* estimation of redox potentials for particular protein residues).

7.3 Bioinformatics Approaches for Prediction of Reactive Cys in Proteins

As introduced above, Cys may serve different functions in proteins. These residues can act as stabilizing elements, e.g. by binding metals or forming stable structural disulfides, or serve as posttranslational modification sites. Thus, a common classification of Cys residues in proteins is based on their function: different functional classes of Cys have different salient features determining their ability to complete the specific function. For example, Cys residues serving catalytic functions have to be accessible to their substrates, and they often show features of high reactivity, such as low pKa or high electrostatic perturbation of their functional groups (Marino and Gladyshev 2009).

Different functional categories of Cys include: (i) catalytic Cys residues, (ii) structural cystine residues (i.e., stable disulfide-bonded Cys), (iii) metal-coordinating Cys residues, and (iv) Cys residues, which serve as sites of posttranslational modifications (often called regulatory Cys). However, it has to be noted that while this classification is certainly useful, not all Cys residues can be unambiguously assigned to one of the above functions (e.g., redox switchable

Cys). In the following paragraphs, we briefly introduce relevant biological aspects of each Cys functional category. Then, for each category, a brief discussion of how bioinformatics approaches can be used to investigate the subject, and with which tools, is provided (for an overview of most relevant methods discussed in this chapter, see Table 7.1). Finally, we comment on the challenges to bioinformatics analyses posed by redox switches.

7.3.1 Catalytic Cys Residues

In many enzymes, Cys plays a critical role as a nucleophile in enzyme-catalyzed reactions. Such Cys residues belong to the functional category of catalytic Cys residues. Depending on whether or not Cys residues change redox states during catalysis, a further division can be made between redox and non-redox catalytic Cys functions. Examples of enzymes with non-redox catalytic Cys are protein tyrosine phosphatases, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cys peptidases, various members of the deubiquitination system, and dCMP hydroxymethylases. It has to be noted that, while in their normal catalytic cycle the active site Cys of these enzymes do not change redox state, some of them (e.g., GAPDH) are redox sensitive (i.e. they are easily targeted by various oxidant, such as hydrogen peroxide) and experience redox regulation. Other enzymes, called thiol oxidoreductases possess an active site nucleophilic Cys that, during the catalytic function, changes redox state. Here, the catalytic Cys function involves substrate oxidation or reduction, disulfide bond isomerization and detoxification of various compounds. To our knowledge, no computational approaches for the detection of catalytic non-redox Cys residues have been developed. So, we further focus on thiol oxidoreductases, for which better progress has been made.

Thiol oxidoreductases are the only known enzymes that make use of Sec as the catalytic residue (Fomenko et al. 2007). In cases where Sec is not employed, Cys is used in its place. Corollary considerations follow: (i) Sec in active sites of enzymes is always redox-active, (ii) in thiol oxidoreductases, homologous Sec/Cys pairs of residues (i.e., Cys substituting Sec in orthologous proteins) indicate that both residues ought to be considered redox-active. The latter observation was used as a foundation for designing an algorithm (Fig. 7.2) for high-throughput identification of catalytic redox Cys in protein sequences. The method searches for sporadic Cys/Sec pairs in homologous sequences (Fomenko et al. 2007). First, it identifies unique Cys/Sec pairs flanked by homologous sequences within a pool of translated nucleotide sequences. These pairs then serve as seeds for sequence analysis at the level of protein families and subfamilies. In other words, because Sec is exclusively used in redox catalysis, an alignment between two protein sequences where Sec and a conserved Cys are paired points to the catalytic role for the Cys. A useful characteristic of the method (Fig. 7.2a) consists of it ability to identify thiol oxidoreductases through their catalytic residues (e.g. not only thiol oxidoreductases, but also the exact identity of their redox active Cys). Together with

Table 7.1 Methods for prediction and analysis of functional Cys

Method	Input	Methodology	Usage[a]/type of prediction	Availability
PROSITE	Protein sequence	Regular expression search	Cys in MBS or in DB[b]	Web accessible[d]
METAL DETECTOR	Protein sequence	SVM prediction	Cys in MBS or in DB	Web accessible[e], Standalone Program
FOLDX	Protein structure	Search for energetically avorable MBS geometries	Cys in MBS	Standalone Program
DISULFIND	Protein sequence	SVM prediction	Cys in DB	Web accessible[f]
DCPB	Protein sequence	Automated homology modeling, SVM prediction	Cys in DB or MBS[c]	Web accessible[g]
FINDSITE-metal	Protein structure	Automated protein threading, SVM prediction	Cys in MBS	Web accessible[h], Standalone Program
PROPKA	Protein structure	Heuristic pKa calculation	pKa	Web accessible[i], Standalone Program
Naccess	Protein structure	Probe based sampling	atomic exposure	Standalone Program
Q-SiteFinder	Protein structure	Probe based sampling	Active/functional site	Web accessible[j]

[a]Some of the listed programs are not limited to Cys evaluation (but can process all other suitable type of residues)
[b]*DB* disulfide bond, *MBS* metal binding site
[c]The SVM is not trained specifically for MBS, but only for DS. For MBS predicted by DCPB, additional analysis (e.g., with METALDETECTOR) maybe required
[d]http://prosite.expasy.org/ and http://prosite.expasy.org/scanprosite/
[e]http://metaldetector.dsi.unifi.it/
[f]http://disulfind.dsi.unifi.it/
[g]http://biomedical.ctust.edu.tw/edbcp/
[h]http://cssb.biology.gatech.edu/findsite-metal
[i]http://propka.ki.ku.dk/
[j]http://www.modelling.leeds.ac.uk/qsitefinder/

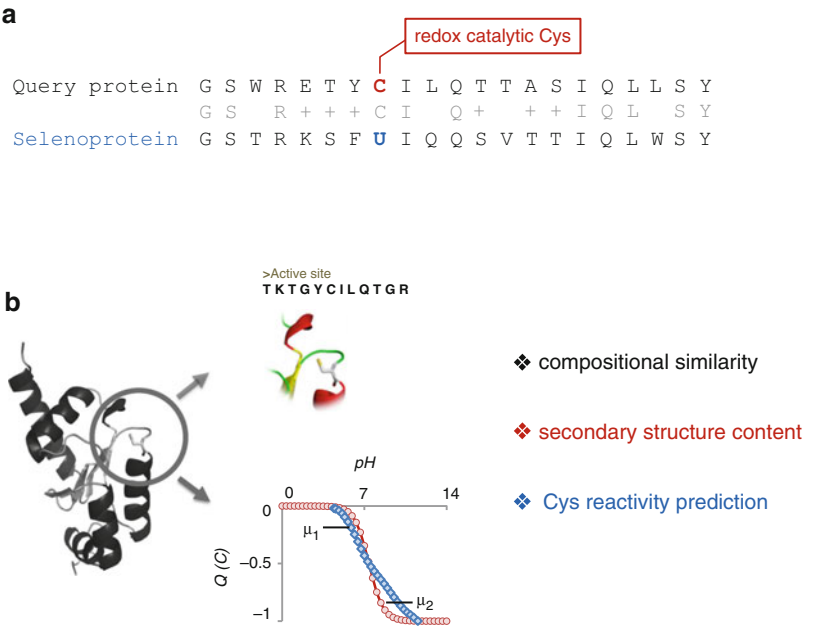

Fig. 7.2 Methods for thiol oxidoreductase prediction. (**a**) Sec2Cys method. A query protein is analyzed with tBlastn against a database of nucleotide sequences. Sec pairing with Cys in the sequence directly points to the catalytic redox function of the Cys. (**b**) Structure-based prediction of thiol oxidoreductases. By analyzing sequence and structural homology with known thiol oxidoreductases, and by theoretical estimation of Cys reactivity, a query protein is evaluated. Important parameters include amino acid and secondary structure composition within 8 Å from the catalytic Cys, and the deviation from the Henderson–Hasselbach (HH) behavior. A typical representation of the latter is shown in the *bottom part* of panel B, where the deviation ($\mu_1 + \mu_2$) reflects the difference between the theoretical titration curve calculated from the structure (*blue curve*), and the corresponding curve (*red curve*) for a standard HH behavior

sensitivity, a key advantage of this approach is speed. High-throughput analyses are possible in reasonable amount of time, allowing genome-wide analyses of thiol oxidoreductases. When tested, the method was capable of correctly recognizing nearly all known thiol oxidoreductases and predicted several new families, such as the one within a superfamily of AdoMet-dependent methyltransferases, as having thiol-based redox functions. This specific family (arsenic methyltransferases) was then experimentally verified to contain the catalytic redox-reactive Cys (Fomenko et al. 2007), further validating the approach. More recently, this approach was applied to assess the general use of thiol-based oxidoreduction in biology (Fomenko and Gladyshev 2012). These analyses showed that approximately 1% of all proteins are thiol oxidoreductases and that these proteins are used in all living organisms. Thus, thiol-based redox control is an essential feature of all cellular life.

A different computational approach, developed principally to investigate common structural features of catalytic residues in thiol oxidoreductases, was also

employed for predictive purposes (Fig. 7.2b) (Marino and Gladyshev 2009). Features describing the majority of catalytic residues in thiol oxidoreductases included: (i) weak, yet detectable sequence similarities between different families of thiol oxidoreductases, (ii) preference for an α-helix or helix/coil secondary structure, (iii) theoretical titration spectra deviating from the corresponding Henderson-Hasselbalch (HH) behavior (this feature described catalytic redox Cys better than the pKa, particularly when compared to non-redox catalytic Cys), and (iv) docking affinity for small and uncharged molecular probes, used as mimics for generic substrates (Laurie and Jackson 2005). These features were combined into a multi-parameter scoring function and implemented in the form of a predictive algorithm. The method was examined with different test cases, correctly predicting known thiol oxidoreductases. Additionally, the method predicted some new candidates, among which particularly interesting was 6-O-methylguanine-DNA methylase (MGMT). This protein showed high scores, higher than those for many known thiol oxidoreductases. Because some MGMTs also have sporadic Cys/Sec pairs in homologous sequences, these enzymes can be predicted as interesting thiol oxidoreductase candidates.

The main advantages of the structure-based approach described above reside in its ability to specifically trace back the contribution of each component to the overall prediction. The contribution of each sub-part of the algorithm can be separately analyzed, and therefore the weight of each to the final output value can be immediately retrieved. As each sub-part corresponds to specific physical, chemical or biological aspects of Cys reactivity (e.g., exposure and accessibility, titration curve and its deviation from HH, sequence homology with known thiol oxidoreductases), the user can easily extract biological information from the final output of scoring function. In addition, it showed an ability to detect new thiol oxidoreductases. On the other hand, the main disadvantage of the method is its speed: several different structure-based calculations are needed, some of which are computationally demanding. Therefore, the method is not well suited for extensive high-throughput analyses.

Bioinformatics approaches applied to the study of thiol oxidoreductases are certainly not limited to their prediction. Indeed, computational investigations examined a variety of aspects of thiol oxidoreductase evolution, distribution and functional classification. We further discuss several specific cases. Babbitt and co-workers developed computational strategies to analyze the structure and sequence landscape of thiol oxidoreductases and examined how, in particular within the thioredoxin fold, they evolved into different protein families and functionalities (Atkinson and Babbitt 2009a). For example, the authors inferred the evolution of a protein from a thioredoxin-like common ancestor of peroxiredoxins (Copley et al. 2004); the two protein families shared the same fold, but evolved different catalytic mechanisms (with peroxiredoxin forming sulfenic acid intermediates on the catalytic Cys, a feature not present in thioredoxins) and rescue modality (i.e., reductants, which in the case of peroxiredoxin are type- and cell-specific, while a single enzyme, thioredoxin reductase, evolved in the case of thioredoxin) (Copley et al. 2004). The effect of evolutionary divergence in shaping functional specificities

can be assessed with Blast-based evaluation of sequence distance, and the functional consequences of such variation can be traced to the protein fold through structural superimpositions.

In another study, evolution of glutathione transferases (GSTs) was assessed though evaluation of both structure and sequence similarity networks (Atkinson and Babbitt 2009b). The study showed the effects of sequence variability (e.g., the loss, in most members, of the nucleophilic catalytic Cys, typically found in CxxC like motifs in thiol oxidoreductases with the thioredoxin fold) in term of structure and function, a process that ultimately produces proteins with different functions. Other bioinformatics approaches to redox biology include the use of functional active site profiling to classify different subfamilies of peroxiredoxins (Nelson et al. 2011; Soito et al. 2011). By using active site signatures, this method allowed researchers to define six peroxiredoxin subfamilies, each of them with specific functionalities. A carefully curated database for peroxiredoxin sub-families classification (PREX) has been developed and is publicly available (http://csb.wfu.edu/prex/).

7.3.2 Structural Disulfides

As discussed above, Cys is one of only two naturally occurring amino acids (the other one being Sec) capable of covalent interaction with other residues of the same type. This property provides fundamental advantages, including structural stabilization and folding guidance. Prior to discussing the relevant computational methods, an important note is due: all the approaches described address (and, thus, apply to) the case of stable, structurally relevant disulfides. In fact, the prediction of transient disulfides (e.g., those present in reducing compartments of the cell) represents a very challenging task because of protein movement associated with disulfide formation or reduction. Therefore, to date, their identification still relies on the case by case in depth analysis, either through theoretical analysis of protein mobility (e.g., molecular dynamics approaches), or by direct solution of protein structure at different oxidation states (e.g., crystal structures of both reduced and oxidized forms).

Computational approaches to predict stable structural disulfides can be divided into sequence-based and structure-based (Fig. 7.3). The former strategy has been, and still is, the elective choice for large-scale analysis of proteomes, where structural information is not available. In turn, structure-based algorithms, albeit slower and inapplicable to proteins, where no structural information is available, can provide distinct advantage in terms of accuracy and precision. The approach to structure-based computation of disulfides can be very straightforward. Starting from the atomic coordinates, the distance between each Cys sulfur is computed. As a common practice, the pairs found to lie within 2.5 Å are considered safe candidate for residues engaged in covalent bonds (Fig. 7.3b). Disulfide bonds detected by analyzing the S-S distance often can be found already annotated in the PDB repository (they can be found by directly accessing the PDB file header).

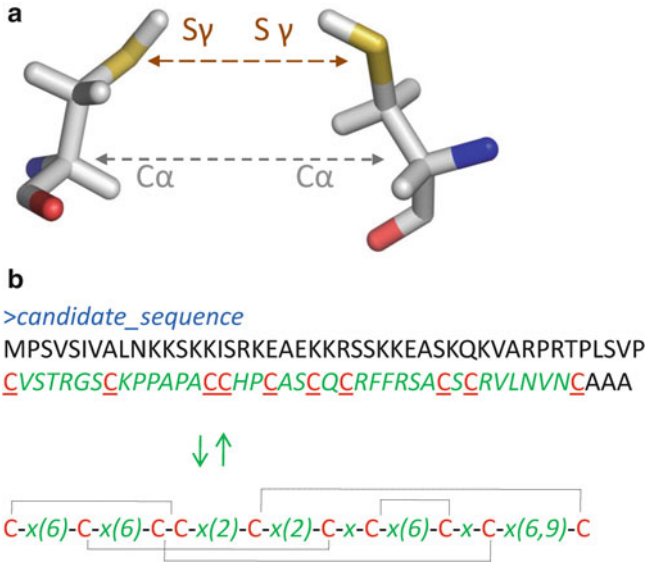

Fig. 7.3 Sequence- and structure-based approaches for disulfide detection. (**a**) A simple structure-based approach for disulfide identification. Two distances are routinely considered: (i) sulfur (Sγ) to sulfur distance, with the usual cut-off distance of 2.5 Å; and carbon alpha (Cα) to carbon alpha distance, with the usual cut-off of 8 Å. (**b**) A typical PROSITE based approach is shown. A candidate sequence under investigation is parsed with PROSITE patterns describing known disulfides. A match is found for the pattern PDOC60024 (also called, Agouti domain signature). For explanation of the regular expression vocabulary for PDOC60024, see the main text. Conserved Cys are shown in *red*, which form disulfides in a sequence specific arrangement (shown as *dotted lines*, connecting Cys in the figure)

A powerful modification of this method consists of the analysis of distances between carbon alpha atoms. In this case, a common cut-off distance is 8 Å (Fig. 7.3b). The main advantage in doing this sort of analysis is the fact that the prediction of "only" the alpha-trace represents a considerable computational advantage. Disregarding positioning side chains and focusing only on the main chain folding, it allows (i) prediction for very difficult homology model cases (e.g., very low homology with a template, using threading approaches), and (ii) reduction of computational time needed to obtain the model. This approach is well suited for large-scale comparative analyses (Beeby et al. 2005; Marino and Gladyshev 2011a), which benefit from a reasonable speed of calculations, along with the ability to analyze scarcely refined structural models.

In some cases, however, it is desirable to work without structural information at all: the structural coverage of natural proteins is still largely incomplete. In this area, various computational approaches have been developed. As a common theme, they search protein sequences for recurring motifs, as found in the primary structure. Usually, these features are discovered by manual curation (i.e., investigating known cases), and are then compiled in the form of rigorous patterns. An example of one such pattern is C-x(6)-C-x(6)-C-C-x(2)-C-x(2)-C-x-C-x(6)-C-x-C-x(6,9)-C

(Fig. 7.3a), where C is Cys, x denotes any amino acid, and the number in parenthesis indicates the number of such amino acids. This is an example of a pattern, which includes several disulfides, compiled, curated and maintained by PROSITE (Sigrist et al. 2002). Indeed, perhaps the simplest approach for sequence-based prediction of disulfides is the direct implementation of sequence patterns and profiles found in PROSITE. These patterns are annotated regular expressions that describe relatively short portions of protein sequence that may have biological meaning or function. They can be directly implementable in simple programming scripts for relatively quick searches of entire proteomes. Alternatively, for non-specialist users, the PROSITE web server provides a simple access interface (ScanProsite, de Castro et al. 2006) to browse for S-S patterns in any input sequence. However, although many S-S patterns with perfect specificity have been compiled, PROSITE profiles and regular expressions can detect only a minority of disulfide-bonded Cys. It has been estimated that only one out of four of them are matched by a pattern with high specificity (Passerini and Frasconi 2004).

Improved sequence-based approaches have been developed in recent years that could overcome this limitation. To enhance performance, machine learning approaches were implemented (Chen et al. 2004; Cheng et al. 2006; Ceroni et al. 2006), which employ various levels of sequence-based information, such as nature of adjacent amino acids, conservation of flanking residues, etc. Very generally, the scheme includes the definition of (i) a set of true positive cases (i.e., known disulfides), and (ii) a set of true negatives (known non-covalently bonded Cys residues). Then, the algorithm is trained to distinguish the two groups. Rules and parameters allowing for a better resolution are kept and become part of the search program. In other words, starting from the training set of manually collected known cases, the algorithm can learn a classification function, which can then be used for prediction purposes. One such approach is called DISULFIND (Ceroni et al. 2006): it uses support vector machine (SVM) and neural networks to classify and rank different Cys in protein sequence. It is available as a user-friendly web service, at http://disulfind.dsi.unifi.it/. The algorithm is fast and performs, overall, better than PROSITE (Ceroni et al. 2006). A viable alternative is represented by the machine learning approach called EDBCP (Lin and Tseng 2010). Its main feature is that it employs structural information through automatic modeling (starting from an input consisting of a protein sequence) via the Modeller package (Lin and Tseng 2010). After the model is created, an SVM-based algorithm uses the information to score and rank all Cys in proteins. These values are then compared with reference scores for true positive candidates. Therefore, only Cys pairs that show structural resemblance to known disulfides are predicted as cystine residues. Similarly to DISULFIND, EDBCP is publicly available as a web accessible service (http://biomedical.ctust.edu.tw/edbcp/).

7.3.3 Metal-Binding Cys

Metal-binding Cys residues are found in structurally and evolutionary distinct groups of proteins, present in all branches of life. Together with histidine (His), Cys

is the most frequently employed amino acids in metal coordination (Dokmanić et al. 2008), especially for binding zinc, cadmium, copper, iron, and nickel. A particularly common occurrence is the coordination of Zn: biologically relevant assets include tetra-Cys coordination (Zn-Cys_4) and mixed Cys and His binding (e.g. His_2-Zn-Cys_2). These structures are present in zinc fingers motifs, where they exert a crucial role in scaffolding the local structure of the broad variety of proteins that use this motif to bind DNA (Klug 2010). In the case of Zn-Cys_4 complexes, four thiolate-Zn^{2+} bridges act as stabilizing elements for protein structure (Kröncke and Klotz 2009). This structure can be thought of as a sort of "substitutive" for structural disulfide in cellular compartments characterized by overall reducing conditions, such as the cytosol, where the formation of disulfides is disfavored while Zn-Cys_4 complexes can form and be stable.

Besides structural roles, metal binding sites carry out many other important functions, such as serving as catalysts and regulatory sites. In this regard, Cys properties make this amino acid an excellent site for redox-dependent regulation of metal binding. For example, Zn^{2+}-Cys complexes allow for tight binding, but at the same time the involved Cys are potentially amenable to oxidation, which would destabilize the complex and release the metal. From a computational perspective, metal-Cys sites can be studied with various approaches, somewhat mirroring the case of disulfides. Thus, the simplest approach uses manually curated sequence patterns/profiles recurrently found in different classes of metal-binding sites. These motifs can be found, meticulously curated, at the PROSITE website. In spite of their speed and simplicity, pattern-based approaches can be affected by low performance. Furthermore, while several patterns provide perfect specificity (e.g., PS00198, PS00190, PS00463, at the PROSITE website), other patterns are much less specific, and some Cys residues can match more than one pattern (i.e., circa 12% of metal bound Cys can be detected by multiple patterns, Passerini et al. 2006). Obviously, another limitation includes their inapplicability for detecting new types of metal-binding sites.

Thus, more sophisticated approaches have been developed based on machine learning (Passerini and Frasconi 2004; Passerini et al. 2006) and non-linear statistical methods (Lin et al. 2005). A generic (and simplified) presentation of similar approaches could be as follows: for each candidate Cys, feature vectors are derived that include sequence information in the form of (i) conservation, for example, through a position specific substitution matrix (PSSM), and (ii) distance, in primary structure, between pairs of candidates (e.g.$[C]_1$-x(d)-$[C]_2$, where $[C]_i$) denotes a candidate site, x any amino acid, and d the number of amino acids separating the two Cys. Various additional layers can be added to the analysis, such as conservation of amino acids within a certain distance from the candidate site. A particularly interesting approach of this type is MetalDetector (Passerini et al. 2006). It considers only a subset of binding sites, where Cys and/or His are involved. Here, feature vectors include PSSM based information and global descriptors, such as protein length and amino acid composition. The feature vector is first classified by an SVM, and then a bi-directional recurrent neural network is used to distinguish metal and non-metal binding sites. These steps are meant to help separate the two types of

structural Cys, i.e. those present in the disulfide form from those in metal-binding sites. The latter is a prominent feature of MetalDetector: for any candidate site, a likelihood score is provided that reflects the probability that this Cys is metal bound, as opposed to disulfide bonded (or vice versa). The method is freely available both as a web accessible service and as a standalone program (http://metaldetector.dsi.unifi.it/). Various other SVM ranking methods have been developed for predicting metal-binding Cys (Shu et al. 2008). These approaches usually outperform simpler sequence based descriptors (e.g. pattern-based, such as PROSITE, or homology-based, such as Blast-derived comparative analyses) while maintaining many of the advantages, such as speed (Shu et al. 2008).

Like in the case of disulfide prediction, structure-based approaches can be a valuable alternative for the prediction of metal-binding Cys. One interesting method involves the use of empirical force field FoldX (Schymkowitz et al. 2005). The search algorithm uses geometric information typically found in metal-binding sites as a starting point for prediction of new sites. It first analyzes typical (e.g. recurrent) arrangements of Cys ligands around zinc coordination sites. The method can recognize similar geometrical and compositional patterns, such as the nature of ligands clustered in space and their relative geometries. The information gained for each type of metal binding site is then stored, and in its final form, the procedure is capable of scanning a candidate protein structure recognizing potential metal-binding sites and identifying residues and types of metal involved (Schymkowitz et al. 2005). To be noted, FoldX, available free of charge for academic users at http://foldx.crg.es/, is not specific for Cys, or His or zinc. In turn, it can work with almost all common metal-binding site types. However, due to the use of structure-based information and employment of energy-based calculations, it has its limitations: it heavily depends on the quality of structural data (highly resolved experimental structure is the ideal situation for such analysis). Thus, it may be not the best choice for large scale analyses of protein datasets, or for the analysis of models with low refinement.

Alternative structure-based strategies that could overcome this issue are now available, such as the algorithm implemented in FINDSITE-metal (Brylinski and Skolnick 2011). This method allows prediction of metal-binding sites in weakly homologous proteins. This is achieved by combining the power of threading techniques for structural modeling based on distantly related templates with evolutionary information (e.g. through comparative analysis of conservation patterns) and, finally, machine learning evaluation and classification of feature vectors. This program is capable of working with entire proteomes in a reasonable time (Brylinski and Skolnick 2011) and is freely available to the community at http://cssb.biology.gatech.edu/findsite-metal.

7.3.4 Regulatory Cys

As discussed above, Cys residues are subject to different types of redox posttranslational modifications (PTMs), with sulfenic acid (Cys-SOH), disulfide bonds (both

intramolecular and intermolecular), S-nitrosylation (NO-Cys) and glutathionylation being the most common. Additionally, Cys can react with endogenous hydrogen sulfide (H_2S), a modification that can lead to significant physiological (Yang et al. 2004; Johansen et al. 2006) and structural effects (Jiang et al. 2010). All of the above can be classified as redox-based PTMs and are reversible. However, other important Cys modifications, which are stable and do not involve a change in the redox state, also occur. They include, for example, the formation of thioether bonds with farnesyl or geranylgeranyl groups, leading to protein lipidation and membrane anchoring (Zhang and Casey 1996) or covalent binding of protein cofactors, such as heme. These Cys modifications could be classified into a separate category of functional Cys residues. In the following text, we discuss the role of bioinformatics in the study of reversible Cys modifications that can affect protein properties, function and interaction networks. For these reasons, they are often referred to as regulatory Cys (Marino and Gladyshev 2011a, b).

Large-scale computational investigation of regulatory Cys was hindered in the past by scarcity of experimental data. However, in recent years several proteomic approaches have been developed, which provided substantial improvements with regard to experimental data (Fratelli et al. 2004; Hao et al. 2006; Leonard et al. 2009; Michelet et al. 2008). Benefitting from this large influx of information, bioinformaticians could start addressing the existence of recurrent patterns defining the specificity of Cys to regulatory modifications. To the day, no sequence-based predictive pattern has been found, for any type of Cys PTM sites. Instead, computational studies revealed heterogeneity of sequence features around different Cys PTMs. In some cases, enriched motifs were detected (Greco et al. 2006; Wu et al. 2010), like adjacent negatively charged residue in the case of some NO-Cys sites, but these could not describe the majority of regulatory sites of this type (Marino and Gladyshev 2010b).

Better results were obtained with structure-based approaches, particularly in the case of NO-Cys (Greco et al. 2006; Marino and Gladyshev 2010b). First, a quantum mechanics (QM)-based study demonstrated that NO modification induces a significant charge redistribution, which largely affects its side chain atoms in a manner inversely proportional to the distance from the modification sites (e.g., carbon alpha is the least affected). At the same time, the effect is negligible for main chain atoms of the same residue (Han 2008). NO-Cys specific force field parameters and charge schemes for NO-Cys were developed, by using a restrained electrostatic potential (RESP) approach (Cieplak et al. 1995). Structural restrains for dihedral angles for NO-Cys sites were derived from the analysis of crystal structures, and geometrical optimization was conducted at the Hartree-Fock (HF) level of theory (HF/6-31G*). With these modifications, molecular dynamics (MD) simulation tests were performed and found to be in good agreement with previously available data (e.g., analysis of human thioredoxin 1, for which the structures of both unmodified and modified protein forms are available in PDB) (Han 2008).

These theoretical findings provide the basis for docking simulation studies with NO-Cys containing proteins or substrates. A first computational approach, using the new and *ad hoc* developed parameters for NO-Cys to investigate a database of potential Cys targets for GSNO mediated trans-nitrosylation, was recently reported

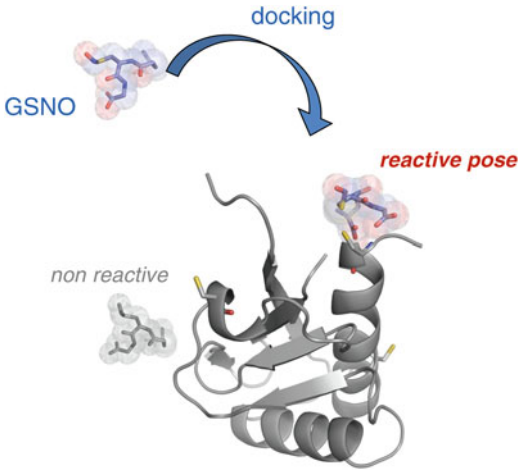

Fig. 7.4 Docking-based approaches for identification of S-nitrosylation sites. Availability of *ad hoc* parameters for S-nitrosylation of Cys residues in proteins (see main text) allowed their implementation for NO-modified proteins. In the example in the figure, S-nitrosylated glutathione (GSNO) is shown. As a putative trans-nitrosylating agent, GSNO is docked to a protein. Among the best ranked docking complexes, one (or more) poses might be consistent with reactivity (e.g. GSNO approaches an exposed Cys with a favorable interaction, and a sulfur to sulfur distance is less than 3 Å with no steric clashes). In the example, a reactive pose is found (labeled "reactive pose"). The Cys is, in this docking based assay, a positive target for GSNO mediated trans-nitrosylation. In addition to GSNO, similar approaches can be applied to many other agents (e.g. Cys-NO, or even small S-nitrosylated proteins)

(Marino and Gladyshev 2010b). This docking approach was able to address the issue of prediction of a specific subset of NO-Cys sites, i.e. Cys modified via trans-nitrosylation with GSNO. Similarly, other trans-nitrosylating agents could be explored with this strategy (Fig. 7.4). Indeed, docking calculations could be a valid computational alternative for detection of specific Cys amenable to modification with different NO-sylated substrates (NO-Cys, S-nitrosylated small peptides, etc.). Particularly challenging, but certainly feasible, is the investigation of the role of protein-protein interactions in the transfer of NO groups from one protein to another, the so-called protein interaction-based trans-nitrosylation. This process has so far escaped detailed computational studies. However, the steady development of suitable docking software (e.g., Rosetta dock) and the information gained from previous studies (Han 2008) may make it soon possible to investigate protein interaction-based trans- nitrosylation.

7.3.5 Cys as Redox Switches

Classification of Cys residues based on their biological roles is certainly reasonable in the context of a general overview. However, in some known cases (and, perhaps,

in many still unknown) the situation is more promiscuous, with the division between different Cys types being much less clear cut, i.e. Cys residues could belong to two or more of these categories. For instance, catalytic non-redox Cys in GAPDH also undergoes regulation by reactive oxygen species and thiols (Fermani et al. 2007; Hara et al. 2005; Hook and Harding 1997). Indeed, thiol redox regulation of enzymes may act on different Cys types, such as catalytic and metal binding (Ilbert et al. 2007; Jakob et al. 1999). Whenever the modification leads to a change in protein structure and/or function, the Cys can be viewed as a sort of switch. In cases where the modification is redox, it is often called the redox switch (Klomsiri et al. 2011). Redox switches are widely employed in a variety of processes and proteins, e.g. transcription factors OxyR and Yap1, kinases, phosphatases, chaperones, mitochondrial branched chain aminotransferase and other proteins. For a detailed discussion on the subject, we refer the reader to representative and updated reviews (Maret 2006; Brandes et al. 2009; Kumsta and Jakob 2009; Nagahara 2010; Klomsiri et al. 2011).

A very interesting case is represented by Hsp33 (Jakob et al. 1999, 2000). This protein protects bacteria from oxidative stress that results in protein unfolding and aggregation. In unstressed conditions, the protein is monomeric and inactive. It has four Cys residues (Cys232, Cys234, Cys265, and Cys268) involved in binding a zinc atom. After exposure to increasing levels of oxidants, e.g. hydrogen peroxide, one of the four Cys is oxidized to sulfenic acid. This transient state leads to the formation of a disulfide, and the loss of the zinc atom. Ultimately, the process triggers a large change in structure (tertiary and quaternary) and activates the chaperone function of Hsp33.

In humans, up to 10% of proteins are believed to possess Zn-binding sites (Kröncke and Klotz 2009; Maret 2006). The majority of these sites contain Cys residues. As discussed in various sections of this chapter, when Cys binds zinc, a very relevant property is the tight binding, but also susceptibility to oxidative modification. This property, together with reversibility of binding as a function of the intracellular redox state, open up the possibility that many Cys-coordinated Zn sites are candidate redox switch sites. The distribution, function and general physiological relevance (i.e. beyond the few known cases) of metal-based redox switches have yet to be thoroughly explored.

Currently, no computational methods can properly deal with Cys redox switches. Sequence-based computational predictors for metal-binding Cys are often unable to distinguish them from Cys capable of disulfide bonding, and vice versa. For instance, when *E. coli* Hsp33 sequence is scanned with a state-of-the-art machine learning-based metal-binding site prediction program (Passerini et al. 2006), Metal detector (http://metaldetector.dsi.unifi.it/), two of its Cys (Cys265, Cys268) are classified as metal binding, whereas the other two Cys are not. Additionally, the program predicts that all Hsp33 Cys residues have a negligible tendency to form disulfides. But we know that Cys265 and Cys268 form the first Hsp33 redox switch, and thus are indeed capable of disulfide bonding, even if only after one of them is oxidized. This is not surprising as the algorithm has not been trained to distinguish redox switches, and training is the most essential part of machine learning approaches.

Therefore, it is perhaps possible that similar approaches, properly devised and implemented, could provide a starting point to initiate bioinformatics to this very important, intriguing and still largely unexplored area of redox biology.

7.4 Concluding Remarks

The main subject of this chapter is Cys, a relatively scarce, but often functional, residue in proteins. Cys residues are leading characters in the play of redox regulation, and their many unique properties make them a very interesting, albeit challenging, subject to study. Believing that many aspects of the biology of Cys are tightly linked to its unique chemical and structural features, we dedicated the first part of this review for discussion of fundamental properties of this amino acid, as well as theoretical descriptors employed by researchers to describe Cys reactivity. In our opinion, a better understanding of basic chemical and physical features of Cys is crucial in order to improve currently available tools for recognition and subsequent functional annotation of reactive thiols in proteins. In the second part of the chapter, we shifted the discussion to the various functional roles played by reactive Cys residues in proteins. Moreover, we reviewed the current state of computational methods for investigation and prediction of Cys functions. In some cases, bioinformatics provided important insights and tools, especially for catalytic redox Cys, metal-binding Cys and disulfide bonds. In other cases, progress has been limited, e.g. for regulatory Cys, sites of stable posttranslational modifications and catalytic non-redox Cys.

On the other hand, experimental advances, especially in proteomics and structural and post-translational datasets, provide researchers with new opportunities to address critical issues in thiol-based redox control. We expect a steady growth of the use of bioinformatics in redox biology, a boost that will help filling the gap between different types of Cys reactivity, ultimately enabling the community to have a more extensive array of tools for large scale investigation (and prediction) of all main types of Cys function, including the ones which have been so far more elusive (e.g. regulatory Cys and redox switches).

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Chapter 8
Redox Regulation in Plants: Glutathione and "Redoxin" Related Families

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Abstract Photosynthetic organisms and especially terrestrial plants contain expanded redox regulatory pathways. Proteins involved in these redox chains and detoxification reactions often evolved on a thioredoxin fold structural basis. We discuss in this review the proteins associated with this superfamily, their modes of reduction/regeneration and their known protein targets, focusing in particular on the protein disulfide isomerase and glutathione transferase families. We also discuss the evolution of the redoxin and target families along the plant kingdom and provide information on the systems biology of the redox circuitry.

8.1 General Background

In general, redox reactions involve changes in the oxidoreduction state of participating compounds. Single redox reactions are combined to redox chains or pathways. In this respect, the electron transfer chain components are redox actors, with transitions at the oxidation state of metals like copper and iron for example. In this review, however, we will consider only the oxidation/reduction reactions that involve cysteine or methionine residues. In this context, the most documented redox reactions in plants concern the dithiol-disulfide exchange cascades involving components of the thioredoxin (TRX) and glutaredoxin (GRX) systems. Some

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glutathione S-transferases (GST) have the capacity to reduce disulfides in the presence of glutathione. Likewise, protein disulfide isomerases (PDI) belong to that category being able to oxidize, reduce or isomerize disulfide bonds. Besides these dithiol-disulfide exchange reactions, we will also consider those reactions, which are catalyzed by peroxiredoxins (PRX) or methionine sulfoxide reductases (MSR) and in which the redox state of catalytic cysteines varies. Overall, the changes in cysteine oxidoreduction state are essential for the catalysis of certain enzymes (e.g., formation of sulfenic acid on the catalytic cysteine of methionine sulfoxide reductases and peroxiredoxins) but they can also induce structural transitions needed to control the catalytic rates of enzymes (e.g., dithiol-disulfide control of fructose-1,6-bisphosphatase or NADP malate dehydrogenase in the chloroplasts) and of many other target proteins with diverse functions in transcription, translation, signalling and regulation.

8.2 Why Is There Redox Regulation in Plants?

It has become apparent in the last decades that redox reactions, as defined above, are particularly exacerbated in plants. For example, there are between 3 and 6 thioredoxin and glutaredoxin genes in non-photosynthetic prokaryotes and mammalian systems but about 40 genes of each family in photosynthetic eukaryotes. The PDI, MSR and PRX families are also extended but to a minor extent. The reasons for this expansion of redoxins are probably multiple. Part of it could be related to the existence of specialized tissues in plants, like the phloem and xylem for transport, epidermal and stomatal cells for leaf protection and gas exchange, stamen and pistil for reproduction etc. A more likely explanation is related to the physiology of plants. Unlike animal cells, plants contain plastids/chloroplasts, which play an essential energetic role by providing ATP and NADPH needed for CO_2 fixation. In animal cells, the energetic demands are mostly met by mitochondria, which are also present in plant cells. Chloroplasts not only fix CO_2 but they also release O_2 in a process called water photolysis at the level of photosystem II. As a result, in oxygenic photosynthetic organisms, in addition to mitochondria, NAD(P)H oxidases and peroxisomes, the chloroplast is an additional site of reactive oxygen species (ROS) generation. In plant mitochondria, ROS are generated at the level of complex I, ubiquinone and complex III (Navrot et al. 2007). In the chloroplasts, ROS are essentially generated at the level of photosystem II and photosystem I (Asada 2006). One plausible reason for the need of elevated redoxin systems in oxygenic photosynthetic organisms might therefore be related to the increased production of ROS in the light. Another specificity of plants is that unlike animal systems, they rely on light as an energy source, and this energy is converted into utilisable chemical energy by chlorophyll, a pigment specifically present in the chloroplasts. The presence of this specific additional compartment in plants has several consequences. First, for a number of reasons, it is essential to switch on selected enzymes in the light and turn them off in the dark. This could prevent futile cycles, like the one potentially

occurring between fructose-1,6-bisphosphatase (FBPase) and phosphofructokinase. Would such a futile cycle occur, it would induce the consumption of ATP with no net gain for the plant. Another observation is that plants lack in general efficient alternative splicing that allows in animal cells for the multiplication of isoforms from a single gene. Thus, possible explanations for the expansion in the number of genes would be first to keep "security copies" of a given gene, second to generate variants with improved selectivity or efficiency without having to rely on alternative splicing. Another relevant observation is that there is much duplication in plant genomes, which are often polyploïd. Finally the sessile lifestyle of plants requires integration of many environmental input parameters to optimize plant performance and fitness. It appears that redox cues are sensed and extensively exploited to modulate or switch developmental and acclimation processes.

8.3 Glutathione and Redoxin-Linked Pathways in Plants

Several regulatory or detoxification pathways depend on glutathione (GSH) or TRX and other thiol-linked systems in plants. In general, the proteins that have the capacity to mediate dithiol-disulfide exchange reactions (redox transmitters) possess a so-called TRX fold, namely a central pleated β sheet with 5β-strands surrounded by three or more α-helices. In TRX itself (classically about 110 amino acids), the active site with the conserved sequence WC[G/P]PC is rather surface exposed which favors the reactivity of the catalytic cysteine. Its reactivity is further enhanced by an adjacent helix dipole effect, which lowers the pKa of the thiol. In plants, TRXs are reduced either by light and the ferredoxin-thioredoxin system in plastids, or by NADPH-dependent thioredoxin reductases (NTR) in the cytosol and mitochondria. An alternative pathway in plastids consists of a fused enzyme (NtrC), composed of a NTR and a Trx module (Spínola et al. 2008). Ferredoxin thioredoxin reductase (FTR) is essentially restricted to photosynthetic organisms and is absent in mammals and non-photosynthetic organisms. This heterodimeric enzyme is uniquely shaped to accommodate on one side the substrate ferredoxin and on the other side TRX. At the interface between the two subunits of FTR lies a critical [4Fe4S] cluster and a catalytic disulfide in close proximity (Dai et al. 2007). The convergent structural evolution between FTR and NTR has been discussed in a recent review together with an exhaustive listing of the thioredoxin reductases in photosynthetic organisms (Jacquot et al. 2009). The thioredoxin structural model has given rise to a number of variants, which differ in their active site sequences, their modular organization, and/or their regeneration mechanisms. GRXs are proteins with a size similar to TRXs but with active sites that have been modified into C[P/G/S][Y/F][C/S] (class 1), CGFS (class 2), CCx[C/S] (class 3) or Cxx[C/S] (class 4) (Couturier et al. 2009). Most GRXs are reduced by the tripeptide glutathione, which itself is maintained in its reduced form via NADPH-dependent glutathione reductase. As mentioned previously, in land plants, the TRX and GRX repertoires have been greatly enhanced with about 40 genes of each category identified. TRXs, GRXs and their reducing

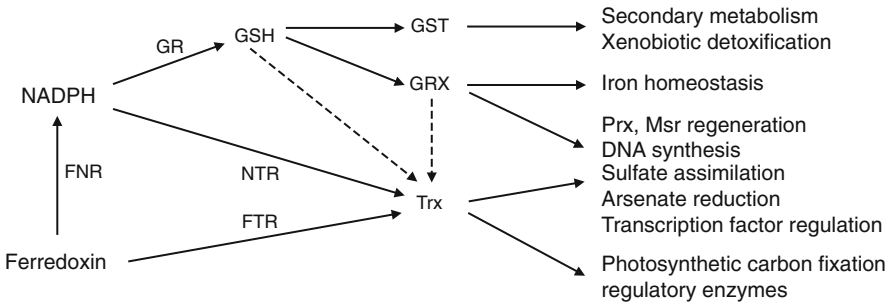

Fig. 8.1 Major functions associated with TRX and GSH/GRX reducing pathways. TRXs are usually reduced by the NADPH/NADPH-thioredoxin reductases (*NTR*) or the Ferredoxin/ferredoxin thioredoxin reductase (*FTR*) systems but a few isoforms have been shown to rely on GSH and/or GRX for their regeneration (Koh et al. 2008). GSH, maintained in its reduced form by the NADPH/glutathione reductase (*GR*) system, provides electrons to both GSTs and GRXs. FNR: ferredoxin NADP reductase

pathways have been discussed extensively in a number of recent reviews (Dietz 2003; Gelhaye et al. 2005; Rouhier et al. 2008; Chibani et al. 2009; Couturier et al. 2009; Meyer et al. 2009) and we will not detail them any further in this section. Figure 8.1 depicts the reducing systems of TRXs and GRXs in plants and some of the known functions associated to each system. The reader is invited to consult these papers and the chapter by Collet and Messens for more information on the biochemical and structural properties of these two protein families.

At least two other types of enzymes, glutathione S-transferases and protein disulfide isomerases, contain thioredoxin domains. The protein disulfide isomerases are located predominantly in the ER and the most common forms contain four thioredoxin-like modules, with an overall organization of the a-b-b′-a′ type. The a and a′ domains display CGHC active sites whereas the b and b′ modules present a TRX fold but lack the active sites. However, they are essential for some aspects of PDI activities, particularly the b′ module, which is involved in substrate recognition (Byrne et al. 2009). In the ER, the functions of PDIs are to create and isomerise disulfide bonds to ensure correct protein folding. Consequently, PDIs have both an oxidizing and reducing activity. After PDI oxidizes its protein substrates, its own disulfide bonds become reduced and need to be reoxidized. Based on results published on human and yeast proteins, different pathways apparently lead to PDI oxidation. The first candidates are the sulfhydryl oxidases Ero1 α and β flavoproteins, which catalyze the formation of disulfide bonds on PDIs with concomitant reduction of oxygen to hydrogen peroxide (Sevier and Kaiser 2008). The produced H_2O_2 can in turn be reduced by ER-located thiol-peroxidases, such as 2-Cys peroxiredoxins or glutathione peroxidases, which, once oxidized, also contribute to PDI re-oxidation (Tavender et al. 2010; Nguyen et al. 2011). Hence, the consumption of one oxygen molecule can serve to form two disulfides, allowing a tight control of ROS accumulation into the ER. Finally, it was also proposed that the proportion of oxidized glutathione, which is elevated in the ER compared to other

subcellular compartments, could be thermodynamically sufficient to reoxidize PDI active sites. It should be mentioned that these different modes of reoxidation might not equally target all PDI isoforms and that there might be specificities linked to the variety of PDI representatives, especially in photosynthetic organisms. A recent genomic analysis of PDIs in photosynthetic organisms has demonstrated that apart from the classical multimodular L-type PDI (that contains the a-b-b′-a′ modules), 8 additional PDI classes exist, which differ in their modular composition, some being monomodular, some trimodular and some others containing thioredoxin unrelated modules. Six of the PDI classes are present in terrestrial plants but three of them are restricted to algae. The detailed phylogenetic analysis can be found in Selles et al. (2011).

Another example of enzymes, which contain a TRX-like module, is glutathione-S-transferase. These enzymatic systems are often ignored although some of these enzymes possess GRX-like and peroxidase activities. For this reason, we will give a comprehensive overview of these enzymes in plants in this review. Glutathione transferases, better known as glutathione S-transferases, have been identified more than 40 years ago in rat liver (Booth et al. 1961) and in plants through their roles in the detoxification of herbicides in annual species (Lamoureux et al. 1970; Shimabukuro et al. 1971). GSTs are present in both eukaryotes and prokaryotes and constitute a multigenic superfamily that is widely distributed in nature. GSTs are the main players involved in the metabolism of electrophilic compounds within the cell. Through the addition or the substitution of a glutathione molecule (GSH, a non-ribosomally synthesized γ-Glu–Cys–Gly tripeptide) to an electrophilic center present in a small acceptor molecule, these enzymes are able to metabolize a broad range of endogenous and exogenous substrates for their detoxification, subsequent transport, export or secretion. In addition, they are also able to reduce peroxides and to recycle antioxidant molecules (ascorbate in particular) thereby providing protection against endogenous or xenobiotic substances with genotoxic and carcinogenic effects. GSTs are also involved in several other cell functions through their capacity to bind non-catalytically to a large number of endogenous and exogenous compounds.

Based on their primary sequence, 3D structure, function and cellular localization, GSTs have been initially classified into three main subfamilies: (i) the cytosolic (or soluble) GSTs, which form the largest family, (ii) the mitochondrial and peroxisomal GSTs also known as kappa GSTs, and (iii) the microsomal GSTs belonging to the superfamily of the trimeric Membrane Associated Proteins involved in Eicosanoids and Glutathione metabolism (MAPEG) (Pearson 2005). A further distinct family is represented by the plasmid-encoded bacterial Fosfomycin-resistance GSTs (FosA), a manganese-containing metalloglutathione transferase conferring resistance to the antibiotic fosfomycin (Bernat et al. 1997). The ambiguity here is that kappa GSTs are also soluble enzymes and that many GSTs included in the cytosolic/soluble class are located in organelles (nuclei, mitochondria, peroxisomes and plastids in the case of plants). Hence, although cytosolic GSTs, kappa GSTs and MAPEGs have indeed evolved separately and have to be distinguished, this classification is clearly no longer fitting (Sheehan et al. 2001).

Kappa GSTs have been discovered and characterized in both prokaryotes and eukaryotes and fulfill, in the latter, specific functions associated with mitochondria and peroxisomes, particularly in energy production and lipid metabolism, respectively (Morel and Aninat 2011). Microsomal MAPEG GSTs are enzymes, which differ substantially in size and structure from other GST families, having an average length of 150 amino acids comprising four possible membrane spanning domains and a less than 10% identity with other GST classes (Jakobsson et al. 1999, 2000; Bresell et al. 2005). In mammals, these membrane-bound trimers are involved in eicosanoid, prostaglandin and leukotriene synthesis by catalyzing GSH-dependent transferase and peroxidase reactions (Jakobsson et al. 1999).

Other GST classes initially classified as members of the cytosolic GST subfamily are present in mammals, plants, insects, parasites, fungi and bacteria. They are notably involved in primary and secondary metabolisms, and in stress response through their herbicide and xenobiotic detoxification capacities. Whereas some GST classes are found only in certain kingdoms or phyla (lambda (L), phi (F) and tau (U) in plants; delta (D), epsilon (E) in insects; beta (B) in prokaryotes, rho (R) in fishes; nu (N) in nematodes; gamma (G), etherases (GTE) and glutathione transferase (GTT) in fungi), some others are present in several kingdoms (mu (M), alpha (A), pi (P), theta (T), sigma (S), zeta (Z), omega (O), Ure2p-like as well as tetrachlorohydroquinone dehalogenase (TCHQD) and S-glutathionyl hydroquinone reductase (GHR) classes) (Table 8.1).

Although GSTs share less than 30% identity among different classes, almost all GSTs are homo- or heterodimeric enzymes with subunits of 25–35 kDa (i.e., 200–250 amino acids). Structural analyses have shown that GSTs can be divided into two domains: a highly conserved N-terminal GSH-binding domain (G-site), which is structurally similar to, and has evolved from, the TRX-fold (Atkinson and Babbitt 2009), and a less conserved C-terminal alpha-helical domain (H-site), which allows binding of a wide range of hydrophobic co-substrates (Sheehan et al. 2001). Amino acids from both domains form the catalytic site of the protein. Most typical GSTs have serine, tyrosine or cysteine as catalytic residue, but some atypical GSTs seem to use arginine or asparagine. In dehydroascorbate reductase (DHAR) and lambda GSTs, for example, the residue responsible for the enzymatic activity is a cysteine, included in a CPxA motif reminiscent of the CPxC motif found in GRXs. As a consequence, the presence of this cysteine completely changes the biochemical properties of these GSTs. Instead of promoting the formation of the reactive thiolate anion of GSH necessary for its reaction with electrophilic compounds, the presence of the cysteine provides the capability to reduce glutathionylated substrates in a manner similar to GRXs.

In plants, GSTs (encoded by 83 genes in *Populus trichocarpa* and 54 in *Arabidopsis thaliana*) can be divided into at least seven classes: theta, zeta, phi, tau, lambda, DHAR and tetrachlorohydroquinone dehalogenase (TCHQD) (Smith et al. 2004; Basantani and Srivastava 2007; Lan et al. 2009). Genes encoding GSTs are generally close to each other and grouped by class in genomes forming gene clusters. In *A. thaliana*, GSTs are divided into 34 class-specific clusters, with isolated genes representing less than 30% of the GST gene panel (Dixon et al. 2002b).

Table 8.1 Classification of GSTs

GST class	Symbol	Origin	Subunit structure	Catalytic residue
Alpha	A	Mammals	Dimer	Tyr
Beta	B	Bacteria	Dimer	Cys
Gamma	G	Fungi	Dimer	Tyr
Delta	D	Insects	Dimer	Ser
Epsilon	E	Insects	Dimer	Ser
Zeta	Z	Animals, plants and bacteria	Dimer	Ser
Theta	T	Animals, plants and bacteria	Dimer	Ser
Kappa	K	Eukaryotes and prokaryotes	Dimer	Ser
Lambda	L	Plants	Monomer	Cys
Mu	M	Mammals	Dimer	Tyr
Nu	N	Nematodes	?	Tyr
Pi	P	Mammals	Dimer	Tyr
Rho	R	Fishes	Dimer	?
Sigma	S	Animals, red and brown algae	Dimer	Tyr
Tau	U	Plants	Dimer	Ser
Phi	F	Plants	Dimer	Ser
Chi	C	Bacteria	Dimer	?
Omega	O	Mammals, fungi, insects	Dimer	Cys
DHAR	–	Plants	Monomer	Cys
GHR	–	Fungi and prokaryotes	Dimer	Cys
GTE	–	Fungi	Dimer	?
GTT	–	Fungi	Dimer	?
MAPEG	–	Eukaryotes and bacteria	Trimer	Arg
TCHQD	–	Bacteria, plants	Dimer	?
Ure2p-like	–	Fungi and bacteria	Dimer	Asn

GSTs are classified according to several criteria: (i) two GSTs belong to the same class if they share more than 40% identity, (ii) less than 25% identity separate classes in mammals, and less than 20% separate classes of GST in plants. When the percentages of identity are between 25 and 40%, other techniques are used to refine the classification such as gene structure and their number in a given species, size and position of introns and exons, immunological properties and finally enzyme activity. A few other protein families are usually classified as GSTs (EF1Bγ, MAK16, CLIC), but in general this is related to structural similarities, not to the existence of a glutathione-dependent activity. This table has been essentially constructed from the references, Pearson (2005) and Morel et al. (2009)

As an example, seven Tau GST genes are located on a fragment of 14 kb on chromosome 2 (Lin et al. 1999). In rice, less than 25% of GST genes (15 genes) are present in isolated loci in the genome. The most important cluster includes 23 Tau GSTs on a 239 kb genomic fragment of chromosome 10 (Soranzo et al. 2004). In poplar, 37 of the full-length tau GSTs and 20 tau-type fragments are arranged in six clusters (clusters I to V and VII) on five chromosomes (1, 8, 10, 11 and 19) and 4 phi GST genes constitute one cluster (cluster VI) on chromosome 14. Members of minor GST classes are sparsely distributed at single loci on different chromosomes (Lan et al. 2009). Interestingly in mammals, human alpha, mu and theta (Morel et al. 2002) and murine pi GST genes (Henderson et al. 1998) are located on specific

sections of the genome whereas delta GSTs form a single compact block of genes in insect genomes (Ding et al. 2003). The almost systematic presence of GST gene clusters in plant and animal genomes reveals common evolutionary mechanisms allowing the generation of gene redundancies.

Phi and tau GSTs are the most abundant in plants and are plant-specific. Tau GSTs catalyze conjugation reactions not only on conventional substrates but also on herbicides, such as atrazine, metolachlor, fluorodifen and thiocarbamates (Edwards and Dixon 2005). These GSTs seem to be involved in cell protection by playing a role in tolerance to environmental stress by enhancing the detoxification of herbicides (Kawahigashi 2009). Tau GSTs are also able to transfer GSH on anthocyanins, allowing their import into the vacuole by ABC transporters (Edwards et al. 2000). Members of the phi class have peroxidase and transferase activity, notably on herbicides (Marrs 1996). In addition, expression of phi GSTs is induced in response to auxin. Interestingly, AtGSTF2, the best-studied Phi GST of *A. thaliana*, is strongly induced by oxidative stress and phytohormone treatment and it is able to bind auxin non-enzymatically. Thus, this protein is involved not only in response to stress, but also in growth and development (Gong et al. 2005; Smith et al. 2003). A GST class present both in plants and animal systems is the theta class. Theta GSTs function as GSH-dependent peroxidases using glutathione to reduce organic hydroperoxides into the corresponding alcohols during oxidative stress (Frova 2003). Zeta GSTs, highly conserved GSTs in eukaryotes, act as GSH-dependent isomerases in the catabolism of tyrosine by catalyzing the isomerization of maleylacetoacetate to fumarylacetoacetate (Dixon et al. 2000). In contrast, lambda and DHAR classes show neither transferase nor peroxidase activity but exhibit GRX-like activities through their capacity to reduce disulfides (hydroxyethyldisulfide) and dehydroascorbate using, as described below, a conserved reactive cysteinyl residue. Thus, like GRXs, they might be involved in the maintenance of the cellular redox balance (Dixon et al. 2002a). The ability of GSTs to bind a large range of small molecules led to hypothesize that these enzymes could be involved in various metabolic pathways. Supporting this view, *Arabidopsis* lambda GSTs were recently shown to bind S-glutathionylated flavonols, porphyrins or tocopherols (Dixon and Edwards 2010; Mueller et al. 2000; Dixon et al. 2011). However, their exact functions *in planta* remain unknown, mostly because the study of knock-out mutants is complex considering the existence of multigenic families. TCHQD, initially discovered in the soil bacterium *Sphingobium chlorophenolicum*, which can grow on pentachlorophenol as carbon source, are found in animals, terrestrial plants and bacteria. In *S. chlorophenolicum*, TCHQD catalyses the reductive dehalogenation of tetrachlorohydroquinone to trichlorohydroquinone and then to 2,6-dichlorohydroquinone during the biodegradation of pentachlorophenol (Xun et al. 1992), an environmental xenobiotic used as fungicide for wood preservation. Members of the TCHQD class, which are closely related to zeta class, present an unusual ability to metabolize chlorinated xenobiotics. Finally, plants also possess members with similarities to microsomal MAPEG or prostaglandin E synthase families, but their function and properties have not been explored to date.

8.4 Thioredoxin Targets

A number of plant thioredoxin targets are extremely well described both in terms of physiology and structure/function. Most of these redox-regulated target enzymes are involved in carbon fixation and metabolism, and are located in plastids (Jacquot et al. 1997). Interestingly, many of these enzymes (e.g., NADP-malate dehydrogenase, fructose-1,6-bisphosphatase, CF1 ATPase) have non-regulatory counterparts in other subcellular compartments. Sequence comparisons have led to the identification of redox regulatory elements that contain critical cysteine residues (Schürmann and Jacquot 2000). These elements vary from one enzyme model to another, both in their position (sometimes as N- or C-terminal extensions, sometimes as insertions), their length (generally between 5 and 30 amino acids) and their amino acid sequence. The multiplication of known regulatory sequences and structures of redox-regulated enzymes led to the following concept: there is no "magic regulatory module" for redox control in enzymes. In fact, some of these enzymes do not even contain sequence insertions or additions but simply harbor cysteine residues that have been introduced at critical positions in their sequence. It appears that the amino acid sequence evolution of each enzyme responds to structural constraints specific for each individual case. 3D-structures have revealed at least for two of these enzymes (NADP-MDH and FBPase) that the regulatory disulfides are distant from the active site and that large molecular movements are needed to open the active sites in order for catalysis to proceed (Ruelland and Miginiac-Maslow 1999). As a consequence, these enzymes are reduced quickly via the FTR system but their catalytic activity appears on a much slower time scale. This property, linked to the additional observation that the rate of reduction is much slower than the rate of catalysis in these enzymes, classifies them as hysteretic enzymes (Hertig and Wolosiuk 1983). Of significant interest is the observation that redox regulation has been acquired along with the evolution of the green lineage and especially together with the onset of oxygenic photosynthesis. One very interesting example is NADP-MDH. In terrestrial plants, each of the two subunits of the homodimeric enzyme has amino acid extensions at both N- and C-terminus, each containing two regulatory cysteines (overall the dimeric enzyme contains four disulfide bonds in its oxidized, inactive state). In the green alga *Chlamydomonas reinhardtii*, however, the enzyme lacks the N-terminal disulfide but contains the C-terminal disulfide and is hence less tightly redox controlled. The *Chlamydomonas* NADP-MDH has been proposed to represent an evolutionary step leading to the terrestrial plant model, where full redox control has been achieved with the subsequent addition of the N-terminal disulfide (Lemaire et al. 2005). Other well-recognized targets of thioredoxins in plants participate in the detoxification of peroxides or repair of their molecular damages. PRXs are thiol peroxidases that are present in various plant cell compartments and detoxify a broad range of peroxides into alcohol, whereas methionine sulfoxide reductases are enzymes catalyzing the reduction of methionine sulfoxide into methionine. Atypical type II PRX, 2 Cys PRX, PRX Q and MSRA are all regenerated by TRXs (Rouhier et al. 2002, 2004,

2007). Interestingly, so-called glutathione peroxidases lack selenocysteines in plants and they are in fact thioredoxin-dependent enzymes (Navrot et al. 2006). A large number of additional thioredoxin targets have been identified by various methods, but principally by fishing them using TRX variants, in which the second cysteine of the active site has been substituted by site-directed mutagenesis into serine. These TRX engineered variants attack proteins with disulfides but cannot release their substrates. Client proteins are then dissociated with dithiothreitol and analyzed with proteomics techniques. This approach has led to the discovery of hundreds of putative targets involved in many different physiological pathways (Balmer et al. 2003, 2004; Montrichard et al. 2009). Some of these target enzymes have been confirmed biochemically but for many of them experimental confirmation is still lacking. A comforting observation is that a large number of these sequences contain conserved cysteines, which could potentially be involved in regulatory sites.

8.5 Glutaredoxin Targets

The first glutaredoxin-linked enzymatic system recorded is probably the ribonucleotide reductase in *E. coli*, and in fact GRX was discovered in a *trx* mutant strain still able to reduce ribonucleotides into deoxyribonucleotides (Holmgren 1976). It is likely that GRXs play a similar role in plants but there is not much evidence about it yet. A second enzymatic system linked to GRX is involved in sulphur assimilation. In terrestrial plants, the step generating reduced sulfide from adenosine phosphosulfate is catalysed by APS reductase. Higher plant APS reductases contain a built-in GRX module located at the C-terminus of the polypeptide (Bick et al. 1998). Glutaredoxin was then identified as a good electron donor to atypical type II PRXs (Rouhier et al. 2001, 2002). Interestingly, it was later found that *prx* and *grx* genes are fused in several pathogenic bacteria and cyanobacteria lending further support to this proposal (Rouhier and Jacquot 2003). GRX was also demonstrated to be a preferred electron donor to a specific subclass of B-type methionine sulfoxide reductases (Tarrago et al. 2009). Besides those "direct" biochemical observations, the need for GRX was also evaluated using a strategy similar to the one employed with TRXs, i.e. generation of GRX variants containing a single active site cysteine, formation of a stable disulfide between GRX and its targets, release of the target proteins with a reductant and identification of target proteins by mass spectrometry. This has led to the identification of nearly 100 potential targets, some of them being redundant with TRX but some others specific to GRX. This work confirmed PRXs as possible targets and also that β-carbonic anhydrase was indeed redox-regulated via GRX *in vitro* (Rouhier et al. 2005). Recent advances have indicated that in addition to their redox role, plant glutaredoxins especially of the CGFS type are involved in iron sulphur cluster assembly and iron homeostasis. We have not detailed these exciting developments in this chapter as they are included in the paper by Messens et al. in this volume. More details can also be obtained in recent reviews published by Rouhier et al. (2008) and Rouhier et al. (2010).

8.6 Evolution of the Redox Systems in Photosynthetic Organisms

Genome comparisons allow the identification of orthologous and paralogous genes and to construct robust phylogenetic relationships. This approach has been used extensively to identify and categorize components of plant redox systems. It became clear that the number of gene family members of redox proteins in plants usually exceeds the one found in non-photosynthetic multicellular organisms as impressively demonstrated when comparing TRX and GRX diversification in plants and humans. Thus, mammalian genomes code for two dithiol GRXs (Grx1 and 2) and two monothiol GRXs (Grx3 and Grx5) while 33 gene loci coding for GRXs and GRX domain-containing proteins have been identified in *A. thaliana* (Couturier et al. 2009). The largest expansion of GRX occurred in the CC-type GRX subfamily, which took over specific functions in developmental control and interact with transcription factors (Ziemann et al. 2009). Likewise 44 open reading frames for TRXs and TRX-like proteins in *A. thaliana* compare with two human TRXs. Regarding peroxiredoxins, a detailed comparison of the fully annotated genomes of *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis PPC6803* allows us to define the minimum PRX inventory in plants and green alga (Table 8.2). Thus, plastids contain at least one 2-Cys PRX, one PRX Q and one type II PRX named PRX IIE. One 1-Cys PRX localizes to the higher plant nucleus and cytosol, the PRX IIF to the mitochondrion and another type II PRX to the cytosol (Dietz 2011). Thus 7–10 *prx* genes in higher plant genomes compare with 6 *prx* genes in humans. In contrast to some animal glutathione peroxidases (GPXs), plant GPXs do not employ a selenocysteine in their catalytic peroxidatic site but, like PRXs, use a Cys residue instead. Plant GPXs are localized in plastids, mitochondria, cytosol and the secretory pathway (Navrot et al. 2006). After each peroxide reduction reaction, PRXs and GPXs are oxidized and need to be regenerated by thiol-dependent electron donors such as GRX or TRX. Usually there is specificity or at least strong preference for specific electron donors, although type II PRXs generally accept electrons from both GRXs and TRXs (Rouhier et al. 2001). In conclusion, the present knowledge suggests that redox systems in plants have evolved to particular complexity, which is linked to the multiplicity of stress factors that act on sessile land plants during their life cycle.

8.7 Systems Biology of the Redox Regulatory Circuits

The thiol/disulfide redox system of the cell can be considered as a network of components linked by electron flow. In its central part, thiol/disulfide exchange reactions take place in a cascade like arrangement (Fig. 8.2). The thiol/disulfide network is connected to other redox reactions in metabolism, such as to photosynthetic

Table 8.2 Compilation of gene families encoding GRX, TRX, PRX, GPX and SRX in *Synechocystis pcc6803*, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, *Populus trichocarpa* and *Oryza sativa*

Gene families		*S. pcc6803*		*C. reinhardtii*		*A. thaliana*		*P. trichocarpa*		*O. sativa*	
PRX	2-CysPrx	5	1	7	2 + 1[a]	9	2	9	2	7	1
	1-CysPrx		1		–	10[b]	1		1		1
	PrxIIB		–		1		3		2		1
	PrxIIE		1		1		(+1)		1		2
	PrxIIF		–		1		1		1		1
	PrxQ		2		1		1		2		1
GPX	Cys	2	2	5	3	8	8	6	6	5	5
	SeCys		–		2		–		–		
SRX				1		1		1		1	
TRX	Trx-f	4		10	2	44	2	44[c]	1	30[d]	
	Trx-m		1		1		4		8		
	Trx-x		1		1		1		1		
	Trx-y		1		1		2		2		
	Trx-z				1		1		1		
	Trx-h				2		8		7		
	Trx-o				1		2		1		
	Others		1		2		24		23		
GRX[e]	Class I (CPY/FC)	3[f]	2	7	2	33	6	38	6	29	5
	Class II (CGFS)		1		4		4		5		5
	Class III (CCxx)				1		21		24		17
	Class IV						2		3		2

[a]The *C. reinhardtii* genome codes for a fusion protein consisting of 2-CysPRX and TRX domains (Dayer et al. 2008)
[b]PrxIIA appears to be a non functional gene (Dietz 2011)
[c]Chibani et al. (2009)
[d]Nuruzzaman et al. (2008); the TRX assignment in *O. saliva* to subfamilies is still incomplete
[e]Ziemann et al. (2009)
[f]Marteyn et al. (2009). The numbers in the first column assigned to each organism give the total sum of family members, and the numbers in the second column depict the numbers of each isoform within the family

electron transport by means of electron input elements (Dietz and Pfannschmidt 2011). Redox transmitters, as described before, mediate electron transfer to redox target proteins. Ultimately the electrons are passed on to electron acceptors such as reactive oxygen species and reactive nitrogen species. During oxidative stress when ROS and RNS accumulate to high levels, they may directly react with some regulatory targets converting them into oxidized disulfide, sulfenylated, sulfinylated, sulfonylated, nitrosylated, nitrated or glutathionylated forms (Jacob and Anwar 2008). However, during regular metabolism, most ROS and RNS are safely detoxified minimizing uncontrolled redox reaction. This role is achieved

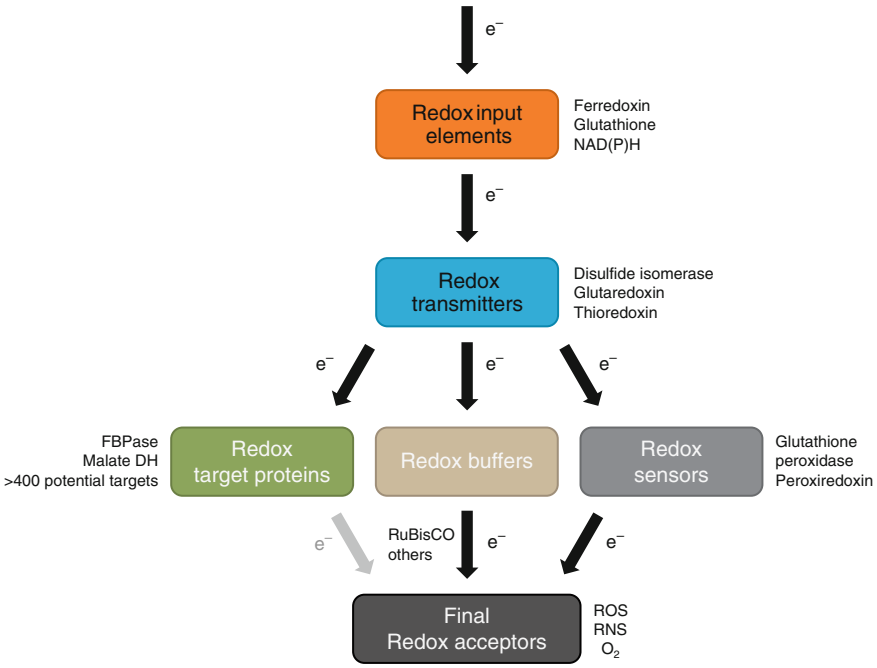

Fig. 8.2 Simplified schematics of the principle structure of the redox regulatory network of the cell. Redox input elements, transmitters, target proteins, sensors, buffers and ROS and RNS constitute a thiol/disulfide redox regulatory network that controls essentially all aspects of metabolism and development. Examples for each type of component are indicated

by the antioxidant systems of the cell. PRXs and GPXs are both antioxidants and elements of the thiol/disulfide network. Therefore, they function as redox sensors, reducing peroxides and withdrawing electrons from the thiol-based redox transmitters, thereby assisting the system to adjust to the prevailing redox condition of the cell. During more severe oxidative conditions, ROS and RNS not only react with protein thiols of these sensors or of less preferred regulated target proteins but also with other protein thiols that likely buffer ROS bursts. Thus, the concept of redox input elements, transmitters, targets, sensors and final electron acceptors, needs to be extended to redox buffer proteins (Fig. 8.2). Proteomics approaches have identified more than 400 potential thiol-oxidation targets in plants (Lindahl and Kieselbach 2009). The distinction between regulatory targets and redox buffer proteins is often difficult, as illustrated by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). With its stromal concentration of 0.5 mmol/L and 8 large and 8 small subunits with $9 + 4$ Cys residues in *A. thaliana*, RubisCO represents the largest thiol pool of any compound in the chloroplast. Moreno et al. (2008) titrated RubisCO with redox reagents and observed two effects; moderate oxidation inhibited RubisCO carboxylation activity while strong oxidation triggered proteolysis. The increasing wealth of information on plant redox regulation and

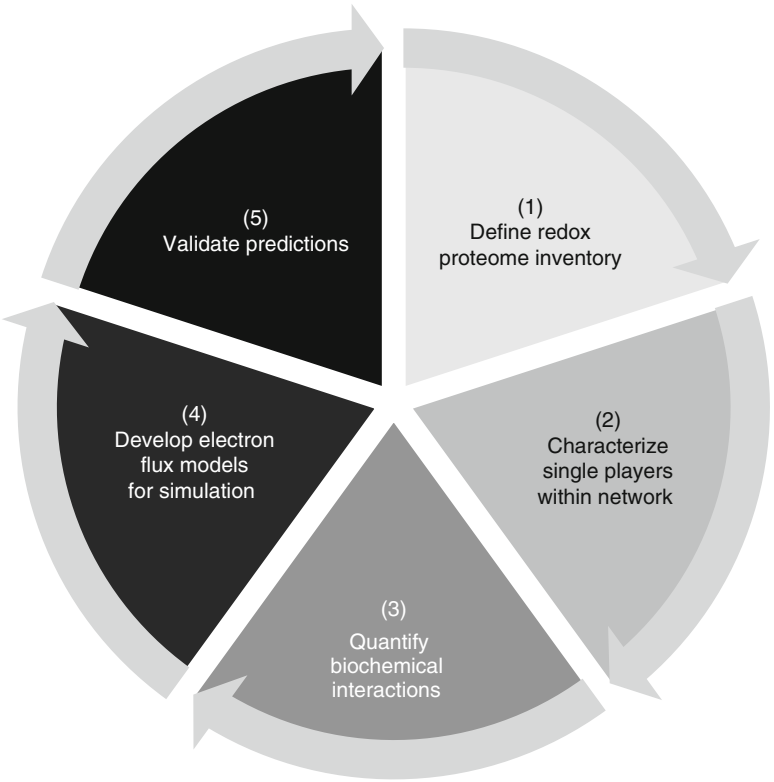

Fig. 8.3 Systems biology approach needed for complete understanding of the thiol/disulfide redox regulatory network (see text for details)

its unique complexity, calls for the next step in analytical pervasion. Figure 8.3 depicts the work flow of a typical systems biology approach, applied to redox biology (in this respect, theoretical and practical concepts concerning hubs and bottlenecks in signalling networks can be found in a recent review by Dietz et al. in 2010). As pointed out above, the basic inventory of participating proteins but also first sets of qualitative and quantitative interactions are available as well as genetic approaches for functional validation. In each work module, additional experimental efforts are still needed, for instance to identify less abundant thiol/dithiol transition proteins and to deepen our quantitative understanding on interactions. In addition, the development of descriptive qualitative and quantitative models for simulation and prediction are needed to understand the competition for electrons and the functionality of feedback and feed forward regulation achieved through the redox network. First models on H_2O_2 reduction by the ascorbate-dependent water-water cycle have been developed by Polle (2001) and were combined with photosynthesis by Laisk et al. (2006). However these models do not include the thiol-disulfide network as central controlling mechanism. Adimora et al. (2010) constructed a

predictive model of the rat liver cell thiol system and analyzed steady-state fluxes. The simulation indicates an unequal flux through the thioredoxin-dependent and glutathione-dependent antioxidant pathways. In human cells, TRX is predicted to bear the major antioxidant defense load due to the function of peroxiredoxins and protein disulfide oxidation and their regeneration (Adimora et al. 2010). Similar attempts are needed for photosynthesizing cells.

8.8 Concluding Remarks

A nagging question remains in photosynthetic organisms: why are there so many TRX and GRX isoforms and what about their possible redundancy? We have given a number of arguments in this paper that provide possible answers to this question but many are awaiting experimental confirmation. The questions are of course difficult to answer because of the complexity of the redox network but also because genetic tools for gene knockout are lagging behind in land plants compared to yeast. In this respect, photosynthetic bacterial models such as *Synechocystis* are of interest. Another possibility would be to use *Physcomitrella patens*, a moss in which gene knockout is much more feasible. Of course high throughput gene sequencing will also help us compare the redoxin equipment along the evolution of the green lineage and we will certainly obtain useful information by comparing the photosynthetic genomes in the future. Finally, comparing redox regulation networks in perennial photosynthetic organisms (*e.g.*, trees) *versus* annual species will also be of interest, and comparing the poplar and *Arabidopsis* models is certainly a first step in this direction.

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Chapter 9
Prokaryotic Redox Switches

John D. Helmann

Abstract Reduction-oxidation (redox) reactions are ubiquitous in metabolism. In microbial systems, individual cells monitor the internal and external environment with a diverse array of regulatory redox sensors that control the expression of appropriate adaptive responses. These sensors detect the presence of redox active molecules in the environment as well as those produced by metabolic processes within the cell. Molecular oxygen is the prototypic oxidant, and sensors of O_2 help coordinate the switch between anaerobic and aerobic lifestyles. The overall redox balance of the cell can be sensed by proteins monitoring the ratio of oxidized and reduced cofactors, such as low molecular weight thiols, the NADH pool, or membrane-associated quinones. Reactive oxygen and nitrogen species, produced endogenously or present in the environment, are also important signal molecules that can be readily sensed by their redox activity. This suite of sensor proteins is as diverse as their activating signals. Some sensors contain redox active metal centers that may include mononuclear iron, iron-sulfur clusters, and heme cofactors. Other redox switches rely on the facile oxidation and reduction of bound flavin cofactors or cysteine thiolates to effect changes in protein activity. Finally, some regulators assess redox-related signals by reversibly binding to molecules that signal redox status. A survey of these diverse mechanisms provides insights into the manifold pathways that enable cells to adapt to a range of environmental conditions that collectively comprise redox stress.

Keywords Flavoprotein • Heme sensor • Iron-sulfur cluster • Metal-catalyzed oxidation • Thiol

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9.1 Electron Transfer Reactions Within the Cell: The Flavors of Biological Electron Transfer

Life processes are inextricably linked to the transfer of electrons. Photosynthesis relies on the generation of high potential electrons that serve to generate NAPDH cofactors for the reduction of CO_2 to organic molecules. Conversely, catabolism often relies on the oxidation of organic molecules to yield both energy (in the form of ATP) and reducing equivalents (in the form of NADH). These two currencies (or their equivalents) fuel anabolic reactions. Redox reactions therefore help drive many of the central processes that underlie metabolism.

Most electron transfers within the cell involve either a metal center or an organic electron carrier as cofactor. Iron is particularly well suited for cellular redox chemistry, although both manganese and copper also play key roles (Frausto da Silva and Williams 2001). Redox active iron centers may include a single ion (a mononuclear iron site), multiple ions, iron-sulfur clusters, or heme iron (Outten and Theil 2009). The local protein environment serves to tune both the redox midpoint of the metal center and the specificity of interaction with redox partners. In addition to metal centers, redox active proteins may use protein thiols (or more accurately, the thiolate ion) or a bound flavin cofactor (Antelmann and Helmann 2011; Becker et al. 2011). These protein-bound redox centers equilibrate with diffusible electron carriers in the cell including low molecular weight thiols and thioredoxins, the NAD^+/NADH couple and, within membranes, the quinones (Green and Paget 2004). While most of the time electrons are transferred to their intended recipients, aberrant reactions also occur and can give rise to reactive oxygen and nitrogen species (RNOS). These species can, in turn, react with protein thiols, cofactors, or metal centers (Imlay 2003).

9.2 Bacterial Redox Sensors and Switches

Redox reactions provide the molecular mechanism for a wide variety of redox sensors and switches. Here, we provide a broad survey of the best-characterized examples within prokaryotes (Bacteria and Archaea), many of which are paradigms for large classes of redox sensors. As defined here, a *redox sensor* has a regulatory role: it detects redox active compounds (e.g., O_2, RNOS) or compounds that report on a change in cellular redox status (e.g., NADH, quinones) and regulates the expression or activity of other proteins in response. Sensing may result from the reversible binding of redox active compounds or by reactions leading to redox changes in the sensor protein or its bound cofactors. Classic examples include the OxyR and Fnr-based redox sensors in *Escherichia coli*. A *redox switch* can be defined as a reversible modification (switch) that turns the activity of a protein on or off. Thus, redox sensors often contain an embedded redox switch. In addition to their role in redox-based regulators, redox switches also occur in

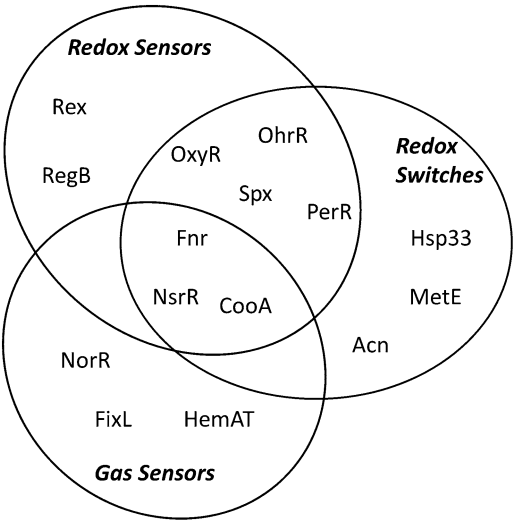

Fig. 9.1 A Venn diagram illustrating a conceptual framework for thinking about redox sensors and switches and their relationship to gas sensors. *Redox sensors* are those proteins that both sense redox changes and in response control gene expression. *Redox switch* refers to a reversible modification of a protein or protein-bound cofactor to control protein activity. Many, but not all, redox sensors contain an embedded redox switch as indicated by the representative examples and discussed in the text. However, the term redox switch is more often associated with non-regulatory proteins (e.g. Hsp33). Some proteins that sense redox active cofactors (e.g. sensing of NADH/NAD^+ ratios by Rex) do not function as redox switches. Bacterial gas sensors use some of the same cofactors as redox sensors, although many do not undergo a redox change upon ligand-binding

redox-regulated enzymes. Classic examples include the redox regulation of the *E. coli* Hsp33 chaperone (Kumsta and Jakob 2009) and the reversible inactivation of *E. coli* methionine synthase by S-glutathionylation (Hondorp and Matthews 2004). Bacteria also contain gas sensors that, at least in some cases, also rely on redox chemistry to sense their ligands (Fig. 9.1).

Redox sensors and switches play a variety of roles within the cell and, as a group, they take advantage of nearly the full range of redox chemistry that is used in metabolic pathways (Fig. 9.2). First, they can sense the redox balance within the cell by monitoring the levels of reduced NADH and quinones within the electron transfer chain. Second, they can sense the oxidation of normally reduced species, including protein and low molecular thiols (disulfide stress), that results from the depletion of reducing equivalents within the cytosol or exposure to oxidizing agents. Third, redox sensors can sense molecular oxygen, RNOS, and non-specific oxidants using a variety of metal centers, flavin cofactors, and redox active thiols (reviewed in Green and Paget 2004).

Of the various types of redox reactions and cofactors in the cell, there are relatively few that have not yet been described as also serving a regulatory role

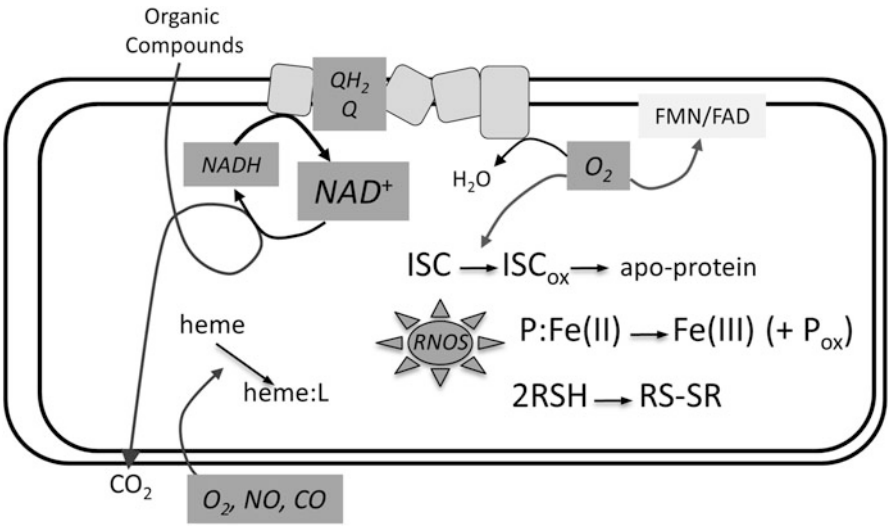

Fig. 9.2 Conceptual overview of redox sensing within a typical bacterial cell. The major classes of redox signals in the cell are in each case shown against a shaded background. Catabolism of organic compounds generates reduced electron carriers (e.g., NADH) which delivers electrons to the electron transport system. With the help of membrane soluble organic carriers (the quinones, Q), electrons are transferred to a terminal electron acceptor (here, O_2). Oxidative metabolism and redox-cycling compounds give rise to ROS, and RNS such as NO are generated during denitrification or by NO synthases. Collectively, these RNOS species are sensed by heme-containing gas sensors (which sense primarily NO and O_2 by formation of a heme:ligand complex; heme:L), ISC-containing proteins (NO, O_2, and RNOS), and thiol-containing redox proteins (RNOS). For non-heme Fe(II)-containing proteins, MCO may lead to protein oxidation (P_{ox}). Proteins containing FMN/FAD cofactors can also sense O_2 and other redox signals

within a prokaryotic redox switch or sensor protein. For example, there are few if any regulatory switches that utilize the redox activity of either copper or manganese. Another notable example is oxidation of methionine (Met) to methionine sulfoxide, a reversible protein modification that would seem well suited to provide a redox switch. Met oxidation is widespread in response to strong oxidants like hypochlorite and it has been suggested that this is a significant contributor to cell killing by activated neutrophils (Rosen et al. 2009). Since oxidized Met can be repaired by two distinct and diasterospecific methionine sulfoxide reductases, this could regulate protein activity. Indeed, such a mechanism has been described for calmodulin in mammals (Bigelow and Squier 2011), but has not yet been documented in the Bacteria.

Here we provide a survey of redox sensors and switches organized according to the chemical nature of the primary redox switch. For each class of sensors, more focused reviews are cited that provide much greater detail on both the mechanistic and structural basis of the switch mechanism and the resulting conformational changes. For a broad overview, the reader is referred specifically to an excellent

2004 review (Green and Paget 2004). The present work takes inspiration from this earlier review and includes an update on some of the same regulatory systems as well as additional systems that have come to prominence in the interim.

9.3 Redox Regulation by Mononuclear Metal Centers

The redox activity of metal ions, and iron in particular, has been co-opted by numerous regulators as a facile mechanism to signal the presence of RNOS (Helmann 2010; Outten and Theil 2009; Green and Paget 2004). Four representative examples include the PerR, NorR, IrrR, and HbpS sensor proteins (Table 9.1). In each of these cases, a redox active species is sensed by coordination and often chemical reaction with an iron atom leading to a conformational change in the regulator that leads to changes in gene expression.

9.3.1 Bacillus subtilis PerR

Bacillus subtilis PerR is a dimeric, metal-dependent repressor of the Fur (ferric uptake repressor) family (Herbig and Helmann 2001; Lee and Helmann 2007). In response to sub-micromolar levels of H_2O_2, PerR is functionally inactivated leading to the induction of protective functions including catalase, alkylhydroperoxide reductase, and an iron sequestration protein (Lee and Helmann 2006b; Helmann et al. 2003). PerR has evolved to be highly sensitive to oxidative inactivation to ensure that protective functions are induced before damage to other macromolecules is widespread. PerR orthologs are widespread in the Bacteria and, together with OxyR, represent one of the most prevalent mechanisms for regulating adaptation to H_2O_2 (Duarte and Latour 2010).

PerR senses H_2O_2 by metal-catalyzed oxidation (MCO) of the protein at one of two distinct metal-binding sites (Lee and Helmann 2006b). The first site plays a structural role and contains Zn(II) held with four Cys ligands. This thiolate rich site is not reactive with even high concentrations of H_2O_2 (Lee and Helmann 2006a), but can be oxidized by the strong oxidant, hypochlorite (Chi et al. 2011). This contrasts with some other Zn(II) sites in which coordinating thiolates are highly reactive with ROS (e.g., RsrA; see below). The second metal-binding site serves to regulate DNA-binding activity and can coordinate either Mn(II) or Fe(II). The iron-loaded form of PerR (PerR:Fe) is highly sensitive to H_2O_2 with rates of inactivation in excess of 10^5 M^{-1} s^{-1}. In contrast, the Mn(II) form (PerR:Mn) is non-reactive (Lee and Helmann 2006b). Thus, the PerR regulon can be derepressed by very low levels of H_2O_2 unless the cytosol has very low Fe(II) and comparatively high Mn(II) levels (Fuangthong et al. 2002). This provides a mechanism whereby PerR can tune the induction of peroxide stress genes to the bioavailability of Fe(II) in the cell. This is likely adaptive since the major toxic effects of H_2O_2 arise from

Table 9.1 Representative iron-containing redox sensors (Fe, ISC, heme)

Organism	Sensor	Targets and/or function	Class	Signal(s)	Mechanism
B. subtilis	PerR	Peroxide stress response	Fe(II)	H_2O_2	MCO (2-oxo-His)
E. coli	Fur	Iron homeostasis	Fe(II)	H_2O_2	Unknown
E. coli	Fur	Iron homeostasis	Fe(II)	NO	DNIC
E. coli	NorR	NO detoxification	GAF:Fe(II)	NO	Mononitrosyl iron complex
S. reticuli	HbpS	Catalase regulation	Fe(II)?	ROS?	MCO (protein crosslinking)
E. coli	Fnr	Anaerobic metabolism	$[4Fe\text{-}4S]^{2+}$	O_2	Cluster degradation
E. coli	SoxR	Chemical protection	$[2Fe\text{-}2S]^{+}$	Oxidants	$[2Fe\text{-}2S]^{+} \gg [2Fe\text{-}2S]^{2+}$
E. coli	IscR	FeS cluster homeostasis	$[2Fe\text{-}2S]^{+}$	Oxidants	ISC disassembly
E. coli	NsrR	NO detoxification	$[2Fe\text{-}2S]^{+}$	NO	DNIC
E. coli	AcnB	mRNA stability/translation	$[4Fe\text{-}4S]^{2+}$	Fe, ROS	Cluster degradation
B. japonicum/ *S. meliloti*	FixL	N_2 fixation	PAS:heme	O_2	Heme:O_2-binding
E. coli	DOS-RNC	mRNA turnover (DOS-RNC = DosP + DosC + RNases)	PAS:heme (DosP) GCS:heme (DosC)	O_2	Heme:O_2-binding
M. tuberculosis	DosS	Dormancy functions	GAF:heme	O_2, MQ, NO?	Heme:ligand-binding
M. tuberculosis	DosT	Dormancy functions	GAF:heme	O_2	Heme:O_2-binding
V. fisheri	H-NOX	Symbiosis functions	H-NOX:heme	NO	Heme:NO-binding
B. japonicum	Irr	Iron homeostasis	Heme	heme, ROS	MCO
R. rubrum	CooA	CO oxidation	CRP:heme	CO ($-O_2$)	Heme:CO-binding

Fenton chemistry in which H_2O_2 reacts with Fe(II) generating hydroxyl radicals that damage macromolecules including DNA, proteins, and potentially the cell membrane (Imlay 2003).

Both biochemical and structural studies support a model in which H_2O_2 is sensed by reaction with Fe(II) in PerR:Fe leading to the generation of a reactive hydroxyl radical that modifies one of three His residues coordinated to the Fe(II) atom (Lee and Helmann 2006b; Traore et al. 2009). The most frequent reaction is at His37 in *B. subtilis* PerR, with a lesser degree of oxidation at His91. His93, which is also an Fe(II) ligand, is refractory to modification apparently due to its own position opposite the site of approach of the incoming H_2O_2 (Jacquamet et al. 2009). Oxidation of His to 2-oxo-histidine and the concomitant oxidation of Fe(II) to Fe(III), which binds much less tightly to PerR, leads to derepression of the peroxide stress response (Lee and Helmann 2006b). Although multiple cycles of oxidation may be possible *in vitro*, in the cell the predominant product is PerR oxidized on a single amino acid. Since His37 is located in the DNA-binding domain, whereas the bulk of the Fe(II) coordinating residues are in the dimerization domain, loss of Fe(II) leads to a reorientation of the DNA-binding domains and PerR adopts a conformation no longer suited for DNA-binding (Jacquamet et al. 2009; Duarte and Latour 2010; Giedroc 2009).

The PerR-regulated peroxide stress response is known to be transient (Helmann et al. 2003), yet the mechanisms enabling the cell to re-establish repression are not well defined. There is no known repair pathway for 2-oxo-His residues, and it is most likely that oxidized PerR is degraded in the cell. Repression is presumably re-established by newly synthesized PerR, which is autoregulated (Fuangthong et al. 2002). PerR thereby functions as a sacrificial regulator. While unusual, this is not unique to PerR: the LexA repressor of the SOS response is regulated by its self-catalyzed degradation (Pruteanu and Baker 2009) and the activity of Ada as a transcription factor for the adaptive response to alkylation stress is regulated by irreversible protein modification (He et al. 2005).

9.3.2 *Sensing of H_2O_2 and NO by Fur Family Proteins*

PerR regulators have evolved to be exquisitely sensitive to H_2O_2-mediated protein oxidation. In contrast with *B. subtilis* PerR, the Fur protein in this organism functions physiologically as an Fe(II) sensor and is less sensitive to inactivation by H_2O_2 (Lee and Helmann 2006b; Helmann et al. 2003). Since both PerR and Fur are homologs with similar Fe(II)-binding sites, it will be of interest to determine how these proteins have evolved to enhance or suppress, respectively, their reactivity with H_2O_2. These proteins bind and sense Fe(II) by reversible binding to a solvent exposed site. In PerR, Fe(II) is five-coordinate with an open coordination site for the incoming H_2O_2. The details of Fe(II) coordination by *B. subtilis* Fur are unknown, but Fe(II) may be six-coordinate, which could account for the lower reactivity

with oxidants. Managing the reactivity of this potentially exposed ferrous ion to endogenous oxidants therefore presents a challenge.

In contrast with *B. subtilis* Fur, *E. coli* Fur is inactivated by sub-micromolar levels of H_2O_2 and, in this sense, resembles PerR (Varghese et al. 2007). The chemical mechanism of this inactivation has not been reported, but is suggested to be due to oxidation of the bound ferrous ion, the protein, or both. Paradoxically, inactivation of Fur leads to derepression of iron uptake under precisely those conditions of oxidative stress where this would be disadvantageous. To counter this effect, the OxyR transcription factor upregulates Fur protein levels to re-establish effective repression of the Fur regulon (Zheng et al. 1999; Varghese et al. 2007). This represents one evolutionary solution to the problem of the intrinsic reactivity of protein-bound Fe(II).

At first glance, *B. subtilis* appears to have a similar regulatory circuitry since PerR is a repressor of the *fur* gene (Fuangthong et al. 2002). However, the *fur* gene itself is not peroxide inducible even though it is regulated by PerR (Fuangthong et al. 2002). This is due to the fact that PerR:Mn is the favored repressor for the *fur* gene and PerR:Fe, the form that responds to H_2O_2, is ineffective. The significance of the PerR regulation of the *fur* gene is not entirely clear, but it may be related to modulating the expression of iron uptake pathways in response to the relative levels of Mn(II) and Fe(II) in the cell.

PerR and Fur proteins also respond to nitric oxide (NO). In the presence of NO, the Fe(II) atom in *E. coli* Fur is dinitrosylated (generating a dinitrosyl iron complex; DNIC) and this results in a conformational change that leads to derepression (D'Autreaux et al. 2004). In *E. coli*, Fur and NorR together regulate most genes that are strongly induced by NO stress (Mukhopadhyay et al. 2004). Similarly, in *B. subtilis* NO is known to derepress both the PerR and Fur regulons, presumably by a similar mechanism of iron nitrosylation (Moore et al. 2004). This is apparently a gratuitous induction since there is no apparent benefit to the cell of inducing the Fur and PerR regulons in response to NO stress. Indeed, *B. subtilis fur* mutants are more sensitive to NO than wild-type under at least some growth conditions (Moore et al. 2004). Although nitrosylation of the non-heme iron of Fur family proteins does not confer any obvious benefit to the cell, a related mechanism is used by the NO-specific sensor, NorR.

9.3.3 Sensing of NO by NorR

E. coli has a complex adaptive response to RNOS that involves multiple regulatory proteins (Mukhopadhyay et al. 2004; Pullan et al. 2007). These include OxyR, a sensor of H_2O_2 (and perhaps other RNOS species) that utilizes a highly reactive thiolate, and two sensors of NO: the non-heme iron-dependent NorR and the iron-sulfur containing NO sensor, NsrR. In addition to these dedicated sensors, RNOS perturb the regulation of numerous other regulons by affecting the integrity or activity of regulatory proteins (Flatley et al. 2005; Pullan et al. 2007).

The ability of NorR to sense NO relies on the mononitrosylation of a non-heme iron atom (Bush et al. 2011) and is, in this respect, similar to the described derepression of the Fur and PerR regulons. Nitrosylation of NorR leads to up-regulation of the *norVW* operon encoding a flavorubredoxin and associated oxidoreductase that detoxify NO by reduction to N_2O (nitrous oxide) under anaerobic conditions.

NorR is an activator protein that functions with the σ^{54} RNAP to enhance transcription (reviewed by Bush et al. 2011). Activators functioning as partners for the σ^{54} RNAP are distinct from most other bacterial transcription factors. Much like some eukaryotic transcription factors, these ATP-dependent enhancer binding proteins (bEBP) can function from a variety of positions relative to the core promoter elements (Shingler 2011). At most promoters, σ^{54} RNAP requires a bEBP in order to effectively engage the promoter as an open complex competent for transcription initiation (Wigneshweraraj et al. 2008). Like other bEBPs, NorR is an ATPase that couples the energy of ATP hydrolysis to the remodeling of the σ^{54} RNAP pre-initiation complex.

NorR is a complex protein with three distinct functional domains (Bush et al. 2011). An amino-terminal GAF domain binds the non-heme iron atom and senses NO. In the absence of NO, this GAF domain inhibits the activity of the AAA^+ ATPase domain and thereby prevents promoter activation. A carboxy-terminal domain contains a DNA-binding helix-turn-helix motif. As described in detail elsewhere (Bush et al. 2011), the current model for NorR activation at the *norVW* promoter region posits that an oligomer (likely a hexamer) of NorR is bound in a higher order complex that is functionally inactive. Sensing of NO relieves the GAF-domain mediated repression of the ATPase domain, and enables the hexameric protein to engage productively with RNAP. It is presently unknown whether binding of NO to a single subunit of the oligomer is sufficient for activation, whether activation is modulated by the extent of NO modification of the oligomer, or whether there is cooperativity in ligand binding.

9.3.4 Sensing of ROS by HbpS

HbpS is an extracellular, oligomeric protein that functions in transducing redox signals to the SenS-SenR two-component system (TCS) in the cellulose-degrading actinomycete *Streptomyces reticuli* (Siedenburg et al. 2011). HbpS is a heme-binding protein that is exported through the twin-arginine transport (TAT) system. Structural studies reveal both a hemin-binding site and a bound iron. It is suggested that iron would normally be provided by the non-enzymatic oxidation of heme (Ortiz de Orue Lucana et al. 2010). In the absence of ROS, extracellular HbpS interacts with the SenS sensor kinase to inhibit autophosphorylation. In the presence of compounds likely to lead to metal-catalyzed protein oxidation (including heme, iron, or H_2O_2), conformational changes within the HbpS octamer activate the SenS sensor kinase (Ortiz de Orue Lucana et al. 2010). These findings support a model in which HbpS alters the activity of the SenS-SenR TCS in response to redox stress.

Studies conducted *in vitro* reveal that HbpS oxidation leads to protein-protein crosslinks, likely due to dityrosine formation (between Tyr77 in two protomers) as well as protein carbonylation (Ortiz de Orue Lucana et al. 2010). The nature of the oxidized products of HbpS in cells is not yet clear. The relevant oxidation event may be the decomposition of the bound heme, the formation of protein-protein crosslinks, or other modifications of the HbpS redox sensor.

Once activated by exposure to ROS in the presence of free (or hemin-associated) iron, the HbpS-SenS-SenR signaling cascade activates transcription of the *senS, senR,* and *hbpS* genes themselves, the *cpeB* catalase-peroxidase, and *furS* encoding a Fur family redox sensor (Ortiz de Orue Lucana and Groves 2009). Although the details are not yet clear, the current model suggests that oxidation of HbpS relieves the inhibition of the SenS-SenR TCS. Newly synthesized HbpS then serves to downregulate SenS activity if, upon secretion, it is not also oxidized. The CpeB catalase is proposed to protect newly synthesized HbpS from being oxidized and to enable the system to reestablish equilibrium. This feedback mechanism may help ensure that the cell produces sufficient catalase-peroxidase to protect against ROS. If correct, this mechanism is similar in some ways to the sacrificial oxidation of PerR leading to the induction of catalase and peroxidases in *B. subtilis*.

FurS is itself a Fur family repressor, which is redox regulated by H_2O_2-mediated disulfide bond formation between two Zn(II) ligands, Cys93 and Cys96, leading to derepression of the *furS-cpeB* operon (Ortiz de Orue Lucana et al. 2003, 2012). Thus, expression of CpeB catalase is proposed to respond to both extracellular redox signals, transduced by the HbpS-SenS-SenR cascase, and intracellular RNOS sensed by FurS.

9.3.5 Mononuclear Iron Centers Function as Redox Switches for Regulating Enzyme Function

The susceptibility of protein-associated Fe(II) sites to oxidation by H_2O_2 and/or nitrosylation by NO varies enormously. As noted above, PerR and *E. coli* Fur react rapidly with H_2O_2 leading to changes in gene expression, whereas *B. subtilis* Fur is less reactive. Bacterial cells contain many enzymes that are known or likely to use Fe(II) as a cofactor for catalysis, although the full extent of Fe(II) metallation of enzymes is poorly understood (Anjem and Imlay 2012). This is due, in part, to the fact that many enzymes will function (albeit at variable levels) with more than one divalent metal cofactor and *in vitro* studies conducted aerobically may not include Fe(II) when testing metal specificity (Anjem et al. 2009).

Enzymes that have a mononuclear Fe(II) center are potentially susceptible to inactivation by RNOS (Sobota and Imlay 2011). However, there are no clear examples where oxidation of a mononuclear iron center is used as a redox switch for directly regulating enzyme function. Indeed, Fe(II) oxidation seems ill-suited for monitoring H_2O_2 levels since oxidation leads to the production of highly reactive hydroxyl radicals, which can covalently modify and irreversibly inactivate the protein

(as seen with PerR). Some mononuclear Fe(II) enzymes appear to use a Cys residue as a sacrificial ligand that, in response to H_2O_2, is reversibly oxidized to the cysteine sulfenate (Anjem and Imlay 2012) and this also has the potential to regulate enzyme activity. Fe(II) centers are also adept at sensing other oxidants (e.g., NO), leading to the loss or inactivity of the catalytically essential metal center and thereby altering metabolism. One solution to the problem of redox sensitivity of Fe(II) centers is the substitution with a different metal ion. In *E. coli*, low levels of H_2O_2 activate OxyR-dependent transcription of the MntH Mn(II) uptake system (Kehres et al. 2002). The resulting increase in cytosolic Mn(II) levels metallates normally Fe(II) dependent enzymes (Anjem et al. 2009; Anjem and Imlay 2012). In some cases this may allow the enzyme to function as a mangano-enzyme, whereas in others this may simply be a way of excluding redox-active Fe(II) from the active site and thereby protecting the protein against destruction by MCO reactions (Sobota and Imlay 2011).

9.4 Sensing of Redox with Iron-Sulfur Clusters

Iron-sulfur clusters (ISC) play a variety of roles in protein structure and function (Johnson et al. 2005). In some proteins, an ISC seems to play a primarily structural role analogous to the widespread utilization of Zn(II) coordination in stabilizing zinc finger domains. In other enzymes, the ISC functions in coordination or activation of substrates but without redox changes in the cluster (analogous to the use of Zn(II) as a Lewis acid). Finally, ISCs may serve as a conduit for electrons, often as part of a multistep electron transport process (Johnson et al. 2005). Here, we focus on those examples where the ISC serves as a redox switch to control the activity of regulatory proteins. ISCs are particularly well suited for the detection of molecular oxygen (FNR), NO (NsrR), and other one-electron oxidants (SoxR) (Outten and Theil 2009; Crack et al. 2012).

9.4.1 FNR as a Sensor of Molecular Oxygen

The fumarate and nitrate reduction regulator (FNR) senses molecular oxygen and functions as a global regulator to effect large changes in cellular metabolism including the activation of alternative respiratory pathways. The *E. coli* FNR protein is the best-understood example of this class of sensor, which is widespread in the Bacteria (Dufour et al. 2010). Molecular oxygen is sensed by the oxidation of a $[4Fe\text{-}4S]^{2+}$ cluster within FNR as discussed in detail elsewhere (Crack et al. 2008). Many of our insights into the biochemical mechanisms of oxygen sensing by FNR were sparked by the development of techniques for the purification of active FNR under strictly anaerobic conditions (Yan and Kiley 2009) and the subsequent application of a variety of spectroscopic and biochemical techniques for monitoring iron-sulfur cluster chemistry.

In the current model for FNR function, the dimeric FNR protein contains a single $[4Fe\text{-}4S]^{2+}$ cluster per protomer and binds tightly to the promoter regions of target operons to activate transcription. In the presence of molecular oxygen, FNR is oxidized to an intermediate $[3Fe\text{-}4S]^{1+}$ cluster with release of Fe^{2+} and superoxide anion ($O_2^{-\cdot}$). This initial oxidation is sensitive to relatively low levels of oxygen (a second order rate constant of $k_1 \sim 250\ M^{-1}\ s^{-1}$). In a second step ($k_2 \sim 0.008\ s^{-1}$), the cluster decays further to form a relatively stable $[2Fe\text{-}2S]^{2+}$ with the release of 2 sulfide ions and Fe^{3+} (Crack et al. 2008). Further decomposition of the $[2Fe\text{-}2S]^{2+}$ to generate apo-FNR is likely to occur *in vivo* since the major form of FNR in aerobic *E. coli* is the monomeric apo-protein. The inactivation of FNR by oxygen is reversible, since the $[4Fe\text{-}4S]^{2+}$-cluster can be reassembled into inactive apo-protein in the cell (Mettert et al. 2008; Dibden and Green 2005). ISC assembly is thought to be essential for dimerization by helping neutralize charge repulsion between monomers (Moore et al. 2006).

While the broad outlines of O_2-sensing by FNR are now established, some features of this regulatory switch are still poorly understood. It has been noted, for example, that a product of O_2-sensing is superoxide anion, which can dismutate to yield H_2O_2 and O_2. Dismutation of superoxide occurs spontaneously but is greatly accelerated by superoxide dismutase (SOD) and, *in vitro*, FNR has also been reported to have SOD activity. These observations have been interpreted to indicate that a single O_2 molecule can potentially oxidize up to four $[4Fe\text{-}4S]^{2+}$-clusters (Green et al. 2009). An additional complexity derives from the observation that the presence of iron chelators affects the rate of cluster decomposition. Since iron chelators are ubiquitous in the cell, this could affect the kinetics of the cluster conversion processes *in vivo* (Crack et al. 2008). It is also not clear whether the mechanisms established for *E. coli* FNR will apply, in whole or in part, to other FNR orthologs. For example, *B. subtilis* FNR has a $[4Fe\text{-}4S]^{2+}$-cluster with three (rather than four) Cys ligands together with an Asp (Gruner et al. 2011), suggesting that it may be functionally divergent (Reents et al. 2006). Indeed, unlike *E. coli* FNR, the *B. subtilis* ortholog does not require cluster assembly for dimerization.

9.4.2 SoxR as a Sensor of One-Electron Oxidants

The *E. coli* SoxR protein is a $[2Fe\text{-}2S]^{+}$-containing transcription factor originally described as a sensor of superoxide stress (Storz and Imlay 1999). SoxR is a dimeric activator protein and a member of the MerR family of transcription factors. In response to redox-cycling compounds, oxidized SoxR activates the expression of SoxS, a MarR family protein that regulates the expression of genes that protect *E. coli* against redox-cycling compounds. The SoxRS regulon includes genes encoding the SodA superoxide dismutase, ISC assembly proteins, and proteins implicated in modification and efflux of redox-cycling compounds.

The activity of SoxR is regulated by the reversible, one electron oxidation of a solvent-exposed $[2Fe\text{-}2S]^{+}$ cluster (Watanabe et al. 2008). Since SoxR was initially

described as a regulator of SodA, and SoxR is activated by various, structurally diverse redox-active compounds, it had long been assumed that superoxide itself was sensed directly by SoxR. However, more recent studies suggest that SoxR reacts directly with redox active compounds and that superoxide is not a physiologically relevant mediator of SoxR activation (Gu and Imlay 2011). This revised model of SoxR redox sensing is supported by several observations. First, cells lacking SOD have significantly elevated fluxes of superoxide, sufficient to damage iron-sulfur containing enzymes, but do not activate the SoxRS regulon. Second, the rate of reaction of SoxR with superoxide anion is insufficient to be relevant at the concentrations of superoxide likely to be present in the cell. Third, induction of the SoxRS regulon is observed even under anaerobic conditions when superoxide cannot be formed (Krapp et al. 2011; Gu and Imlay 2011). The notion that SoxR has a much broader role in sensing the presence of redox active compounds by their direct oxidation of the exposed $[2Fe\text{-}2S]^+$ cluster, also fits with the observation that SoxR does not even regulate SOD in several non-enteric bacteria (Dietrich and Kiley 2011).

9.4.3 *IscR as a Sensor of Redox Stress Affecting Iron-Sulfur Cluster Proteins*

E. coli IscR is a $[2Fe\text{-}2S]^+$-containing repressor that regulates the expression of the *isc* operon encoding one of two major pathways for ISC assembly. The requirement of a $[2Fe\text{-}2S]^+$ cluster for IscR-mediated repression allows IscR to repress the *isc* operon in response to the catalytic potential for ISC assembly. The IscR $[2Fe\text{-}2S]^+$ cluster is coordinated by three conserved Cys residues in the C-terminal domain of the protein. IscR also has a regulatory role when the $[2Fe\text{-}2S]^+$ cluster is not present: apo-IscR acts as a transcription activator of the second, stress-induced pathway for ISC assembly encoded by the *suf* operon (Yeo et al. 2006). This system is essential under conditions of even low level oxidative stress due to the intrinsic redox sensitivity of the Isc-mediated pathway (Jang and Imlay 2010). Remarkably, the apo-form of IscR binds to a distinct set of target operons. Thus, the IscR regulon includes two sets of genes: those regulated by metallated IscR and those regulated by apo-IscR (Nesbit et al. 2009).

In addition to the feedback regulation of the *isc*-encoded pathway, IscR also contributes to regulation of the alternative, stress-induced *suf* pathway for ISC assembly. Induction of the *suf* operon upon oxidative stress reflects the coordinate action of several regulatory proteins. Fur binds as a repressor, and inactivation of Fur by ROS is a prerequisite for activation of *suf* transcription (Lee et al. 2008). Activation of *suf* transcription can be mediated by OxyR (Outten et al. 2004; Lee et al. 2004b), which appears to play the major role and is essential for achieving sufficient levels of the SUF machinery to support robust growth (Jang and Imlay 2010), and by IscR (Yeo et al. 2006). The integration of signals sensed by these various transcription factors ensures that *suf* operon induction is enabled when ISC assembly pathways are limited in the cell or when oxidants are present that destroy existing ISCs.

9.4.4 NsrR: Sensing of NO with an Iron-Sulfur Cluster

E. coli NsrR is, together with IscR, a member of the Rfr2 family of regulatory proteins. NsrR shares with NorR the property of being a direct NO-sensor that controls NO detoxification functions. NsrR was discovered in *E. coli* in an attempt to understand the NO-specific induction of *hmp* encoding an NO-detoxifying flavohemoglobin (Bodenmiller and Spiro 2006). Genomic-based analyses indicate that the *E. coli* NsrR regulon is comprised of ~60 genes and includes functions for NO-detoxification, for nitrogen respiration, as well as for the other dedicated NO-sensor, NorR (Partridge et al. 2009). NsrR orthologs are found in many bacteria and representatives have been purified from *E. coli, Neisseria gonorrhoeae, B. subtilis,* and *S. coelicolor* (Tucker et al. 2010).

Studies of the detailed mechanism of NO-sensing by NsrR proteins have yielded conflicting results. Each purified *E. coli* NsrR protomer contains a $[2Fe\text{-}2S]^{+}$ cluster which, like IscR, is coordinated by three Cys residues. The fourth ligand in the case of NsrR is predicted to be a His residue (Tucker et al. 2010). The NsrR $[2Fe\text{-}2S]^{+}$ cluster is dinitrosylated (to a dinitrosyl iron complex; DNIC) in the presence of NO leading to derepression. Similarly, both the *N. gonorrhoeae* and *S. coelicolor* NsrR proteins were purified with a $[2Fe\text{-}2S]^{+}$ cluster, which responds to NO with formation of a DNIC and loss of DNA-binding activity (Isabella et al. 2009; Tucker et al. 2008). In contrast, *B. subtilis* NsrR purified anaerobically contains a $[4Fe\text{-}4S]^{2+}$ cluster (Yukl et al. 2008). Since the *N. gonorrhoeae, B. subtilis,* and *S. coelicolor* proteins were all purified after overexpression in *E. coli*, and only the *B. subtilis* protein was purified under anaerobic conditions, this raises the possibility that the observed $[2Fe\text{-}2S]^{+}$ clusters may be an artifact of heterologous expression or a product of the aerobic decomposition of an originally present $[4Fe\text{-}4S]^{2+}$ cluster. Indeed, anaerobic purification of *S. coelicolor* NsrR was recently reported to yield protein with a $[4Fe\text{-}4S]^{2+}$ cluster although it is unclear whether this form of the protein can sense NO (Tucker et al. 2010). It is possible that some NsrR proteins use a $[2Fe\text{-}2S]^{+}$ cluster and others use a $[4Fe\text{-}4S]^{2+}$ cluster to sense NO, but this would be unusual given the overall similarity in protein sequence and function.

9.4.5 Aconitase: A Metabolic Enzyme with a Redox-Sensitive Switch

Aconitases catalyze the interconversion of citrate and isocitrate in the tricarboxylic acid (TCA) cycle. This reaction occurs when the substrate binds to a $[4Fe\text{-}4S]^{2+}$ cluster coordinated by three Cys residues with one exposed iron atom. Like other, related $[4Fe\text{-}4S]^{2+}$ dehydratases, aconitase functions as a dehydratase, converting citrate to cis-aconitate, followed by rehydration to generate the isomer, iso-citrate. As a class, $[4Fe\text{-}4S]^{2+}$-containing dehydratases are sensitive to redox stress and, in some cases, to iron depletion due to the presence of a weakly bound iron atom

(Varghese et al. 2003). In the presence of superoxide, oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster leads to an unstable $[4Fe\text{-}4S]^{3+}$ cluster, which decomposes with loss of Fe^{2+} to a $[3Fe\text{-}4S]^{+}$ cluster (Flint et al. 1993). Thus, oxidation of this ISC constitutes a switch that, minimally, converts aconitase from an active to an inactive form. What makes this switch particularly noteworthy is that, in at least some systems, the oxidized form of aconitase has other roles in the cell.

In mammalian cells, a cytosolic aconitase functions as a redox- and iron-regulated RNA-binding protein to control both translation initiation and mRNA stability of target mRNAs (Rouault 2006). This Iron-Responsive Element (IRE)-binding protein (IRP1), together with a homolog IRP2, responds to iron status, oxidative stress, and NO to control the expression of iron homeostasis functions. Detailed structural and functional characterization of IRP1 demonstrate that this cytosolic aconitase has a $[4Fe\text{-}4S]^{2+}$ cluster and, upon loss of the ISC, functions as an mRNA binding protein (reviewed in Wang and Pantopoulos 2011).

There is suggestive evidence that at least some bacterial aconitases may also have an additional regulatory role. *E. coli* encodes two, differentially regulated aconitases. The major enzyme in non-stressed cells is aconitase B (AcnB), which is sensitive to inactivation by one-electron oxidants and under conditions of iron-limitation (Varghese et al. 2003). The inactive form of AcnB is not well characterized *in vivo* but is presumed to be the $[3Fe\text{-}4S]^{+}$ form. This conversion not only inactivates the enzymatic activity of AcnB, but the demetallated protein binds to the 3′-untranslated region of its own mRNA thereby stabilizing the message against degradation (Tang and Guest 1999).

A second aconitase, AcnA, is expressed at a lower level but further induced under iron-replete conditions (as sensed by Fur) and as part of the SoxRS-regulon. Unlike AcnB, this aconitase isozyme is relatively resistant to both oxidative stress and iron fluctuations. These observations have led to a model in which one function of AcnB is to sense iron availability in the cell (Varghese et al. 2003). Under iron-restricted conditions, AcnB will be inactive leading to the accumulation and excretion of citrate, which can serve as a siderophore. Conversely, if iron levels are sufficient but AcnB is inactivated by redox stress, induction of AcnA can provide aconitase activity (Varghese et al. 2003). AcnB also reacts rapidly with nitric oxide to generate the DNIC. This reaction occurs faster than the competing reactions of NO with either molecular oxygen (which generates peroxynitrite) or glutathione. This suggests that aconitase, and related $[4Fe\text{-}4S]^{2+}$ dehydratases (e.g., IlvD) may be primary targets of NO toxicity in *E. coli* (Duan et al. 2009).

The ability of demetallated aconitases to bind to their own mRNA suggests that aconitases may comprise a molecular switch (Tang and Guest 1999). Initial studies suggest that this switch may be mediated by $[4Fe\text{-}4S]^{2+}$ cluster oxidation, much like mammalian IRPs. Both *E. coli* aconitases affect the stability of their own mRNAs. They also tune the translation of *sodA*, encoding manganese superoxide dismutase, with AcnA playing a positive role and AcnB a negative role (Tang et al. 2002). AcnB also affects translation of *ftsH* and thereby affects motility (Tang et al. 2004). Although the $[4Fe\text{-}4S]^{2+}$ cluster is implicated in this regulatory switch, structural studies of AcnB revealed that this protein contains an additional HEAT domain

that may mediate iron-responsive protein dimerization and be part of the functional switch (Williams et al. 2002; Tang et al. 2005).

Further studies are required to determine whether aconitases routinely play a dual role in the Bacteria, serving both as a central metabolic enzyme and regulatory switch. To date, mRNA binding activity has been reported for aconitases from *B. subtilis* (Alen and Sonenshein 1999) and *Mycobacterium tuberculosis* (Banerjee et al. 2007). However, it is not yet clear whether these switches respond physiologically to iron depletion, RNOS, or both and the scope and significance of the changes elicited by the RNA-binding activity have yet to be determined. Ultimately, deciphering the regulatory role of aconitases will require a concerted effort to define their global impact on gene expression under various stress conditions.

9.4.6 WhiB-Like Regulators: A Family of Redox Sensors in Actinobacteria

In addition to the relatively well-characterized model systems described above, there is suggestive evidence that iron-sulfur clusters may play a regulatory role in several other contexts. One emerging area of research is the role of the WhiB protein and, more generally, the WhiB-like (Wbl) family of regulators in the actinobacteria (reviewed in den Hengst and Buttner 2008). The *whiB* gene was originally described in *S. coelicolor* as the locus of a mutation affecting development, antibiotic production, and pigmentation. The extended WhiB family of proteins includes 14 members in *S. coelicolor* and it is generally assumed, based largely on their pleiotropic phenotypes, that *wbl* genes encode transcription factors although detailed biochemical characterization of their DNA-binding activity and regulatory potential is just beginning to emerge.

The first Wbl protein found to have an associated $[4Fe\text{-}4S]^{2+}$ cluster was *S. coelicolor* WhiD (Jakimowicz et al. 2005). Subsequently, several Wbl proteins from another member of the actinobacteria, *M. tuberculosis*, were also found to have associated ISCs although, in this case, several were found to contain $[2Fe\text{-}2S]^{+}$ clusters (Alam et al. 2009). The *M. tuberculosis* WhiB4 protein appears to purify with a bound $[2Fe\text{-}2S]^{+}$ cluster, but can be reconstituted with a $[4Fe\text{-}4S]^{2+}$ cluster. In this case, WhiB4 was proposed to function as a protein disulfide reductase in which the active site is occluded, in non-stress conditions, by an assembled ISC (Alam et al. 2007). If confirmed, this will be the first example of a redox-gated switch in a bacterial protein disulfide reductase. However, this activity was not shared with the demetallated *S. coelicolor* WhiD (Crack et al. 2009).

We are still far from understanding the presumed regulatory functions of the iron sulfur clusters in the extended Wbl family of proteins. Current evidence suggests a role for Wbl proteins in sensing RNOS. *M. tuberculosis* WhiB3 appears to respond to NO by oxidation of its $[4Fe\text{-}4S]^{2+}$ cluster (Singh et al. 2007) and has been demonstrated to bind directly to the promoter regions of target operons (Singh et al. 2009). Recent biochemical results indicate that both *S. coelicolor* WhiD and

M. tuberculosis WhiB1 react extremely rapidly with NO leading to complete cluster dissolution. Together with genetic evidence linking Wbl proteins to nitrosative stress, this rapid reaction suggests a likely regulatory role in NO signaling (Crack et al. 2011).

9.4.7 Iron-Sulfur Clusters as Redox Switches to Regulate Protein Function

As the exploration of the aconitase and Wbl proteins illustrates, the assembly, modification, and ultimate dissolution of ISCs may serve as regulatory switches to control enzyme activity. In the case of *E. coli* AcnB, the loss of the labile iron atom from the $[4Fe\text{-}4S]^{2+}$ cluster inactivates enzyme function potentially leading to the accumulation of citrate, a known siderophore (Varghese et al. 2003). This would thus appear to be a biologically relevant switch, albeit not necessarily a redox switch.

Bacterial metabolism relies on a large number of enzymes that utilize ISCs. It has been estimated that perhaps 5% of all proteins encoded by *E. coli* contain ISCs (predominantly $[4Fe\text{-}4S]^{2+}$) (Fontecave 2006). Although in many cases, the role of the ISC is apparent from the mechanism of the enzyme, in other cases its presence is seemingly unrelated to catalysis. It seems likely that some ISCs will play a role in redox-sensing instead of, or in addition to, any role in catalysis. One example of such a mechanism is *B. subtilis* PurF (glutamine phosphoribosylpyrophosphate amidotransferase) encoding the first enzyme of *de novo* purine biosynthesis. This enzyme contains an oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster proposed to function as a redox switch controlling protein stability (Grandoni et al. 1989; Switzer 1989). Another example is *B. subtilis* CysH1 (adenylyl sulfate/phosphoadenylyl sulfate reductase). This enzyme has a $[4Fe\text{-}4S]^{2+}$ cluster also proposed to function as an oxygen-sensitive redox switch (Berndt et al. 2004). It is widely appreciated that ISCs are very sensitive to oxidative degradation and this can have profound impacts on cellular physiology (Py et al. 2011; Imlay 2006). The more challenging question is to ascertain those specific examples where this sensitivity has been exploited by the cell to an adaptive advantage.

9.5 Redox Regulation by Heme Proteins

The third class of iron-containing redox sensors are those containing heme as a prosthetic group (Gilles-Gonzalez and Gonzalez 2005). Heme is bound non-covalently to a hydrophobic pocket in many heme sensors with the heme iron coordinated to four nitrogen atoms from the porphyrin macrocycle and one or two protein ligands. Typically, the fifth coordination site of the heme iron is a histidine imidazole nitrogen (designated the proximal ligand) with the sixth coordination site

interacting, often weakly, with an additional (distal) ligand and available for the binding of a regulatory ligand. The distal ligand to the heme iron can be an amino acid, or even a low molecular weight thiol (Gusarov and Nudler 2005).

Heme proteins are particularly well suited for sensing of small diatomic gases such as O_2, NO, and CO (reviewed in Gilles-Gonzalez and Gonzalez 2005; Aono 2012). In a subset of these sensors, there is a redox change in the heme iron that accompanies ligand binding, but in many cases heme proteins function as gas sensors but not, strictly speaking, as redox sensors. However, the detailed mechanism of sensing is not, in all cases, established and, in any event, we have here extended the idea of redox sensors to include those proteins that sense redox active molecules. Therefore, we will consider several of the best-characterized examples of heme-based gas sensors.

As a class, most heme-based gas sensors have a proximal histidine that binds the heme ferrous ion and a relatively apolar pocket where a gas molecule can bind as a distal ligand. It has been noted that, for a wide-range of heme-based sensors, the ratio of binding affinities are similar with highest affinity for NO, a 1000-fold decreased affinity for CO, and a roughly 10^6-fold decreased affinity for O_2 (Tsai et al. 2012). However, individual sensor proteins can increase or decrease their ability to discriminate amongst these diatomic gases by a variety of chemical mechanisms including variation of the proximal ligand or altering the structure and properties of the ligand-binding pocket.

9.5.1 Oxygen Sensing by FixL

FixL was the first described representative of a class of heme-based oxygen sensors that use a PAS domain-associated heme (Gilles-Gonzalez et al. 1994). FixL is the sensor kinase of the FixL-FixJ TCS found in the alpha-proteobacteria. FixL proteins have been best characterized in *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*, two organisms that establish a symbiotic relationship with the roots of specific host plants and are responsible for nitrogen fixation. In both systems, O_2 inhibits the activity of FixL, which in turn regulates a phosphorylation signaling cascade. The kinase activity of FixL leads to phosphotransfer to FixJ which then activates transcription of nitrogen fixation and microaerobic respiration genes (Green et al. 2009). Inhibition of FixL by O_2 ensures that the highly O_2 sensitive nitrogenase is not synthesized under conditions where it would be oxidatively destroyed.

The specificity of FixL for O_2 derives from the presence of a key Arg residue in the ligand-binding pocket. In the absence of O_2, this key Arg residue interacts with a proprionate side chain on the edge of the porphyrin macrocycle and the kinase is active. When bound to O_2 ($K_d \sim 50$–$140\ \mu M$) the heme iron adopts a low spin state (Green et al. 2009) and the electronegative oxygen attracts the Arg guanidinium group and leads to inhibition of kinase activity (Dunham et al. 2003). Although FixL can bind to CO and NO, these ligands do not inhibit kinase activity.

9.5.2 Oxygen Sensing by the E. coli Dos Ribonucleoprotein Complex (DOS-RNC)

The *E. coli* direct oxygen sensor (Ec DosP; formerly EcDos) protein is, like FixL, an oxygen sensor that utilizes a heme group bound to a PAS domain (reviewed in Sasakura et al. 2006). In DosP, the heme is bound to one of two PAS domains (PAS-A) and regulates the activity of a carboxyl-terminal region with similarity to GGDEF/EAL domains related to cyclic-di-GMP signaling (c-di-GMP). In this case, the relevant activity is presumed to be c-di-GMP hydrolysis, which is enhanced in the presence of oxygen (Kobayashi et al. 2010). Thus, DosP likely serves to help modulate levels of the key second messenger, c-di-GMP (Mills et al. 2011).

Recent work suggests that DosP functions in *E. coli* as part of a multiprotein complex (Tuckerman et al. 2011). This complex includes the DosP c-di-GMP phosphodiesterase and a co-transcribed, heme-regulated c-di-GMP cyclase (DosC). Together, DosC and DosP co-purify with polynucleotide phosphorylase (PNPase), which is part (together with enolase and RNase E) of an RNA degradosome. Collectively, these proteins have been described as the Dos ribonucleoprotein complex (DOS-RNC). Both DosC and DosP are regulated by binding to oxygen. Under anaerobic conditions, the DosC cyclase is active and c-di-GMP activates PNPase in the complex. Conversely, when O_2 is present, the DosP phosphodiesterase hydrolyzes c-di-GMP (and the cyclase is now inactive) thereby stabilizing target mRNAs against degradation (Tuckerman et al. 2011; Mills et al. 2011). The mechanisms by which mRNAs might be targeted for O_2-dependent regulation are presently unknown.

Although both DosP and DosC are heme-based O_2 sensors, they are structurally distinct. The DosP sensor domain contains a heme-binding PAS domain but is unusual in that the heme iron is coordinated by both a proximal His residue and a distal Met. Binding to O_2 therefore requires displacement of Met. DosP binds O_2 with an affinity (K_d) of ~ 74 μM (Tuckerman et al. 2009). In contrast, DosC contains heme bound to a globin-like domain (a globin-coupled sensor; Hou et al. 2001), which binds oxygen with a K_d of 20 μM (Tuckerman et al. 2009).

9.5.3 Mycobacterium DosS and DosT: Sensing by Heme-GAF Domain Proteins

The mycobacterial DosS and DosT proteins are paralogous sensor kinase proteins containing heme bound within a GAF domain. When activated, these kinases phosphorylate the response regulator DosR, which controls the expression of genes required for the entry of *M. tuberculosis* into the dormant state that allows for long term persistence within the host (Gerasimova et al. 2011). These three proteins (DosRST; also known as DevRST) are therefore thought to be key regulators for

coordinating gene expression with environmental signals during the process of infection. These signals include O_2, and perhaps also CO and NO.

The DosS and DosT sensor kinases are thought to respond differently to O_2 and both are necessary for the full activation of the DosR regulon. DosT is more sensitive to decreasing oxygen levels and is the first activated during the transition to anaerobiosis (Kim et al. 2010; Honaker et al. 2009). The sequential activation of these two kinases leads to a gradual increase in active DosR, which may account for the sequential induction of various dormancy regulon target genes depending on the number and affinity of DosR binding sites (Chauhan et al. 2011). DosT functions as a direct gas sensor: the kinase is active in the absence of bound oxygen and binding of O_2 to the heme inactivates the kinase. In contrast, DosS is easily oxidized and binds H_2O at the distal position in the oxidized (Fe^{3+}) state. The DosS heme is reduced to the ferrous form under anoxic conditions, and this serves as a redox sensor (the heme iron changes valence). These differences may be due to the structure of the channel leading to the heme-iron within the GAF domain: point mutations that affect the size of the channel affect the access of O_2 to the heme iron and control whether the protein functions as a direct gas sensor or a redox sensor (Cho et al. 2011; Kim et al. 2010).

The nature of the signals that elicit changes in DosS and DosT activity *in vivo* are not yet fully understood. It is likely that O_2 is the relevant signal for DosT. Recent evidence indicates that a reduced menaquinone pool may also activate DosS, thus providing a mechanism to sense changes in activity of the electron transport chain and thereby regulating induction of the dormancy regulon (Honaker et al. 2010). Further, when coordinated with NO, the DosS Fe(III)-heme is much more easily reduced to the active, Fe(II) state suggesting the NO may be a physiologically relevant signal in cells (Yukl et al. 2011). Finally, it has been noted that the DosRST system is responsible for up-regulation of dormancy-associated genes in response to CO, which can be produced from the host heme-oxygenase system of macrophages (Shiloh et al. 2008). Defining the full range of signals that are active *in vivo* during the various stages of infection, and understanding how these signals work with the DosRST system and other convergent signaling pathways (Chao et al. 2010), presents a formidable challenge for future work.

9.5.4 Sensing by H-NOX Proteins

NO came to prominence as a second messenger in mammalian systems, where it exerts its effects, in part, by interactions with a soluble guanylate cyclase (sGC). A combination of genomics and structural studies led to the appreciation that related, heme-containing sensors are widespread in the Bacteria. These domains can bind either NO or oxygen as ligands and were christened as H-NOX (heme-NO and oxygen binding) domains (Pellicena et al. 2004).

Structural studies of bacterial H-NOX proteins have focused on the representatives from *Thermoanaerobacter tengcongensis* (Tt H-NOX), an anaerobic

thermophile, and one from the cyanobacterium *Nostoc punctiforme* (NpH-NOX). Detailed biophysical studies of these proteins have led to a model in which distortion of the porphyrin ring by the H-NOX protein modulates the electron density on the heme iron and alters the iron redox potential (Olea et al. 2010; Spiro 2008).

While several biophysical studies have focused on the properties of bacterial H-NOX proteins, the output of these sensors, and their physiological roles are largely unknown. Recent results suggest that one H-NOX protein, from the squid symbiont *Vibrio fischeri*, functions to detect NO during colonization of the squid light organ (Wang et al. 2010). In this system the H-NOX protein is encoded adjacent to a gene encoding a histidine protein kinase suggesting that the output of NO-sensing might be an alteration in kinase activity. In the presence of NO and the H-NOX protein, *V. fisheri* downregulates the expression of genes involved in the utilization of heme iron. Since, as noted above, NO leads to nitrosylation of Fur it can lead to derepression of iron uptake functions. The pathway controlled by the H-NOX protein in this system may serve, in part, to counter this derepression (Wang et al. 2010).

9.5.5 Sensing of Heme Iron by Irr

In the alpha-proteobacteria, Fur proteins play a relatively minor role in regulating gene expression in response to iron status (Johnston et al. 2007; Rudolph et al. 2006). Indeed, in several species within the Rhizobiales and the Rhodobacterales the Fur-like protein functions instead as a manganese responsive regulator (Mur). In *Bradyrhizobium japonicum*, Fur responds to both manganese (Hohle and O'Brian 2009, 2010) and iron (Yang et al. 2006b). However, since iron and manganese regulation are closely intertwined (Puri et al. 2010), the apparent Fe(II) responsiveness in cells may be indirect.

Instead of Fur, many alpha-proteobacteria sense iron in the form of heme by the iron-responsive repressor (Irr). This is likely expedient since it is estimated that one-half of all iron in *Bradyrhizobium japonicum* is in the form of heme (Small et al. 2009). Irr contains two heme-binding sites and obtains heme directly from the site of synthesis on ferrochelatase (Qi and O'Brian 2002). Binding of heme not only signals iron sufficiency to Irr, but also primes the protein to function as a sensor or oxidative stress. Irr binds a ferric heme with an N-terminal heme-recognition motif (HRM) and a ferrous heme in an internal His-rich domain. Irr normally represses gene expression when iron is limited (the opposite of a typical Fur protein) and in response to iron (heme) sufficiency, Irr is inactivated. The inactivation of Irr is associated with heme-dependent protein oxidation, which targets the protein for rapid degradation (Small et al. 2009; Yang et al. 2006a). This MCO reaction is reminiscent of the inactivation of *B. subtilis* PerR by reaction of peroxide at a non-heme iron center, although the molecular details of this heme-mediated event are not yet clear. The inactivation of Irr by heme is not obligately coupled to protein degradation since this regulated degradation is not observed with the *Rhizobium leguminosarum* Irr protein (Singleton et al. 2010).

9.5.6 Sensing of CO and Redox by CooA

The heme-based *Rhodospirillum rubrum* CO-specific sensor, CooA, is a remarkable example of a protein with dual roles as both a redox and gas sensor (Roberts et al. 2005). Unlike other examples discussed, CooA is a member of the CRP family of DNA-binding activators. In response to CO-binding, CooA activates the transcription of genes involved in CO oxidation. Analysis of the CooA sensing mechanism has been aided by the availability of a crystal structure of the sensor protein (Lanzilotta et al. 2000). Upon binding of CO, a distal iron ligand is displaced resulting in a conformational change of CooA. Structural studies revealed that, in the off state, the heme iron is coordinated by both a proximal His ligand (H77) with the amino-terminal Pro residue (P2) from the other protomer serving as distal ligand. Remarkably, the proximal ligand is regulated by the oxidation state of the heme iron with H77 being replaced by Cys75 in Fe(III) CooA. It has been suggested that this redox switch is biologically relevant: under aerobic conditions CooA would be in the inactive Fe(III)-heme state, which would thereby prevent activation of the CO reduction system which is known to be oxygen labile. CooA would activate transcription only in response to CO and in the absence of O_2 (Bonam et al. 1989). *Pseudomonas aeruginosa* DNR is another member of the CRP superfamily that utilizes a heme moiety to sense a diatomic gas, NO (Giardina et al. 2011). In response to NO, DNR activates the expression of denitrification genes, but whether this is strictly a gas sensor, or additionally involves a redox change, is not yet resolved (Rinaldo et al. 2012).

9.5.7 Other Heme-Based Gas Sensors

We have here reviewed some of the best-characterized examples of heme-based sensor proteins. Recent work has led to the identification of numerous other examples that further emphasize the utility of heme as a sensor of environmental changes. Two additional families of heme-based sensors worth noting are represented by the *Burkholderia xenovorans* RcoM proteins (Marvin et al. 2008) and the globin-coupled sensor (GCS) proteins HemAT (Hou et al. 2000).

The *B. xenovorans* RcoM-1 and RcoM-2 proteins contain heme in a PAS domain and regulate CO-dependent transcription. In the absence of bound ligand, the heme is in the Fe(III) oxidation state with six ligands (including a proximal His and a distal Cys thiolate). Upon reduction to Fe(II), the Cys ligand is displaced and the Fe(II) is coordinated instead by a Met residue (Marvin et al. 2008). Coordination of either CO or NO can displace the weakly bound Met residue resulting in activation of transcription. This is analogous to the O_2-sensing mechanism employed by DosP, as noted above.

The best characterized O_2-binding heme proteins are, of course, myoglobin and hemoglobin. A widely distributed family of sensor proteins, found in both Bacteria

and Archaea, use a related heme-containing domain and define the globin-coupled sensor (GCS) family (Hou et al. 2001). GCS family proteins include *E. coli* DosC (see above) and the aerotaxis sensor (HemAT) involved in coordinating motility in response to O_2 availability. The HemAT proteins were originally described in the archaeon *Halobacterium salinarum* (HemAT-HS) and in *B. subtilis* (HemAT-BS) (Hou et al. 2000). HemAT proteins contain an amino-terminal domain related to methyl-accepting chemotaxis proteins (MCPs) and a carboxy-terminal GCS domain. In response to increasing cytosolic O_2, binding to the heme iron leads to movement of the iron into the heme plane, which leads to movement of the proximal histidine. Selectivity for O_2 is derived, in part, from its ability to form a hydrogen bond with Thr95, which is coupled to bonding between His86 and a heme propionate side chain (Yoshimura et al. 2006). The dimeric HemAT protein is thought to bind O_2 with negative cooperativity. Activation of HemAT-coupled CheA protein initiates the chemotaxis signal transduction cascade that ultimately couples changes in O_2 levels to alterations in the direction of rotation of the flagellum and hence motility.

9.6 Thiol-Based Redox Regulators

The cysteine side-chain contains a thiol (−SH) group that provides a redox-switch for a variety of sensor proteins and enzymes. Ionization of the thiol (to form the thiolate anion) greatly enhances the reactivity of proteins with RNOS. Oxidation of cysteine (Cys) leads in many proteins to a disulfide in which two Cys residues are covalently linked (the oxidation of two Cys residues to form the disulfide generates cystine). Disulfide bond formation is initiated when one partner reacts with an oxidant to generate a sulfenic acid (R-SOH). Condensation of the initially formed sulfenate with another thiol (such as another Cys residue) generates a disulfide. Protein disulfides may form between two Cys residues in a single polypeptide chain (an intramolecular disulfide), between two subunits of a multimeric protein (an intersubunit disulfide), or between a reactive Cys thiolate and a low molecular weight thiol in the cell (a mixed disulfide).

There are a large and ever-growing number of thiol-containing redox sensors in both prokaryotes and eukaryotes and this area has been the focus of numerous recent reviews. These include reviews focused on cysteine chemistry and efforts to monitor redox status *in vivo* (Klomsiri et al. 2011; Leonard and Carroll 2011), thiol-based sensors (Antelmann and Helmann 2011), and thiol-based switches in enzymes (Klomsiri et al. 2011). Related topics are also addressed elsewhere in this Volume. Here, we will briefly review the key features of some of the best-characterized prokaryotic thiol-based redox sensors with a specific emphasis on the unique features of each system (Table 9.2).

Table 9.2 Thiol-based redox sensors and switches

Organism	Sensor (class)	Targets and/or function	Signal(s)	Mechanism
E. coli	OxyR	Peroxide stress response	H_2O_2	Intrasubunit disulfide
B. subtilis	OhrR (1-Cys)	Peroxidase regulation	ROOH, NaOCl	S-bacillithiolation
X. campestris	OhrR (2-Cys)	Peroxidase regulation	ROOH,	Intersubunit disulfide
S. aureus	SarZ (1-Cys)	Virulence gene expression	ROS	S-thiolation
B. subtilis	HypR	Hypochlorite resistance	NaOCl	Intersubunit disulfide
S. coelicolor	RsrA (Cys_4:Zn)	Anti-σ for σ^R thiol homeostasis regulon	ROS	Intrasubunit disulfide
B. subtilis	Spx	Thiol homeostasis regulon	ROS	Intrasubunit disulfide
S. enterica	SsrB	SPI-2 regulation (virulence)	RNOS	Cys S-nitrosylation
E. coli	Hsp33 (Cys_4:Zn)	Protein chaperone (holdase)	NaOCl	Intrasubunit disulfide
E. coli	MetE	Methionine synthase	NaOCl	S-glutathionylation
B. subtilis	MetE	Methionine synthase	NaOCl	S-bacillithiolation

9.6.1 *E. coli OxyR: An Intramolecular Disulfide Switch in a Transcription Activator*

OxyR was the first regulatory protein shown to be controlled by reversible disulfide-bond formation (Storz et al. 1990). In the presence of sub-micromolar levels of H_2O_2, OxyR is converted from a reduced form to an oxidized form in which two Cys residues (C199 and C208) are disulfide-linked. OxyR is a tetrameric DNA-binding protein of the LysR family and binds to its target DNA in both the reduced and oxidized forms. However, the oxidized form is altered in conformation and often activates transcription from adjacent promoter sites (Toledano et al. 1994). The targets of OxyR include enzymes for degradation of peroxides (catalase and alkylhydroperoxide reductase), maintenance of thiol homeostasis (glutathione reductase and a glutaredoxin), and for metal homeostasis (the Fur regulator, the MntH manganese uptake channel, and the iron-sequestration protein Dps).

The molecular mechanism of peroxide-sensing by OxyR has been characterized both structurally and through detailed biochemical analysis (Lee et al. 2004a; Choi et al. 2001). The initial oxidation occurs on C199 (the peroxidatic Cys) to yield the Cys-sulfenate (Cys-SOH), which then reacts with the C208 thiol to generate the disulfide. This is accompanied by a significant conformational change in the protein since these two thiols are normally 17 Å apart. The C199 thiolate is ionized at neutral pH due to an abnormally low pKa and this accounts, in part, for its high reactivity. This thiolate can also react with other RNOS including nitrosating agents or by trapping of the initially produced C199-sulfenate by reaction with glutathione (S-glutathionylation). The physiological relevance of these other types

of modifications is a matter of debate. It has been shown, however, that the OxyR regulon can be induced by depletion of reduced thiols in the cytosol (disulfide stress, Aslund et al. 1999).

9.6.2 OhrR Proteins as Oxidation-Sensitive Repressors

The OhrR family of repressors bind DNA in their reduced state, typically as transcription repressors, and oxidation of the protein leads to derepression (Duarte and Latour 2010; Zuber 2009). OhrR family repressors represent a subset of the MarR family of regulators and are characterized by a conserved Cys residue in the amino-terminal, DNA-binding domain of the protein and often one or more Cys residues in a carboxyl-terminal domain. OhrR proteins were first characterized in *B. subtilis* (Fuangthong et al. 2001) and in *Xanthomonas campestris* (Sukchawalit et al. 2001) where they serve as repressor of a peroxidase (OhrA).

B. subtilis OhrR ($OhrR_{BS}$) is the prototype for the 1-Cys family of OhrR redox sensors, so-named since each protein subunit contains only a single redox active Cys residue. The structure of the complex of $OhrR_{BS}$ bound to operator DNA reveals a typical winged-helix, helix-turn-helix architecture (Hong et al. 2005). $OhrR_{BS}$ has a single Cys residue per protomer (Cys15) that reacts with organic peroxides to generate a sulfenate (Fuangthong and Helmann 2002). However, this reaction leads to only a small conformational change (Hong et al. 2005) and is not sufficient to inactivate the repressor (Lee et al. 2007). Since there is no nearby Cys residue (from either subunit of the dimer), protein disulfide bond formation does not occur. Instead, the initial sulfenate reacts rapidly with low molecular weight (LMW) thiols in the cytosol to generate a mixed disulfide. Isolation of oxidized OhrR from cells treated with organic hydroperoxide led to the identification of mixed disulfides between OhrR-Cys15 and both free cysteine and a novel thiol of 398 Da (Lee et al. 2007). This latter molecule was subsequently characterized as bacillithiol and is now appreciated as the major LMW thiol in many low GC Gram positive bacteria (reviewed in Helmann 2011).

Oxidation of $OhrR_{BS}$ can lead to a variety of products. When thiols are not available, the initial sulfenate slowly condenses with a backbone amide to generate a cyclic sulfenamide and this also inactivates DNA-binding activity. There is evidence that this reaction may also occur *in vivo* under some conditions. Both the mixed disulfide and sulfenamide form of OhrR can be reduced by thiol reducing agents to regenerate active repressor (Lee et al. 2007). Oxidation of OhrR to the mixed disulfide form has been studied with cumene hydroperoxide (a model organic peroxide) and also occurs in cells treated with the strong oxidant sodium hypochlorite (NaOCl) (Chi et al. 2011). When linoleic acid hydroperoxide was used as oxidant, the protein was instead rapidly overoxidized to the sulfinic and sulfonic acid forms (Soonsanga et al. 2008b). Unlike the mixed disulfide form, these cannot be repaired in the cell and, in this case, $OhrR_{BS}$ appears to function as a sacrificial regulator. Studies using single-chain variants demonstrate that oxidation of a single

active site per dimer is likely sufficient to mediate induction (Eiamphungporn et al. 2009), which may be important to allow the cell to respond to even very low levels of ROS.

Xanthomonas campestris OhrR ($OhrR_{XC}$) is homologous to $OhrR_{BS}$ but has additional Cys residues in the C-terminal domain of the protein. In this case, initial oxidation of the N-terminal Cys residue leads to the rapid formation of an intersubunit disulfide (Newberry et al. 2007). This 2-Cys mechanism differs from the 1-Cys mechanism in that the regulator is much more resistant to overoxidation and does not rely on the LMW thiol pool for modification of the peroxidatic Cys (Soonsanga et al. 2008a). In $OhrR_{XC}$ the reactive Cys (C22) is separated by > 15 Å from its redox partner (C127) on the opposing subunit of the dimer. The redox active thiolate is held in a hydrogen-bonding network with two Tyr residues from the opposing subunit (as also seen with $OhrR_{BS}$) and disulfide bond formation is accompanied by disruption of these contacts leading to a reconfiguration of the dimer interface. As a result, the two DNA-binding domains undergo a rigid body rotation of 28° relative to the core of the protein dimer. A similar conformational change is likely to lead to DNA dissociation of other redox-active MarR regulators (Poor et al. 2009).

Redox-regulated MarR family regulators related to OhrR are present in many bacteria and there are often multiple paralogs within a single cell (Chen et al. 2011). Many of these repressors appear to be redox-regulated and this may involve either formation of an intersubunit disulfide (2-Cys mechanism) or a mixed disulfide (1-Cys mechanism). Regulators of this family include *S. aureus* MgrA and SarZ and *P. aeruginosa* OspR and MexR (reviewed in Chen et al. 2011). These regulators likely play important roles in coordinating the expression of virulence genes in response to RNOS. While some are closely related to OhrR, others are members of the structurally related MarR/DUF24 family of proteins (Antelmann and Helmann 2011). Of particular note, the redox-sensing mechanism of the *S. aureus* SarZ protein has been visualized by structure determination of the protein in the reduced, sulfenic acid, and mixed-disulfide forms (Poor et al. 2009). The structural basis for redox-sensing by the *B. subtilis* DUF24 family sensor, HypR, has also been determined. HypR is oxidized by hypochlorite to an intersubunit disulfide linking Cys14 of one subunit with Cys49 of the other and the resulting crosslinked protein functions as a transcription activator both *in vitro* and *in vivo* (Palm et al. 2012).

9.6.3 Streptomyces coelicolor RsrA: A Redox-Regulated Anti-sigma Factor

The *S. coelicolor* alternative σ factor, σ^R, regulates gene expression in response to oxidative stress (Paget et al. 2001; Kallifidas et al. 2010). In this system, oxidative stress is sensed by an anti-σ factor (RsrA) that binds and sequesters σ^R in an inactive state (Kang et al. 1999; Paget et al. 1998). When redox-active compounds oxidize

cytosolic thiols, RsrA is inactivated and σ^R is released to activate genes involved in thiol homeostasis, synthesis of LMW thiols (Newton and Fahey 2008), and repair or degradation of oxidatively damaged proteins (Kallifidas et al. 2010).

RsrA is inactivated by reversible disulfide bond formation between 2 Cys residues in a Zn(II)-binding domain (Zdanowski et al. 2006; Bae et al. 2004; Li et al. 2003). In contrast with the other examples described above, the RsrA Zn(II) finger motif serves to activate the reactivity of (at least one) of the thiolate ligands. Zn(II) is bound by Cys11 together with three ligands from a conserved HCC motif (H37, C41, and C44). The primary disulfide in oxidized RsrA joins Cys11 and Cys44 (Zdanowski et al. 2006; Bae et al. 2004).

9.6.4 B. subtilis Spx: An Intramolecular Disulfide Switch

The *B. subtilis* Spx protein regulates a large group of perhaps 120 genes in response to disulfide stress (reviewed in Zuber 2009). Spx is unusual amongst prokaryotic transcription factors in that it lacks an obvious DNA-binding motif and interacts weakly if at all with target operons in the absence of bound RNAP. Indeed, Spx can interact with the C-terminal domain (CTD) of the alpha subunit of RNAP and interfere with activation by some more classical activator proteins (Nakano et al. 2003b). Spx is related in structure to the ArsC family of As(III) reductases and has a CxxC motif near the N-terminus. Oxidation to the disulfide activates Spx to engage with RNAP, which leads to elevated transcription of thiol homeostasis genes, methionine sulfoxide reductase, and various detoxification functions (Nakano et al. 2003a). When functioning as a transcription activator, Spx binds to DNA together with the alpha-CTD of RNA polymerase: a complex that has been characterized by X-ray crystallography (Newberry et al. 2005; Nakano et al. 2010).

The activity of Spx is tightly regulated in the cell at multiple levels. Transcription of Spx is controlled by at least three promoters including one repressed by PerR and the redox-sensitive MarR homolog YodB and one activated by the antibiotic stress inducible alternative σ factor, σ^M (Zuber 2009). The latter contributes to increasing oxidative stress resistance in cells exposed to antibiotics, many of which trigger cell killing pathways dependent on oxidative stress (Kohanski et al. 2007). Spx activity is also regulated by reversible disulfide bond formation. Finally, the levels of Spx are regulated by proteolysis by the ClpXP protease. Indeed, activation of Spx leads to upregulation of YjbH, which serves as an adaptor protein for delivery of Spx to the ClpXP protease (Kommineni et al. 2011). This therefore constitutes a negative feedback loop. Intriguingly, YjbH itself has a redox-sensitive Zn(II)-finger domain, suggesting that redox also regulates its activity. Moreover, YjbH activity is also regulated by an anti-adaptor protein, YirB (Kommineni et al. 2011). Thus, the Spx regulon is regulated by numerous known or potential redox-sensing proteins including Spx itself, PerR, YodB, and YjbH. The mechanisms by which all these signals are integrated to control the Spx stress response is presently unknown.

9.6.5 *Salmonella SsrB: A Reactive Cys Residue as a Sensor of Nitrosative Stress*

Salmonella enterica serovar Typhimurium is an intracellular pathogen that uses a type III secretion system to deliver effector proteins into the host cell (Fass and Groisman 2009). The genes encoding this secretion system are encoded on Salmonella Pathogenicity Island-2 (SPI-2) and are regulated by the two-component system SsrAB. The presence of RNOS is one of the signals that regulate virulence gene expression during infection. Remarkably, SsrB is a direct sensor of RNOS and is S-nitrosylated on Cys203 in the C-terminal dimerization domain. Modification of C203 inhibits the DNA-binding activity of SsrB. *In vivo* studies indicate that this modification is physiologically relevant: a C203S mutant protein retains DNA-binding activity but is insensitive to RNOS. Further, the ability of SsrB to respond to RNOS is correlated with attenuated growth in a mouse disease model (Husain et al. 2010).

9.6.6 *Hsp33: A Redox Regulated Chaperone*

The *E. coli* heat shock protein, Hsp33, functions as a protein chaperone, whose activity is regulated by a reversible thiol-dependent redox switch. This system, recently reviewed in detail (Kumsta and Jakob 2009), is an excellent example of a redox switch devoted to control of protein activity rather than effecting changes in gene expression. Hsp33 is an abundant protein "holdase" that helps prevent protein aggregation under stress conditions. Hsp33 activity is strongly upregulated by ROS (Jakob et al. 1999) and can thereby compensate for the inactivity of DnaK under severe stress conditions (Winter et al. 2005). Although it was initially noted that Hsp33 activity was activated by the simultaneous presence of high levels of H_2O_2 and high temperatures (Ilbert et al. 2007), the physiological relevance of this was unclear. The realization that Hsp33 is strongly and selectively activated by comparatively low levels of bleach suggests that this is likely to be a physiologically relevant oxidant for Hsp33 activation (Winter et al. 2008). Indeed, Hsp33 protects proteins against hypochlorite-induced aggregation and thereby confers resistance to this strong oxidant.

The mechanism of Hsp33 activation has been defined through detailed biochemical and structural studies (Kumsta and Jakob 2009). In non-stressed conditions, Hsp33 is a monomer with a C-terminal Cys_4-Zn regulatory domain. Oxidation leads to release of Zn(II), formation of two intramolecular disulfide bonds, and dimerization of Hsp33 to an active chaperone. Once the stress conditions have been alleviated, Hsp33 can be reduced by the thioredoxin or glutaredoxin systems. The reduced Hsp33, together with any bound client proteins, interacts with the ATP-dependent foldase, DnaK, to release refolded proteins and allow release of the inactive Hsp33 chaperone.

9.6.7 *Redox Regulated Enzymes*

Disulfide stress can lead to oxidation of thiols in numerous proteins as monitored by a variety of redox proteomics technologies (Leonard and Carroll 2011; Lindahl et al. 2011). In some cases, it is likely that these oxidation events play a regulatory role. For example, oxidation of a Cys in or near a protein active site can potentially provide a redox switch for reversible regulation of enzyme function, analogous to the regulation of aconitase activity in response to Fe(II) availability noted above. One example of such a mechanism is methionine synthase.

Exposure of *E. coli* to H_2O_2 leads to the oxidation of Cys residues in numerous proteins including MetE, encoding cobalamin-independent methionine synthase (Hondorp and Matthews 2004). Oxidation of MetE leads to methionine deficiency (Hondorp and Matthews 2009). Proteomic analyses indicated highly specific S-glutathionylation of MetE on Cys645, which is near the entrance to the active site. This oxidation event, which can be triggered by H_2O_2 or the thiol-specific oxidant diamide, can quantitatively inactivate MetE (Hondorp and Matthews 2009). The reduction of MetE to an active form is correlated with resumption of growth. Mutation of C645 to Ala eliminated the Met auxotrophy imposed by diamide, demonstrating that this modification is necessary for the observed physiological effects. Remarkably, this same mechanism is conserved in *B. subtilis* (Chi et al. 2011). In this case, exposure to bleach leads to the highly specific S-bacillithiolation of MetE (and a paralog, YxjG). In addition to MetE, several other enzymes are S-bacillithiolated in response to NaOCl stress in *B. subtilis* (Chi et al. 2011). It is presently unclear whether this is a general protective mechanism or whether this serves a regulatory role by acting as a redox switch to control enzyme function, as shown for MetE. It can be anticipated that the increasing power of redox proteomics will uncover additional examples of post-translational modifications of enzymes, some of which will be manifestations of redox processes. Deciphering which of these are adventitious events, and which have regulatory consequences, will require careful physiological and genetic studies of each modification event.

9.7 Redox Regulation Mediated by Flavin Cofactors

Flavin cofactors, including both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are involved in a wide variety of one- and two-electron transfer reactions in the cell. There are now numerous examples where the flavin cofactor serves a regulatory function in addition to, or instead of, a catalytic function. The roles of flavins in the redox switching of protein functions has recently been reviewed in depth (Becker et al. 2011), and we will here summarize some of the highlights from this field. Unlike many of the other redox switches described, flavoproteins do not typically function as DNA-binding regulatory proteins, although they can control transcription indirectly.

9.7.1 Flavin-Containing PAS Domains: The LOV Subfamily

The PAS domain is one of the most versatile ligand-binding motifs in bacterial sensor proteins and is associated with binding of flavins in several different classes of sensor proteins (Henry and Crosson 2011). Flavin-based sensors utilizing a PAS domain include the LOV domain photosensors, the *E. coli* Aer aerotaxis receptor, and the *Azotobacter* NifL protein.

The most thoroughly characterized FAD/FMN-based light sensors are the Vivid (VVD) protein from the fungus *Neurospora* and the *B. subtilis* YtvA protein that ultimately contributes to the regulation of the σ^B general stress response (Losi and Gartner 2012; Herrou and Crosson 2011). Here, we focus on the latter example. YtvA is thought to be a dimeric protein that associates as part of a multiprotein "stressosome" complex that ultimately helps to regulate activity of σ^B. YtvA itself has two domains: a LOV domain with an FMN chromophore for signal perception and a STAS (sulfate transporter and anti-sigma factor) domain that binds GTP and is involved in signal transmission. YtvA senses blue light as a signal and this sensing results in the formation of a covalent adduct between a protein Cys residue and the bound FMN cofactor. Absorption of blue light excites the bound FMN cofactor to triplet state, which decays with the generation of a FMN-cysteine C(4a)-thiol adduct (Losi and Gartner 2012; Herrou and Crosson 2011). This covalently bound (light) state (referred to as LOV390 to indicate the peak of absorbance at 390 nm) reverts, on a time-scale that varies dramatically from one photosensor to another, to regenerate the (dark) state (LOV450). For YtvA, this process takes about one hour, making it one of the slower LOV-based photoswitching events. Detailed studies of the YtvA protein in the dark and light states have provided insights into signal propagation in this system as described elsewhere (Losi and Gartner 2012; Herrou and Crosson 2011). The physiological significance of light-mediated regulation of the σ^B general stress response is not entirely clear.

Genomic studies have uncovered numerous examples of LOV-domain associated signaling systems, including several associated with pathogens that await further study. One notable example, from the anaerobic bacterium *Erythrobacter litoralis*, is EL222. This protein, notable as the first example of a full length LOV domain signaling protein to have a high resolution structure (Nash et al. 2011), has an N-terminal LOV domain and C-terminal DNA-binding domain related the LuxR family or repressors. In this system, light is proposed to directly activate the DNA-binding function of the EL222 protein.

9.7.2 Flavin Containing PAS Domains Involved in Redox Signaling

PAS domains play an integral role in numerous bacterial signal transduction systems (reviewed in Taylor and Zhulin 1999; Becker et al. 2011). In the nitrogen fixing

bacterium *Azotobacter vinelandii*, NifL provides a redox sensor that controls the activity of the NifA transcription factor. When oxygen is present, and NifL is in the oxidized conformation, it binds to NifA preventing the expression of the *nif* genes, which encode the O_2-sensitive nitrogenase enzyme. In the absence of O_2, FAD bound to one of two PAS domains in NifL is in the reduced state and NifA is released to activate gene expression (Martinez-Argudo et al. 2004). Oxidation of FAD in one PAS domain propagates a signal through the second domain and ultimately affects the NifA sequestration function as defined by recent genetic and physiological studies (Little et al. 2011). The pathway by which NifL is reduced is less well understood.

A second example of redox-sensing role for a PAS-domain associated FAD is the *E. coli* aerotaxis receptor, Aer (Taylor 2007). This transmembrane chemotaxis receptor forms a four-helical bundle, stabilized by adjacent HAMP domains, in which the cytosolic FAD-containing domains monitor cellular redox status (Taylor 2007). The redox state of the PAS-FAD domain alters the conformation of the HAMP helical bundle, as monitored in detail using structure-based protein-protein crosslinking studies, and ultimately leads to activation of the CheA kinase and a chemotactic response (Watts et al. 2011). This FAD domain coordinates aerotaxis in response to changes in the reduction potential of electron carriers, and stands in contrast the HemAT protein of *B. subtilis*, which senses O_2-binding directly.

9.7.3 Redox Regulation of Flavin-Containing Enzymes

The oxidation and reduction of a bound flavin cofactor is necessary for catalysis by many enzymes, but may also serve a regulatory function. Two well characterized examples, of what is perhaps a much broader phenomenon, are the *E. coli* pyruvate:quinone oxidoreductase (PQO) and proline dehydrogenase (PutA) involved in proline utilization. In both of these systems, changes in FAD redox status alter protein localization and function within the cell.

PQO localizes to the inner surface of the cytosolic membrane when the flavin is reduced. This form of the enzyme is activated for catalysis and transfers electrons from pyruvate, via the flavin, to ubiquinone in the membrane. In contrast, when PQO is oxidized the enzyme is cytosolic rather than membrane-associated (Becker et al. 2011). The cytosolic PQO is not known to have a separate function, so this redistribution can be viewed as a simple on:off switch for enzyme activity.

A more dramatic example of a FAD-mediated redox switch is the PutA enzyme needed for proline utilization. PutA has an N-terminal DNA-binding domain and when the FAD is oxidized PutA localizes to five binding sites in the regulatory region controlling transcription of the divergent *putP* and *putA* genes. In contrast, when proline is present PutA relocalizes to the inner surface of the membrane where it catalyzes electron transfer from proline to ubiquinone (Becker et al. 2011).

Table 9.3 Redox sensors using or sensing organic cofactors (FMN, FAD, NADH/NAD^+, quinones)

Organism	Sensor (cofactors)	Targets and/or function	Signal(s)	Mechanism
B. subtilis	YtvA (LOV:FMN)	Regulation of σ^B general stress response	Blue light	FMN-Cys adduct
A. vinelandii	NifL (PAS:FAD)	Regulation of NifA and nitrogen fixation	O_2	FAD redox
S. coelicolor	Rex	Regulation of respiratory pathways	NADH/NAD^+	Cofactor binding
E. coli	ArcB	ArcA response regulator; anaerobic respiration	Q, MK	Cofactor binding; thiol redox?
Rhodobacter spp.	RegB	RegA response regulator; photosynthesis	Q/QH_2 ratio	Cofactor binding; thiol redox

9.8 Direct Sensing of Electron Carriers (NADH/NAD^+ and Quinone Pools)

The final group of prokaryotic redox sensors we will consider are those proteins that sense cellular redox status by the direct binding to, or reaction with, electron carriers in the cell (Table 9.3). The Rex protein, initially described in *S. coelicolor*, is the prototype for a family of redox sensors that monitor the ratio of reduced to oxidized NADH in the cytosol. The *E. coli* ArcB transmembrane sensor kinase is an example of a family of sensors that monitor the status of the membrane quinone pools. The ability of regulatory proteins to sense electron carriers has been recently reviewed (Delgado et al. 2012).

9.8.1 Sensing of the NADH/NAD^+ Ratio by Rex

S. coelicolor Rex regulates respiratory processes by binding to operator sites controlling transcription of the cytochrome bd oxidase (*cydABCD*), the proton translocating NADH dehdyrogenase (*nuoA-N*), and the *rex-hemACD* operon encoding both Rex and heme biosynthetic enzymes (Brekasis and Paget 2003). Rex proteins from several organisms (including *Thermus aquaticus, S. coelicolor, B. subtilis,* and *S. aureus*) have been interrogated using a variety of structural, genetic and biochemical approaches. These analyses support a mechanism of Rex regulation in which the protein DNA-binding activity is controlled by the relative ratio of NADH and NAD^+ in the cytosol.

Rex binds DNA in the presence of NAD^+ and binding is inhibited by NADH. Indeed, *B. subtilis* Rex (Rex_{BS}) binds NAD^+ 30 times tighter when bound to DNA, implying that the converse is also true: NAD^+ enhances DNA-binding affinity (Wang et al. 2008). Normally, the rapid oxidation of NADH by transfer of electrons to the respiratory chain results in a much greater level of NAD^+ than NADH in the

cell and Rex is tuned to sense changes in this ratio. Indeed, since Rex represses the NADH dehydrogenase which is the major pathway by which NADH is oxidized by ongoing respiration, this serves as a feedback loop (Gyan et al. 2006). Consistent with this notion, Rex_{BS} binds NADH 20,000 tighter than NAD^+ (K_d's of 24 nM for NADH vs. 0.5 mM for NAD^+) (Wang et al. 2008). A similar difference is noted for *S. aureus* Rex with a K_d of 95 nM for NADH and 150 μM for NAD^+ (Pagels et al. 2010). For *Thermus aquaticus* Rex (T-Rex), the first protein of this family to be structurally characterized (Sickmier et al. 2005), binding of NADH leads to a significant 40° rotation between the two subunits of the dimer leading to a conformation incompatible with DNA-binding (McLaughlin et al. 2010). The regulons controlled by Rex proteins have been at least partially defined and efforts are underway to better understand how the action of this regulator is integrated with other sensors monitoring the availability of electron acceptors (including oxygen) and other redox signals.

9.8.2 Two-Component Systems That Monitor Quinone Pools

After electrons are transferred to the electron transport system (ETS) from the soluble NADH pool, the major non-protein redox carriers are the quinones. *E. coli* synthesizes ubiquinone (Q), which is the major carrier for respiration with O_2 and NO_3 as electron acceptors, and menaquinones (MK) that are more commonly used for other types of anaerobic respiration. In *E. coli*, the oxidation status of the Q pool is thought to be directly sensed by the ArcB membrane kinase, which is inhibited in response to oxidized Q (Georgellis et al. 2001). Conversely, when Q is largely in the reduced form (QH_2) the ArcB kinase is active and phosphorylates the ArcA response regulator. ArcA, in turn, activates expression of an estimated 30 operons involved in respiration. The precise mechanism of regulation of ArcB by changes in redox status is still incompletely understood. One model posits that ArcB binds directly to oxidized Q and this allosterically regulates kinase activity. This binding may also lead to protein oxidation leading to disulfide bond formation between two ArcB monomers. The implicated Cys residues are in a linker domain between the transmembrane domains (where Q presumably interacts) and the C-terminal transmitter and kinase domains. It has also been noted that regulation of ArcB is not limited to the sensing of Q; the redox state of the MK pool also appears to play an important role (Bekker et al. 2010). Finally, transcriptional analyses combined with modeling approaches have led Poole and colleagues to question whether Q is a relevant signal for ArcA. They propose instead that the relevant signal, at least under their experimental conditions, is inhibition of the ArcB phosphatase activity by fermentation products (Rolfe et al. 2011). This system is further complicated by the ability of the ArcBA system to activate transcription of a small RNA (sRNA), ArcZ, that is encoded convergent with and overlapping the *arcB* gene. This sRNA is proposed to function, in part, as a negative feedback loop on the expression of the ArcB (Mandin and Gottesman 2010).

A conceptually related regulatory system is present in several photosynthetic bacteria and we here focus on the RegBA two-component system from *Rhodobacter* spp. In this system, RegB is the membrane-spanning sensor kinase and phosphorylates the RegA response regulator (Elsen et al. 2004). Together, RegBA regulate numerous metabolic processes including photosynthesis, carbon and nitrogen fixation, and respiration. Current models for RegB regulation propose that the transmembrane domain senses the ratio of Q/QH_2 in the membrane. In this system, both Q and QH_2 bind to RegB, but only Q inhibits the kinase activity. Although RegB also contains potentially redox active Cys in the linker region, studies with a Cys to Ser mutant demonstrate that this site is not required for regulation (Wu and Bauer 2010). Two-component systems related to RegBA are widespread in the Bacteria and are likely involved in redox regulation of central metabolic processes, although the biochemical details of these regulatory switches are still under emerging.

9.9 Concluding Remarks

Bacteria encode a remarkable diversity of redox-sensing regulators as evidenced by the broad survey presented in this chapter. In light of the central role that redox reactions in metabolism, it should perhaps not be surprising that redox-sensing regulators are tightly interwoven into the regulatory network of the cell. In many cases the biochemical basis of sensing is now well understood and this is increasingly supported by high resolution structural studies. An equally formidable challenge is to define the physiological role of each sensor and how multiple sensor proteins work together to integrate information about cellular redox status. Genetic and physiological studies, often buttressed by genomics-enabled approaches, are allowing rapid progress in this area as well. Redox-sensing regulators play important roles in maintaining homeostasis during major metabolic transitions, such as from aerobic to anaerobic growth. Moreover, many redox active gases (O_2, NO) and secondary metabolites (including several antibiotics) are involved in cell-cell signaling. As a result, redox regulators play key roles both in pathogenesis and the establishment of symbiotic interactions in a variety of systems. Here, the reader has been introduced to many of the best known types of regulators which will hopefully provide a useful framework for ongoing studies of these and yet to be discovered redox regulators.

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Chapter 10
Combating Oxidative/Nitrosative Stress with Electrophilic Counterattack Strategies

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Abstract Redox stress is thought to contribute to neurodegenerative disorders, including Alzheimer's and Parkinson's diseases. Cysteine-based redox regulation, via glutathione- and thioredoxin-mediated pathways, represents an acute defense system. Additionally, with prolonged oxidative stress, cells mount a 'counterattack' to activate transcription-dependent pathways, including the Keap1/Nrf2 and HSP90/HSF-1 pathways, which induce phase 2 enzymes and heat-shock proteins, respectively. Oxidative/nitrosative stress itself is an activator of the Keap1/Nrf2 pathway via cysteine thiol oxidation. Moreover, stress-induced oxidation of endogenous compounds can generate electrophiles, including active aldehydes, nitroso-compounds, fatty acids, nitro-guanosine, and quinone-based dopamine derivatives. These electrophilic compounds were considered toxic, but recently have been shown to be neuroprotective under certain conditions. These endogenously-produced electrophiles signal an "electrophilic counterattack," binding to specific cysteines of Keap1 and HSP90 to activate these pathways. Finally, we describe novel pro-electrophilic drugs (PEDs) that are activated by the very oxidative/nitrosative stress that they subsequently counteract. One example is carnosic acid (CA), found in the herb rosemary. CA itself is not electrophilic, but in response to oxidation becomes electrophilic, and then activates the Keap1/Nrf2 pathway. PEDs appear to have minimal side effects, in part because they are generated preferentially in cells

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experiencing oxidative stress. In contrast, in the absence of oxidative stress, true electrophiles, unlike PEDs, react with and thus deplete glutathione, paradoxically rendering these normal cells susceptible to damage.

Keywords Electrophile • Nrf2 • HSF-1 • Keap1 • HSP90 • Pro-electrophilic drug • Pathologically-activated therapeutic

Abbreviations

ABCC	ATP-binding cassette, sub-family C
AD	Alzheimer's disease
ARE	antioxidant response element
BDNF	brain-derived neurotrophic factor
CA	carnosic acid
CAB	biotinylated carnosic acid
CNTF	ciliary neurotrophic factor
DA	dopamine
EP	electrophile
ER	endoplasmic reticulum
GCLM	glutamyl cysteine ligase modifier subunit
GCLC	glutamyl cysteine ligase catalytic subunit
GSSG	disulfide form of glutathione
GPX	glutathione peroxidase
GR	NADPH-dependent GSSG reductase
GST	glutathione-S-transferase
HHE	*trans*-4-hydroxy-2-hexenal
HNE	*trans*-4-hydroxy-2-nonenal
H_2O_2	hydrogen peroxide
HO-1	hemeoxygenase-1
HSE	heat-shock factor response element
HSF-1	heat-shock factor-1
HSP	heat-shock protein
HO	hydroxyl radical
NEPP	neurite outgrowth-promoting prostaglandin
NMDA	*N*-methyl-D-aspartate
NO_2	nitric dioxide
NO	nitric oxide
NQO1	NADPH quinone oxidoreductase 1
NGF	nerve growth factor
O_2^-	superoxide radical anion
$ONOO^-$	peroxynitrite
PAT	pathologically activated therapeutic
PUFA	poly-unsaturated fatty acids
PRX	peroxyredoxin

PED	pro-electrophilic drug
RNS	reactive nitrogen species
ROS	reactive oxygen species
SRXN	ATP-dependent reductase, sulfiredoxin
TBHQ	*tert-butyl* hydroquinone
TRX	thioredoxin
TRXR	thioredoxin reductase
xCT	Na^+-independent cystine-glutamate exchanger

10.1 Direct Effects of ROS/RNS and Electrophilic Counterreaction

10.1.1 Oxidative and Nitrosative Stress

The brain is sensitive to oxidative and nitrosative damage (Coyle and Puttfarcken 1993; Halliwell and Gutteridge 1999). In neurodegenerative diseases, increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) within specific brain regions lead to selective neurodegeneration (Fig. 10.1), thereby suggesting that ROS/RNS can contribute to the development of disease through primary or secondary pathological mechanisms (Coyle and Puttfarcken 1993; Pacher et al. 2007; Bredesen 2008; Hardingham and Lipton 2011). Many ROS and RNS contain one or more unpaired electrons in their outer pi molecular orbitals, and are thus characterized as free radicals with high reactivity. The insults promulgated by ROS and RNS are termed oxidative stress and nitrosative stress, respectively. One mechanism for such damage in biological systems involves dysregulation of cysteine thiol-based redox regulation (Fig. 10.2) due to overproduction or altered spatiotemporal distribution of ROS/RNS (Coyle and Puttfarcken 1993; Pacher et al. 2007; Bredesen 2008).

10.1.2 Direct Effects of ROS/RNS and Electrophilic Counterreactions

There are at least two modes of counterreaction against excessive ROS/RNS; the first is mediated directly by ROS/RNS themselves, and the other occurs via endogenous electrophiles (EPs; Talalay 2000; Satoh and Lipton 2007). The broad definition of an EP is a molecule having one or more electron-poor atoms that can accept electrons from electron-rich donor molecules (nucleophiles). ROS/RNS can directly oxidize critical amino acids, such as cysteine thiols, and this oxidation can result, for example, in reaction with critical thiols to activate Keap1/Nrf2 and HSP90/HSF-1 transcriptional pathways. These reactions are termed "direct counterreaction," as is shown in Fig. 10.1B (Fourquet et al. 2010; Um et al.

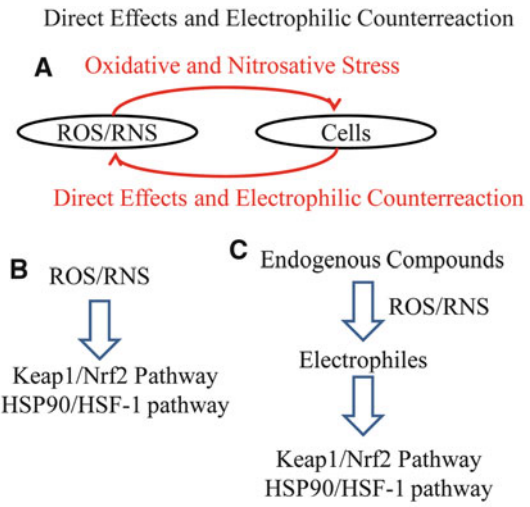


Fig. 10.1 Direct effects of ROS/RNS and electrophilic counterreaction. *A* Interaction between ROS/RNS and cells. Although ROS/RNS exert toxic effects by oxidation of cellular components, cells have two counterreactions to remove ROS/RNS. ROS/RNS and EPs cause direct effects and electrophilic counterreactions, respectively. *B* Direct counterreactions. ROS/RNS can directly modify critical cysteines of modulatory proteins such as Keap1 and HSP90, and thus induce activation of the Keap1/Nrf2 and HSP90/HSF-1 pathways. *C* Electrophilic counterreactions. ROS/RNS oxidize endogenous compounds to produce bioactive EPs, which then activate the Keap1/Nrf2 and HSP90/HSF-1 pathways

2011). In some cases, this type of counterreaction provides cells full activation of pathways that afford efficient protection against oxidative and nitrosative stress. From studies conducted over the past 20 years, we know that ROS/RNS oxidize certain endogenous compounds to produce bioactive EPs (Groeger and Freeman 2010; Akaike et al. 2010). These ROS/RNS-produced EPs can oxidize specific cysteine thiols much more effectively than ROS/RNS themselves, and also activate the same signaling pathways activated by the direct counterreaction. Hence, this mode of activation is termed "electrophilic counterreaction," as shown in Fig. 10.1C. Biologically relevant EPs can be generated during tightly controlled metabolic processes or during dysregulated pathological processes as byproducts of oxidation (Groeger and Freeman 2010; Akaike et al. 2010). Some EPs are toxic in and of themselves, e.g., due to their indiscriminate reaction with thiols such as glutathione, thus depleting cellular defensive reserves against oxidative stress. In response to such EP stress, some cells mount a second kind of 'electrophilic counterreaction,' characterized by a system that detoxifies EPs and removes them immediately (Satoh and Lipton 2007). This electrophilic counterreaction usually lies relatively dormant, with only mild baseline activity, but becomes activated by EPs themselves (Satoh and Lipton 2007). This type of counterreaction can prevent neurodegeneration and tumor growth because it eliminates not only the EPs themselves but also ROS (Mattson and Cheng 2006; Satoh and Lipton 2007). Thus, EPs could possibly be

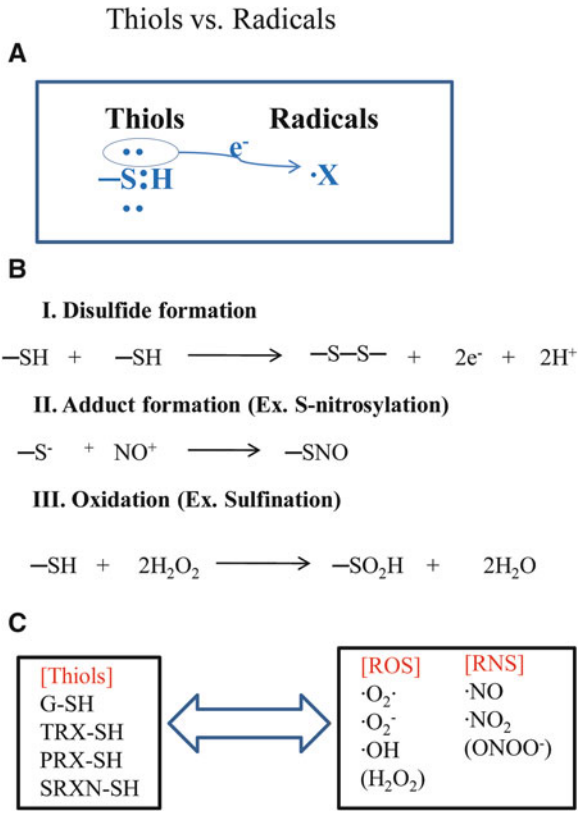


Fig. 10.2 Thiols *vs.* ROS/RNS. *A* Basic reaction. Thiols have two lone pairs of electrons and donate electrons to radicals, which have an unpaired electron(s), although the reaction mechanism(s) and other participating molecules are still under contention. *B* Three modes of reaction. There are three enzymatic reactions (I. Disulfide formation, II. Adduct formation, and III. Oxidation) that allow the basic reaction in panel *A*. *C* Thiols and ROS/RNS. There are various molecules representing thiols, ROS, and RNS

used as neuroprotective agents if depletion of glutathione and other critical thiols could be avoided. In this review, we will discuss four molecules that are typical endogenous EPs produced by ROS/RNS:

1. Active aldehydes including *trans*-4-hydroxy-2-nonenal (HNE) and *trans*-4-hydroxy-2-hexenal (HHE) produced by a series of lipid peroxidations of $n = 3$ and $n = 6$ poly-unsaturated fatty acids (PUFA), respectively (Uchida 2003; Catalá 2009; Long and Picklo 2010);
2. Nitrated fatty acids, including nitrated derivatives of oleic and linoleic acids produced by nitration of fatty acids in phospholipids (Groeger and Freeman 2010; Kansanen et al. 2011);
3. Nitrated-guanosines, including 8-nitro-cGMP produced by nitration of guanosine-containing compounds (Akaike et al. 2010; Fujii et al. 2010);
4. Quinone-formation of catecholamines, including dopamine (DA), in the presence of Cu^{2+} and O_2 (Shih et al. 2007).

Free radical formation is involved in the production of endogenous EPs, and is initiated by reactive radicals or related molecules (·OH, NO_2, and O_2^-). Hence, until recently, these compounds were assumed to produce only toxic effects so they were studied in neurodegenerative conditions (Coyle and Puttfarcken 1993; Halliwell

and Gutteridge 1999). However, an emerging concept in cellular stress responses is that under certain conditions these EPs can be neuroprotective. The pivotal event in developing this concept was the discovery that EPs could influence the activity of the Keap1/Nrf2 and HSP90/HSF-1 pathways (Talalay 2000; Itoh et al. 2004; Bukau et al. 2006; Morimoto 2008; Satoh et al. 2011). In the context of the Keap1/Nrf2 pathway, EPs are now considered physiological activators (Mattson and Cheng 2006; Satoh and Lipton 2007). Furthermore, these "electrophilic counterreactions" can be used to develop neuroprotective drugs (Satoh and Lipton 2007). Along these lines, we have been developing Pro-Electrophilic Drugs (PEDs) that are activated to their electrophilic form by oxidative/nitrosative stress, the very insult that they then counteract. Since activation of PEDs occurs preferentially in cells undergoing such stress (and therefore that are already relatively depleted of glutathione), PEDs are innocuous in normal, unstressed cells. We have found that such drugs can afford neuroprotection in animal models of chronic neurodegenerative diseases such as Alzheimer's and Parkinson's (Satoh et al. 2008a, 2011).

10.2 Thiols *vs*. Radicals

10.2.1 Basic Reaction

As shown in Fig. 10.2A, the basic reaction for cysteine-based redox regulation against oxidative/nitrosative stress is the electron donation from cysteine thiols to free radicals (Halliwell and Gutteridge 1999; Arteel and Sies 2001). Thiols have two lone pairs of electrons and tend to donate electrons. In contrast, free radicals have an unpaired electron and thus accept electrons from donors. Thus, an electron may potentially be donated from thiol to radical, as shown in Fig. 10.2A. Cells contain high concentrations of reduced thiols, such as reduced glutathione (GSH), thioredoxin (TRX), peroxiredoxin (PRX), and sulfiredoxin (SRXN; Immenschuh and Baumgart-Vogt 2005; Arteel and Sies 2001; Holmgren and Lu 2010), which serve to combat free radicals such as molecular oxygen (O_2), superoxide anion (O_2^-), hydroxyl radical (·OH), nitric oxide (·NO) and nitric dioxide ($\cdot NO_2$) radicals. Critical thiols on a number of proteins can also undergo reaction with GSH to form glutathionylated cysteine residues (Dalle-Donne et al. 2009). H_2O_2 and $ONOO^-$ do not have unpaired electrons and thus are not free radicals, but they may produce related free radicals (Radi 2004; Pacher et al. 2007; Butterfield et al. 2011), ·OH and $\cdot NO_2$. Therefore, they can be included as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Importantly, however, the reaction mechanisms are not well characterized and are most probably not direct. For example, reaction of cysteine with an NO group may possibly proceed via thiolate anion (RS^-) reacting with a nitrosonium intermediate (NO^+, with one less electron than NO) in the presence of an electron acceptor such as a transition metal to balance the electron equation (Lipton et al. 1993; Lipton and Stamler 1994).

Cells continuously have to combat ROS and RNS, and thus cysteine-based redox regulation, as shown in Fig 10.2B, is an effective frontline defense for cells. Protein

and glutathione cysteine thiols (−SH) are some of the most powerful nucleophilic compounds, having two lone pairs of electrons; thus, they can donate electrons to oxygen, ROS, and RNS. There are basically three types of reduction pathways used for redox regulation, as shown in Fig. 10.2B, i.e., disulfide formation, adduct formation, and oxidation.

10.2.2 ROS

Molecular oxygen requires a total of four electrons for reduction to water, and reacts slowly with many biomolecules (Halliwell and Gutteridge 1999). If reduced, however, O_2 becomes more reactive (hence, a ROS) (Fig. 10.2C). One-electron reduction results in O_2^-; two-electron reduction, in hydrogen peroxide (H_2O_2); and three-electron reduction, in the highly destructive hydroxyl radical (·OH). ROS are frequently interconverted, for example, superoxide into peroxide (by dismutase enzymes) and peroxide into ·OH (the so-called Fe^{2+}-catalyzed Fenton reaction).

10.2.3 RNS

Nitric oxide (NO) is a gaseous free radical with a relatively short half-life, and the NO group also exists, at least at very low pH, as a nitrosonium cation (NO^+), with one less electron, or nitroxyl anion (NO^-), with an additional electron in its outer pi molecular orbital. In the case of NO^-, the electron pair can be in a high-energy singlet state or a lower energy triplet state, with distinctive biological reactivity (Kim et al. 1999). These various NO species regulate an ever-growing list of biological processes in the brain (Hess et al. 2005; Hara and Snyder 2007; Nakamura and Lipton 2009, 2011). NO represents a pleiotropic signaling molecule, regulating diverse cellular processes and is produced endogenously from L-arginine by NO synthases. The classical NO signaling pathway involves reaction with heme in soluble guanylate cyclase to activate the enzyme and generate cGMP, which in turn stimulates protein kinase G (Hess et al. 2005; Hara and Snyder 2007). However, in the past several years, protein S-nitrosylation, which is the covalent attachment of an − NO group to a cysteine thiol (or more properly thiolate anion), has been recognized as a reversible posttranslational modification by which NO regulates the function of many target enzymes, transcription factors, and ion channels. These targets include protein disulfide isomerase (Uehara et al. 2006), matrix metalloproteinases (Gu et al. 2002), parkin (Chung et al. 2004; Yao et al. 2004), cyclin-dependent kinase 5 (Cdk5, Qu et al. 2011), peroxiredoxin 2 (PRX2, Fang et al. 2007), dynamin related protein 1 (Drp1, Cho et al. 2009), X-linked inhibitor of apoptosis protein (Nakamura et al. 2010), phosphatase with sequence homology to tensin (PTEN, Numajiri et al. 2011), and many other molecules (Hess et al. 2005; Hara and Snyder 2007; Nakamura and Lipton 2009, 2011; Nakamura et al. 2011). Interestingly, while some proteins are S-nitrosylated by physiological levels of

endogenous NO as part of a normal signaling pathway, other targets appear to be nitrosylated only under pathological conditions with high levels of NO, as seen in a number of neurodegenerative disorders. These aberrant nitrosylation reactions can lead to abnormal protein activity and thus contribute to the pathogenesis of the disease (Nakamura and Lipton 2009, 2011).

Oxidative metabolism leads to the formation of numerous RNS, including NO_2, peroxynitrite ($ONOO^-$), and *S*-nitrosoglutathione. RNS can react directly or indirectly with proteins and other molecules in the cell, inducing chemical modifications that lead to changes in their structure and function. For example, $ONOO^-$ can act as an effector of NO-dependent signals as a strong oxidizing agent by targeting cysteine thiols, in some cases facilitating disulfide formation in proteins. By targeting cysteine thiols, $ONOO^-$ inhibits tyrosine phosphatases, antioxidant enzymes, and cysteine proteases (Radi 2004; Pacher et al. 2007). $ONOO^-$ also modifies proteins by nitrating several amino acids, including tyrosine residues (Pacher et al. 2007; Butterfield et al. 2011). Much research has focused on peroxynitrite-mediated modification of tyrosine residues, which forms 3-nitrotyrosine, considered a key player in $ONOO^-$-mediated toxicity in animals (Radi 2004). This nitration of tyrosine residues, in counterdistinction to S-nitrosylation of cysteine thiols, is mediated by reaction of $ONOO^-$ with tyrosine not cysteine residues as is a common misconception. Recently, $ONOO^-$ has been reported to also induce nitration (by adding -NO_2) of unsaturated fatty acids and guanosine, which function as intracellular signaling molecules (Niles et al. 2006). Interestingly, these nitrated molecules (nitro-fatty acid and nitro-guanosine) are thought to be physiological activators of the Keap1/Nrf2 pathway and thus function in electrophilic counterreaction (Groeger and Freeman 2010; Kansanen et al. 2011). $ONOO^-$ itself is highly reactive and thus unstable, but may be stabilized when complexed with carbonate radical, which can produce NO_2 radicals (Radi 2004; Pacher et al. 2007). These radicals mediate nitration of fatty acid and guanosine under physiological conditions, suggesting a potential normal signaling function of these molecules in addition to their known toxic effects (Groeger and Freeman 2010; Kansanen et al. 2011).

10.3 Cysteine-Based Redox Regulation

Cysteine-based redox regulation is an effective frontline of defense in cells that is used to combat oxidative and nitrosative stress, as shown in Fig. 10.3 (Winyard et al. 2005; Immenschuh and Baumgart-Vogt 2005; Satoh and Lipton 2007). Importantly, virtually all of the molecules and proteins involved in this defense mechanism are regulated by the Keap1/Nrf2 and/or the HSP90/HSF-1 transcriptional pathways (Takahashi et al. 2009; Tamaki et al. 2010; Satoh et al. 2011). For cysteine-based regulation to work effectively, transcriptional regulation is essential, and EPs are effective activators of these transcriptional pathways. Three of the cysteine-based redox regulatory cascades that are activated by these transcriptional pathways are summarized below, and they include (i) the glutathione peroxidase (GPX) system, (ii) the PRX/TRX system, and (iii) the PRX/SRXN system.

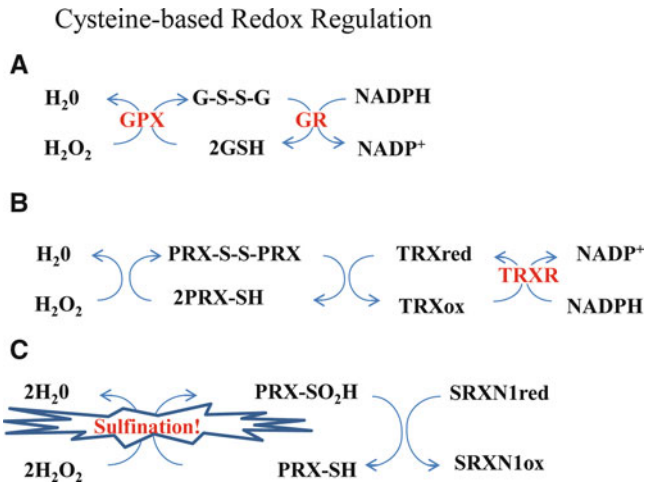


Fig. 10.3 Cysteine-based Redox regulation. Cells manifest three cascades for cysteine-based redox regulation: the GPX/GSH (A), PRX/TRX (B), and PRX/SRXN (C) systems

10.3.1 GPX/GSH System

Glutathione is amongst the most important intracellular antioxidants and is present at millimolar concentrations within human cells (Arteel and Sies 2001). As shown in Fig. 10.3A, reduced glutathione (GSH) exists in equilibrium with its disulfide form (GSSG), and the ratio of GSH to GSSG can be used as an indicator of the redox status of the cell. In addition to catalase and PRX, the antioxidant system composed of the selenium-dependent enzyme GPX and GSH constitute the major cellular enzymatic mechanisms to reduce hydrogen peroxide to water (Arteel and Sies 2001). GSH is also used by glutathione-S-transferase (GST), which catalyzes the conjugation of GSH to various electrophiles, thereby removing these toxic products of free radical reactions. GSH-dependent defenses rely, of course, on the availability of GSH. Therefore, systems involved in the reduction of GSSG are essential, including NADPH-dependent GSSG reductase (GR), as well as GSSG transporters. GR achieves reduction of GSSG by a combination of a thiol–disulfide exchange reaction, two-electron transfer (from $FADH_2$ to reduce the GR disulfide) and hydride transfer from NADPH to FAD (Arteel and Sies 2001).

10.3.2 PRX/TRX System

TRX has the capacity to repair oxidized proteins by reducing protein disulfides at the expense of the oxidation of its own redox-active cysteine residues, as shown in

Fig. 10.3B (Chang et al. 2004; Immenschuh and Baumgart-Vogt 2005; Holmgren and Lu 2010). The oxidized TRX cysteines are then reduced back to the free thiol form by thioredoxin reductase (TRXR). TRXR contains redox-active cysteine and selenocysteine residues, and regenerates the reduced form of TRX by a series of thiol–disulfide and seleno–disulfide exchange reactions and electron transfer from NADPH. PRX reacts with peroxides to detoxify them to water, forming disulfides on PRX, which are then reduced by TRX to regenerate active PRX with free thiols. The TRX-PRX pathway is arguably the most important antioxidant system to limit accumulation of intracellular peroxides via redox reactions at critical cysteine residues. Expression of TRX-PRX is controlled by the Keap1/Nrf2 system (Talalay 2000; Itoh et al. 2004). In particular, the TRX-PRX system is extremely abundant in the brain. In summary, the TRX-PRX system detoxifies peroxides by transferring reducing equivalents from NADPH to peroxides via TRX reductase, TRX, and finally PRX (Chang et al. 2004; Immenschuh and Baumgart-Vogt 2005; Holmgren and Lu 2010). Recently, we showed that excessive NO can react with the free thiols of PRX to prevent its reaction with peroxides, thus increasing susceptibility to neuronal cell death and injury (Fang et al. 2007).

10.3.3 PRX/SRXN System

In response to fulminant oxidative stress, PRX forms PRX-SOH via reaction with peroxide, and can be further oxidized to a sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid derivative (Rhee et al. 2007; Soriano et al. 2008, 2009). This hyperoxidation of PRX causes inactivation of its peroxidase/neuroprotective activity. Although PRX-SO_2H cannot be reduced by TRX, it can be reduced back to the catalytically active free thiol form in eukaryotic cells including neurons by an ATP-dependent reductase, sulfiredoxin (SRXN). The activity of SRXN1 restores inactive PRX-SO_2H back to the TRX cycle and prevents permanent oxidative inactivation of PRX by strong oxidative insults, as shown in Fig. 10.3C (Rhee et al. 2007; Soriano et al. 2008, 2009). However, SRXN1 must be generated by transcriptional activation in cells in order for it to be produced in sufficient quantities to reverse hyperoxidation of PRX, and this transcriptional activation requires activation of the Keap1/Nrf2 pathway (Soriano et al. 2008).

10.4 GSH Metabolism as a Key to Protection

The level of GSH is important for protection against oxidative/nitrosative stress. Through the study of various EPs, we found that the intracellular GSH level is a key in determining whether an EP will be protective or toxic to neurons (Fig. 10.4). EPs can exert opposite effects on the level of GSH: they can directly react with GSH to deplete it (action #1) as well as induce phase 2 enzymes that synthesize GSH (action

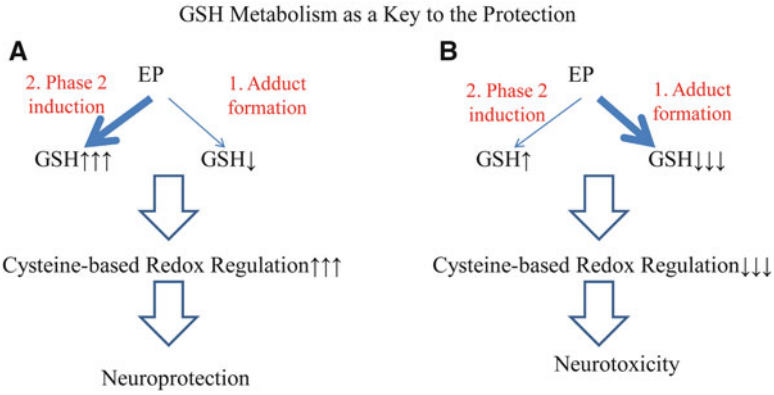


Fig. 10.4 GSH metabolism as a key to redox protection. EPs do not have unpaired electrons like ROS/RNS, but have atoms (carbon, nitrogen, and sulfur) with electron deficiency. EPs react in facile fashion with GSH. In the case of neurotoxic EPs, the depletion of GSH can proceed by adduct formation. In contrast, an increase in GSH is dominant in neuroprotection via transcriptional activation by EPs. Note that this increase in GSH is due to the induction of phase 2 enzymes, which is involved in the cysteine-based redox regulation described in Fig. 10.3

#2). If action #1 overwhelms action #2, then the EPs will be toxic and actually render the cells more susceptible to oxidative and nitrosative stress. In contrast, if phase 2 induction overcomes adduct formation, then the EP is neuroprotective (Fig. 10.4A). When adduct formation overcomes phase 2 induction, the EP will be neurotoxic (Fig. 10.4B). Cysteine-based redox regulation is a powerful defense against acute oxidative and nitrosative stress only if the various forms of cysteines (GSH, TRX, PRX, SRXN and other proteins) are constitutively supplied, i.e., they are available at the time of the insult. If they have to be transcriptionally induced, then the cell may not survive an acute fulminant episode of oxidative/nitrosative stress since the insult may overwhelm the number of reduced cysteines that are present; the same caveat exists if a milder stress is present for a prolonged time. However, in the latter case, cells may have time to generate additional cysteine-based redox compounds by activating various phase 2 enzymes such as the glutamyl cysteine ligase modifier subunit (GCLM), glutamyl cysteine ligase catalytic subunit (GCLC), or Na^+-independent cystine-glutamate exchanger (xCT).

As a master transcriptional regulator of redox activity, Nrf2 can regulate the expression of many proteins relevant to the cell's reducing capacity, including phase 2 enzymes. The Keap1/Nrf2 pathway can be activated by various agents, including ROS/RNS themselves as well as various forms of electrophilic compounds. In the context of continued oxidative/nitrosative stress, the continued supply of reduced cysteine-containing compounds via the Keap1/Nrf2 pathway is a key event for the regulation of cysteine-based redox regulation.

The importance of GSH metabolism for the neuroprotective effects of EPs has also been implicated in many experimental paradigms (Ahlgren-Beckendorf et al. 1999; Lee et al. 2003; Kraft et al. 2004; Sun et al. 2005; Satoh et al. 2008a, b,

2009a, b; Takahashi et al. 2009; Tamaki et al. 2010). Here we discuss carnosic acid (CA) as an example of a compound that can be converted to a neuroprotective EP, and the protective mechanism can be understood at least in part in terms of GSH metabolism. We recently found that CA induces transcription of four phase 2 genes encoding GST (A2 subtype), GCLC, and two ATP-binding cassette family members, represented by ATP-binding cassette sub-family C member 4 (ABCC4) and multidrug resistance-associated protein 1 (ABCC1). All of these are involved in GSH regulation. GSTA2 catalyzes the conjugation of GSH with electrophilic compounds, GCLC is involved in the synthesis of GSH, and ABCC1 and ABCC4 actively transport GSH-conjugated electrophilic compounds. The induction of these genes is known to up-regulate GSH metabolism and hence increases the redox capacity of the cell against oxidative stress.

10.5 Two Transcriptional Pathways for Electrophilic Counterattack

As shown in Fig. 10.5, the Keap1/Nrf2 and HSP90/HSF-1 transcriptional pathways provide an effective 'backup system' for cysteine-based redox regulation by activating endogenous gene networks that are involved in antioxidant defense. The work of Satoh et al. (2011) and Zhang et al. (2011) showed that several activators of Nrf2 via reaction with Keap1 also covalently bind to cysteines of HSP90 to activate HSF-1, thereby inducing molecular chaperones, including additional HSPs. These results suggest that S-alkylation of critical cysteine residues by EPs can activate both of these transcriptional pathways, representing electrophilic counterreactions. Since neurons have little capacity for redox regulation, these backup systems become very important in the nervous system. After electrophilic activation, the transcription factors Nrf2 and HSF-1 bind to the antioxidant response element (ARE)- and heat-shock factor response element (HSE), respectively, to initiate transcription of genes whose products provide neuroprotection from oxidative and nitrosative insults (Satoh et al. 2011).

10.5.1 The Keap1/Nrf2 Pathway

Nrf2 is a member of a small family of transcription factors that share a conserved bZIP dimerization/DNA-binding domain and the ability to bind ARE-like DNA sequence motifs. Characterization of mice that lack Nrf2 has revealed an essential role for this transcription factor in both basal and inducible expression of classical phase 2 genes (Talalay 2000; Itoh et al. 2004). The ability of Nrf2 to activate transcription of its target genes is regulated, in large part, through association with a cytoplasmic protein termed Keap1 (Talalay 2000; Itoh et al. 2004). Keap1 was first identified by two-hybrid screening that used the N-terminal regulatory domain of Nrf2 as bait (Talalay 2000; Itoh et al. 2004). Keap1 is a member of a large family of proteins that contain an N-terminal Broad complex, Tramtrack, and Bric a brac

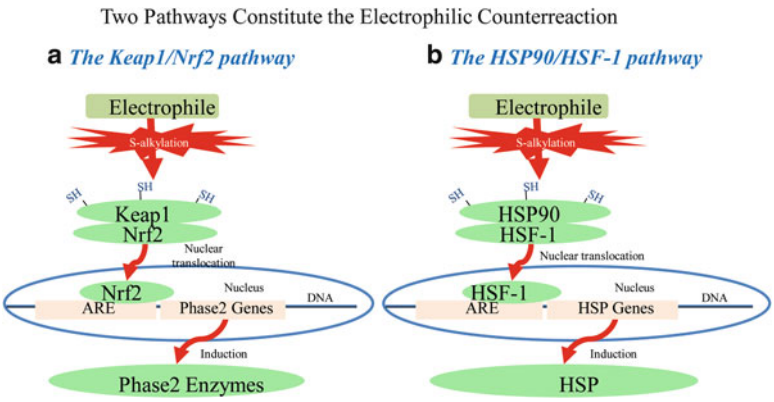


Fig. 10.5 Two pathways for electrophilic counterattack. Two essential pathways (*A* Keap1/Nrf2 and *B* HSP90/HSF-1) constitute electrophilic counterreactions. Some EPs activate both pathways and others activate one or the other. Note that EPs activate these pathways by triggering S-alkylation of the regulatory thiol proteins, Keap1 and HSP90

(BTB) domain, and a C-terminal Kelch repeat domain (Padmanabhan et al. 2006). Previous reports have demonstrated that Keap1 retains Nrf2 in the cytoplasm via a direct protein-protein interaction between the C-terminal Kelch repeat domain of Keap1 and the N-terminal Neh2 regulatory domain of Nrf2. The role of Keap1 as a physiological regulator of Nrf2 is further supported by the observation that macrophages from mice that lack Keap1 have constitutive nuclear accumulation of Nrf2 (Padmanabhan et al. 2006; Satoh et al. 2009b).

Because EPs are effective activators of the Keap1/Nrf2 pathway, they can induce the expression of a set of metabolic antioxidant enzymes, called 'phase 2 enzymes,' which include hemeoxygenase-1 (HO-1), NADPH quinone oxidoreductase 1 (NQO1), sulfiredoxin (SRXN), glutamyl cysteine ligase modifier subunit (GCLM) and the Na^+-independent-cystine/glutamate exchanger (xCT), all of which provide efficient cytoprotection by regulating the intracellular redox state (Kraft et al. 2004; Satoh et al. 2006, 2008a). Mechanistically, when EPs react with critical cysteine residues on Keap1 protein to form a covalent adduct, they perturb this system, thereby releasing Nrf2 and allowing it to be translocated from the cytoplasm into the nucleus, where it binds to AREs and stimulates the transcription of phase 2 genes (Kraft et al. 2004; Satoh et al. 2006, 2008a). Thus, Nrf2 has emerged as a potential therapeutic target for the treatment of neurodegenerative diseases (Satoh and Lipton 2007; Alfieri et al. 2011).

10.5.2 The HSP90/HSF-1 Pathway

The HSP90/HSF-1 pathway is the principal regulatory pathway leading to expression of heat-shock proteins (HSPs), which rescue cells from endoplasmic reticulum

(ER) stress and related insults (Bukau et al. 2006; Morimoto 2008). Several reports have indicated that ROS/RNS regulate HSP expression by activating this pathway. We and others have recently reported that EPs, which activate the Keap1/Nrf2 pathway through S-alkylation of a critical cysteine thiol on Keap1, also induce HSPs via activation of the HSP90/HSF-1 pathway (Satoh et al. 2011; Zhang et al. 2011). The ER is a reticulated organelle in which proteins are synthesized and modified for proper folding. Approximately 30% of newly synthesized proteins in normal cells are misfolded, but some of these proteins can be refolded to achieve their correct structures. This process of refolding is facilitated by ER chaperones. However, other proteins remain misfolded, accumulate in the ER, and induce ER stress (Bukau et al. 2006; Morimoto 2008). Transcriptional induction of molecular chaperones is governed by the stress-inducible heat-shock transcription factor known as HSF-1, which when bound to HSEs, plays a key regulatory role in activating transcription in response to environmental stress (Bukau et al. 2006; Morimoto 2008). Compounds that modulate HSF-1 to increase HSP expression can exert protective effects against various types of stress including ER stress (Bukau et al. 2006; Morimoto 2008). The HSF-1/HSE system can protect against oxidative stress in addition to ER stress (Kim et al. 2008; Nakamura and Lipton 2009, 2011).

In this background, we recently found that induction of HSPs mediated by D1, a cyclic EP, plays a role in neuroprotection against ER stress as well as against oxidative stress (Satoh et al. 2011). Oxidative stress can also lead to ER stress, which may contribute to several chronic neurodegenerative diseases. Accumulation of misfolded proteins in the ER induces ER dysfunction (Kim et al. 2008; Nakamura and Lipton 2009). To combat such stress, there is a powerful endogenous protective mechanism for induction of molecular chaperones, including heat-shock protein 70 (HSP70) and DnaJ, as well as heat-shock factor-binding protein (HSBP) and heat-shock 105-kDa/110-kDa protein 1 (HSPH1). These molecular chaperones are known to suppress protein misfolding (Bukau et al. 2006; Morimoto 2008). The expression of these chaperones after exposure to various types of cell stress is known to be regulated by HSF-1. Under unstressed conditions, HSF-1 is localized in the cytosol and is inactivated in a protein complex that includes HSP90. As mentioned above, upon exposure to stress-inducing compounds including EPs, HSF-1 dissociates from the HSP90 protein complex, is translocated into the nucleus, and binds there to the HSE in the promoter region of various molecular chaperone genes to induce their expression. For this reason, HSF-1 has been considered as a potential target for the treatment of neurodegenerative diseases (Morimoto 2008).

10.6 Keap1(Cys151) as a Hyperactive Thiol

For EPs to be effective in electrophilic counterreaction, an initial event is the S-alkylation of essential cysteine residues of regulatory proteins such as Keap1 and HSP90. Several publications have studied the critical cysteines of Keap1 that can be S-alkylated, but we will limit our discussion to the critical cysteines of

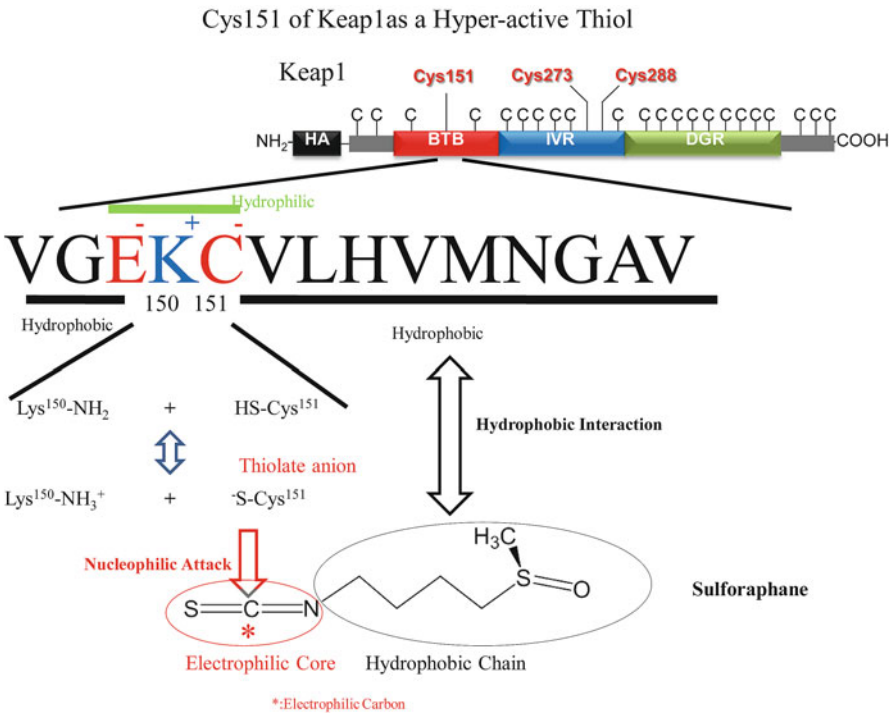


Fig. 10.6 Keap1(Cys151) as a hyperactive thiol. Keap1 has 25 cysteines, and the amino acid sequence surrounding the critical residue at Cys151 is shown in the middle column. The amino acids Glu149 and Lys150 are the only hydrophilic residues in a stretch of hydrophobic amino acids. The NH_2 of Lys150 is thought to remove a proton from the -SH of Cys151, thus forming a thiolate anion ($-S^-$). This hyperactive thiol can bind to the electrophilic core of sulforaphane, represented by isocyanate

Keap1 whose modification leads to activation of Nrf2 (Talalay 2000; Itoh et al. 2004; Padmanabhan et al. 2006). The studies pertaining to Keap1 activation by EPs have concentrated on Cys273, Cys288, Cys151 and Cys434, while activation by ROS/RNS has centered around Cys226 and Cys613. Keap1 actively targets the N-terminal Neh2 domain of Nrf2 for ubiquitination and proteosome-mediated degradation. Two cysteine residues located in the linker domain of Keap1, Cys273 and Cys288, are critically required for both Keap1-dependent ubiquitination and Keap1-mediated repression of Nrf2-dependent transcription under basal conditions (Dinkova-Kostova et al. 2002). A third cysteine residue located in the BTB domain of Keap1, Cys151, is required for stabilization of Nrf2 and for activation of Nrf2-dependent transcription by the EPs tBHQ and sulforaphane, as shown in Fig. 10.6 (Zhang et al. 2004; Hong et al. 2005; Kobayashi et al. 2009). Cys151 provides a link between the ability of chemical inducers to block Keap1-mediated repression of Nrf2 and to induce posttranslational modification(s) of Keap1.

Why is Cys151 of Keap1 distinct from its other cysteines? One possible answer is the formation of a thiolate anion, $–S^-$ (Kobayashi et al. 2009). Cys151 is the direct molecular target of a number of chemical inducers of Nrf2-dependent transcription (Zhang et al. 2004). Kobayashi et al. (2009) reported the importance of Lys150 in addition to Cys151, suggesting that the –SH group of Cys151 donates H^+ to the NH_2 of Lys150, thus forming a thiolate anion ($-S^-$) and an ammonium cation ($-NH_3{}^+$). Thiolate anion, as opposed to a thiol of the sulfhydryl group itself (−SH), displays an increased capacity to donate electrons to ROS, RNS, and EPs. This proposed chemical reaction scheme makes Keap1(Cys151) distinct from other cysteines in Keap1. Furthermore, Lys150 and Cys151 are surrounded by hydrophobic amino-acid residues, suggesting that the reaction of EPs with Cys151 requires an interaction between the nearby hydrophobic amino acids in Keap1 and the hydrophobic chain of EPs. For example, sulphoraphane, which is reported to bind to Cys151, is a typical example of this chemical reaction (Fig. 10.6). The electrophilic core (isocyanate) of sulphoraphane binds to Cys151, and its hydrophobic tail binds to the hydrophobic chain of Keap1, resulting in the release of Nrf2 and explaining the effective activation of the Keap1/Nrf2 pathway by sulphoraphane (Zhang et al. 2004; Hong et al. 2005; Kobayashi et al. 2009). In contrast, ROS and RNS themselves, including hydrogen peroxide (H_2O_2) and NO, have an electrophilic core but lack a hydrophobic chain; thus, they require a much higher concentration to facilitate their reaction with Cys151 (Fourquet et al. 2010; Um et al. 2011).

Although H_2O_2 does not apparently directly oxidize Cys 151 to form a stable sulfenic, sulfinic, or sulfonic acid derivative, it does facilitate disulfide bond formation with a second Cys151. Fourquet et al. (2010) found that in untreated cells, a fraction of Keap1 carries a long-range disulfide linking Cys226 and Cys613. Exposing cells to H_2O_2 or certain NO donors increases the incidence of this disulfide and also promotes formation of a disulfide linking two Keap1 molecules via their Cys151 residues. The involvement of other cysteines was also reported, for example, Cys434 (Fujii et al. 2010). In contrast to Cys151, Cys434 is not surrounded by many hydrophobic amino acids and thus hydrophilic compounds, such as 8-nitroguanosine, can approach it for reaction. In fact, Keap1(Cys434) is one of the cysteine residues that is most sensitive to adduct formation with 8-nitroguanosine. Additionally, nitro-fatty acids can reportedly make an adduct with Keap1 in a Cys151-independent manner (Kansanen et al. 2011).

10.7 Electrophilic Counterreactions by Endogenous Electrophiles

In addition to direct reaction of ROS and RNS with Keap1 or HSP90, endogenous EPs produced by ROS/RNS can activate these pathways, a process termed “electrophilic counterreaction” or “electrophilic counterattack” against oxidative

and nitrosative stress (Groeger and Freeman 2010; Akaike et al. 2010; Satoh and Lipton 2007). Examples of the reactions that generate EPs are illustrated below.

10.7.1 Active Aldehyde

Lipid peroxidation yields multiple aldehyde species. Of these, *trans*-4-hydroxy-2-nonenal (HNE), derived from *n*-6 poly-unsaturated fatty acids (PUFA), is one of the most studied products of lipid peroxidation (Fig. 10.7A) (Uchida 2003; Catalá 2009; Long and Picklo 2010). Additionally, oxidative damage to *n*-3 PUFAs, e.g., docosahexaenoic acid (22:6, *n*-3) and eicosapentaenoic acid, is now recognized as an important effector of oxidative stress and is of particular interest in *n*-3-rich tissues such as the brain and retina (Uchida 2003; Catalá 2009; Long and Picklo 2010). *Trans*-4-hydroxy-2-hexenal (HHE) is a major-unsaturated aldehyde product of *n*-3 PUFA oxidation and, like HNE, is an active biochemical mediator resulting from lipid peroxidation. HHE adducts are elevated in disease states, in some cases to higher levels than the corresponding HNE adduct. HHE has properties in common with HNE, but there are also important differences, particularly with respect to adduct targets and detoxification pathways (Uchida 2003; Catalá 2009; Long and Picklo 2010). During the generation of HHE and HNE, the withdrawal of the π electron from the conjugated double bond by OH is thought to initiate a series of lipid peroxidation steps shown in Fig. 10.7A. Although HNE and HHE activate the Keap1/Nrf2 and HSP90/HSF-1 pathways, this action is also accompanied by toxic effects (Uchida 2003; Mangialasche et al. 2009). The reason for this is that the reaction produces various toxic radicals and lipid hydroperoxides, and thus this pathway is considered toxic rather than beneficial against oxidative stress.

10.7.2 Nitro-Fatty Acid and 8-Nitro-cGMP

As shown in Fig. 10.7B and C, the chemical reaction for the generation of nitro-fatty acid and 8-nitroguanosine is basically via the same pathway, although they are achieved under hydrophobic and hydrophilic conditions, respectively (Groeger and Freeman 2010; Kansanen et al. 2011; Akaike et al. 2010; Fujii et al. 2010). NO_2, as an initiator of the chemical reaction, withdraws a π electron from the double bond and makes an adduct (Radi 2004; Groeger and Freeman 2010). The resultant alkyl radical is attacked by another NO_2, and finally HNO_2 is released from the molecule, and nitro-fatty acid and 8-nitroguanosine are produced. Surprisingly, the production of NO_2 seems to proceed continuously at very low levels. Free and esterified polyunsaturated fatty acids (PUFAs), such as linoleate, are in particular predisposed to formation of both reactive intermediates and bioactive products (Groeger and Freeman 2010; Kansanen et al. 2011). Nitrated derivatives of oleic, linoleic, linolenic, arachidonic, and eicosapentaenoic acids have also been identified

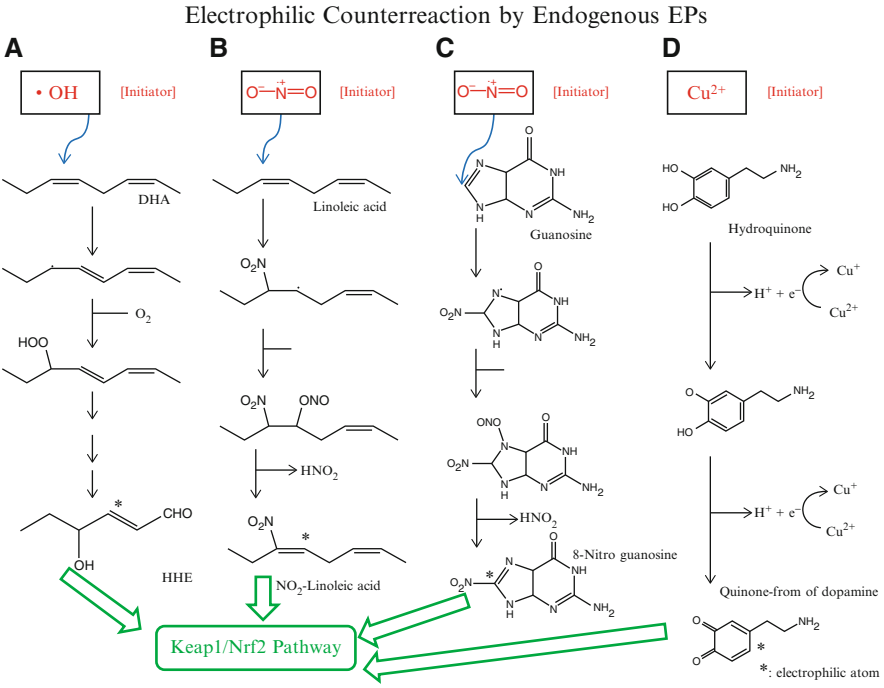


Fig. 10.7 Electrophilic counterreactions triggered by endogenous EPs. Chemical reactions of ROS to generate endogenous EPs are illustrated. The precise chemical reactions 1–3 are not fully understood but are thought to involve free radical-initiated lipid peroxidation. The EPs thus produced activate the regulatory Keap1/Nrf2 and HSP90/HSF-1 pathways

in mammals. These molecules might exist under physiological conditions because nitrated derivatives of unsaturated fatty acids are present in healthy human blood, and nitrohydroxy derivatives of linoleate have been detected in healthy human plasma and lipoproteins (Groeger and Freeman 2010; Kansanen et al. 2011). Acting as an EP, 8-nitro-cGMP reacts with particular protein thiols to generate a unique posttranslational modification (Akaike et al. 2010; Fujii et al. 2010).

10.7.3 Dopamine Quinone

Dopamine (DA) is synthesized from tyrosine via the enzymatic activities of tyrosine hydroxylase and aromatic amino acid decarboxylase (Winyard et al. 2005; Hastings 2009). Once formed, DA is safely stored in high millimolar concentrations in synaptic vesicles. If vesicular storage is disrupted, DA levels increase in the cytoplasm (Winyard et al. 2005; Hastings 2009). The catechol ring of DA can be

oxidized by ROS to form electron-deficient DA quinone (Fig. 10.7D). DA quinone will react readily with cellular nucleophiles, such as reduced thiol groups of small peptides and protein cysteinyl residues (Winyard et al. 2005; Hastings 2009). There appear to be two opposite opinions as to whether DA quinone is protective or toxic to neurons (Hastings 2009; Shih et al. 2007). The classical opinion is that the process produces peroxides and electrophilic quinones, and these events can lead to neurodegeneration via increased oxidative stress (Hastings 2009). A more recent opinion is that this reaction of DA and other catecholamines produces electrophilic quinones that activate the Keap1/Nrf2 pathway and can thus protect neurons (Shih et al. 2007; Takahata et al. 2009). Both protective and toxic effects of DA quinone require high concentrations ($> 100\ \mu M$), and these processes therefore occur under pathological conditions (Shih et al. 2007; Hastings 2009). The critical difference between the opinions is whether DA quinone is to be regarded as toxic waste or as a mediator of electrophilic counterreaction. In fact, both lines of work may be correct. One possible interpretation is that DA quinone is toxic to neurons but protective to glial cells. Because neurons are highly sensitive to oxidative stress, DA quinone appears to be directly toxic to neurons, but, in at least some cases, appears to be tolerated by glia, activating the Keap1/Nrf2 pathway. Thus, the glia may in turn protect themselves as well as nearby neurons in a non-cell autonomous fashion, for example, by generating protective factors such as GSH and neurotrophic factors (Shih et al. 2007; Hastings 2009). Nonetheless, as discussed further below, an EP that is only protective would, in our opinion, be preferred so that potential consequences of toxic side reactions could be avoided entirely.

10.8 Two Distinct Groups of Electrophiles

In our opinion, it is possible to take advantage of the intracellular mechanism of electrophilic counterreaction by endogenous EPs, as described above, in order to develop a novel strategy for drug development against neurodegenerative diseases (Fig. 10.8). The core concept of this drug development strategy is based on the following principles (Satoh et al. 2008a, 2011; Satoh and Lipton 2007):

1. We are developing pro-electrophilic compounds rather than electrophilic compounds, and thus call these new drugs "Pro-Electrophilic Drugs (PEDs)."
2. PEDs become electrophilic (i.e., are converted to the active form to trigger electrophilic counterreaction) in a Cu^{2+}/O_2-recycling system-dependent manner, and thus PEDs preferentially become active in the face of oxidative stress rather than under physiological conditions. Hence, only cells undergoing oxidative stress will generate active EPs, making the PEDs relatively innocuous for normal cells not facing free radical insult.

This approach still takes advantage of an important property of EPs, namely, their superiority to antioxidant molecules because of the sustained action and amplification of the effect of EPs via transcription-mediated signaling pathways

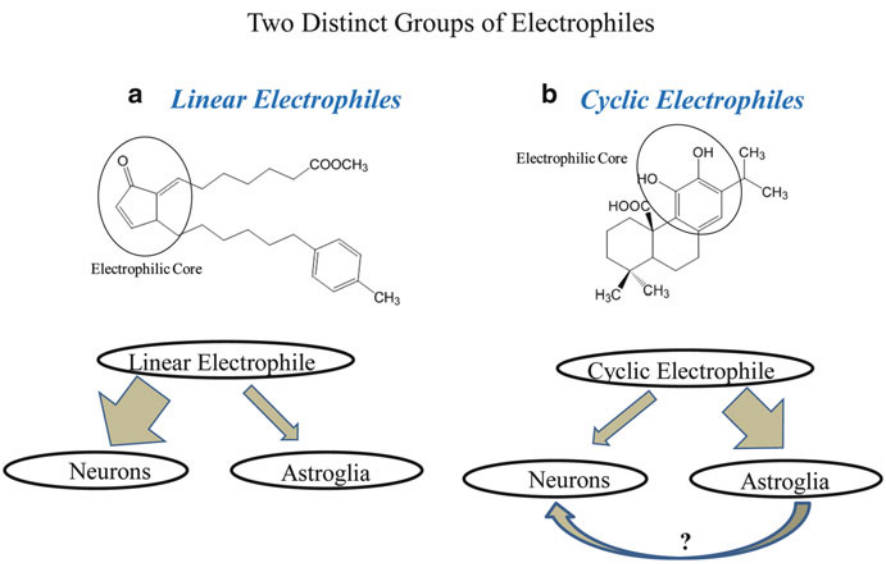


Fig. 10.8 Two distinct groups of electrophiles. There are two types of EPs, one of which has a linear electrophilic core, such as NEPP11, whereas the other has cyclic structures with hydroquinone as the electrophilic core, e.g., CA. After treatment of cells, linear EPs appear to concentrate in neurons and cyclic EPs, in astroglia. Thus, paracrine effects may be involved in the neuroprotective effect of cyclic EPs

(Talalay 2000; Itoh et al. 2004). The neuroprotective EPs reported previously can be divided into two major groups, linear EPs and cyclic EPs (Satoh et al. 2008a). These two types of compounds manifest distinct features. One difference lies in their degree of electrophilicity and thus their ability to activate the Nrf2/ARE pathway. Linear EPs, including neurite outgrowth-promoting prostaglandin (NEPP11) (Satoh et al. 2000, 2001, 2003, 2006), curcumin (Mandel et al. 2009) and sulforaphane (Kraft et al. 2004), are electrophilic in nature; i.e., they readily react with electron donors. In contrast, cyclic EPs are more properly termed PEDs since they are not in and of themselves truly electrophilic, but they become electrophilic via oxidative conversion to their *quinone* form, which occurs when they encounter free radical oxidative stress (Satoh and Lipton 2007). Thus, these cyclic EPs function as pro-drugs, requiring conversion from *hydroquinone* to *quinone* in order to exert their neuroprotective effect (Satoh et al. 2009a; Wang et al. 2010). The cyclic EPs or PEDs include compounds such as carnosic acid (CA), *tert*-butyl hydroquinone (TBHQ), strongylophorine 8 (Sasaki et al. 2011), and D1 (Satoh et al. 2011).

We found that the cellular distribution of linear EPs and cyclic EPs in the brain treated with these compounds appears to be different (Satoh et al. 2006, 2008a). Linear EPs, including NEPP11, appear to act preferentially on neurons, based on our earlier findings that NEPP11 accumulates in neurons as opposed to astrocytes, and consequently induces phase 2 enzymes such as neuronal HO-1 (Satoh et al. 2006).

In contrast, in general cyclic EPs, including CA and TBHQ, appear to preferentially act on astrocytes, as evidenced by the fact that TBHQ activates the ARE in astrocytes to induce phase 2 enzyme synthesis rather than in neurons (Lee et al. 2003: Kraft et al. 2004; Satoh et al. 2008a; Ahlgren-Beckendorf et al. 1999; Vargas and Johnson 2009). In view of these findings, we propose that linear EPs such NEPP11 exert direct protective effects in neurons while cyclic EPs/PEDs such as CA and TBHQ exert paracrine effects to protect neurons (Kraft et al. 2004; Satoh et al. 2008a). In all likelihood, therefore, paracrine or non-cell autonomous effects are the most plausible mechanism for neuroprotection of PEDs, and some candidate mediators of neuroprotection that are released from astrocytes have been identified by microarray analysis (Lee et al. 2003). These non-cell autonomous neuroprotective molecules may include neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF), since we have found that CA potently releases NGF protein from glioblastoma cells. Another possible candidate is GSH since TBHQ potently induces GSH formation in astrocytes (Satoh et al. 2008a; Ahlgren-Beckendorf et al. 1999; Vargas and Johnson 2009). Importantly, tissues undergoing oxidative stress generally involve both neurons and glia, so glia contiguous to stressed neurons may also be expected to have undergone insult. In a number of neurodegenerative diseases, in recent years it has become increasingly clear that it is the nonneuronal cells or glia that mediate the toxic effect on neurons (Clement et al. 2003; Lioy et al. 2011; Garden and La Spada 2012), so in fact it may be the glia that are injured initially by the disease. Hence, mitigating oxidative stress in the glia with PEDs may in fact be more effective than activating the transcriptional pathways in neurons. In any event, after treatment with higher concentrations of PEDs such as CA, drugs may also enter neurons and may therefore exert direct effects on the Nrf2/ARE pathway in neurons as well as in glia (Satoh et al. 2008a, b). Another important point concerning the potential "drugability" of PEDs is that in many cases their catechol chemical formula renders them permeable to the blood–brain-barrier, thus producing excellent levels in the brain (this property of catechols is one of the reasons that L-dopa is used to treat Parkinson's disease rather than dopamine itself).

10.9 Concept of Pro-electrophilic Drugs

Linear EPs have been shown to protect neurons through an electrophilic chemical reaction. However, linear EPs have a serious flaw as potential therapeutic agents because systemic administration can result in reaction with thiol substrates prior to reaching the intended target in the brain (Satoh et al. 2006, 2008a; Satoh and Lipton 2007). It would be far better to have a pro-electrophilic compound that remains non-reactive until it is converted to an electrophile by the oxidative insult at or near the pathological site of its intended action (Satoh and Lipton 2007; Lipton 2007; Satoh et al. 2008a). Such a drug would represent what we have termed a "Pro-Electrophilic Drug," and we have shown that CA constitutes this type of a compound (Fig. 10.9). We have demonstrated that cyclic EPs/PEDs appear to have

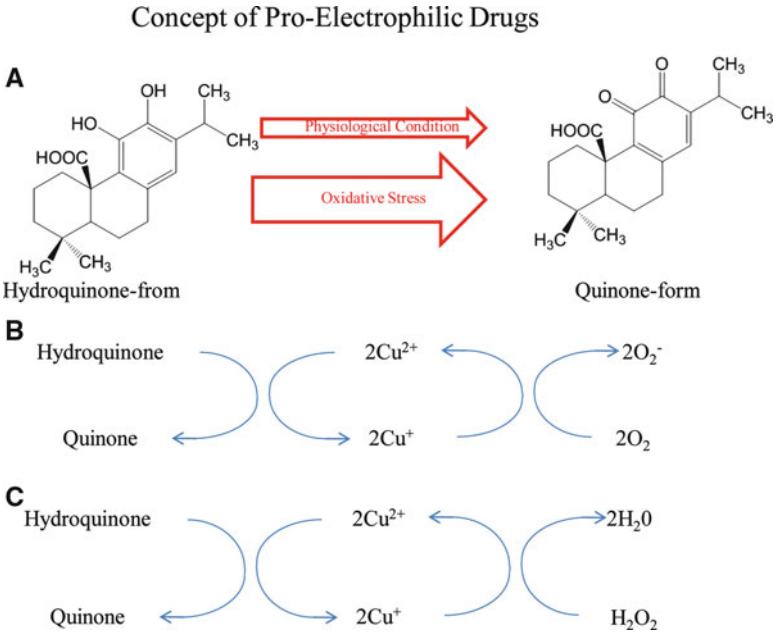


Fig. 10.9 Concept of pro-electrophilic drugs. The *catechol-type* CA that is oxidized to a *quinone-type* form, with the carbon at position 14 [C(14)] becoming electrophilic. This CA quinone is subject to nucleophilic attack by the cysteine thiol of Keap1 to form an adduct. Importantly, via this chemical reaction, the cysteine thiol of Keap1 forms a Keap1-CA adduct, resulting in release of Nrf2 protein from the Keap1/Nrf2 complex. Nrf2 can then be translocated into the nucleus, where it activates transcription of phase 2 enzymes via ARE transcriptional elements of the corresponding genes. These phase 2 enzymes improve the redox state of neurons, contributing to an endogenous anti-oxidant defense system. Note that quinone formation is enhanced under oxidative stress, as described in the text

two clear-cut advantages over linear EPs and similar compounds: *(i)* low potential toxicity due to conversion of the drug to an EP by the oxidative insult at the pathological site, and *(ii)* effective penetrance into brain tissue. These characteristics stem from their chemical structure. Moreover, when the hydroquinone form is oxidized to a quinone, it becomes more hydrophobic and will tend to stay in the injured tissue.

The time course of conversion from *hydroquinone* to *quinone* is rather slow in cell-free systems. How, then, can CA be neuroprotective? One possible answer may lie in the difference between *cell-free* and *cell-based* experiments. Since normal cells have many thiols, including GSH and cysteine-bearing proteins, a quinone would rapidly react with these thiols to form an adduct. Via this mechanism, removal of free quinone would shift the equilibrium between hydroquinone and quinone towards formation of more quinone (Satoh et al. 2008a, b). Thus, the conversion to quinone proceeds much faster in cells than in cell-free systems. Another possible explanation is the difference in reactivity of quinones for the thiols of GSH

versus Keap1. Some proteins have active cysteines, which can be easily converted to thiolate anion if surrounded by basic amino acids, as shown in Fig. 10.6. Keap1(Cys151) is a typical example of such an active cysteine (Zhang et al. 2004; Kobayashi et al. 2009). This cysteine is thus a sensor for electrophilic compounds, and in fact EPs appear to be more reactive with Keap1(Cys151) than GSH. In other words, low concentrations of CA quinone preferentially react with Keap1 over GSH and thus can contribute to activation of cell defense systems while sparing GSH (Satoh et al. 2008a, 2009b, 2011). Other researchers have reported similar results for TBHQ and other simple benzene-type hydroquinones (Nakamura et al. 2003; Wang et al. 2010). Additionally, in cells undergoing oxidative stress, GSH is generally depleted, as it is a first line of defense against free radical insult. Thus, in cells stressed cells where PEDs are converted to the active quinone electrophilic form, the resulting EP would also be more likely to react with Keap1(Cys151) since GSH is already depleted.

As stated above, cyclic EPs are converted from an electrophilic precursor (or pro-electrophilic) compound to an electrophilic form, thereby activating the neuroprotective Keap1/Nrf2 pathway (Fig. 10.9b, C). There are additional factors that influence the rapidity of quinone formation from PEDs, i.e., Cu^{2+} and molecular oxygen (Nakamura et al. 2003; Satoh et al. 2009a, b; Wang et al. 2010). Copper is present in many tissues at micromolar concentrations, and in some tissues, including kidney and liver its concentration exceeds 100 μmol/l (Linder and Hazegh-Azam 1996). In the body, copper exists as either oxidized Cu^{2+} or reduced Cu^{+}. Moreover, it is a redox-active transition metal and can catalyze oxidative activation of a number of phenolic compounds via a Cu^{2+}/Cu^{+} redox cycling mechanism that produces ROS. Under physiological conditions, an electron acceptor from Cu^{+} is O_2 (Fig. 10.9B); whereas under pathological conditions, the electron acceptor is likely be a ROS, for example, H_2O_2, as shown in Fig. 10.9C (Nakamura et al. 2003; Satoh et al. 2009a, b; Wang et al. 2010). It is important to note that ROS are much better electron acceptors from Cu^{+} than from O_2, and thus oxidation of Cu^{+} to Cu^{2+} should proceed more easily under pathological conditions. This also means that conversion of a PED to the quinone form will proceed more rapidly under these conditions. In conclusion, we have developed a platform of drug development using PEDs, which provides us with an important framework for producing neuroprotective drugs that become electrophilic only when they encounter oxidative damage, the very condition that they are designed to combat via transcriptional activation of phase 2 enzyme counterreaction (Satoh et al. 2008a; Satoh and Lipton 2007; Lipton 2007).

Importantly, these PED compounds remain relatively innocuous in their pro-drug form and only become active at the site of oxidative stress when converted from their hydroquinone to quinone form. These compounds therefore represent a type of pathologically-activated therapeutic or PAT drug, representing a more gentle 'tap' than previous attempts to develop neuroprotective drugs that failed because of their lack of clinical tolerability (Lipton 2004, 2006, 2007). As reviewed here, we believe that this chemical conversion is the key to understanding the molecular mechanism of the protective yet well tolerated effects afforded by cyclic EPs (Satoh and Lipton

2007; Satoh et al. 2008a). In our work, we have focused on hydroquinone-type pro-electrophiles because they have the distinct property of being "pro-drug"-like compounds. During neurodegenerative insults, oxidative stress plays a critical role in disease progression. Our published studies have demonstrated that this pathological insult can be used to activate pro-electrophilic compounds via their oxidation, which occurs in the target tissue to provide neuroprotection where it is needed. Thus, this approach represents a novel strategy against neurodegenerative disorders that could activate electrophilic drugs via pathological activity.

We have thus delineated a strategy for the development of novel neuroprotective drugs that are clinically well tolerated (Lipton 2004, 2007; Satoh and Lipton 2007). This strategy is based on the principle that drugs should interact with their target only during states of pathological activation but not interfere with the target if it functions normally. Such drugs should therefore exhibit little inhibition of normal physiological function. We have had previous success developing such a PAT drug, resulting in FDA approval of the NMDA-type glutamate receptor antagonist memantine for moderate-to-severe Alzheimer's disease (Lipton 2004, 2006, 2007). PEDs represent another possible candidate for a PAT drug because conversion to the active quinone is redox-sensitive and thus enhanced in the presence of oxidative insults. For this reason, we have studied the neuroprotective effects afforded by cyclic EPs/PEDs, including CA and D1 (Satoh et al. 2008a, 2011).

10.10 List of Potential Pro-electrophilic Drugs

Based on their chemical structures and biological actions, we list in Fig. 10.10 potential PEDs from among published compounds. The quinone form of each of these cyclic EPs can activate electrophilic counterreactions, including the Keap1/Nrf2 and/or HSP90/HSF-1 pathways. Each drug has been reported to induce phase 2 enzymes via activation of the Nrf2/ARE pathway. They have para- or ortho-hydroquinones as their electrophilic cores and thus have PED properties. These compounds have been reported to protect neuronal cells against oxidative and nitrosative stress, and some of them (e.g., CA, quercetin, fisetin, and TBHQ) can protect the rodent brain in various models of neurodegenerative diseases. In particular, neuroprotective effects of diterpene-type PEDs, including CA (Satoh et al. 2008a, b, 2009a, b; Tamaki et al. 2010), CS (Satoh et al. 2008b; Tamaki et al. 2010), strongylophorine 8 (Sasaki et al. 2011), TBHQ (Murphy et al. 1991; Lee et al. 2003; Kraft et al. 2004; Sun et al. 2005; Shih et al. 2005; Jakel et al. 2007), and D1 (Satoh et al. 2011), are quite striking in their ability to trigger the Keap1/Nrf2 pathway. Flavonoid-type PEDs, such as quercetin (Ishige et al. 2001; Milioli et al. 2007; Bureau et al. 2008; Ahmad et al. 2011; Suematsu et al. 2011), eriodictyol (Hanneken et al. 2006; Johnson et al. 2009), and fisetin (Burdo et al. 2008; Maher 2008; Lewerenz et al. 2010; Maher et al. 2011), also manifest neuroprotective effects, although their action may occur via other pathways, including direct antioxidant effects.

List of Pro-Electrophilic Drugs (PEDs)

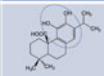
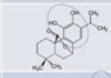
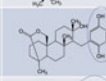
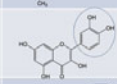
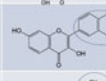
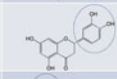
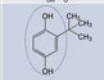
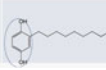
Name	Structure	Origin	Type	Examined cells	Examined models	Ref
Carnosic acid		Rosemary (Rosmarinus officinalis)	Ortho-hydroquinone Diterpene	HT22 PC12 Cortical Neurons	MCAO	Satoh T et al., 2008a Satoh T et al., 2008b Satoh T et al., 2009a Saqtoh T et al., 2009b Takahashi et al., 2010 Tamaki Y et al., 2010
Carnosol		Rosemary (Rosmarinus officinalis)	Ortho-hydroquinone Diterpene	HT22	N.D.	Satoh T et al., 2008a Satoh T et al., 2008b
Strongylophorine 8		Sponge Petrosia (Strongylophora) corticata	Para-hydroquinone Diterpene	HT22	N.D.	Sasaki et al., 2011
Quercetin		Green plant	Ortho-hydroquinone Flavonoid	Cortical Neurons PC12 HT22 SH-SY5Y	MCAO MPTP	Bureau et al., 2008 Milioli EM et al., 2007 Ahmad A et al., 2011 Ishige K et al., 2001 Suematsu N et al., 2011
Fisetin		Ggreen plant	Ortho-hydroquinone Flavonoid	PC12 ARPE-19 Cortical Neurons	HD	Maher P et al., 2011 Lewerenz J et al., 2010 Burdo J et al., 2008 Mahere P 2008
Eriodictyol		Green plant	Ortho-hydroquinone Flavonoid	PC12 ARPE-19	N.D.	Johnson J et al., 2009 Hanneken et al., 2009
TBHQ		Synthetic Compound	Para-hydroquinone	Cortical Neurons HT22 PC12 IMR-32	MCAO MPTP 6-OHDA	Murphy TH et al., 1991 Lee et al., 2001 Kraft AD et al., 2004 Sun X et al., 2005 Shih AY et al., 2005 Jakel RJ et al., 2007
D1		Synthetic Compound	Para-hydroquinone	ARPE-19 HT22	N.D.	Satoh T et al., 2011

Fig. 10.10 List of possible pro-electrophilic drugs. We list possible PEDs based on published data and chemical structures. Structures: The ovals on the chemical structures highlight the electrophilic core. Origins: Origins are given in the case of natural compounds. Type: Isomer type of the electrophilic core on the hydroquinone is given. Examined cells: All of the EPs shown exerted protective effects in neuronal HT22 cells (mouse hippocampal neurons), PC12 (mouse pheochromecytoma cells), SH-SY5Y (mouse neuroblastoma cells), IMR-32 (human neuroblastoma cells), or ARPE (human retinal pigment epithelial cells). Examined models: Some of the compounds (CA, quercetin, fisetin and TBHQ) have been shown to be neuroprotective in rodent neurodegenerative models such as MCAO (Middle Cerebral Artery Occlusion, a mouse model of brain ischemia), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity, a mouse model of Parkinson's disease), HD (mouse Huntington's disease model), or 6-OHDA (6-hydroxydopamine toxicity, a mouse model of Parkinson's disease). N.D., not determined

Interestingly, we have recently shown that *para*-hydroquinones (rather than the *ortho* or *meta* positions for the hydroxyl groups) form more electrophilic compounds and thus display maximal activation of the Nrf2/ARE pathway (Satoh et al. 2009a, 2011); thus, we feel that these drugs may have the greatest clinical potential as neuroprotectants. Importantly, however, the ultimate success of PEDs in the clinic will depend on their balance between anti-oxidant effects and clinical tolerability. Only clinical studies in humans will be able to determine this outcome, but the approach does appear to be a promising one.

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Chapter 11
Reactive Oxygen Species, Kinase Signaling, and Redox Regulation of Epigenetics

Isaac K. Sundar and Irfan Rahman

Abstract Reactive oxygen species (ROS) cause alterations in the cellular redox status (GSH/GSSG ratio) and hence activate various kinase signaling pathways, redox sensitive transcription factors (NF-κB and AP-1), and chromatin modification enzymes leading to increased pro-inflammatory and immunological responses. Chromatin modification enzymes include histone acetyltransferases (HATs), histone and non-histone deacetylases (HDACs, sirtuins), histone methyltransferases (HMTs) and histone demethylases (HDMs). Activation of these enzymes results in transcriptional gene activation/repression. Histone methyltransferases are enzymes that dynamically cause histone mono-, di- or tri-methylation at lysine residues, which either result in gene activation (H3K4, H3K36, and H3K79) or repression (H3K9, H3K27, and H3K20). Histone demethylases catalyze the removal of methyl groups from lysine or arginine residue of histones, hence regulating gene expression. Recent evidences have indicated that oxidative stress and environmental agents can alter nuclear histone acetylation/deacetylation/methylation, allowing access for transcription factor DNA binding and leading to enhanced pro-inflammatory gene expression. Cross-talks between histone modifications and DNA methylation also occur during inflammation. Understanding the mechanisms of ROS and redox epigenetic regulation via stress signaling kinases, redox sensitive transcription factors, the balance between histone acetylation/deacetylation and histone methylation/demethylation may lead to the development of novel therapies based on epigenetics against chronic inflammatory diseases, and cancer.

Keywords Redox • Epigenetics • Oxidative stress • Histone acetylation • Gene expression

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Abbreviations

AP-1	activator protein-1
CBP	CREB-binding protein
COPD	chronic obstructive pulmonary disease
CREB	cAMP-response-element-binding protein
CS	cigarette smoke
ECSOD	extracellular superoxide dismutase
ERK	extracellular signal-related kinase
FOXO	forkhead box O
GR	glucocorticoid receptor
GSH	glutathione
GSSG	glutathione disulfide
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HMT	histone methytransferase
4-HNE	4-hydroxy-2-nonenal
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
IKK	IkappaB kinase
IL-8	interleukin-8
JNK	c-Jun N-terminal protein kinase
LPS	lipopolysaccharide
MMP-9	matrix metalloproteinase-9
MnSOD	manganese SOD
MSK1	mitogen- and stress-activated kinase 1
NAD^+	nicotinamide adenine dinucleotide
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
NO	nitric oxide
NSCLC	non-small cell lung cancer
PCAF	p300-CBP associated factor
$ONOO^-$	peroxynitrite anion
PGC-1α	peroxisome-proliferator-activated receptor (PPAR) gamma co-activator-1 alpha
PI3K	phosphoinositide 3-kinase
PKC	Protein kinase C
RNS	reactive nitrogen species
ROS	reactive oxygen species
SIRT1	sirtuin 1
STAT3	signal transducer and activator of transcription 3
$O_2^{\bullet-}$	superoxide anion
SOD	superoxide dismutase

TGF-β1	transforming growth factor-beta 1
TNF-α	tumor necrosis factor-alpha
TSA	trichostatin A

11.1 Introduction

Lung tissue is a direct target for oxidative injury from reactive oxygen species (ROS) and free radicals derived by cigarette smoke (CS) or environmental agents, which results in increased cellular kinase signaling associated with post-translational modifications (acetylation, deacetylation, methylation, phosphorylation, carbonylation, ubiquitination, sumoylation, and poly-ADP-ribosylation) of histones and non-histone proteins, and redox modification of proteins implicated in chromatin remodeling (Gilmour et al. 2003; Tomita et al. 2003; Yang et al. 2008, 2009; Bast et al. 1991; Rahman et al. 2004; Repine et al. 1997; Sundar et al. 2012).

Lung cells have developed antioxidant defense mechanisms that can efficiently counteract the oxidative challenge, which is continuously imposed by the environment. The most important antioxidant is the non-protein sulfhydryl glutathione (GSH), which plays an important role in maintaining the cellular redox status in lung cells (Forman and Dickinson 2003). The ratio of reduced GSH to glutathione disulphide GSSG in the cytoplasm is a key determinant in the regulation of different intracellular processes, such as signal transduction, gene expression, proliferation and apoptosis (Rahman et al. 2005). The ratio between GSH/GSSG serves as an indicator of the cellular redox state (Ghezzi et al. 2005). This ratio is determined by the rates of H_2O_2 reduction by glutathione peroxidase and GSSG reduction by glutathione reductase. The intracellular redox status is shown to play an important role in the regulation of epigenetic events (Cyr and Domann 2011).

Chromatin remodeling by histone modifications play a key role in the regulation of cellular processes including gene transcription/repression, DNA repair, and proliferation (Sundar et al. 2012). Posttranslational histone modifications predominantly occur via acetylation/deacetylation and methylation/demethylation reactions (Fukuda et al. 2006; Roth et al. 2001; Sundar et al. 2012) (Fig. 11.1). The amino-terminal region of histones is a hotspot for such post-translational modifications, which affect the interaction of histones with DNA leading to chromatin remodeling events on gene promoters (Fischle et al. 2003).

Histone acetyltransferases, HATs (PCAF, p300/CBP and GCN5) and histone deacetylases, HDACs (e.g. HDAC2 and SIRT1) maintain the balance in histone acetylation/deacetylation events (Sundar et al. 2012). HATs affect the binding of DNA sequence-specific transcription factors and subsequently recruit coactivators or corepressors on gene specific regions to form either coactivator or corepressor complexes (Wang et al. 2001). Several reports support the fact that transcriptional regulators possess intrinsic HAT (CBP/p300) and HDAC activities, suggesting that histone acetylation and deacetylation play a causal role in regulating gene transcription in various organisms (Kuo and Allis 1998; Wolffe 1997; Workman and

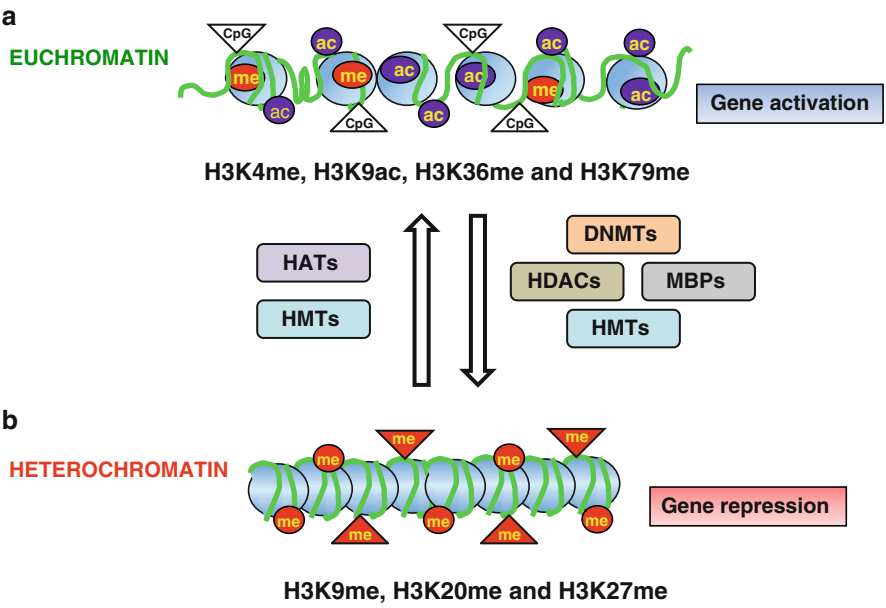

Fig. 11.1 Schematic representation of epigenetic regulation of gene expression by alterations in DNA/histone modifications. (**a**) Open chromatin confirmation with widely spaced nucleosomes (euchromatin) represents active gene transcription, associated with unmethylated CpG residues on DNA and post-translational histone modifications, such as increased acetylation and methylation. The euchromatin structure is readily accessible to redox sensitive transcription factors NF-κB and AP-1, HATs and cofactors/coactivators to regulate gene expression. Acetylation of histone H3K9 and methylation of histone H3K4, H3K36, and H3K79 are the hallmarks for gene activation. (**b**) Closed chromatin confirmation (heterochromatin) represents transcriptional repression characterized by tightly packed nucleosomes with DNA methylation at CpG islands, deacetylation of histones and post-translational histone modifications, such as methylation of specific histone H3 residues. The heterochromatin structure renders the DNA inaccessible to transcription factors but provides access to gene repressive proteins, including histone deacetylases (HDACs), DNA methyltransferases (DNMTs), and methyl-CpG binding proteins (MBPs). Methylation of histone H3K9, H3K20, and H3K27 are associated with gene repression

Kingston 1998). There are several evidences showing that an increase in histone acetylation leads to increased gene transcription while histone hypoacetylation is linked to decreased gene transcription (Kuo and Allis1998; Wolffe 1997; Workman and Kingston 1998). Histone acetylation by HATs and deacetylation by HDACs are also linked to cell-cycle progression, proliferation, senescence, and DNA damage/repair, as well as to inflammatory gene transcription, which can be affected by intracellular redox signaling (Kouzarides 1999; Taplick et al. 1998; Biswas and Rahman 2009). A recent report suggests a novel role for histone carbonylation on specific histone proteins. This may have implications in redox epigenetics, which occurs in proliferating cells (particularly in fibroblasts) during the active phase of DNA synthesis (Garcia-Gimenez et al. 2012). Evidence from recent studies also

suggest that epigenetic events, such as DNA methylation, histone modifications, and non-coding RNAs or microRNAs (miRNAs) affect gene expression/repression in several chronic inflammatory lung diseases, such as asthma, COPD and interstitial lung disease, which are influenced by environment (tobacco smoke and inhaled pollutants/gases), diet, and aging (Yang and Schwartz 2011). Further, the role of these epigenetic events in prenatal or early childhood exposure to environmental stress conditions, including tobacco smoke, pollutants/oxidants, and dietary agents rendering children susceptible to asthma and allergic airways disease later in life has been suggested (Martino and Prescott 2011). Thus, post-translational modifications of histone proteins by oxidants and environmental stresses can trigger genes that are involved in various cellular processes and chronic inflammatory events. This chapter focuses on ROS and redox epigenetic regulators, such as stress signaling kinases, transcription factor (NF-κB), HATs, HDACs, histone methytransferases (HMTs), and histone demethylases (HDMs), and their role in epigenetic regulation of inflammatory response in chronic lung diseases.

11.2 ROS and Intracellular Redox Regulation in Epigenetics

ROS, such as superoxide anions ($O_2^{\cdot -}$) and hydroxyl radicals ($^{\cdot}OH$) are highly unstable oxygen species with unpaired electrons, capable of initiating oxidation. ROS cause oxidation of proteins, DNA and lipids, which may directly affect the lung or induce several other cellular responses through the generation of secondary metabolic reactive species. Similarly, the reactive nitrogen species (RNS) nitric oxide (NO) is generated by nitric oxide synthase (iNOS) and forms potent and damaging peroxynitrite molecules in the presence of superoxide anion. ROS cause alterations in extracellular matrix (ECM) and blood vessels, stimulate mucus secretion, lead to apoptosis, and regulate cell proliferation (Rahman 2005). Ozone, cigarette smoke and inflammatory cells are major sources of ROS/RNS generation. NADPH oxidase is an important ROS-generating enzyme found in inflammatory cells. Additional mechanisms include mitochondrial respiration via the release of reactive oxygen intermediates. Other enzyme systems include xanthine or the xanthine oxidase system and the heme peroxidases, which are also involved in the pathogenesis of COPD (Rahman and MacNee 1996; Pinamonti et al. 1998). Cigarette smoke is the major source for environmentally derived ROS that are involved in pathogenesis of COPD. There are some striking features found in the COPD phenotype, characterized by an inflammatory-immune response, activation of epithelial cells, resident macrophages, and the recruitment and activation of neutrophils, monocytes and B and T lymphocytes into the lung. Inflammatory cells are activated in response to cytokines/chemokines/chemoattractants after recruitment into the airspace, thereby generating various ROS. ROS are highly reactive and, when generated close to cell membranes, can induce lipid peroxidation (oxidation of membrane phospholipids) and the accumulation of malondialdehyde, 4-hydroxy-2-nonenal, acrolein, and F_2-isoprostanes (Rahman and Adcock 2006; Rahman et al.

2002b). Alveolar repair responses and immune modulation in the lung may also be influenced by ROS (Rahman and MacNee 1998), suggesting the role of ROS/RNS in epigenetic regulation of chronic lung diseases where oxidant/antioxidant imbalance is a common phenomenon (Rahman and MacNee 1996).

11.2.1 Kinases and Redox Signaling

ROS generated from cellular metabolic response (inflammatory cells) and environmental sources (air pollutants or cigarette smoke) can cause activation of a number of signal transducing molecules via oxidation-prone cysteine rich domains, resulting in active gene transcription (Rahman and MacNee 1998; Adler et al. 1999). Among these are members of the MAPK family, extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase and PI3K/Akt. Based on the oxidative/redox status of the cells, members of the MAPK family are activated, leading to a multifaceted transactivation of redox sensitive transcription factors (c-Jun, ATF-2, CREB-binding protein (CBP) and Elk-1) (Adler et al. 1999; Thannickal and Fanburg 2000). Furthermore, activation of upstream kinases, such as NF-κB-inducing kinase (NIK), mitogen-and stress-activated kinase 1 (MSK1), and IκB kinase-α (IKKα) results in downstream chromatin remodeling events (Chung et al. 2010, 2011; Yang et al. 2008, 2009; Sundar et al. 2012), which in turn modulates a series of specific pro-inflammatory gene transcription events that mediate cellular functions, such as apoptosis, proliferation, transformation, and differentiation. Lipid peroxidation by cigarette smoke generates aldehydes, which have been shown to trigger the inflammatory response-mediated signal transduction and gene activation (Uchida et al. 1999). Cigarette smoke-mediated oxidative stress specifically induces the JNK pathway, leading to endothelial cell injury (Hoshino et al. 2005). Cigarette smoke also induces activation of protein kinase C (PKC), mediating regulation of intracellular signaling pathways (Wyatt et al. 1999). PKCζ-knockout mice exposed to cigarette smoke or LPS showed a reduction in lung inflammatory response via chromatin modifications suggesting that PKCζ is an important modifier of lung inflammatory response (Yao et al. 2010b). Hence, activation of kinases by ROS and aldehyde/lipid peroxidation products can lead to a pro-inflammatory response by chromatin remodeling in eukaryotic cells (Sundar et al. 2012).

Stress signaling kinases, such as phosphoinositide 3-kinase (PI3K) and p38 are shown to be involved in the pathogenesis of COPD and lung cancer (Doukas et al. 2009; Engelman and Janne 2008; Gustafson et al. 2010). Inhibition of PI3K/p38 lowers tobacco/cigarette smoke-induced lung inflammatory response (Doukas et al. 2009). Interestingly, PI3K inhibition significantly increased the steroid efficacy to inhibit an inflammatory response under CS-induced oxidative stress in the lung (Marwick et al. 2009; To et al. 2010; Bhavsar et al. 2010). Overexpression of p110, a subunit of PI3K, in type II alveolar epithelial cells is involved in pathogenesis of lung adenocarcinomas, which can be repressed by dual pan-PI3K and mTOR

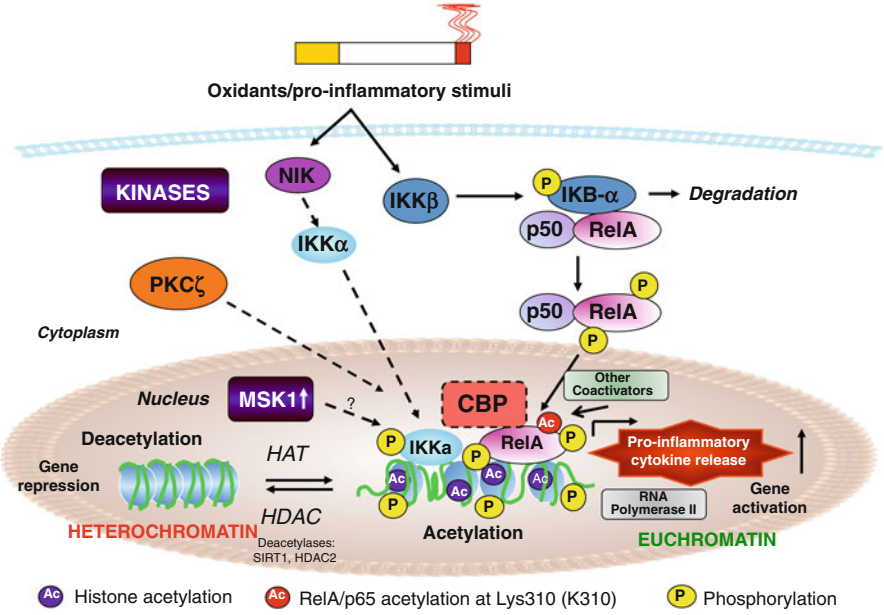

Fig. 11.2 Role of redox kinase signaling in chromatin modifications. Cigarette smoke-derived oxidants/aldehydes activate kinase signaling mechanisms, such as NIK, IKKα, MSK1, and PKCζ by redox modulation, and induce pro-inflammatory gene transcription via chromatin modifications

pharmacological inhibitors (Engelman and Janne 2008). However, treatment of mice bearing lung adenocarcinomas with rapamycin, an inhibitor of mTOR, was unable to shrink the tumors. Furthermore, PI3K is required for Kras-induced lung tumorigenesis (Engelman and Janne 2008). Therefore, in addition to p38 MAPK and PKCζ, oxidants/cigarette smoke-mediated activation of PI3K signaling pathway may be a potential target for the intervention of COPD and other chronic inflammatory lung diseases. These oxidant and redox sensitive pathways activate various kinases such as NIK, MSK1, PI3K, PKCzeta and IKKα resulting in histone acetylation events (Yang et al. 2008; Chung et al. 2011; Sundar et al. 2012) (Fig. 11.2).

11.2.2 NF-κB and Redox Regulation of Inflammation

Among transcription factors, NF-κB remains to be of particular interest due to its role in regulating inflammatory mediators (Christman et al. 2000). The important NF-κB-dependent proinflammatory mediators include IL-1, IL-6, IL-8, MCP-1, and TNFα, which are known to be involved in lung airway inflammation in COPD. Nuclear accumulation of NF-κB RelA/p65 was increased in smokers and patients

with COPD as well as in lung epithelial cells and macrophages exposed to cigarette smoke (Di Stefano et al. 2002; Yagi et al. 2006; Rajendrasozhan et al. 2008b). Furthermore, the number of RelA/p65-positive epithelial cells and macrophages correlated with the severity of airflow limitation in COPD patients (Di Stefano et al. 2002), suggesting a role of NF-κB activation in chronic inflammatory lung diseases. NF-κB activating upstream kinases are redox-sensitive, thus oxidative stress is associated with NF-κB activation in the lungs of patients with COPD (Bowie and O'Neill 2000; Pantano et al. 2006). Phosphorylation of NF-κB RelA/p65 is regulated by several redox-regulating kinases, such as protein kinase A (PKA), MSK1 at Ser276, IKKβ at Ser536 and protein kinase C (PKC) ζ at Ser311, and p38 further leading to increased RelA/p65-mediated cytokine release (Yang et al. 2003; Chen and Greene 2004; Sundar et al. 2012) (Fig. 11.2).

Phosphorylation of the RelA/p65 subunit facilitates co-activator CBP/p300 binding that causes acetylation of RelA/p65 at residues K218, K221 and K310. Importantly, acetylation of NF-κB RelA/p65 at Lys310 is essential for proinflammatory gene transcription. Earlier studies have demonstrated cigarette smoke-induced inflammatory cellular influx was associated with NF-κB-mediated proinflammatory cytokine release in the lungs, and in peritoneal macrophages and monocytes (i.e., MonoMac6 cells). However, IKKβ inhibitors attenuated cigarette smoke extract-induced NF-κB-dependent proinflammatory mediators (Yang et al. 2006; Yao et al. 2008a). Specific knockdown of NF-κB subunit in epithelial cells or IKKβ deficiency in macrophages significantly attenuated lung tumorigenesis in mice (Stathopoulos et al. 2007; Takahashi et al. 2010). Alternative or non-canonical pathways require NIK, along with IKKα to induce the processing of p100 C-terminus (termed IκBδ), resulting in nuclear translocation of p52/RelB (Senftleben et al. 2001; Yin et al. 2001; Xiao et al. 2004). Cigarette smoke-mediated increase in the levels of p52, RelB, IKKα and NIK, as well as interaction of RelB with p52 and NIK occur in mouse lung (Yang et al. 2008, 2009). RelB was recruited to the promoters of proinflammatory genes signifying the role of NF-κB alternative pathway in cigarette smoke-mediated lung inflammatory response (Yang et al. 2008). In addition, cigarette smoke-mediated RelA/p65 NF-κB phospho-acetylation (ser276 and lys310) increased nuclear levels of RelB, and augmented IL-8 release in MonoMac6 cells transfected with IKKα (Yang et al. 2008) implicating the functional role of IKKα in NF-κB activation by cigarette smoke. These findings are in agreement with earlier reports demonstrating a marked decrease in proinflammatory cytokine expression in IKKα deficient fibroblasts, though IκBα degradation and *in vitro* NF-κB DNA binding activity were normal in these cells (Sizemore et al. 2002; Anest et al. 2003; Yamamoto et al. 2003). A recent study demonstrated the protective role of lung-targeted overexpression of the NF-κB member RelB in attenuating cigarette smoke-mediated lung neutrophil infiltration, inflammatory cytokine and chemokine production, cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in mice (McMillan et al. 2011). The mechanism of this protection observed in acute cigarette smoke-induced pulmonary inflammation was due to decreased expression of redox sensitive NF-κB-dependent intercellular adhesion molecule 1, through which RelB regulates inflammatory cell migration into the vasculature

(McMillan et al. 2011). Therefore, activation or increased expression of RelB could be a novel therapeutic strategy against cigarette smoke-mediated acute lung inflammation and other chronic lung inflammatory diseases including COPD (Yang et al. 2009). Another recent report showed a novel role of NIK in cigarette smoke and TNFα-induced RelA/p65 activation and histone H3K9 acetylation. Human bronchial epithelial (H292) cells treated with cigarette smoke extract and TNFα showed increased nuclear accumulation of NIK, and recruitment of NIK onto proinflammatory gene promoters associated with sustained NF-κB-dependent gene transcription (Chung et al. 2011).

Pharmacological administration of NF-κB inhibitors attenuated cigarette smoke-induced acute lung inflammation in rodents (Rajendrasozhan et al. 2010b). Further studies are required to assess whether NF-κB inhibitors are effective in controlling cigarette smoke-induced chronic inflammation, as well as overcoming steroid resistance, and subsequent pathological changes in patients with COPD. Interestingly, deficiency of p50, another subunit of NF-κB, enhances cigarette smoke-induced lung inflammation and emphysema, suggesting a differential role of p50 and RelA/p65 in inflammation (Rajendrasozhan et al. 2010a; Cao et al. 2006). Furthermore, pharmacological administration of NF-κB inhibitor (e.g. pyrrolidine dithiocarbamate) and IκB protease inhibitor (tosylphenylalanylchloromethane) reduced TGF-β1-induced human lung cancer cell migration (Fong et al. 2009). Therefore, down-regulation of the redox sensitive IKKβ-NF-κB pathway may provide the therapeutic targets in inflammation that occurs in lung cancer. Activated STAT3 also induces tumor-promoting local inflammation by activating NF-κB, as shown by a study demonstrating the occurrence of lung adenocarcinomas in alveolar epithelial cells of mice overexpressing STAT3 (Li et al. 2007). Indeed, STAT3 is activated in the lungs of patients with lung carcinomas and COPD (Qu et al. 2009). Hence, the multiple redox-sensitive signaling pathways can regulate NF-κB in response to oxidative stress in chronic inflammatory lung diseases.

11.2.3 HATs and Redox Epigenetics

Histone acetyltransferease (HATs) transfer acetyl groups from acetyl-CoA to the N-terminal lysine residues of histones (H3 and H4), thereby uncoiling the DNA resulting in transcription factor binding followed by gene transcription (Imhof and Wolffe 1998; Tan 2001; Rahman et al. 2004; Ito et al. 2007; Sundar et al. 2012). HATs and HDACs play opposing roles in regulating acetylation of core nucleosomal histones. HATs are classified into five distinct families (Torchia et al. 1998; Tan 2001). Each of these HAT families has diverse roles in regulating chromatin structure (via histone acetylation/deacetylation) as they share a common enzymatic activity (Racey and Byvoet 1971).

Cigarette smoke/oxidant-mediated acetylation of histone H3 in macrophages and epithelial cells, and in lungs of humans and rodents suggests that histone acetylation plays an important role in chromatin remodeling (Sundar et al. 2012).

Cigarette smoke-mediated alterations in chromatin remodeling are shown to be involved in sustained lung inflammatory responses which occurs in patients with COPD (Marwick et al. 2004; Yang et al. 2008; Szulakowski et al. 2006; Yao et al. 2010b). CREB-binding protein (CBP) and p300 are the known coactivators that possess intrinsic HAT activity. They are regulated by the p38 MAP and other kinase pathway, which activates redox sensitive transcription factors, such as NF-κB and AP-1 (Thomson et al. 1999). Therefore, histone acetylation by CBP/p300 has an impact on the activation of specific proinflammatory mediators linked with NF-κB/AP-1-mediated gene expression (Kamei et al. 1996; Carrero et al. 2000; Rahman et al. 2002a). Transcription factor NF-κB is acetylated by other HATs (CB/p300) but also cause histone acetylation in a temporal manner by recruiting other cofactors/coactivators and chromatin remodeling complexes to activate proinflammatory gene transcription (Ito et al. 2000; Ghosh and Karin 2002; Lee et al. 2006). Ito et al. have reported that (CBP/p300) induced acetylation of histone H3, but not histone H2A, H2B or H3, occurs in epithelial cells on specific lysine residues (Lys8 and Lys12) at NF-κB-responsive regulatory elements on proinflammatory genes in human cells (Ito et al. 2000). Mutations and deletions in CBP gene has been detected in patients with lung cancer, suggesting the role of CBP in the tumorigenesis and/or progression of human lung cancer (Kishimoto et al. 2005). Oxidative stress and cellular GSH/GSSG levels regulate histone acetylation/deacetylation and thereby chromatin remodeling. For example, H_2O_2 and cigarette smoke activate NF-κB, HAT, CBP/p300 leading to specific histone acetylation in human macrophages and lung cells (Adenuga et al. 2009; Ito et al. 2005; Moodie et al. 2004; Yang et al. 2006; Yao and Rahman 2011). Glutathione depletion is associated with increased histone acetylation (Rahman et al. 2002b, 2004).

Corticosteroid resistance is also associated with increased oxidative stress imposed by cigarette smoke. Corticosteroids do not block inflammatory response when there is an increased oxidative stress, such as in severe asthmatics and patients with COPD. This inefficacy is not associated with alterations in nuclear HAT activity, (Hew et al. 2006). The corticosteroid insensitivity to inhibit inflammatory response is due to the reduction in HDAC2 levels/activity (a redox sensitive protein), which is lost in lungs of patients with COPD and severe asthmatics (see below under HDAC2 and steroid resistance section). Therefore, development of small molecule inhibitors (HAT inhibitors) against coactivators, such as CBP/p300, PCAF, and GCN, or activators of HDAC2 may be the key targets for therapeutic intervention of COPD and asthma as well as other chronic inflammatory lung diseases where oxidative stress occurs.

11.2.4 HDACs and Redox Epigenetics

Acetylation of histones by HATs is not an irreversible post-translational modification (Sundar et al. 2012). Histone deacetylases play a crucial role in removing the acetyl groups from histones (Hay and Candido 1983). Presence of HDACs within

the gene regulatory regions leads to formation of closed chromatin complexes resulting in epigenetic gene silencing (chromatin condensation). So far 18 mammalian HDAC enzymes are known, which are classified into four classes based on their homology to a prototypical HDAC found in yeast (de Ruijter et al. 2003). Class I HDACs (HDACs 1, 2, 3, and 8) are ubiquitously expressed with the possible exception of HDAC3 and HDAC10, which are predominantly localized in the nucleus (Taplick et al. 2001). Class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) are expressed in a tissue specific manner, and they have the ability to shuttle between the nucleus and cytoplasm during specific cellular signals (Bertos et al. 2001; Grozinger et al. 1999). The shuttling of class II HDACs from the nucleus is mainly regulated by nuclear export signaling 14-3-3 proteins (Bertos et al. 2001; Johnstone 2002). The class III HDACs or sirtuins are named based on their homology to the yeast Sir2 gene, which is a highly conserved gene family. In humans, sirtuins comprise seven members, sirtuin (SIRT) 1–7 (Michan and Sinclair 2007). Class IV deacetylases may undergo redox modifications and carbonylation due to the presence of cysteine, lysine, and histidine moieties in their catalytic sites (Caito et al. 2010; Yang et al. 2006, 2007).

HDACs are also reported to deacetylate non-histone proteins, such as the redox sensitive transcription factor NF-κB (RelA/p65) and FOXO3, thereby regulating transcription of NF-κB-dependent proinflammatory genes (Sengupta and Seto 2004; Sundar et al. 2012). The levels and activities of HDACs are significantly decreased in macrophages, lung cells *in vitro*, and in lungs of COPD patients in response to oxidative/carbonyl stress and via alterations in intracellular GSH/GSSG redox ratio (caused by H_2O_2, PM_{10}, and cigarette smoke-derived oxidants/free radicals), and are associated with COPD severity, asthma, and steroid resistance in asthmatics, who smoke tobacco (Adenuga et al. 2009; Ito et al. 2005; Moodie et al. 2004; Yang et al. 2006; Yao and Rahman 2011). Intracellular levels of GSH retain HDAC2 in a reducing environment since an increase in intracellular reduced GSH levels can inhibit the oxidative post-translational modifications of HDAC2 (Adenuga et al. 2009; Yang et al. 2006; Meja et al. 2008). Furthermore, a decrease in levels of HDACs is due to post-translational modifications, such as oxidation/carbonylation, nitrosylation, acetylation, and phosphorylation in response to oxidants and aldehydes derived from cigarette smoke (Adenuga et al. 2009; Ito et al. 2004; Moodie et al. 2004; Yang et al. 2006; Adenuga and Rahman 2010). Hence, HDAC2 levels/activities and the status of redox/oxidative post-translational modifications dictate the effects of coactivators on the chromatin status whether to drive the gene activation or repression in chronic inflammatory diseases and cancer (Sundar et al. 2012).

11.2.4.1 HDAC2 and Steroid Resistance

Cigarette smoke/oxidants/carbonyl-mediated reduction in HDAC2 is associated with activation of NF-κB RelA/p65 subunit (increased levels of total and acetylated RelA/p65). RelA/p65 interacts with HDAC2 (possibly HDAC1 and HDAC3) and nuclear accumulation of RelA/p65 results in transcription of proinflammatory genes

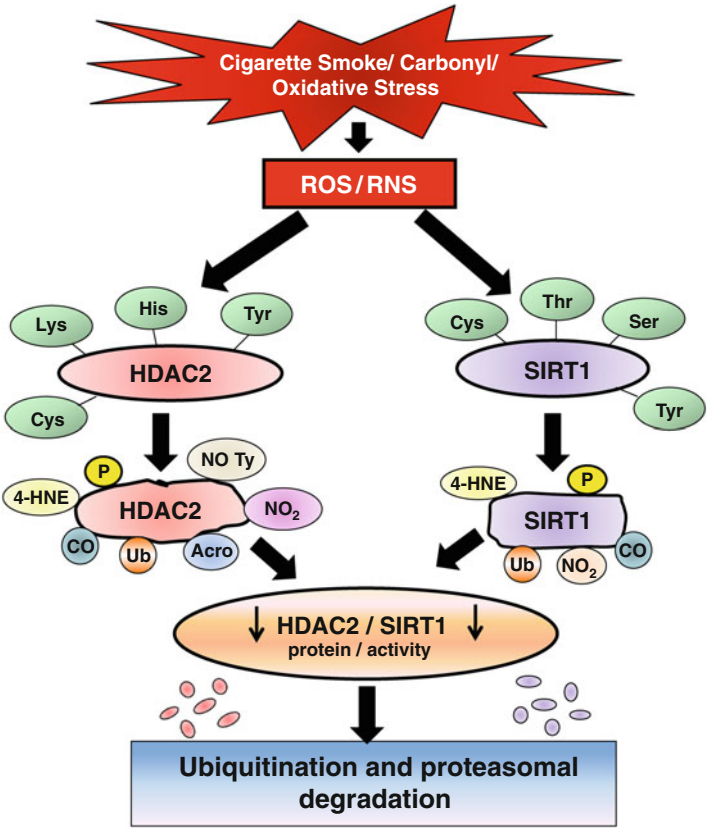

Fig. 11.3 Oxidative post-translational modifications of deacetylases (HDAC2 and SIRT1). Cigarette smoke or carbonyl/oxidant stress-mediated reactive oxygen species (ROS)/reactive nitrogen species (RNS) and/or alterations of the GSH/GSSG ratio cause various post-translational modifications of HDACs (HDAC2 and SIRT1), including phosphorylation (P), nitration (NO_2), carbonylation (CO), adduct formation (4-HNE: 4-hydroxy-2-nonenal, Acro: Acrolein), nitrotyrosine (NO-Ty), and ubiquitination (Ub) leading to ubiquitination-proteosomal degradation of HDACs. Post-translational modifications of HDAC2 and SIRT1 can include (i) direct nitration/nitrosylation on cysteine and tyrosine residues, (ii) covalent adduct formation on cysteine, lysine, and histidine residues, or (iii) phosphorylation of serine and threonine residues. Covalent modifications of the deacetylases on cysteine, histidine, or lysine residues can be irreversible, whereas other modifications can be reversible. The oxidative covalent modifications can lead to protein ubiquitination and degradation

when HDAC2 is oxidatively modified, ubiquitinated, and degraded (Yang et al. 2006; Yao et al. 2008b; Londhe et al. 2011; Yao and Rahman 2011) (Fig. 11.3). HDAC2 modification is dependent on the intra cellular thiol redox status (Yang et al. 2006; Meja et al. 2008). *In vitro* studies using trichostatin A (HDAC inhibitor) in different cell lines showed enhanced NF-κB-driven inflammatory gene transcription (Ito et al. 2000; Chen et al. 2001). Hence, alteration of HDACs by

cigarette smoke/oxidative stress leads to increased acetylation of histones (histone H3 and H4) and activation of transcription factor (NF-κB), thus enhancing transcription of proinflammatory genes (Sundar et al. 2012; Yang et al. 2006; Adenuga et al. 2008a, b; Rajendrasozhan et al. 2009). In humans, a marked reduction of HDAC2 expression/activity in lung parenchyma, bronchial biopsies, and alveolar macrophages of patients with COPD is correlated with the severity of inflammation and the disease state (Ito et al. 2005). The underlying mechanism for reduced HDAC2 levels/activities includes post-translational modifications (i.e., nitrosylation, phosphorylation and ubiquitination) further leading to proteasome-dependent degradation particularly in response to cigarette smoke/oxidants/reactive aldehydes (Galasinski et al. 2002; Adenuga et al. 2009; Rajendrasozhan et al. 2009; Sundar et al. 2012), and/or oxidative/carbonyl modifications of HDAC2 (Yang et al. 2006). HDAC2 is also required for the anti-inflammatory effects of glucocorticoid efficacy as reduced levels/activity of HDAC2 are reported in lungs of patients with COPD that are resistant to corticosteroids to inhibit proinflammatory mediators (Ito et al. 2005). Increasing the activity of HDACs by curcumin and theophylline (HDAC2 activators) significantly enhanced the suppression of IL-8 release by steroids in monocytes and alveolar macrophage from patients with COPD, and was blocked by HDAC inhibitor (Cosio et al. 2004; Meja et al. 2008). Furthermore, deacetylation of glucocorticoid receptor (GR) by HDAC2 enhanced the association of GR and RelA/p65, which resulted in the attenuation of proinflammatory gene transcription (Ito et al. 2006). Consequently, restoration or attenuation of decreased HDAC2 levels/activities will further enhance the glucocorticoid sensitivity by deacetylating RelA/p65 and GR to inhibit transcription of proinflammatory genes (Sundar et al. 2012). This can possibly be achieved by reversing the post-translational modifications of HDAC2, such as decarbonylation or dephosphorylation, via inducing Nrf2-dependent aldehyde dehydrogenases/reductases, thioredoxin reductase, and phosphatases, or inducing the antioxidant buffer systems by using Nrf2 activators and ECSOD mimetics (Adenuga et al. 2010; Yao et al. 2010a). Hence, HDAC2 is a redox sensitive protein, whose levels/activities are decreased under oxidative stress/carbonyl conditions, resulting in chromatin modifications associated with steroid resistance leading to lung inflammatory response (Sundar et al. 2012) (Fig. 11.4).

11.2.4.2 SIRT1 Deacetylase, Inflammation, and Senescence

Sirtuin 1 (SIRT1) belongs to class III HDACs, which possess anti-inflammatory, anti-aging/senescence, and anti-apoptotic properties due to their ability to deacetylate both histones and non-histone proteins, including transcription factors and other signaling molecules (NF-κB, FOXO3, p53, Ku70, and PGC-1α) (Sundar et al. 2012; Yang and Sauve 2006) (Fig. 11.5). Earlier reports showed that SIRT1 levels and activities were decreased in monocytes (MonoMac6 cells), bronchial epithelial cells (Beas-2B), mouse lungs exposed to cigarette smoke, as well as

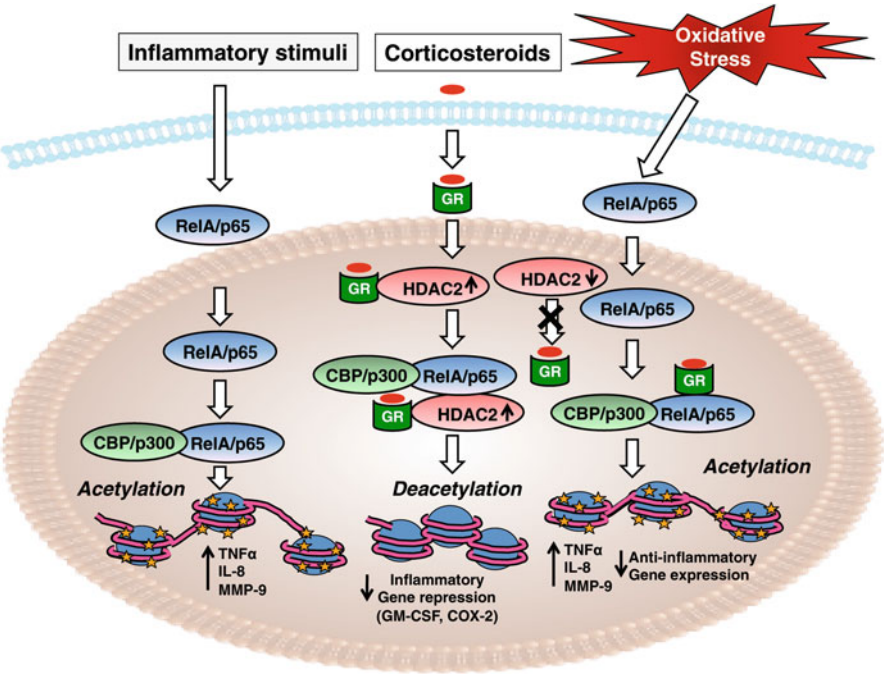

Fig. 11.4 Oxidative depletion of HDAC2 and redox-mediated chromatin modifications of pro-inflammatory genes in chronic inflammation and steroid resistance. Stimulation of alveolar macrophages and epithelial cells activates RelA/p65 (NF-κB) leading to recruitment of CBP/p300-RelA/p65 onto the promoters of proinflammatory genes (TNFα, IL-8, and MMP9), thereby resulting in proinflammatory gene transcription. Corticosteroids reverse this process by binding to gluococorticoid receptors (GR) and recruiting HDAC2 into the transcriptome complex, thereby reversing histone acetylation and RelA/p65 activation and causing transcriptional repression. Cigarette smoke-mediated degradation HDAC2 leads to the disruption of GR and NF-κB complex, and results in NF-κB activation and histone acetylation on promoters of pro-inflammatory genes. This leads to augmented gene expression of pro-inflammatory mediators

in the lungs of COPD patients and smokers (Rajendrasozhan et al. 2008a, b; Yang et al. 2007; Hwang et al. 2010), implicating the involvement of SIRT1 in the pathogenesis of COPD (Sundar et al. 2012). The mechanism for SIRT1 reduction involves post-translational modifications, such as phosphorylation, nitrosylation, and oxidation/carbonylation by ROS/carbonyls in response to cigarette smoke/oxidative/carbonyl stress, ultimately leading to SIRT1 oxidative modifications and degradation (Caito et al. 2010; Rajendrasozhan et al. 2008a, b) (Fig. 11.3). SIRT1 is also regulated by the intracellular redox thiol (GSH/GSSG) pool (Yang et al. 2007; Caito et al. 2010). SIRT1 activators or endogenous SIRT1 regulators (Milne and Denu 2008; Milne et al. 2007) are implicated in the therapeutic intervention of COPD (Rajendrasozhan et al. 2008b). Nakamaru et al. showed that administration of the SIRT1 activator SRT2172 attenuated cigarette smoke-induced lung inflammation in mice (Nakamaru et al. 2009). Furthermore,

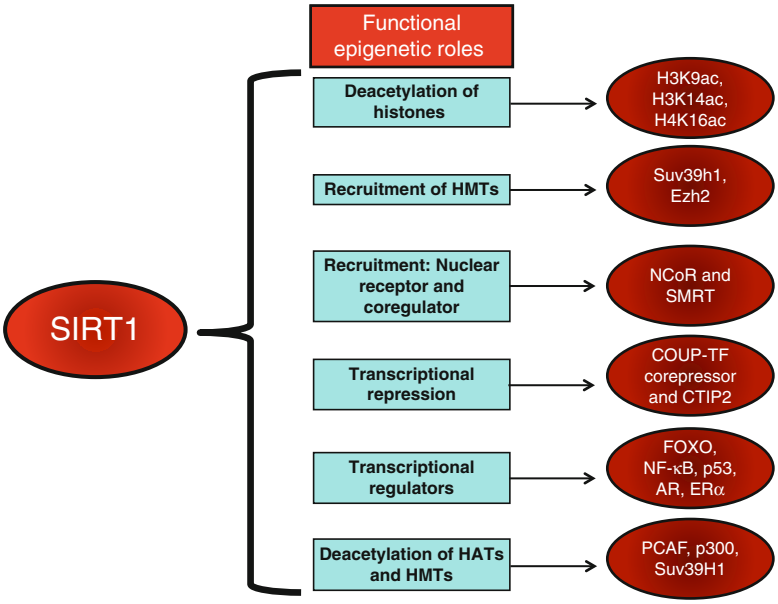

Fig. 11.5 Functional epigenetic roles of Sirtuin 1. SIRT1 regulates chromatin modifications and transcriptional activation/repression through different mechanisms. SIRT1 interacts with several factors and regulatory molecules, particularly those which are closely associated with chromatin regulation events. SIRT1 deacetylates histones H3K9, H3K14, and H4K16. Deacetylation of histones by SIRT1 synergizes with increased tri-methylation of H3K9 (gene repressive modification), thus resulting in transcriptional repression. SIRT1-mediated deacetylation of histone H3K9 activates histone methyltransferase Suv39h1, Ezh2 to further repress transcription. Similarly, SIRT1 interacts with nuclear receptor NCoR and coregulator SMRT, and transcriptional repression by COUP-TF corepressor, CTIP2, is also enhanced during deacetylation of histone H3 and H4 by SIRT1. SIRT1-mediated deacetylation of transcription regulators (FOXO, NF-κB, p53, AR, ERα), HATs (PCAF, p300) and HMT (Suv39h1) also cause transcriptional repression. Suv39h1 suppressor of variegation 3–9 homolog 1, Ezh2 Enhancer of Zeste 2 gene, NCoR nuclear corepressor, SMRT silencing mediator for retinoid or thyroid-hormone receptors, COUP-TF chicken ovalbumin upstream promoter transcription factor, CTIP2 COUP-TF-interacting protein 2, FOXO Forkhead Box class O, NF-κB nuclear factor-kappaB, AR androgen receptor, ERα estrogen receptor alpha, PCAF p300/CBP associated factor

SIRT1-mediated protection in response to cigarette smoke/oxidative stress against lung inflammatory response and injury are linked to the deacetylation of NF-κB RelA/p65 and negative regulation of MMP-9 (Chen et al. 2002; Yang et al. 2007; Nakamaru et al. 2009). Further studies are required to investigate the role of SIRT1 regulation and progression of COPD/emphysema using genetic/pharmacological approaches.

SIRT1 also deacetylates other transcription factors, such as forkhead box class (FOXO3), p53, and NF-κB, thereby regulating oxidative stress-induced cell cycle arrest, apoptosis and cellular senescence, which play important roles in the pathogenesis of COPD (Yao and Rahman 2011; Sundar et al. 2012) (Fig. 11.5). FOXO3

is regulated by its phosphorylation and acetylation status. SIRT1 deacetylates FOXO3, leading to its activation and to the induction of cell cycle arrest. Hence, increased SIRT1 activity promotes cell survival (Motta et al. 2004; You and Mak 2005). FOXO3 is acetylated when SIRT1 levels/activity are reduced in response to cigarette smoke in mouse lung (Rajendrasozhan et al. 2008a). Furthermore, FOXO3 has a regulatory role in lung inflammatory response and regulation of antioxidant genes (MnSOD and catalase). Targeted disruption of FOXO3 results in downregulation of antioxidant genes in mouse lungs and increases the susceptibility for the development of COPD/emphysema (Hwang et al. 2011). Further studies on the SIRT1-FOXO3 pathway will elucidate the underlying pathological mechanisms in response to oxidative/carbonyl stress, and will provide the possible therapeutic interventions in treatment of COPD.

SIRT1 interacts with p53, and deacetylates lysine residue at the C-terminal regulatory domain (Vaziri et al. 2001), whereas SIRT1 reduction leads to increased p53 acetylation, thus increases its pro-apoptotic function and cellular senescence (Vaziri et al. 2001; Luo et al. 2004). Oxidative stress is known to accelerate cellular senescence, due to increased p53 acetylation via decreasing the function of SIRT1 through NAD^+ depletion (Furukawa et al. 2007; Ota et al. 2007). Furthermore, blockade of p53 by antisense oligonucleotides reversed the inhibitory effect of SIRT1 on cellular senescence (Ota et al. 2007). Earlier studies from the authors laboratory have shown that the nuclear levels of SIRT1 were decreased in response to CS both *in vitro* and *in vivo* (Yang et al. 2007), but still it remains unclear whether SIRT1-mediated regulation of p53 (acetylation/deacetylation) and possibly p21 plays an important role in oxidants/CS-mediated apoptosis, and cellular senescence/lung aging (Sundar et al. 2012). Other members of sirtuins (eg. SIRT6 have also shown to modify cellular functions under various oxidative stress conditions) (Mao et al. 2011; Michishita et al. 2008; Kawahara et al. 2009; Van Gool et al. 2009). Therefore, other sirtuin members possibly gain similar credence as SIRT1 in understanding the pathogenesis of COPD and other chronic lung diseases (Sundar et al. 2012).

SIRT1 is reported as a key regulator of vascular endothelial homeostasis controlling angiogenesis, vascular tone and endothelial dysfunction by regulating endothelial nitric oxide synthase (eNOS) activity (Wright and Churg 2008; Potente and Dimmeler 2008a; Potente and Dimmeler 2008b). Endothelial dysfunction plays an important role in pathogenesis of emphysema, which is associated with decreased expression of eNOS (Edirisinghe et al. 2008; Ferrer et al. 2009). SIRT1 interacts with eNOS and deacetylates lysines at position 496 and 506 in the calmodulin-binding domain of eNOS, leading to enhanced nitric oxide (NO) production (Mattagajasingh et al. 2007; Arunachalam et al. 2010). Hence, the activation of SIRT1 by small molecules may help in resetting the eNOS activity during endothelial dysfunction, e.g. in susceptible smokers, where NO availability is limited (Michaud et al. 2006). Another study demonstrated that SIRT1 overexpression attenuated cigarette smoke-induced apoptosis and inflammatory response in cultured coronary arterial endothelial cells (Csiszar et al. 2008). Therefore, SIRT1 acts as a possible redox regulator in the treatment and prevention of chronic lung

and cardiovascular diseases including COPD (emphysema) and atherosclerosis by protecting endothelial cells from stress-induced premature senescence, apoptosis and inflammatory response (Sundar et al. 2012).

11.2.5 Histone Acetylation/Deacetylation and Its Cross-Talk with Histone Methylation in Redox Epigenetics

Unlike histone acetylation, which drives gene activation, histone methylation can either serve as signal for activation or repression of gene transcription, depending on the sites of histone methylation (Zhang and Reinberg 2001; Bannister and Kouzarides 2005). HMTs utilize S-adenosylmethionine (SAM) as a cofactor during transmethylation and produce S-adenosylhomocysteine (SAH) as by-product (Sundar et al. 2012). The substrate specificity and activity of HMT depends on SET domains and associated motifs (Zhang et al. 2003). HMTs are deregulated in several chronic lung diseases, thereby affecting the global methylation status. Histone H3K4, H3K9, and H3K27, and H4K20 are frequently and preferentially methylated as mono-, di- or tri- methylated histone H3 and histone H4 (Seligson et al. 2009). Methylation at H3K4, H3K36, and H3K79 is linked to gene activation, whereas H3K9, H3K20, and H3K27 methylation is associated with gene repression (Gosden and Feinberg 2007). Histone modifications are governed by specific chromatin modification enzymes, which have a residue-specific role in regulating gene expression with respect to histone modifications in epigenetic events (Table 11.1). Only lysine and serine modifications are shown in this table due to the focus of this review. Furthermore, the role of these enzymes in regulation of the histone code is nebulous. Barlési et al. demonstrated two key epigenetic histone modifications, H3K4me2 ($\geq$85% tumor cells) and H3K9ac ($\geq$68% tumor cells) that may contribute to the development and progression of Non-Small Cell Lung Cancer (NSCLC) (Barlesi et al. 2007). Cancer cells have a very different epigenotype compared to the cells from normal tissues from which they arise. This epigenetic switch in cancer cells is due to changes in DNA methylation and histone modifications caused by increased oxidant burden. Cancer cells have increased expression of epigenetic modification enzymes, yet their reaction products do not correlate, suggesting that there may be other factors affecting their activity (Ehrlich et al. 2006). A recent study has demonstrated a distinct mechanism by which histone methyltransferase G9a domains and different molecular mechanisms (ligand-dependent ERα binding function) regulate G9a-mediated coactivator versus corepressor function (Purcell et al. 2011). Transient reporter gene assays using MCF-7 breast cancer cells showed an involvement of endogenous G9a in contribution of estrogen estradiol (E_2)-dependent activation of estrogen receptor (ER)α target genes while concurrently limiting the expression of E_2-induced expression of other ERα target genes (Purcell et al. 2011). *In vitro* exposures to cigarette smoke condensate or extract and oxidants using airway epithelial cells and immortalized bronchial epithelial cells showed reduced H4K16ac and H4K20me3 but increased H3K27me3 levels, which may

Table 11.1 Post-translational histone modification sites (serine and lysine residues), chromatin modification enzymes, and their proposed functional role in gene regulation

Histone/ modification type	Post-translational modification sites	Chromatin modification enzymes	Proposed function
Histone H3 phosphorylation	H3S10	Aurora-B kinase, MSK1, MSK2, IKKα, Snf1	Mitosis, meiosis, immediate-early gene activation, transcriptional activation (proinflammatory genes)
	H3S28	Aurora-B kinase, MSK1, MSK2	Mitosis, immediate-early gene activation
Histone H3 acetylation	H3K4 (*S. cerevisiae*)	Esa1	Transcriptional activation
		Hpa2	Unknown
	H3K9	Gcn5, SRC-1	Transcriptional activation and histone deposition
	H3K14	Gcn5, PCAF, Esal, Tip60, SRC-1, Elp3, Hpa2, hTFIIIC90, TAF1, Sas2, Sas3, p300	DNA repair, transcriptional activation, RNA polymerase II & III transcription, euchromatin
	H3K18	Gcn5, CBP/p300	Transcriptional activation, DNA replication and repair
	H3K23	Gcn5, Sas3	Transcriptional activation (elongation) and DNA repair
	H3K27	Gcn5	Transcriptional activation
	H3K56 (*S. cerevisiae*)	Spt10	Transcriptional activation and DNA repair
Histone H3 methylation	H3K4	SET1 (*S. cerevisiae*), SET7/9 (vertebrates), MLL, ALL01,	Permissive euchromatin (di-me), transcriptional activation (tri-me)
	H3K9	SUV39h, Clr4, G9a, SETDB1	Transcriptional repression (tri-me)
	H3R17	CARM1	Transcriptional activation
	H3K27	Ezh2, G9a	Transcriptional repression, X-inactivation (tri-me)
	H3K36	Set2	Transcriptional activation (elongation)
	H3K79	Dot1	Euchromatin, transcriptional activation (elongation), checkpoint response
Histone H4 phosphorylation	H4S1	CK2	Mitosis, chromatin assembly and DNA repair

(continued)

Table 11.1 (continued)

Histone/ modification type	Post-translational modification sites	Chromatin modification enzymes	Proposed function
Histone H4 acetylation	H4K5	Hat1, Esal, Tip60, ATF2, Hpa2, p300	Histone deposition, transcriptional activation and DNA repair
	H4K8	Gcn5, PCAF, Esal, Tip60, ATF2, Elp3, p300	Transcriptional activation (elongation) and DNA repair
	H4K12	Hat1, Esal, Tip60, Hpa2, p300	Histone deposition, telomeric silencing, transcriptional activation and DNA repair
	H4K16	Gcn5, Esal, Tip60, ATF2, Sas2	Transcriptional activation, DNA repair, euchromatin
Histone H4 methylation	H4R3	PRMT1, PRMT5	Transcriptional activation/repression
	H4K20	PR-SET7, SUV4-20h	Transcriptional repression (mono-me), heterochromatin (tri-me)

have implications in tumor development in a wide array of cancer types (Liu et al. 2010). However, it remains to be seen whether the regulation of these enzymes and specific histone modifications via modulation by oxidative stress and/or redox status of the cells also occur in redox epigenetics regulation of pro-inflammatory genes particularly in chronic inflammatory diseases (Sundar et al. 2012).

The cross-talks between different histone methylation sites also epigenetically control the transcriptional activation of target genes (Cheung and Lau 2005; Wang et al. 2008). The positive and negative cross-talks ultimately generate the complex pattern of gene- or locus-specific histone marks, which are linked with distinct chromatin states (euchromatin or heterochromatin), leading to transcriptional repression or activation (Sundar et al. 2012). Cross-talk exists between various epigenetic markers on histones and DNA methylation (Fig. 11.6). For example, the methyl-cytosine binding protein, MeCP2, recruits HDACs to methylated DNA as well as to methylated histones (Gilliland et al. 2002; Ng et al. 1999). It has been shown that inhibition of HDACs and histone acetylation/methyation, and/or DNA methylases leads to upregulation of pro-inflammatory mediators, such as GM-CSF by IL-1β in lung epithelial cells (Kagoshima et al. 2001). Histone H3K4 tri-methylation has been reported to play a role in tumorigenesis and X-chromatin inactivation in human cells (Lachner and Jenuwein 2002). Furthermore, hypermethylation of histone H3K4 was found to be unrelated to acute TNF-α-induced gene expression (Saccani and Natoli 2002). In contrast, the involvement of H3K4 tri-methylation in IL-1β-induced gene expression has been reported (Wada et al. 2005). Furthermore, H3K4 tri-methylation plays a critical role in IL-1β stimulated secretory leukocyte protease inhibitor (SLPI) expression (Wada et al. 2005). However, it remains to be

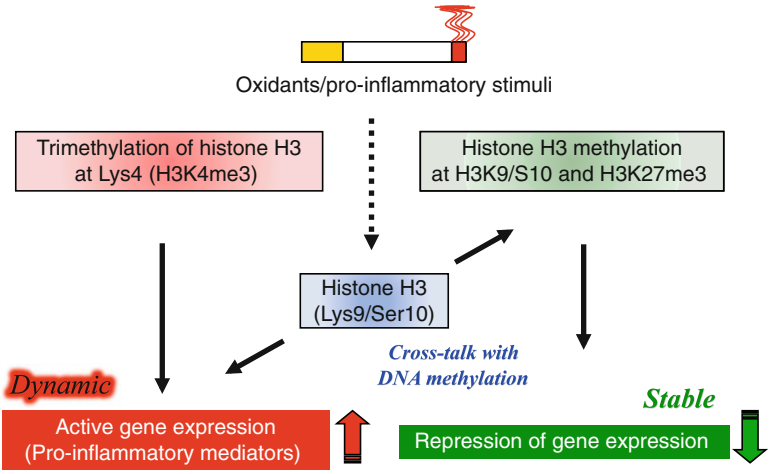

Fig. 11.6 Cross-talk between histone acetylation and methylation. Oxidative/carbonyl stress can activate histone acetylation on specific lysine residues. Trimethylation of histone H3 at K4 (H3K4me3) can lead to dynamic gene transcription, whereas histone H3 methylation at H3K9 and H3K27me3 can result in stable gene repression. Histone H3 acetylation at K9 and serine 10 (S10) phosphorylation can culminate in gene transcription. Histone acetylation/deacetylation and histone methylation can cross-talk with DNA methylation on CpG islands to activate or repress gene transcription

unknown whether the cross-talk occurs between histone acetylation and methylation in response to cigarette smoke/oxidant during lung inflammatory response. Studies on sequential events of histone acetylation/histone methylation will provide the understanding on the new epigenetics-based biomarkers and/or treatment for chronic inflammatory diseases including COPD/emphysema and cancer (Sundar et al. 2012) (Fig. 11.7).

11.2.6 HDMs and Redox Epigenetics

Histone demethylases catalyze the removal of methyl groups from lysine or arginine residue of histones (Sundar et al. 2012). They are classified into two types, the lysine specific demethylase 1 (LSD1) and Jumonji C (JmjC) domain family proteins involved in the regulation of gene expression (Tian and Fang 2007). LSD1 specifically demethylates histone H3K4me2, and is an important member of HDMs among other transcriptional repression complexes (Shi et al. 2004). JmjC demethylates mono-, di- or trimethyl lysine residues by a different mechanism that requires cofactors, such as molecular oxygen, α-ketoglutarate, Fe^{2+}, and ascorbate via a redox modulating process (Klose et al. 2006a, b). Aberrant expression of HDMs occurs during the course of tumor initiation and progression (Lim et al. 2010). However, the role of ROS/redox GSH status in modulation of HDMs in COPD

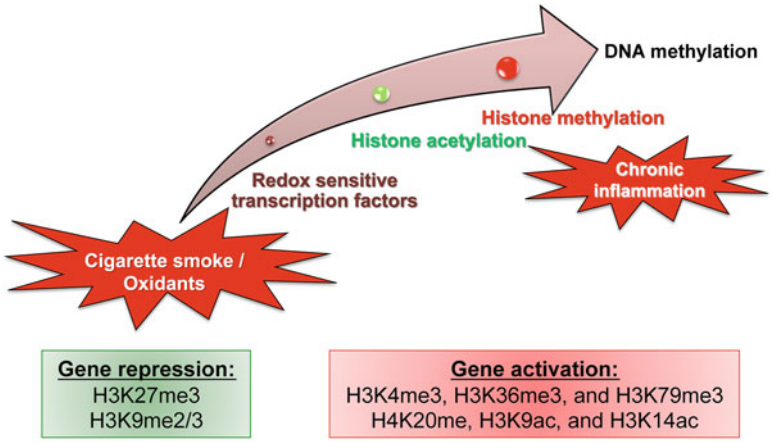

Fig. 11.7 Redox chromatin modifications in pathogenesis of chronic inflammatory diseases. Specific histone modifications can cross-talk with CpG methylation to activate or repress gene transcription in inflammation involved in chronic inflammatory diseases

and other chronic lung diseases remains unknown (Sundar et al. 2012). Hypoxia is known to occur in tumor microenvironments, as well as in lungs of patients with COPD and other chronic inflammatory lung diseases. The level of JMJD2B is upregulated in response to hypoxia, which depends on hypoxia-inducible factor 1 alpha (HIF-1α) (Yang et al. 2010). Inhibition of H3K4 demethylase JMJD1A sufficiently reduces tumor growth *in vivo* demonstrating its role in regulating histone methylation in hypoxia (Krieg et al. 2010). In contrast, hypoxia increases global levels of H3K4me3 in alveolar A549 and bronchial Beas-2B cell lines, due to the inhibition of demethylation process, particularly demethylase (JARID1A) (Zhou et al. 2010). The involvement of JARID1B (KDM5B) in growth of cancer cells has been shown through the E2F/RB1 cell cycle regulation pathway in different cancer cell lines. Lung tumor samples from NSCLC and SCLC showed elevated expression of JARID1B compared to non-neoplastic tissues confirmed by microarray analysis and immunohistochemistry suggesting JARID1B activation plays an important role in lung carcinogenesis (Hayami et al. 2010). Thus, identification of target specific histone demethylase inhibitors may be a viable tool in epigenetic therapeutics or via potentiating the activity of hypomethylating agents in cancer (Grant 2009).

11.2.7 DNA Methylation in Redox Epigenetics

DNA methylation is a nonhistone epigenetic event that plays an important role in transcriptional regulation. Mostly in all eukaryotes, methylation occurs on cytosine residues, producing 5-meC (5-methylcytosine), but the local context of the methylation event differs from one organism to the other (Suzuki and Bird

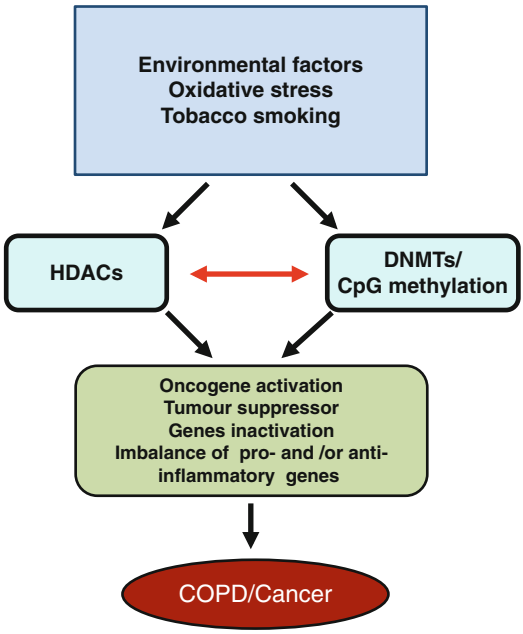

Fig. 11.8 Epigenetic regulation of gene expression in COPD and cancer. Environmental factors, oxidative stress, diet, genetic predisposition factors, and aging can increase promoter CpG methylation by recruitment of MeCP2 and DNMTs on various promoters along with HDACs leading to alterations in tumor suppressor, onocogenes, pro- and anti-inflammatory genes. DNA methylation is associated with recruitment of HDACs on promoters of various genes. These processes are intertwined in pathogenesis of COPD and lung cancer

2008). DNA methylation is associated with epigenetic silencing, and this effect is in part mediated by recruitment of HDACs through the methyl-DNA binding motifs, including components of several HDAC-containing complexes (Nan et al. 1998). Environmental factors/agents that trigger oxidative stress, such as diet, genetic predisposition factors, and aging can gradually affect the promoter CpG methylation by recruiting MeCP2 and DNMTs to various promoters along with HDACs (Sundar et al. 2012). This leads to alterations in the expression of tumor suppressor, onocogenes, and pro- and anti-inflammatory genes (Fig. 11.8). Promoter methylation in multiple genes has been demonstrated in adenocarcinomas and NSCLC, and they are linked with tumor progression (Zochbauer-Muller et al. 2001). Hence, identifying gene specific DNA methylation will provide further insights in the development of useful markers as tools for early detection and treatment of different cancers. There are several reports available that describe the promoter hypermethylation and associated gene-silencing of various genes in lung cancer (Belinsky 2004; Anglim et al. 2008). However, the relationship between gene-specific DNA methylation and smoking history is not known (Tsou et al. 2002). Georgiou et al. reported methylation of p16 promoter in sputum of patients with COPD that significantly correlated with heavy cigarette smoking (Georgiou et al. 2007). Gene ontology analysis suggested genes involved in immune and inflammatory system pathways, stress response, as well as wound healing and coagulation cascades. Significant association was shown between SERPINA1 hypomethylation and pathogenesis of COPD with lower lung function (Qiu et al.

2012). This suggests that DNA methylation/hypomethylation, which is caused by oxidative stress in specific genes may be a epigenetic mark in the pathogenesis of COPD (Qiu et al. 2012).

Cigarette smoke downregulates DNMT3B in adenocarcinoma alveolar A549 epithelial cells resulting in demethylation of the CpG island in the oncogene SNCG and leading to high expression of this prometastatic oncogene in A549 cells (Liu et al. 2007). Promoter specific hypermethylation in genes, such as *APC1* (Brabender et al. 2001), *DAPK* (Kim et al. 2001) and *p16* (Sanchez-Cespedes et al. 2001) from lung tissue of NSCLC patients showed no significant association between smoking history. On the contrary, significant association between promoter hypermethylation and smoking history has been observed in NSCLC patients in *CDKN2A* (Kim et al. 2001) *HIC1* (Eguchi et al. 1997), *HtrA3* (Beleford et al. 2010), and *CHFR* (Takeshita et al. 2008). Promoter hypermethylation leads to gene-silencing of various genes in lung cancer (Belinsky 2004; Anglim et al. 2008). Sood et al. identified the promoter methylation from sputum samples of patients demonstrating wood smoke exposure associated with COPD phenotype (decline in lung function, airflow obstruction and chronic bronchitis), and smokers with aberrant *p16* and *GATA4* methylation (Sood et al. 2010). Epigenetics in human asthma is not well understood, particularly in older adults. Investigating the methylation profiles in sputum DNA among older adults with asthmatics, who are smokers revealed methylation of selective genes such as Protocadherin-20. Hence, the use of novel demethylating agents may possibly help to prevent and treat asthma among older smokers (Sood et al. 2012). Suzuki et al. demonstrated methylation of *IL-12Rb2* and *Wif-1* in COPD patients compared to non-COPD group, suggesting the molecular epigenetic events that influence COPD-related NSCLC (Suzuki et al. 2010). An interesting report described the association of prenatal exposure to tobacco smoke with significant changes in two types of DNA methylation (global methylation and promoter CpG island methylation) (Breton et al. 2009). Hence, the findings from this study supports that epigenetic effects of *in utero* exposures may be due to alterations in DNA methylation patterns (Breton et al. 2009).

A recent study demonstrated the influence of cigarette smoke extract on WW domain containing oxidoreductase (WWOX) expression and methylation status in human bladder cancer cells (T-24). Cigarette smoke extract treatment significantly increased the levels of DNMT1 but not DNMT3A and DNMT3B. The expression levels of DBMT1 corroborate with hypermethylation of WWOX induced by cigarette smoke which may be the underlying mechanism for the loss of WWOX expression in bladder cancer (Yang et al. 2012). Another study on gender-specific methylation differences in relation to prenatal exposure to cigarette smoke demonstrates two differentially methylated regions (DMRs) regulating insulin-like growth factor 2 (IGF2) and H19. There was a positive correlation between smoking-related increased DNA methylation at the IGF2 DMR in male offsprings, who had smoking-related low birth weight (approximately 20%). Hence, these observations suggest that IGF2 DMR plasticity is an important mechanism that regulates *in utero* adaptation to environmental toxicants (Murphy et al. 2012). Coordinated changes in DNA methylation particularly at the aryl hydrocarbon receptor repressor

(AHRr) was observed in lymphoblasts and alveolar macrophages from smokers suggesting the implications of epigenetic effects mediated by cigarette smoke on carcinogenesis and other related co-morbidities (Monick et al. 2012). The DNA methylation status on susceptibility genes may have implications in smokers, who are at high risk for chronic lung diseases including COPD. The role of various histone modifications versus DNA methylation, particularly in response to oxidative stress in inflammatory conditions is an emerging field of further research (Sundar et al. 2012).

11.3 Conclusions

The role of ROS and redox epigenetic regulation in pro-inflammatory/anti-inflammatory gene transcription is discussed in this chapter. ROS produced by extracellular or by intracellular processes have the potential to induce considerable post-translational modifications of intracellular proteins. Oxidative stress by cigarette smoke/oxidants is critical for chronic lung inflammatory response via activation of stress signaling kinases, redox-sensitive transcription factors, and modulation of epigenetic chromatin modifications, which result in gene transcription (Sundar et al. 2012). Epigenetic events, such as histone acetylation, deacetylation, phosphorylation, and histone/DNA methylation play an important role in oxidative stress-induced chronic inflammatory diseases, such as asthma, COPD, and cancer. HDAC2 is required for glucocorticoids to function as anti-inflammatory agents. Thus, activation of HDAC2 or reversal of oxidative post-translational modifications of HDAC2 is another avenue to devise epigenetic based therapy in treatment of severe asthma and COPD (Sundar et al. 2012). Further studies are required to unravel the mechanisms of redox epigenetics for the development of epigenetics-based therapies in chronic inflammatory diseases and cancer.

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Chapter 12
Redox Regulation of Stem Cell Function

Heinrich Jasper and Dirk Bohmann

Abstract Stem cells are critical for tissue regeneration and homeostasis, and their regenerative potential has to be carefully managed to prevent degenerative diseases and cancer. In recent years, it has emerged that stem cells are uniquely sensitive to changes in intracellular reactive oxygen species (ROS) (Hochmuth et al., Cell Stem Cell 8:1–12, 2011a; Ito et al., Nature 431:997–1002, 2004; Owusu-Ansah and Banerjee, Nature 461:537–541, 2009; Smith et al., Proc Natl Acad Sci USA 97:10032–10037, 2000; Tothova et al., Cell 128:325–339, 2007; Tsatmali et al., Brain Res 1040:137–150, 2005). Dynamic regulation of intracellular ROS seems to be critical to control the regenerative potential and to avoid loss of self-renewal capabilities and pluripotency of stem cells. Here, we review these findings, highlighting recent observations in genetic model organisms that clarify the role of ROS in stem cell regulation.

12.1 Introduction

Two major stem cell types can be found in most metazoans: germline stem cells (GSCs) and somatic stem cells (SSCs). GSCs are the precursors for egg and sperm cells, which, after fertilization, can give rise to all cell types that constitute the organism. They are therefore truly totipotent. SSCs, on the other hand, are more restricted in their developmental potential, i.e. they are normally

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not totipotent, but rather pluripotent with a limited set of cell types that can arise from the differentiation of SSCs. Accordingly, multiple distinct SSC types, such as hematopoetic, neuronal or epithelial stem cells have been identified. SSCs serve as a reservoir to replenish somatic cells that have been lost or rendered dysfunctional, and thus contribute critically to tissue homeostasis.

SCs are characterized by the ability to undergo asymmetric cell division, whereby they give rise to one daughter cell that recapitulates the stem cell characteristic of the mother cell (self-renewal), and one daughter that initiates a process towards terminal differentiation. This latter cell may already express markers and phenotypes of a terminally differentiated cell, or represent an intermediary, mitotically active, but specified state. It is currently being debated, if SC division is invariably asymmetric, or whether the stem cell pool in a tissue can be maintained through a process called "population asymmetry", where symmetric and asymmetric divisions are in balance to ensure proper self renewal and differentiation rates (Biteau et al. 2011; Simons and Clevers 2011; Snippert et al. 2010; Tian et al. 2011).

A thorough understanding of SC molecular and cell biology is a paramount priority for biomedical research. The integrity of adult stem cell reservoirs is critical for health and homeostasis, and preserving this integrity requires a well-regulated balance between stem cell self renewal and differentiation. Declining stem cell numbers and function cause loss of regenerative potential and lead to degenerative diseases. Conversely, unchecked stem cell amplification can disrupt stem cell homeostasis and cause hyperplasia and/or aberrant differentiation, a condition that may favor the initiation and progression of malignant diseases and cancer (Biteau et al. 2011; Radtke and Clevers 2005; Radtke et al. 2006; Rando 2006).

Self-renewal and multipotency set stem cells apart from the "mortal" differentiated cells of the soma. Reflecting these special properties, stem cells have unique molecular and signaling characteristics that distinguish them from their fellow cells of the organism. One such unique aspect of stem cell physiology and molecular biology is the relationship of stem cells to oxidative stress and redox homeostasis. This chapter will review stem cell specific functions of redox signaling and stress response that have emerged as general features shared among many types of stem cells, including adult as well as germ line stem cells. To illustrate the research that led up to the current understanding of redox signals in the regulation of stem cell differentiation and maintenance, we will describe studies in specific models of stem cell biology in greater detail. We will concentrate this discussion on research that was conducted in *Drosophila melanogaster*, which is emerging as a powerful, genetically tractable model system for the study of germline and adult stem cell biology (Biteau et al. 2011; Jasper and Jones 2010; Jiang and Edgar 2011; Wang and Jones 2010). For readers interested in research related to redox regulation of vertebrate stem cells, we refer to an excellent recent review by T. Finkel and colleagues (Liu et al. 2011).

12.2 REDOX and Stem Cells: A Special Relationship

Several observations highlight the special role of redox regulation in stem cells.

1. *Stress resistance.* Undifferentiated, pluripotent stem cells and multipotent progenitor cells are generally resilient against oxidative damage.
2. *Reducing conditions favor stemness.* Research on a number of specific stem cell systems has revealed that a reducing intracellular environment characterizes the undifferentiated state of stem and progenitor cells. Conversely, an increasing oxidized state correlates with, and in some cases has been shown to be required for, differentiation and loss of stemness, the state in which stem cells retain the potential to self renew and give rise to differentiated descendants.
3. *Stem cell specific oxidative stress responses.* Specific signaling pathways permit eukaryotic cells to sense and respond to increased oxidative stress caused either by changes in metabolism or by extracellular conditions. Stem cells also employ these signaling pathways, but, interestingly, they differ from many other cell types in the way they respond to them. Such stem cell specific signal responses may explain some of the unique biological properties of stem cells.

Below we will elaborate on these three points:

12.2.1 Stress Resistance

Germ line cells have unlimited replicative potential and therefore are, at least in principle, immortal. Adult stem cells are also long-lived. Many adult stem cells divide rarely or remain quiescent for months or years. Over such protracted periods of time the genetic and physical integrity of stem cells has to be safeguarded. A major threat to any cell, including stem cells, are reactive oxygen species (ROS), which can emerge as byproducts of mitochondrial respiration or originate from external sources, for example due to exposure to xenobiotic insults or as a consequence of microbial infection. ROS can damage proteins, lipids and nucleic acids. Importantly, they can cause DNA damage, which, if not repaired, can result in mutations. Loss or genetic damage of stem cells would have fatal consequences for the organism as damage to stem cells might comprise their regenerative and developmental potential or might cause the descendants of these cells to inherit deleterious mutations. It appears, therefore, that stem cells should be more "precious" to the organism than expendable somatic cells. Consistent with this notion, they are generally especially well protected. Stem cells frequently reside in special "niches" (Jones and Wagers 2008; Voog and Jones 2010; Walker et al. 2009). Such stem cell niches not only provide a reservoir for regeneration and release the cues that control stem cell identity and function, they are also a protective environment. Niches such as those for hematopoetic stem cells in the bone marrow are located in areas of low oxygen tension, reducing the probability of oxidative damage (Jang and Sharkis 2007;

Parmar et al. 2007). In addition to their residence in such sheltered niches, stem cells are themselves well equipped to handle extraneous ROS that might cause damage. An effective mechanism to avoid oxidative damage to vital cell components is to keep the intracellular milieu reducing. Indeed many stem and progenitor cells have been found to have more reducing intracellular conditions than the differentiated cells that they generate (Hochmuth et al. 2011b; Noble et al. 2003; Smith et al. 2000).

12.2.2 Reducing Milieu = Stemness

A reducing intracellular environment that was introduced in the preceding paragraph as a characteristic of stem cells that may confer oxidative stress resistance has also been suggested as a cue for regulating stem cell differentiation. Multiple examples, some of which are discussed in more detail below, have shown that the transition from a reduced to an oxidized state occurs during stem cell differentiation, the departure from the state of stemness. In many cases, this change of redox state is not only a consequence but a condition for stem cell differentiation (see schematic in Fig. 12.1). It is now a broadly accepted view that regulated changes of redox state are central parts of the mechanisms that control stem cell maintenance and differentiation (Hochmuth et al. 2011b; Ito et al. 2004; Liu et al. 2011; Owusu-Ansah and Banerjee 2009; Ray et al. 2012; Smith et al. 2000; Tothova and Gilliland 2007; Tsatmali et al. 2005). The molecular mechanisms that control stem cell fate via changes in redox state are intensively investigated in the stem cell field.

12.2.3 Stem Cell Specific Signal Responses

Stem cell are distinct from other cells in terms of their developmental potential and their unlimited self-renewal. They are different in other respects too. One example is their response to stress signaling pathways. Multiple conserved signaling systems function to inform eukaryotic cells of changes in oxidative load. Well-known components of these signaling pathways include protein kinases such as JNK, p38 and ATM (Bhatti et al. 2011; Biteau et al. 2011; Cuadrado and Nebreda 2010; Weston and Davis 2007) as well as transcription factors like Nrf2, p53 and Fos (Biteau et al. 2010; Borras et al. 2011; Maher and Yamamoto 2010).

Interestingly, cells differ in the biological responses that they mount upon signaling through such pathways. When a typical body cell is exposed to acute oxidative stress, it will arrest the cell cycle attempting to limit damage and avoiding damage to newly replicated DNA. Stem cells are different. They often respond to oxidative stress by leaving quiescence and embarking on an expansion and

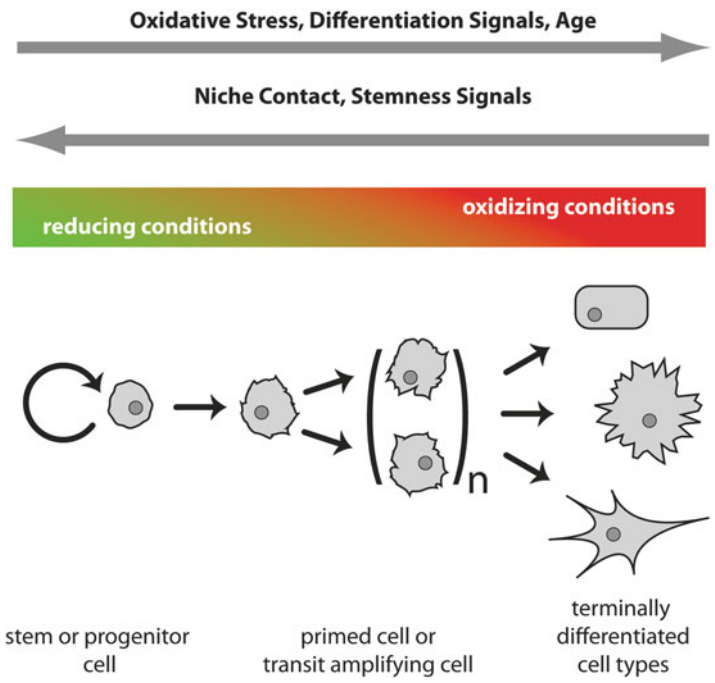

Fig. 12.1 Cartoon depicting the correlation between redox state and stem cell function. Undifferentiated, pluripotent or multipotent stem and progenitor cells keep their intracellular milieu reducing. In this state they are less vulnerable to oxidative damage, and stay quiescent or divide very infrequently. Regenerative responses to stress or tissue injury invoke increased stem cell differentiation and/or expansion. The transition from a state of stemness to terminal differentiation can involve intermediary steps such cell types that are primed for differentiation or transit amplifying cells that expand the population of differentiated descents of a limited number of stem cells. The progression along the path from a stem cell to a terminally differentiated cell coincides with shift in redox balance from a reduced to an oxidized state

differentiation program (Abou-Khalil and Brack 2010; Biteau et al. 2008, 2011; Hochmuth et al. 2011a; Tothova et al. 2007). This response can be viewed as the onset of a regenerative response. The wiring and regulatory logic of a stem cell appears in many aspects to be fundamentally different from a non-stem cell. A molecular and genetic description of such differences is now beginning to emerge. It is important to understand the unique rules by which stem cells interpret oxidative inputs and other signals from their environment.

Next, we will discuss several specific examples that have illustrated this concept and advanced our understanding of redox mechanisms that control in the maintenance and differentiation of stem and progenitor cells.

12.3 Redox Mechanisms in Stem Cells

12.3.1 EXAMPLE 1: Hematopoetic Stem Cell in Drosophila

The ancestors of red and white blood cells are arguably the best understood of all stem cells. Decades of research on viral and non-virally caused blood malignancies have yielded important insights into the lineage relationships and regulators of hematopoesis (Orkin and Zon 2008). Blood cell lineages are comparatively easy to monitor using well-established cytological criteria and markers. Hematopoetic stem cells are also the only stem cells that are currently in widespread therapeutic use. Bone marrow transplantation is an established treatment to replenish the loss of hematopoetic stem cells in myeloma and leukemia patients after chemotherapy (Burt et al. 2008; Gratwohl et al. 2010).

While *Drosophila* has no red blood cells, the fly's hemolymph contains three types of mature white blood cell types that resemble mammalian myeloid cells: the plasmatocytes, crystal cells and lamellocytes (Evans et al. 2003). These cells mediate innate immune responses to various types of infectious agents and parasites. All of these cells originate in the lymph gland, the fly's hematopoietic organ. The lymph gland forms during larval development from mesodermal cell lineages (Evans et al. 2003). As the organ matures, multipotent stem-like cells emerge that can differentiate into the three terminally differentiated immune cells mentioned above. The differentiation process can be stimulated by microbial infection or by parasite invasion. The generation of the immune cells is accompanied by a shift to an oxidized cell state that can be visualized by redox dyes (Owusu-Ansah and Banerjee 2009). This increase in redox levels is critical for myeloid differentiation. Experiments in which the intracellular milieu of the hematopoetic progenitor cells is forced towards a more reducing level, for example by overexpression of antioxidant scavenger proteins or catalase, prevent immune cell formation, proving that the oxidative shift is necessary for the response. Conversely, increasing the levels of ROS in the stem cells by mutations in electron transport chain components causes ectopic myeloid differentiation, even in the absence of immune challenges (Owusu-Ansah and Banerjee 2009). Thus, oxidative stimuli are sufficient to trigger stem cell differentiation. Interestingly, *Drosophila* hematopoesis follows a two-step redox mechanism to control differentiation. As the lymph gland matures, the myeloid progenitors become increasingly oxidized, which confers to them a "primed state" in which they are receptive to immune signals that push ROS levels across a threshold and stimulates the differentiation and release of mature myeloid cells.

Molecular analysis has shown that a key signaling pathway that triggers myeloid differentiation in response to increased ROS levels in the Drosophila hematopoetic lineage is comprised of the JNK MAP kinase cascade and its downstream target, the FoxO transcription factor (Owusu-Ansah and Banerjee 2009). Oxidative stress will increase JNK's enzymatic activity causing it to phosphorylate FoxO. FoxO,

when phosphorylated by JNK, is transcriptionally competent and can activate a gene expression program that drives myeloid cell differentiation. Active JNK will also cause the repression of polycomb-like transcriptional repressors, and this regulation contributes to progenitor cell differentiation independently of Foxo.

The mechanism that causes stimulation of JNK activity after oxidative stress exposure is complex and still not completely worked out. A heterogeneous group of JNKKKs can connect JNK activity to a range of upstream signals and stresses. The JNKKK Ask1, for example can respond to changes in thioredoxin levels and/or oxidation state. Tak1 has been implicated in responses to inflammatory signals and microbial challenges. Which one of these, if any, are operative in the *Drosophila* hematopoietic system is still an open question. The activation of FoxO by oxidative stress and JNK mediated phosphorylation is not unique to stem cells and has been described as a ubiquitous mechanism in many cell types (Essers et al. 2004; Salih and Brunet 2008; Wang et al. 2005). Interestingly, however, the biological readout to FoxO activation seems to be markedly different in stem cells and non-stem cells. Rather than inducing differentiation, FoxO activation in the developing eye causes growth arrest and, eventually, apoptosis (Owusu-Ansah et al. 2008; Biteau et al. 2010). The comparison between the genetically well-described *Drosophila* lymph gland and eye systems might thus provide insights into the unique biology and the idiosyncrasies of stem cells.

The impact of redox changes on hematopoietic progenitor function is likely to be conserved in vertebrates: Interestingly, FoxO seems to play a critical role in managing reactive oxygen species in mouse HSCs. Loss of FoxO in these cells results in elevated ROS levels, triggering exit from quiescence, and in consequence depletion of the HSC pool and reduced long-term regenerative potential (Miyamoto et al. 2007; Miyamoto et al. 2008; Tothova and Gilliland 2007; Tothova et al. 2007). This exit from quiescence of HSCs is accompanied by increased myeloid differentiation, suggesting that Foxo acts in HSCs primarily to prevent excessive ROS accumulation, while Foxo-independent redox responses govern hematopoietic progenitor cell differentiation in mice. While the general response of stem and progenitor cells to increased ROS levels may thus be conserved between flies and mice, it is possible that the molecular regulation of these responses has diverged. The role of Foxo in vertebrate HSCs, however, needs to be further examined. It remains unclear, for example, whether activation of FoxO in mammalian HSCs or restricted progenitors is also sufficient to promote myeloid cell expansion. Interestingly, a recent study suggests that this might indeed be the case, as FoxO and/or JNK/Jun activity is found to be high in patient samples of acute myeloid leukemia (AML). In mouse models for AML, it was found that FoxO and JNK/Jun signaling maintain a differentiation blockade that is critical for the progression of AML. While FoxO proteins are expressed at the transition from HSCs to myeloid progenitors, it remains to be established precisely at which stage in the HSC to myeloid progenitor lineage FoxO activity responds to ROS or controls ROS levels to direct cell cycle arrest, cell expansion, or differentiation (Sykes et al. 2011; Tothova and Gilliland 2007).

12.3.2 EXAMPLE 2: Redox-Dependent Control of Germline Stem Cell Function

The *Drosophila* male and female germlines have served as productive model systems to characterize the molecular mechanisms governing the interaction between stem cells and their niches (reviews Fuller and Spradling 2007; Ohlstein et al. 2004; Spradling et al. 1997, 2001; Voog and Jones 2010). A particularly active field of study in this context is the characterization of age-related changes in GSC function, which are caused by both stem cell intrinsic and extrinsic factors, including oxidative stress (Cheng et al. 2008; Jasper and Jones 2010; Pan et al. 2007; Wang and Jones 2010). In the female germline, GSCs are attached to niche cells through adherens junctions, in which E-cadherin plays a major role. Proper niche attachment is critical for self-renewal, and prevents differentiation of GSCs. Interestingly, the E-cadherin-mediated interaction between GSCs and their niche declines with age, causing an age-related decrease of GSC numbers (Pan et al. 2007). This age-related decline can be rescued by over-expression of superoxide dismutase, indicating that the accumulation of ROS in GSCs plays a major role in this loss of function.

A role for reactive oxygen species in influencing progenitor cell attachment to other cells has recently been established in primordial germ cells (PGCs) of the fly. In these cells, the peroxiredoxin Jafrac1 plays a critical role in promoting E-cadherin protein stability and thus PGC attachment and motility (DeGennaro et al. 2011). The exact mechanism by which E-cadherin stabilization is achieved remains unclear. Nevertheless, the common role of reactive oxygen species in modulating stem and progenitor cell adhesiveness through this particular molecular interaction suggests that ROS-mediated changes in E-cadherin function play a critical and general role in stem cell regulation.

12.3.3 EXAMPLE 3: Intestinal Stem Cells

A particularly illustrative example for the intersection between regulatory, physiological and deleterious functions of redox signaling is observed in intestinal stem cells (ISCs) of Drosophila. ISCs are critical for tissue regeneration along the gastrointestinal tract of flies, and their regulation and function have been subject of intense investigation in recent years. Best understood so far are ISCs of the posterior midgut epithelium. These cells regenerate the two functional cell types of the posterior midgut, enterocytes (ECs) and enteroendocrine cells (EEs) (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). ISCs commonly divide asymmetrically, producing a new stem cell and an undifferentiated progenitor cell, an enteroblast (EB). ISCs are the only proliferating cells in the intestinal epithelium and direct EB differentiation by activating the Notch signaling pathway in these cells.

ISC proliferation is regulated dynamically according to the needs of the intestinal epithelium. In conditions in which the intestine is healthy and not exposed to excessive stress, most ISCs are found in a quiescent state, as characterized by the fact that few of these cells incorporate BrdU in a window of 24 h (Hochmuth et al. 2011a). When challenged by a variety of stressors (including infection, cell death, tissue damage, DNA damage, and oxidative stress), ISCs embark on a regenerative program that is characterized by a high rate of proliferation in the intestine.

The signaling pathways responding to these challenges and inducing ISC proliferation include the Jun-N-terminal Kinase (JNK) and Jak/Stat signaling pathways. These inductive pathways control ISC proliferation in coordination with signals that are essential for proliferative competence of ISCs, including insulin, Epidermal Growth Factor Receptor (EGFR), and Wnt signaling (Amcheslavsky et al. 2009; Biteau et al. 2008; Biteau and Jasper 2011; Buchon et al. 2009; Choi et al. 2008; Lee et al. 2009; Lin et al. 2008). The proliferative response to JNK signaling is an illustration of the unique signaling characteristics of stem cells. The more common response to JNK activation in non stem cells is growth arrest or apoptosis (Liu and Lin 2005; McEwen and Peifer 2005; Weston and Davis 2007).

Stimulation of ISC proliferation represents the first step in a regenerative response that is required to recover intestinal integrity after a challenge, and as such can be viewed as a mechanism that should benefit the organism. However, this process needs to be tightly regulated to ensure intestinal homeostasis (Biteau et al. 2008; Jiang et al. 2009). Accordingly, the stress-induced increase in ISC proliferation rates declines rapidly after the stress subsides. When excessive proliferation is induced in the gut, either artificially, or through chronic of excessive damage, disruption of epithelial homeostasis ensues. This is caused by the accumulation of misdifferentiated ISC daughter cells (Amcheslavsky et al. 2009; Biteau et al. 2008; Choi et al. 2008).

Multiple recent studies have established that modulation of the redox balance in the intestinal epithelium significantly influences ISC proliferation rates and epithelial homeostasis. Increased proliferation is observed in response to treatment with the ROS-inducing compound paraquat (Biteau et al. 2008), as well as in mutants for the ROS detoxifying enzyme catalase (Choi et al. 2008), while treating flies with N-acetyl-cysteine (NAC) or glutathione is sufficient to restrain ISC proliferation (Buchon et al. 2009). Interestingly, loss of proliferative homeostasis in the intestine of aging flies limits Drosophila lifespan (Biteau et al. 2008, 2010; Hochmuth et al. 2011a). Excessive ISC proliferation in aging intestines seems to be caused by dramatically increased oxidative load due to a chronic inflammatory state. This state is triggered by age-related changes in the intestinal commensal flora that induce dual oxidase (DuOX)-mediated innate immune responses, generating large amounts of reactive oxygen species (Biteau et al. 2008; Buchon et al. 2009; Choi et al. 2008; Ha et al. 2005, 2009b; Hochmuth et al. 2011b).

Given the potential exposure of ISCs to an adverse oxidative environment, and the critical regenerative role of ISC in such an environment, it is not too surprising that proliferative activity of these cells is regulated by a well-known master regulator of the antioxidant response, the transcription factor Nrf2.

12.4 Nrf2: Stem Cell Control by the Master Regulator of the Antioxidant Response

Nrf2 is a central regulator of the intracellular redox state in vertebrates and invertebrates. This transcription factor is a member of the 'cap-and-collar' (Cnc) family that counteracts excessive ROS accumulation in cells by inducing genes encoding key antioxidant molecules, such as enzymes involved in glutathione (e.g., γ-glutamyl-cysteine-ligase catalytic subunit, Gclc) and thioredoxin metabolism (Hayes and McMahon 2009; Lee et al. 2005; McMahon et al. 2001; Motohashi et al. 2002), and antioxidant enzymes such as peroxiredoxins (Lee et al. 2005; Maher and Yamamoto 2010; Motohashi et al. 2002).

This function of Nrf2 significantly influences overall stress sensitivity and lifespan of several tested model organisms: gain-of-function conditions for the *Drosophila* homologue of Nrf2, CncC, result in significantly increased stress tolerance and longevity, and activation of the *C. elegans* homologue SKN1 has similar consequences in worms (Inoue et al. 2005; Sykiotis and Bohmann 2008; Tullet et al. 2008). Interestingly, gene expression analysis has suggested that SKN1 increases tissue homeostasis and extends lifespan by promoting a germcell – like environment in somatic cells (Curran et al. 2009).

In vertebrates and in *Drosophila*, Nrf2 is negatively regulated by the cytoplasmic repressor Kelch-like ECH-associated protein 1 (Keap1) (Hayes and McMahon 2009; Nguyen et al. 2009; Sykiotis and Bohmann 2008, 2010; Toledano 2009). Keap1 acts as an adaptor of a Cul3-ubiquitin ligase complex that promotes Nrf2 degradation. Accordingly, loss of Keap1 results in lifespan extension and increased oxidative stress tolerance in flies (Sykiotis and Bohmann 2008).

In somatic cells, oxidative stress results in disruption of the interaction between Keap1 and Nrf2, causing nuclear accumulation of Nrf2, and activation of gene expression. In contrast, recent studies in *Drosophila* suggest that the *Drosophila* homologue of Nrf2, CncC, is constitutively active in ISCs and that oxidative stress results in a Keap1-mediated inhibition of Nrf2 in these cells. Constitutive Nrf2 activity is required to maintain low ROS concentrations in ISCs. This activity is therefore required to prevent spontaneous activation of ISC proliferation, promoting epithelial homeostasis. Inhibition of Nrf2 activity, on the other hand, is critical to engage in regenerative responses, as artificially sustaining Nrf2 activity in ISCs exposed to stress inhibits such a response (Hochmuth et al. 2011b). These results suggest that constitutive Nrf2/CncC activity in ISCs prevents excessive ROS accumulation in response to challenges, setting a threshold for ROS levels at which a regenerative response is warranted. The Keap1/CncC regulatory module thus emerges as central in the control of ISC proliferation and intestinal regeneration.

The origin of redox regulation in the control of ISC proliferation remains unclear. It can be speculated, however, that repression of CncC activity by Keap1 is required to induce a permissive state in ISCs that ensures sufficient ROS accumulation in response to a mitogenic signal engaging receptor tyrosine kinases (RTKs). ROS accumulation is critical for signal transduction and signal stabilization in

growth factor signaling pathways (Bashan et al. 2009), as RTK signal transduction requires transient inactivation of peroxiredoxins to promote local ROS accumulation at the membrane (Woo et al. 2010). The increased local ROS concentration is believed to cause oxidation and inactivation of redox-sensitive protein tyrosine phosphatases, thus strengthening the signal downstream of the receptor (Lambeth 2004; Toledano et al. 2010; Tonks 2006). Supporting this view, CncC overexpression inhibits insulin receptor-mediated proliferation of ISCs (Hochmuth et al. 2011b).

This model predicts that appropriate regulation of ROS production and detoxification is critical to ensure proper cellular responses to mitogenic and survival signals (Bashan et al. 2009). At the same time, this regulatory system would be sensitive to external oxidative challenges, promoting regenerative stress responses, but also promoting excessive proliferation under chronic stress conditions. This is particularly prevalent in barrier epithelia like the intestinal epithelium, where a vigorous oxidative burst response is employed as a defense against pathogenic bacteria (Ha et al. 2005, 2009a, b).

Interestingly, Keap1 mutant mice show significant hyperkeratosis of the esophageal epithelium, suggesting that Keap1/Nrf2 also influences cell differentiation and proliferation in intestinal epithelia of higher organisms (Wakabayashi et al. 2003). This function is reminiscent of the requirement for SKN1 in intestinal development in *C. elegans* (An and Blackwell 2003), highlighting a potential evolutionary conservation of this function, but the exact role of Nrf2 in the regulation of stem cell activity in vertebrates remains to be established.

12.5 Perspectives and Outlook, Therapeutics

The examples listed above highlight the critical role of redox signaling in the regulation of stem cell function. It emerges that regulated changes in redox state affect stem cell function at different levels in addition to simply providing a defense against ROS inflicted damage to this precious cell type. Redox changes also modulate the specificity of signal response and even the adhesive properties of stem cells and therefore their interaction with specific niches. The observation that stem and progenitor cells are controlled by regulatory networks that distinguish the response of these cells to redox signals from the response of differentiated cells has been unexpected. Stem cell-specific signaling and regulatory networks are therefore attractive targets for therapy, both in efforts to boost stem cell maintenance and self-renewal, as well as in efforts to limit the expansion of aberrant stem-like cells. Interestingly, a role for redox changes in the biology of cancer stem cells has emerged in recent years (Diehn et al. 2009; Sykes et al. 2011), supporting this idea. A particularly interesting target for such therapies is Nrf2, as it is a central regulator of the intracellular redox state and has been the target of numerous clinical trials with a wide range of pharmacological activators and inhibitors (Glintborg

et al. 2006; Yu and Kensler 2005). For a productive approach to Nrf2-based therapies, it will be critical to clearly establish the role of Nrf2 in the regulation of mammalian stem cell lineages, following the conceptual insight generated by the *Drosophila* model.

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Chapter 13
Oxidative Stress in Infectious Diseases

Esther Jortzik and Katja Becker

Abstract The "big three" infectious diseases HIV/AIDS, tuberculosis, and malaria were collectively responsible for nearly 260 million infected people in 2010. HIV, *Mycobacterium tuberculosis*, and *Plasmodium falciparum*, the causative agents of AIDS, tuberculosis, and malaria, are continuously exposed to reactive oxygen and nitrogen species endogenously produced or derived from the host immune system in response to infection. Oxidative stress has a key function in the pathogenesis of many infectious diseases, and represents moreover a promising strategy for chemotherapeutic development. Understanding the redox interactions and redox signaling mechanisms of pathogens and their hosts is crucial for developing (1) drugs that support the host antioxidant defense in order to protect cells from oxidative damage, (2) drugs that enhance specific reactive oxygen or nitrogen species to improve the host defense against pathogens, and (3) drugs that interfere with the redox system of the pathogen in order to block its growth and survival.

Keywords Antioxidants • HIV/AIDS • Malaria • Tuberculosis • Redox system

Abbreviations

AhpC	alkyl hydroperoxide reductase
AIDS	acquired immunodeficiency syndrome
AOP	antioxidant protein
ASK	apoptosis signal-regulating kinase
G6PD	glucose 6-phosphate dehydrogenase
GR	glutathione reductase

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GST	glutathione S-transferase
gp120	glycoprotein 120
Grx	glutaredoxin
GSH	reduced glutathione
GSSG	oxidized glutathione
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
KatG	catalase-peroxidase
iNOS	nitric oxide synthase
LTR	long terminal repeat
Mca	mycothiol *S*-conjugate amidase
MscR	mycothiol-*S*-nitrosoreductase/-formaldehyde reductase
MSH	mycothiol
MSNO	*S*-nitrosomycothiol
MSSM	mycothiol disulfide
Msr	methionine sulfoxide reductases
Mtr	mycothiol reductase
NAC	N-acetylcysteine
NFκB	nuclear factor κB
NO	nitric oxide
NOX	NADPH oxidase
nPrx	nuclear peroxiredoxin
Plrx	plasmoredoxin
Prx	peroxiredoxin
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
Tat	transactivator protein
TNF	tumor necrosis factor
TPx	thioredoxin peroxidase
Trx	thioredoxin
TrxR	thioredoxin reductase

13.1 Introduction

Oxidative stress is not a disease, but an unspecific pathological state that can be worsened by depletion of antioxidants, immunosuppressants, zinc and selenium deficiency, xenobiotics, and infections, thus being involved in the pathogenesis of a variety of diseases (Stehbens 2004). Mammalian cells of the immune system use the production of reactive oxygen and nitrogen species (ROS, RNS) to control and defend themselves against infections with bacterial pathogens, parasites, or viruses. Infectious agents counteract oxidative stress derived from the host immune system by highly efficient antioxidant defense systems. Inhibiting the antioxidant

defense of parasites, bacteria, and viruses is an intensely studied strategy in order to develop effective anti-infectious drugs. AIDS (HIV), tuberculosis (*Mycobacterium tuberculosis*), and malaria (*Plasmodium falciparum*) are the three leading infectious diseases worldwide. In their pathogenesis, oxidative stress plays a major role, which will be comprehensively discussed in this review.

13.2 Oxidative Stress in HIV/AIDS

According to the WHO, 2.7 million people were newly infected and 34 million people were living with the human immunodeficiency virus (HIV) in 2010 (WHO 2011a). HIV infection leads to a complex disease with broad clinical symptoms and complex pathogenic mechanisms. Dysregulation of the immune system is caused by a constant decline of $CD4^+$ T cells (Pantaleo et al. 1993), which has been intensely studied. ROS and oxidative stress are critically involved in the pathogenesis of HIV infection, with the most important aspects being discussed in the following paragraphs.

13.2.1 Mechanisms of Oxidative Stress in the Pathogenesis of HIV Infection

A role of ROS in viral infections had already been described in 1970 (Belding et al. 1970) and has been intensely investigated since that time. Oxidative stress, ROS, and antioxidant defense systems appear to be implicated in many aspects of HIV infection and AIDS such as immune function, inflammatory response, virus replication, and apoptosis. Humans infected with HIV show chronically increased levels of oxidative stress, decreased concentrations of antioxidants, and perturbations in the antioxidant systems (Gil et al. 2010). Furthermore, high plasma concentrations of hydroperoxides and malondialdehyde were found in HIV patients, indicating increased lipid peroxidation (Suresh et al. 2009). The activation of $CD4^+$ T cells and monocytes/macrophages upon HIV infection is triggered by hydrogen peroxide, superoxide anion, and peroxynitrite. Moreover, HIV infection of macrophages results in enhanced production of peroxynitrite and superoxide (Elbim et al. 1999). Oxidative stress upon HIV infection can be induced by the HIV transactivator protein (Tat) and the envelope glycoprotein gp120 (Price et al. 2005). However, the mechanisms of oxidative stress induction have not been completely unraveled.

13.2.1.1 Viral Replication

Redox signaling has an important function in the regulation of viral gene transcription and replication. Oxidative stress has been found to increase HIV replication

in vitro, while antioxidants show the opposite effect and reduce virus replication (Schreck et al. 1991; Roederer et al. 1990). The promoter of HIV-1 is located in the long terminal repeat (LTR) at the 5′-end and contains binding sites for different transcription factors, including NFκB that mediate the activation of viral transcription in complex interplay with both viral and host factors (for review please see Hiscott et al. 2001). Reactive oxygen intermediates such as H_2O_2 and oxygen radicals disturb the redox balance towards oxidizing conditions and thereby activate the translocation of NFκB into the nucleus, where it binds to the HIV LTR in order to increase the expression of viral genes and replication (Schreck et al. 1991). Moreover, peroxynitrite was shown to play a major role in virus replication, since a synthetic peroxynitrite decomposition catalyst (MnTBAP) had a strong inhibitory effect on virus replication. Peroxynitrite is suggested to also mediate its effects via the NFκB pathway (Aquaro et al. 2007). Overexpression of peroxiredoxin IV in T cells deactivated HIV LTR and thus inhibited HIV transcription by regulating hydrogen peroxide-mediated activation of NFκB (Jin et al. 1997). Both degradation of IκB and binding of NFκB to the LTR are suggested to be redox-controlled. Furthermore, the HIV LTR contains binding sites for the transcription factors p53 and AP-1, which are also sensitive to redox regulation (Pereira et al. 2000).

An HIV-encoded protein required for efficient viral transcription is Tat, a transactivator protein activated upon oxidizing conditions and inhibited upon reduction, thus functioning as a redox sensor (Koken et al. 1994; Washington et al. 2010). Dysfunction of Tat leads to disturbed transcription with the formation of prematurely terminated short transcripts (Kao et al. 1987). HIV Tat protein contains a cysteine-rich domain with several CxxC motifs that form intramolecular disulfide bonds required for the transactivation activity (Koken et al. 1994). *In vitro* inhibition of HIV expression by N-acetylcysteine (NAC) might at least in part be mediated by reduction and deactivation of Tat (Washington et al. 2010). Similarly, thioredoxin reductase has been shown to act as a negative regulator of Tat transactivation activity by reducing critical disulfide bonds (Fig. 13.1) (Kalantari et al. 2008). Moreover, Tat itself enhances oxidative stress by increasing the expression of tumor necrosis factor (TNF α/β) and by repressing the expression of antioxidant defense enzymes such as manganese superoxide dismutase, glutathione peroxidase, and γ-glutamylcysteine synthetase (Romani et al. 2010; Buonaguro et al. 1994; Flores et al. 1993; Richard et al. 2001; Choi et al. 2000). Therefore, Tat contributes to the oxidizing environment during HIV infection by decreasing the levels of several antioxidant enzymes.

13.2.1.2 Apoptosis

HIV manipulates apoptotic pathways of host cells by multiple factors in order to kill non-infected inflammatory or immune cells and infected $CD4^+$ T cells by apoptosis (reviewed in (Gougeon 2005)). Balancing of ROS and redox signaling are suggested to be critically involved in HIV-induced apoptosis (Agrawal et al. 2007; Romero-Alvira and Roche 1998; Buccigrossi et al. 2011; Banki et al. 1998). HIV infection

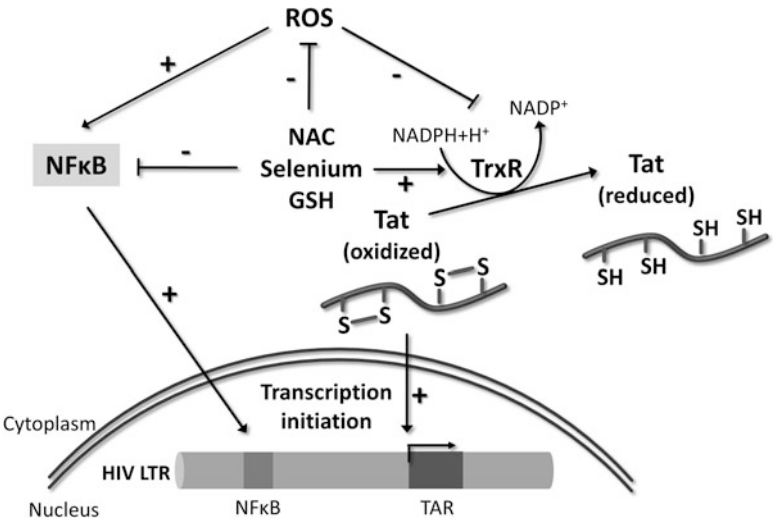

Fig. 13.1 Scheme of the influence of reactive oxygen species on HIV transcription and replication. Increased cellular concentrations of reduced glutathione (GSH) and supplementation with selenium or N-acetylcysteine (NAC) decrease the concentrations of reactive oxygen species (ROS) and activate antioxidant enzymes such as thioredoxin reductase (TrxR). ROS activate NFκB-dependent gene expression, which is decreased under reducing conditions. HIV transactivator protein (Tat) is inhibited by reduction of critical disulfide bonds. TrxR has been shown to reduce Tat and thereby inhibits Tat-mediated HIV transcription (modified after Kalantari et al. 2008)

leads to an increase in mitochondrial ROS that subsequently trigger apoptosis together with other agents such as viral proteins (Tat, gp120) and cytokines (TNFα). Moreover, caspases are cysteine-dependent enzymes and are thus sensitive towards the redox status of the cell (Hampton and Orrenius 1998).

Members of the thioredoxin (Trx) family including Trx itself, glutaredoxin (Grx), and peroxiredoxins have been discussed in the context of apoptotic signaling during HIV infection (Masutani et al. 2005). Trx is upregulated in the plasma of HIV-infected patients (Nakamura et al. 1996), and is known to inhibit the activity of apoptosis signal-regulating kinase 1 (ASK1) under reducing conditions, and thereby blocks induction of apoptosis, an effect that is reversed under oxidative stress and subsequent oxidation of Trx (Saitoh et al. 1998). In contrast, thioredoxin-dependent peroxidases, peroxiredoxins, are able to inhibit apoptosis by scavenging hydrogen peroxide (Kim et al. 2000).

Recently, it has been reported that HIV Tat protein can directly mediate apoptosis in enterocytes via a redox-dependent mechanism, leading to damage of the intestinal mucosa (Buccigrossi et al. 2011). Similarly, ROS have been shown to be crucial for HIV Tat-induced apoptosis in neurons (Agrawal et al. 2007). Tat-induced apoptotic signaling is suggested to be based on an increase in intracellular ROS concentrations and an imbalance in the GSH/GSSG ratio (Buccigrossi et al. 2011; Agrawal et al. 2007; Choi et al. 2000). Moreover, NAC not only prevents oxidative stress by

balancing ROS and GSSG concentrations, but also appears to inhibit Tat-induced apoptosis (Buccigrossi et al. 2011).

13.2.2 Antioxidant Systems During HIV Infection

Several clinical studies showed that the redox balance in HIV-infected patients without highly active anti-retroviral therapy (HAART) is severely disturbed, with perturbations affecting almost all components of the antioxidant defense system including glutathione, tocopherol, ascorbate, selenium, the thioredoxin system, superoxide dismutase, and glutathione peroxidase (Gil et al. 2010), as summarized in the following paragraphs.

13.2.2.1 Thioredoxin Family

In order to enter host cells, HIV requires the viral glycoprotein gp120, which undergoes conformational changes depending on the reduction of disulfides. Reduction of gp120 can be catalyzed by Trx1, Grx1, and protein disulfide isomerase, thus demonstrating that the host redox system is required for virus entry into host cells (Reiser et al. 2012; Azimi et al. 2010; Auwerx et al. 2009).

Dysregulation of the Trx system appears to be involved in apoptosis in HIV-infected cells as outlined above. *In vitro*, HIV infection of T cell lines decreased the expression of Trx3 shortly after infection (Masutani et al. 2005). Similarly, Trx levels were decreased in monocytes from asymptomatic untreated patients, while Trx expression in cells from AIDS patients was shown to be at a higher level when compared to uninfected cells, which has been suggested to limit ROS levels and apoptosis at later disease stages (Elbim et al. 1999). Thioredoxin reductase 1 (TrxR1) negatively regulates the activity of Tat and thereby Tat-dependent transcription in human macrophages by reducing two disulfide bonds within the cysteine-rich motif of Tat (Kalantari et al. 2008). Inhibition of Grx1 activity by anti-Grx antibodies blocks HIV replication *in vitro*, most likely by inhibiting HIV entry into $CD4^+$ T cells (Auwerx et al. 2009).

Peroxiredoxins reduce hydrogen peroxide by using Trx as an electron donor. In HIV-infected T cell lines, peroxiredoxin IV is downregulated, while T cells overexpressing peroxiredoxin IV show a decreased HIV transcription (Jin et al. 1997). Altogether, these results show that the Trx system is intriguingly involved in HIV infection.

13.2.2.2 Glutathione

In HIV-infected patients, reduced levels of glutathione were found in the plasma, lymphocytes, monocytes, as well as in $CD4^+$ and $CD8^+$ T cells (Eck et al. 1989;

de Quay et al. 1992; Roederer et al. 1991; Buhl et al. 1989). Low levels of reduced glutathione (GSH) are associated with a poor survival of HIV-infected patients (Herzenberg et al. 1997). The mechanisms responsible for impaired GSH production and GSSG reduction upon HIV infection are unclear. Depletion in GSH is accompanied by increased concentrations of oxidized glutathione, indicating a shift in the GSH:GSSG ratio (Aukrust et al. 1995), which has been confirmed in infected macrophages (Morris et al. 2012). Progressively depleted intracellular GSH levels are at least partially mediated by reduced GSH synthesis, since HIV Tat protein decreases transcription and protein levels of γ-glutamylcysteinyl synthetase (Choi et al. 2000). This was supported by a recent study showing that expression of GSH biosynthesis enzymes is decreased in HIV patients, which was attributed to chronically enhanced production of inflammatory cytokines (IL-1, IL-17, and TNF-α) that might interfere with GSH biosynthesis (Morris et al. 2012).

13.2.2.3 Superoxide Dismutase and Catalase

The first enzyme in the defense against superoxide anions is superoxide dismutase (SOD), which converts superoxide anions into hydrogen peroxide, which is subsequently reduced by catalase. The expression of mitochondrial manganese superoxide dismutase (MnSOD) is decreased in HIV-infected patients, with the effect being mediated by HIV Tat protein followed by increased oxidative stress with enhanced protein carbonylation and lipid peroxidation (Flores et al. 1993). In contrast, the expression of cytosolic CuZnSOD in macrophages is increased during HIV infection *in vitro*, most likely to counteract elevated superoxide anion concentrations (Delmas-Beauvieux et al. 1996). Hydrogen peroxide produced by SOD is scavenged by catalase, which shows an increasing activity with progressing HIV infection, and most likely compensates GSH deficiency in HIV-infected cells (Leff et al. 1992).

13.2.3 Antioxidants in the Treatment of AIDS

Decreased concentrations of antioxidants observed in HIV-positive patients are associated with deficiencies of micronutrients with antioxidant properties such as vitamins C and E, thiamine, selenium, and zinc. There are many indications that supplementation with antioxidants can have a beneficial therapeutic effect for HIV-infected patients (reviewed e.g. in Lanzillotti and Tang 2005; Singhal and Austin 2002).

13.2.3.1 N-Acetylcysteine

Enhancing cysteine bioavailability in order to increase GSH concentrations is most likely the major effect of the antioxidant NAC. Oral supplementation with NAC was reported to increase the glutathione pool in plasma and muscle (Atkuri et al. 2007).

NAC has been found to efficiently inhibit HIV replication. NAC is a potent inhibitor of NFκB by counteracting the effect of ROS (Staal et al. 1990), but has also been shown to block the TNFα-stimulated replication of HIV-1 (Roederer et al. 1990). Moreover, treatment with NAC can prevent Tat-induced apoptosis by restoring the GSH:GSSG ratio *in vitro* and *ex vivo*, and thereby protects the intestinal mucosa (Buccigrossi et al. 2011). An increase in GSH can be achieved by both NAC and glutamine supplementation, with NAC supplying cysteine and glutamine by delivering glycine (Borges-Santos et al. 2012). Similar to NAC, glutamine supplementation enhances plasma GSH levels and significantly increases lean body mass in patients with HIV infection (Borges-Santos et al. 2012).

13.2.3.2 Selenium

Selenium deficiency is strongly associated with disease progression and mortality in HIV-infected patients. Selenium supplementation of HIV-infected individuals improves the CD4 count, suppresses the virus load, decreases anxiety, and reduces the need for hospitalization (Hurwitz et al. 2007; Shor-Posner et al. 2003; McDermid et al. 2002). However, information on how selenium influences HIV infection is limited. The effect is most likely mediated via selenoproteins such as TrxR and glutathione peroxidase that shift the redox balance towards a reducing environment and thereby inhibit Tat and thus HIV transcription as explained above (Kalantari et al. 2008).

13.3 Oxidative Stress in Tuberculosis

Tuberculosis infected 8.8 million and killed 1.4 million people in 2010 and is after HIV the second leading cause of death from an infectious disease worldwide (WHO 2011b). *Mycobacterium tuberculosis*, its causative agent in humans, infects and multiplies in lung macrophages of the host. In immunodeficient individuals (e.g., HIV-positive individuals), *M. tuberculosis* can multiply to high numbers and induce an active disease. In contrast, the immune system of immunocompetent individuals is in most cases able to control the infection, and more than 90% of the infected remain asymptomatic (Lawn and Zumla 2011). Despite the bactericidal environment, *M. tuberculosis* is able to survive in macrophages, a fact that demonstrates the adaptation of the pathogen to the oxidant burden in the host.

13.3.1 *Mechanisms of Oxidative Stress in the Pathogenesis of Tuberculosis*

The host immune system is able to influence the virulence of *M. tuberculosis* by producing ROS and RNS. As a reaction towards phagocytosis of *M. tuberculosis*, macrophages can induce the expression of NADPH oxidase (NOX) and nitric oxide synthase 2 (iNOS, NOS2) and thereby increase the oxidative burden. NOX generates superoxide by catalyzing the one-electron reduction of O_2 (Leto and Geiszt 2006). iNOS produces $NO^{\bullet}$, which can react with superoxide to form highly reactive peroxynitrite and can kill mycobacteria (Shiloh and Nathan 2000). iNOS expression and release of RNS are suggested to be important for controlling the virulence of *M. tuberculosis* in humans. A range of studies based on murine models of tuberculosis using iNOS inhibitors and iNOS-deficient mice show that iNOS and RNS are required in order to control tuberculosis infection (MacMicking et al. 1997; Chan et al. 1995; Scanga et al. 2001). Moreover, NO appears to be important for latent tuberculosis infection in mice and prevents reactivation together with RNS-independent mechanisms (Flynn et al. 1998). Contrarily, another study reported no influence of a lack of iNOS on tuberculosis infection (Jung et al. 2002). Similarly, the function of ROS produced by NADPH oxidase in controlling *M. tuberculosis* is controversially discussed. Experiments using NOX-deficient mice models showed that the absence of NOX-produced superoxide increased bacterial growth during an early infection stage (Cooper et al. 2000; Adams et al. 1997). In contrast, another study did not show any differences between a murine knockout model lacking NOX and wildtype mice in ability to control *M. tuberculosis* infection (Jung et al. 2002). However, clear experimental evidence on the ROS/RNS-based mechanisms of infection control in humans is rare. Alveolar macrophages from *M. tuberculosis*-infected patients show increased concentrations of iNOS compared to non-infected individuals (Nicholson et al. 1996). Several single nucleotide polymorphisms of the *nos2a* gene in African Americans show associations with susceptibility to tuberculosis, thus supporting the role of iNOS for pathogenesis of tuberculosis in humans (Velez et al. 2009; Gomez et al. 2007).

13.3.2 *Antioxidant Defense Systems of* Mycobacterium tuberculosis

Several studies reported a remarkable resistance of *M. tuberculosis* towards oxidative stress both *in vitro* and *in vivo*; the bacterium can tolerate H_2O_2 concentrations up to 10 mM (Voskuil et al. 2011). In contrast to enteric bacteria, *M. tuberculosis* shows very low but differential transcriptional responses after exposure to a range of H_2O_2 and NO concentrations, and is resistant to DNA damage-mediated killing by H_2O_2 (Voskuil et al. 2011; Garbe et al. 1996). Low H_2O_2 or NO concentrations lead to an induction of few H_2O_2-/NO-responsive genes such as those encoding proteins

involved in H_2O_2 scavenging, repair mechanisms, and iron acquisition (Voskuil et al. 2011). Similarly, treatment with cumene hydroperoxide also did not have a major impact on gene transcription (Garbe et al. 1996). Interestingly, the expression of many genes involved in antioxidant defense did not show major changes after H_2O_2 or NO exposition indicating that they are constitutively expressed at a high level (Voskuil et al. 2011). Several antimycobacterial drugs, such as ethionamide, isoniazid, and the nitroimidazopyran PA-824, have to be reduced for activation and thus depend on the intracellular redox state. Moreover, an increased NADH/NAD^+ ratio, mutations in NADH dehydrogenase, and mutations in mycothiol biosynthesis have been related to resistance against isoniazid (Vilcheze et al. 2005; Xu et al. 2011; Miesel et al. 1998). Therefore, understanding the redox homeostasis of *M. tuberculosis* is important for drug identification strategies. As an intracellular pathogen, *M. tuberculosis* employs different antioxidant systems to counteract oxidative stress of the host defense system, as discussed in the following paragraphs.

13.3.2.1 Response Towards Oxidative Stress

M. tuberculosis shows an unusually low transcriptional response towards oxidative and nitrosative stress when compared to other enteric bacteria (Garbe et al. 1996). In different bacteria, transcriptional response towards oxidative stress is regulated by the transcription factors OxyR and SoxR, which modulate transcription in response to peroxide and superoxide, respectively. Both regulate the expression of a variety of genes, including a range of redox-active proteins (summarized in (Zahrt and Deretic 2002)). OxyR from *M. tuberculosis* is nonfunctional and is not able to sense peroxide stress. Several mutations (deletions and frameshifts) in the *oxyR* gene render the transcription factor inactive, which impairs the oxidative and nitrosative stress response in *M. tuberculosis* (Deretic et al. 1995; Sherman et al. 1995). Moreover, the *soxRS* regulon is missing in the genome of mycobacteria (Cole et al. 1998).

In *M. tuberculosis*, oxidative stress response can be mediated by the regulator *furA* and catalase-peroxidase KatG, which are mutated and dysfunctional in human pathogenic *M. leprae*. *furA* is located upstream of the catalase-peroxidase *katG*, and is supposed to act as a negative regulator of *katG* expression (Zahrt et al. 2001; Pym et al. 2001). This regulation was suggested to be important for virulence of *M. tuberculosis*, since *M. tuberculosis* mutants lacking *katG* showed a severely impaired persistence in mice and guinea pigs (Li et al. 1998). Moreover, KatG is required for the conversion of the prodrug isoniazid into its active form (Zhang et al. 1992).

The expression of *katG* can also be negatively regulated in a FurA-independent manner by the transcriptional regulator OxyS. Via a cysteine residue in its DNA-binding domain, OxyS binds directly to the katG promoter region, with the DNA-binding capacity being diminished upon oxidation of OxyS. Mycobacteria overexpressing OxyS show an increased susceptibility towards oxidative stress, thereby indicating that OxyS functions as a redox sensor (Li and He 2012).

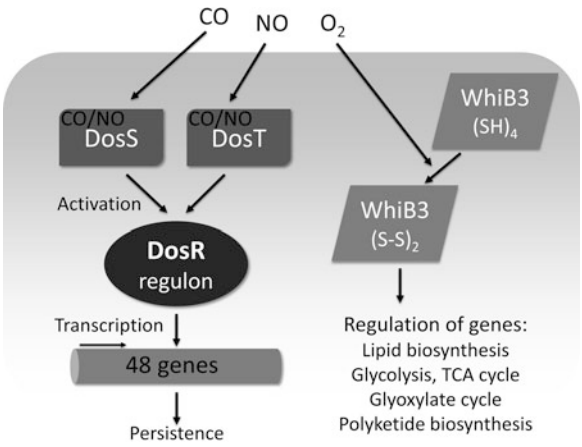

Fig. 13.2 Regulation of oxidative stress response in *Mycobacterium tuberculosis*. The DosR regulon is modulated by the histidine kinases DosS and DosT that sense NO, CO, and O_2. The DosR regulon comprises 48 genes with functions for persistence and stress adaptation. On the basis of a redox switch mechanism, WhiB3 regulates lipid biosynthesis and other central metabolic pathways involved in virulence, persistence, and redox homeostasis in response to oxidative stress (modified after Kumar et al. 2011 and Singh et al. 2009)

The DosR/S/T system (also called DevR system) is a redox-sensing system involved in virulence and persistence of *M. tuberculosis* (Leistikow et al. 2010; Shiloh et al. 2008; Park et al. 2003). The DosR regulon is controlled by DosR (DevR), a response regulator, and DosS and DosT, two sensor histidine kinases. The DosR regulon is induced by conditions that inhibit aerobic respiration, since the heme proteins DosS and DosT can sense NO, O_2, and CO by binding them to iron in their heme group (Shiloh et al. 2008). Subsequently, the DosR dormancy regulon is induced, which includes at least 48 genes suggested to play a role in persistence, latent infection, and stress adaptation (Fig. 13.2) (Park et al. 2003). The DosR regulon enables *M. tuberculosis* to follow and respond to conditions that do not allow aerobic respiration and is required for maintaining the redox balance and energy levels under anaerobic conditions (Leistikow et al. 2010).

Another redox signaling pathway in *M. tuberculosis* is controlled by WhiB3, a redox regulator that senses NO and O_2 from the host and is involved in virulence and pathogenesis of mycobacteria (Fig. 13.2) (Steyn et al. 2002). WhiB3 from *M. tuberculosis* contains four cysteine residues coordinating an iron-sulfur cluster that can specifically bind NO and O_2 (Singh et al. 2007). DNA binding of WhiB3 occurs independently of its iron-sulfur cluster but depends on the oxidation state of its four cysteines: oxidation stimulates DNA binding, while reduction diminishes it (Singh et al. 2009). Oxidation of WhiB3 stimulates DNA binding to several lipid biosynthetic genes and directly regulates production of lipids including poly- and diacyltrehaloses, sulfolipids, and triacylglycerol. WhiB3 is regarded as a redox sensor that connects host redox signals with its intermediary metabolism,

since it induces a metabolic shift to fatty acids by regulating lipid anabolism in response to oxidative stress during tuberculosis infection (Singh et al. 2009). Thus, WhiB3 maintains the intracellular redox homeostasis in part by channeling reducing equivalents into *M. tuberculosis* lipid synthesis, which modulate inflammatory cytokine production (Singh et al. 2009).

13.3.2.2 Thioredoxin System

Intracellular redox homeostasis can be maintained by the Trx family, which can catalyze thiol/disulfide exchange reactions and comprises TrxR, Trx, and thioredoxin peroxidases (TPx). *M. tuberculosis* encodes one TrxR and three Trx (A, B, and C) (Akif et al. 2008). However, no mRNA transcripts of *trxA* have been observed. This is supported by the finding that recombinant TrxA cannot be reduced by TrxR. Thus, *trxA* was suggested to be a cryptic gene in *M. tuberculosis* and is most likely not involved in antioxidant defense (Akif et al. 2008). The TrxR/Trx couple from *M. tuberculosis* is able to reduce peroxides and dinitrobenzenes, while cumene hydroperoxide can only be reduced by TrxR and not by Trx *in vitro* (Zhang et al. 1999). Moreover, TrxC is able to reduce mycothiol, GSSG, and *S*-nitrosoglutathione *in vitro*, which was suggested to be important for defense against host-derived oxidative stress (Attarian et al. 2009). The crystal structure of *M. tuberculosis* TrxC, both oxidized and in complex with an inhibitor, has been solved and can be exploited for structure-based inhibitor development (Hall et al. 2006, 2011)

Thioredoxin peroxidase (TPx) from *M. tuberculosis* shows homology to atypical 2-cys peroxiredoxins but has been characterized as a 1-cys peroxiredoxin with Cys60 being the peroxidatic cysteine (Trujillo et al. 2006). TPx can use TrxB and C as electron donors to efficiently reduce hydrogen peroxide, *t*-butyl hydroperoxide, cumene hydroperoxide, and peroxynitrite (Trujillo et al. 2006; Jaeger et al. 2004). The crystal structure of dimeric TPx revealed a Trx fold similar to that of other peroxiredoxin family members (Rho et al. 2006). *M. tuberculosis* mutants lacking TPx show an increased sensitivity towards hydrogen peroxide and nitric oxide. Moreover, they are unable to survive and grow in macrophages, cannot establish acute infections or maintain persistent infections in murine tuberculosis models, and show an attenuated virulence. This indicates that TPx is an essential component of the antioxidant defense of *M. tuberculosis* (Hu and Coates 2009).

13.3.2.3 Mycothiol

Like other actinomycetes, *M. tuberculosis* lacks detectable levels of glutathione and glutathione peroxidases and employs mycothiol (MSH, AcCys-GlcN-Ins) as its major low molecular weight thiol. MSH is a cysteinyl pseudo-disaccharide consisting of N-acetylcysteine, glucosamine, and *myo*-inositol, and was discovered in 1993 (Newton et al. 1993). In *M. smegmatis* and *M. tuberculosis*, MSH concentrations are in a low millimolar range, comparable to the concentrations of glutathione in

eukaryotes (Newton et al. 1996). MSH is required for the survival of *M. tuberculosis* (Sareen et al. 2003), but not for *M. smegmatis* (Rawat et al. 2002).

The biosynthetic pathway of MSH consists of five steps catalyzed by four enzymes, which cannot be found in eukaryotes or eubacteria and are thus discussed as highly feasible targets for chemotherapeutic interventions against mycobacteria. MshA is an N-acetylglucosamine transferase that forms 3-phospho-GlcNAc-Ins, which is converted into GlcNAc-Ins by dephosphorylation catalyzed by a phosphatase termed MshA2. The deacetylase MshB deacetylates GlcNAc-Ins to form Gln-Ins, which is then ligated to cysteine by MshC, a mycothiol ligase. Cys-Gln-Ins is subsequently acetylated by mycothiol synthase MshD generating MSH (for detailed reviews of MSH biosynthesis please see (Jothivasan and Hamilton 2008; Fan et al. 2009)).

The functions of MshA-D have been intensely investigated by gene knockout studies in *M. smegmatis* and *M. tuberculosis*. MshA and C are indeed essential for growth and survival of *M. tuberculosis* (Buchmeier and Fahey 2006; Sareen et al. 2003), while mutants lacking MshB and D are still viable (Buchmeier et al. 2006). These studies show that only the absence of MshA or C leads to complete depletion of MSH, while still 10 and 2% of normal MSH levels could be detected in the mutants lacking MshC or MshD, respectively (Sareen et al. 2003; Buchmeier and Fahey 2006; Buchmeier et al. 2003, 2006). Moreover, disruption of the MshD gene in *M. tuberculosis* results in high levels of the MshD substrate Cys-GlcN-Ins and N-formyl-Cys-GlcN-Ins, with the latter being maintained in a reduced state. Thus, N-formyl-Cys-GlcN-Ins might function as a surrogate for MSH under normal conditions, but is not sufficient under increased oxidative stress (Buchmeier et al. 2006). These studies indicate that MshA and C might be more feasible as targets for anti-mycobacterial drug development (Fan et al. 2009).

MSH exerts a range of protective functions in mycobacteria by serving as an intracellular redox buffer as a functional analog of GSH and by maintaining the redox balance. Low MSH levels in *M. smegmatis* result in a remarkably increased sensitivity towards free radicals, oxidants, alkylating agents, and antibiotics including erythromycin, rifamycin, and penicillin G (Rawat et al. 2002; Buchmeier et al. 2006). Thus, MSH is involved in protection against oxidative stress and antibiotics. In contrast, MSH-depleted mutants show a 200-fold increased resistance towards isoniazid, demonstrating a function of MSH in the drug mechanism of isoniazid (Rawat et al. 2002). Furthermore, MSH protects against electrophilic xenobiotics by forming MSH-toxin conjugates (Rawat et al. 2004).

MSH-dependent enzymes are involved in several cellular processes, including defense against ROS and RNS and detoxification of electrophilic xenobiotics (Fig. 13.3). Mycothiol disulfide (MSSM) can be reduced by the NADPH-dependent flavoenzyme mycothiol disulfide reductase (Mtr, mycothione reductase), which maintains a high MSH:MSSM ratio by the same disulfide reducing mechanism found in glutathione reductase (Argyrou et al. 2004; Argyrou and Blanchard 2004). MSH-toxin conjugates can be hydrolyzed by mycothiol *S*-conjugate amidase (Mca) forming a mercapturic acid and GlcN-Ins. While the mercapturic acid derivative can be exported from the cell, GlcN-Ins is recycled for re-synthesis of MSH (Newton

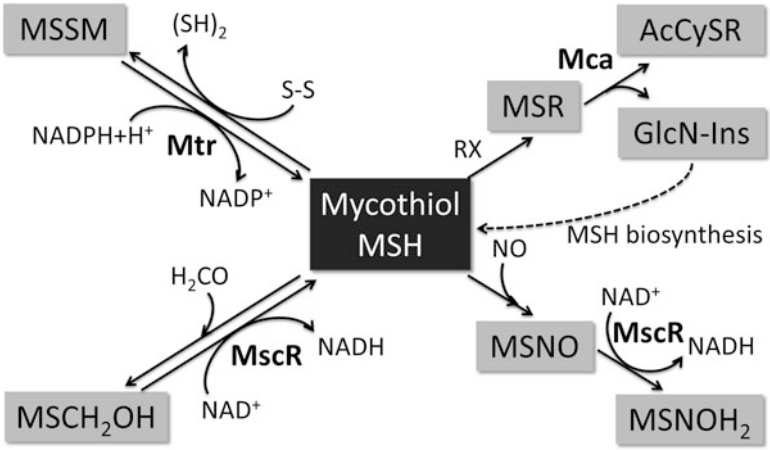

Fig. 13.3 Functions of mycothiol, the major low molecular weight thiol in *Mycobacterium tuberculosis*. Mycothiol (MSH) can reduce proteins and oxidants and is thereby oxidized to mycothiol disulfide (MSSM). MSSM is recycled by mycothiol reductase (Mtr) under consumption of NADPH + H^+. *S*-nitrosomycothiol (MSNO) can be reduced by mycothiol-*S*-nitrosoreductase/-formaldehyde reductase (MscR) to MSH sulfonamide ($MSNOH_2$). MSH can react with formaldehyde (HCOOH) to form the hemothioacetal formylmycothiol ($MSCH_2OH$), which is reduced by MscR. Thiol-reactive reagents (RX) form MSH-toxin conjugates that are hydrolyzed by mycothiol *S*-conjugate amidase (Mca) yielding a mercapturic acid and GlcN-Ins. The latter can enter the MSH biosynthesis pathway to be recycled to MSH (modified after Newton et al. 2008)

et al. 2000). *M. tuberculosis* Mca has a crucial function in drug resistance, a fact that directed interest towards the development of inhibitors against Mca (Nicholas et al. 2003). MSH can rapidly react with formaldehyde forming a hemithioacetal, which is a substrate of mycothiol-*S*-nitrosoreductase/-formaldehyde reductase (MscR) (Vogt et al. 2003). Moreover, MscR can reduce *S*-nitrosomycothiol (Vogt et al. 2003) and is required for growth of *M. tuberculosis* (Sassetti et al. 2003).

13.3.2.4 Further Enzymes of Antioxidant Defense

M. tuberculosis encodes a catalase peroxidase (KatG) that exhibits catalase, peroxidase, and peroxynitritase activity and can therefore detoxify ROS and RNS (Rouse et al. 1996; Manca et al. 1999; Wengenack et al. 1999). Experiments using transgenic murine models indicated that the major role of KatG is the defense against the oxidative burden in the host by metabolizing peroxides generated by the host phagocyte NADPH oxidase (Ng et al. 2004). In order to exhibit its antimycobacterial activity, the most effective and specific antimycobacterial drug isoniazid has to be converted from a prodrug form into its active form by KatG (Zhang et al. 1992). Mutations in the mycobacterial *katG* gene are associated with resistance to isoniazid. At least 130 known mutations of *katG* are characterized by a

decreased or diminished activity of KatG and thus reduced formation of the adduct INH-NAD (reviewed in Vilcheze and Jacobs 2007).

The peroxiredoxin-type alkyl hydroperoxide reductase (AhpC) can directly detoxify hydroperoxides and peroxynitrite (Bryk et al. 2000; Master et al. 2002). Although AhpC is considered to be a 2-cys peroxiredoxin, it involves a third cysteine in catalysis (Koshkin et al. 2004). AhpC employs the Trx-like protein AhpD, a protein with a low alkylhydroperoxidase activity of its own, as an electron donor but cannot be reduced by Trx (Hillas et al. 2000; Bryk et al. 2002). Moreover, *M. tuberculosis* codes for a 1-cys peroxiredoxin named alkyl hydroperoxide reductase E (AhpE), which is involved in peroxide and peroxynitrite detoxification (Hugo et al. 2009). In order to detoxify superoxide radicals, *M. tuberculosis* encodes two SODs, an iron-dependent SOD, and a Cu and Zn-dependent one (Zhang et al. 1991; D'Orazio et al. 2009). Two methionine sulfoxide reductases (MsrA and B) are supposed to reduce methionine sulfoxide to methionine in *M. tuberculosis*. MsrA and B appear to have redundant functions, since only a mutant lacking both genes would be more sensitive to nitrite and hypochlorite compare to wildtype (Lee et al. 2009).

13.4 Oxidative Stress in Malaria

Besides HIV/AIDS and tuberculosis, malaria is one of the world's most devastating diseases. Although the number of reported malaria cases was reduced by more than 50% between 2000 and 2010, the estimated number of malaria-related deaths is with 665,000–1,133,000 people in 2010 still high (WHO 2011c; Murray et al. 2012). Approximately 86% of malaria deaths globally were of children under 5 years of age, and 91% of the deaths were in Africa (WHO 2011c). Tropical malaria is caused by *Plasmodium falciparum*, a unicellular eukaryotic parasite that lives and multiplies both in *Anopheles* mosquitoes and humans. An infected *Anopheles* mosquito injects sporozoites into the subcutaneous tissue of the human host. The sporozoites migrate to the liver, where they develop into merozoites, which subsequently can invade red blood cells. There, the parasites undergo asexual replication until merozoites are formed and released by rupture of the red blood cell membrane. Most merozoites infect new red blood cells and thus restart the intraerythrocytic cycle leading to the typical symptoms of tropical malaria. Some parasites differentiate into the sexual forms required for transmission into the mosquito vector (for review please see Tuteja 2007; Kappe et al. 2010).

13.4.1 Sources of Oxidative Stress in Malaria Parasites

During their intraerythrocytic development, malaria parasites face high concentrations of oxygen and iron and are exposed to intense oxidative stress derived

from different sources. *Plasmodium* parasites degrade host hemoglobin as their major source of nutrients for protein synthesis, which is at the same time also a major source of oxidative stress. Hemoglobin is taken up from the erythrocyte cytoplasm into the acidic food vacuole and is systematically degraded by proteases into free heme and amino acids (Francis et al. 1997). Most of the highly toxic free heme (ferriprotoporphyrin IX) aggregates to crystalline hemozoin, the so-called malaria pigment (Stiebler et al. 2011). Alternative detoxification pathways of heme comprise heme degradation and binding to glutathione or heme-binding proteins (Loria et al. 1999; Ginsburg et al. 1998). However, small amounts of heme escape from the detoxification mechanisms and cause major oxidative damage including lipid peroxidation and DNA damage (reviewed in Kumar and Bandyopadhyay 2005). In the acidic food vacuole, free heme is oxidized from Fe(II) to Fe(III) with concomitant production of superoxide and H_2O_2 (Atamna and Ginsburg 1993). Additionally, malaria parasites have to counteract ROS released by the host immune system in order to fight the infection (Becker et al. 2004).

13.4.2 Oxidative Stress in the Pathogenesis of Malaria

Oxidative stress has been implicated in different aspects of malaria pathogenesis (reviewed in Becker et al. 2004; Hunt and Stocker 1990). Human glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy and confers resistance to infection and/or the development of severe clinical symptoms of malaria (Ruwende and Hill 1998). Protection from malaria is thought to be based on a lack of reducing equivalents in the form of NADPH, leading to oxidation of hemoglobin to membrane-associated hemochromes, which finally lead to early IgG-based detection and degradation of G6PD-deficient parasitized erythrocytes (Cappadoro et al. 1998). Moreover, a reduced multiplication rate was attributed to an intracellular accumulation of toxic oxidized molecules such as oxidized glutathione and hemozoin. Similar protection mechanisms were proposed for glutathione reductase-deficient erythrocytes (Gallo et al. 2009).

During disease manifestation, the redox balance of the patient is disturbed. The levels of glutathione, tocopherol, catalase, and superoxide dismutase in the erythrocyte and the concentrations of ascorbate and albumin in the plasma are significantly decreased in patients with malaria when compared to uninfected erythrocytes (Das and Nanda 1999; Pabon et al. 2003; Narsaria et al. 2011). Reduced α-tocopherol content in the erythrocyte membrane was associated with malaria and may contribute to erythrocyte loss and anemia in severe malaria (Griffiths et al. 2001). Moreover, increased lipid peroxidation has been reported in patients infected with *falciparum* or *vivax* malaria (Polat et al. 2002; Das et al. 1990; Pabon et al. 2003; Narsaria et al. 2011). Micronutrients including vitamins A, E, and zinc may have a beneficial effect on the severity of malaria by modulating immune response and decreasing oxidative stress (Nussenblatt and Semba 2002; Zeba et al. 2008).

The role of macrophage-derived ROS in controlling malaria infection has been controversially discussed. It has been hypothesized that phagocyte-derived ROS are involved in host immunity against malaria infections (Hunt and Stocker 1990). Superoxide produced by NADPH oxidase was reported to be involved in malaria transmission and gametocyte development but not in parasitemia patterns, as shown in murine malaria models lacking NADPH oxidase (Harada et al. 2001). Similarly, another study did not show any effect of the absence of functional NADPH oxidase and thus of phagocyte-derived ROS on parasitemia and parasite burden in murine malaria models. This indicates that ROS generated by phagocytes are not involved in controlling malaria infection (Potter et al. 2005). Furthermore, phagocyte-derived ROS are not associated with the pathogenesis of cerebral malaria in mice malaria models (Sanni et al. 1999) but have been implicated in neuronal damage in humans (Becker et al. 2004).

In uncomplicated and severe malaria, low levels of nitric oxide (NO) were detected and are associated with increased disease severity and mortality. Plasma levels of IL-10, a cytokine that suppresses NO synthesis, were increased with severity, while levels of NOS2 were decreased in cerebral malaria (Anstey et al. 1996). Moreover, severe malaria is associated with depletion of arginine, the precursor of NO, and elevated levels of plasma arginase, which catabolizes arginine (Weinberg et al. 2008). Therefore, suppression of NO was suggested to protect against severe disease rather than to contribute to malaria pathogenesis (Anstey et al. 1996).

A beneficial role of antioxidants as an adjunctive treatment of severe malaria has been discussed. Treatment of adults with NAC increased the rate of normalization of plasma lactate by a TNF-independent mechanism that was attributed to improved red cell deformability or increased GSH levels (Watt et al. 2002). However, NAC as an adjunctive treatment with artesunate did not influence the disease outcome in patients with severe *falciparum* malaria (Charunwatthana et al. 2009). The effect of NAC appears to be concentration-dependent: low doses of NAC decrease H_2O_2 levels and lipid peroxidation by increasing GSH concentrations, while supplementation with high doses of NAC had the opposite effect *in vitro* (Fitri et al. 2011). Thus, putative beneficial effects of NAC treatment remain controversial.

As explained above, NO is supposed to have a significant function in controlling malaria infection (Weinberg et al. 2008). Supplementation with L-arginine, the substrate of NO synthase and precursor of NO, leads to recovery of arginine levels and improvement of endothelial function in patients with severe malaria as shown in a Phase I trial (Yeo et al. 2008).

13.4.3 Antioxidant Defense System

In order to avoid oxidative damage and maintain a redox balance, malaria parasites employ an efficient combination of antioxidant systems based on GSH and Trx as outlined in the following paragraphs. Moreover, *Plasmodium* codes for two SODs,

which convert superoxide radicals to hydrogen peroxide (Gratepanche et al. 2002; Sienkiewicz et al. 2004). However, enzymes with major antioxidant functions in other organisms such as catalase, glutathione peroxidase, and methionine sulfoxide reductase are missing in *Plasmodium* (Sztajer et al. 2001; Clarebout et al. 1998).

13.4.3.1 The Glutathione System

Glutathione is the major low molecular weight thiol in malaria parasites and functions as a thiol redox buffer by cycling between a reduced and an oxidized form (Becker et al. 2003b). GSH is a cofactor for detoxification of electrophilic compounds and methylglyoxal (Harwaldt et al. 2002; Iozef et al. 2003), reduces the dithiol glutaredoxin (Rahlfs et al. 2001), and directly reduces a range of ROS, RNS, protein disulfides, and sulfenates (Becker et al. 2003b). GSH is synthesized by the consecutive activity of γ-glutamyl-cysteine synthetase and glutathione synthetase. A lack of the glutathione biosynthesis pathway leads to decreased GSH levels and a significant growth delay during the intraerythrocytic stage but completely blocks oocyte development in the *Anopheles* vector (Vega-Rodriguez et al. 2009). During the intraerythrocytic stage, the parasites depend on either GSH *de novo* synthesis or efficient reduction of GSSG by glutathione reductase, since a knockout of both enzymes is lethal for the parasites (Pastrana-Mena et al. 2010).

High intracellular concentrations of GSH in malaria parasites are maintained by an NADPH-dependent reaction catalyzed by the flavoenzyme glutathione reductase (GR). A range of studies examined the kinetic mechanism of GR in detail and developed selective inhibitors of the parasite enzymes (e.g. Bohme et al. 2000; Krauth-Siegel et al. 1996; Sarma et al. 2003). As a consequence of its central position in antioxidant defense, *P. falciparum* GR was regarded as a highly attractive drug target (Becker et al. 2003b, 2004). However, recent studies demonstrated that *P. berghei* GR is not essential during the intraerythrocytic stage but is required for sporogony in the *Anopheles* vector (Buchholz et al. 2010; Pastrana-Mena et al. 2010). High functional redundancy in the antioxidant network of *P. falciparum* allows reduction of GSSG by several GR-independent pathways such as direct reduction by Trx, reduction by protein *S*-glutathionylation, and by dihydrolipoamide-dependent reactions catalyzed by Grx (Becker et al. 2003b, 2004; Kanzok et al. 2000). In order to maintain low intracellular GSSG concentrations, the parasite exports GSSG into the erythrocyte cytosol (Atamna and Ginsburg 1997).

A major function of glutathione is the non-enzymatic reduction of Grx, a dithiol protein that catalyzes the reduction of a variety of proteins on the basis of a dithiol exchange mechanism (Holmgren 1989). *Plasmodium* encodes one classic dithiol Grx (i.e., PfGrx) and three monothiol Grx (Rahlfs et al. 2001; Deponte et al. 2005). PfGrx can serve as a hydrogen donor for ribonucleotide reductase (Rahlfs et al. 2001), interacts with several proteins from different metabolic pathways in *Plasmodium* (Sturm et al. 2009), and catalyzes the deglutathionylation of proteins *in vitro* (Kehr et al. 2011).

GSH is a cofactor of glutathione *S*-transferase, an enzyme that catalyzes the conjugation of electrophiles with GSH for detoxification of xenobiotics (reviewed in Eaton and Bammler 1999). *P. falciparum* GST cannot be assigned to any of the described GST classes and has been investigated in detail (Harwaldt et al. 2002; Fritz-Wolf et al. 2003).

13.4.3.2 The Thioredoxin System

Thioredoxins are central proteins in redox homeostasis and reduce a large variety of protein and non-protein substrates. Trx are directly involved in antioxidant defense and furthermore contribute to redox signaling and regulation by modulating the structure and/or activity of many proteins in response to the intracellular redox state (Arner and Holmgren 2000; Holmgren 1989). *Plasmodium* employs three Trx and two Trx-like proteins with distinct subcellular localization (Nickel et al. 2006). Cytosolic Trx1, the most intensely studied *P. falciparum* Trx, detoxifies hydroperoxides and glutathione disulfide directly, functions as an electron donor for peroxiredoxins and plasmoredoxin (see below), and regulates a variety of proteins (Nickel et al. 2006; Sturm et al. 2009; Jortzik et al. 2010).

Thioredoxin reductase (TrxR) maintains Trx in a reduced state in an NADPH-dependent reaction. TrxR regulates antioxidant defense and cell growth either indirectly by reducing Trx or directly by reducing other substrates including hydrogen and lipid peroxides, dehydroascorbate, selenium compounds, Grx, protein disulfide isomerase, and ubiquinone (reviewed in Becker et al. 2000). *P. falciparum* TrxR contains two catalytic centers each consisting of two cysteine residues, while mammalian TrxR contains a selenocysteine-cysteine in its C-terminal active site (Gladyshev et al. 1996; Williams et al. 2000). By using alternative translation initiation, *P. falciparum* expresses two isoforms of TrxR, which are located in the cytosol and in the mitochondrion (Kehr et al. 2010). *P. falciparum* TrxR was discussed as an excellent drug target (Becker et al. 2000; Nickel et al. 2006). Knockout studies demonstrated that TrxR is indeed essential for *P. falciparum* (Krnajski et al. 2002) but not for the rodent malaria parasite *P. berghei* (Buchholz et al. 2010). Whether the discrepancy is due to *in vitro* culture conditions, technical aspects, or indeed major differences in the redox metabolism of *P. falciparum* and *P. berghei* remains to be studied. However, high redundancies in the Trx and GSH systems might allow compensation for the loss of single components. Simultaneous inhibition of the two *Plasmodium* disulfide reductases GR and TrxR provides a good strategy for the development of antimalarial chemotherapeutic interventions (Buchholz et al. 2010).

Trx protects from oxidative damage by serving as an electron donor for Trx-dependent peroxidases, which catalyze the reduction of different peroxides including hydrogen peroxide, peroxynitrite, cumene hydroperoxide, and *tert*-butylhydroperoxide. According to the number of catalytic cysteines, the enzymes are clustered into 1-cys and 2-cys peroxiredoxins (Wood et al. 2003). *Plasmodium* harbors six peroxidases (recently reviewed in Gretes et al. 2012): Prx1a (TPx1) and

Prx1m (TPx2) (Nickel et al. 2005; Komaki-Yasuda et al. 2003), Prx5 (antioxidant protein, AOP) (Sarma et al. 2005), and Prx6 (1-cys peroxiredoxin) (Nickel et al. 2006) are classic peroxiredoxins. Additionally, a glutathione peroxidase-like TPx and a nuclear peroxiredoxin (PrxQ, nPrx) associated with chromatin have been described (Sztajer et al. 2001; Richard et al. 2011). Except for nPrx (Richard et al. 2011), all *P. falciparum* peroxidases prefer Trx as a reducing substrate. Additionally, malaria parasites import human peroxiredoxin 2 as an enzymatic scavenger of hydroperoxides into their cytosol (Koncarevic et al. 2009).

A *Plasmodium*-specific oxidoreductase is plasmoredoxin (Plrx), a dithiol protein belonging to the thioredoxin superfamily. Plrx can be reduced by glutathione but more effectively by Trx and Grx *in vitro*; it transfers electrons to ribonucleotide reductase and glutathione disulfide and interacts with several enzymes involved in different cellular pathways (Becker et al. 2003a; Nickel et al. 2005; Sturm et al. 2009). In *P. berghei*, Plrx is dispensable under unstressed conditions, indicating a high functional redundancy with other redox-active proteins (Buchholz et al. 2008).

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Chapter 14
Role of Oxidative Stress in Aging

D. Knoefler, H.L. Tienson, and U. Jakob

Abstract The question of why we age has given rise to many different theories over the last decades. One of the most popular and long-lasting hypothesis is the free radical theory of aging. It postulates that endogenously generated reactive oxygen species (ROS) accumulate over time, causing damage to cellular macromolecules and eventually leading to physiological decline, disease, aging, and death. Over the years, a multitude of correlative evidence has been collected in favor of this aging theory, including the discovery that aging and many age-related diseases are accompanied by substantial cellular oxidative damage. However, genetic manipulation of components of cellular antioxidant defense systems in model organisms, like *Caenorhabditis elegans*, *Drosophila melanogaster* or mice have generated conflicting results and suggested a more complex interplay between endogenous oxidants, antioxidants, and lifespan. The fact that ROS play important roles as second messengers in signaling processes, in hormesis, and during the oxidative burst in innate immune cells, likely contributes to the complexity of this issue. In this chapter, we present an overview of the most crucial experiments conducted to address the free radical theory of aging. Our conclusion is that ROS are major

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players involved in lifespan and aging but likely not (only) in their role as cytotoxic agents but as regulators of essential physiological processes in the cell.

Keywords Redox regulation • Lifespan • Reactive oxygen species • Caloric restriction • Insulin/IGF signaling • Free radical theory of aging

14.1 The Free Radical Theory of Aging

Max Rubner was the first to suggest that aging might be connected to energy metabolism after he observed that organisms with different lifespans expend the same total amount of energy (Rubner 1908). The idea that organisms have a fixed amount of "vital substances", which, when utilized faster, would shorten lifespan formed the basis of the 'rate-of-living' theory proposed by Raymond Pearl in 1921 (Pearl 1921). Although this theory was never proven to be valid, it drew attention to the concept that oxygen metabolism and lifespan might be connected. When Denham Harman realized that ionizing radiation, which induces the formation of oxygen radicals, causes biological effects that are very similar to the physiological changes that occur during aging, he postulated the 'free radical theory of aging' (Harman 1956). This hypothesis suggested that free radicals, which are generated by cells themselves, accumulate over time, leading to increased cell and tissue damage and eventually causing physiological decline and death. The suggestion that harmful reactive oxygen species (ROS) are endogenously produced was initially received with skepticism but gained acceptance with the discovery of superoxide dismutase (SOD), an enzyme whose sole function is the specific removal of superoxide from cells and organisms (McCord and Fridovic 1969). The 'free radical theory of aging' was later modified to the 'mitochondrial free radical theory of aging' (Harman 1972) to take into account the fact that mitochondria are the major source and also the major target of ROS. To acknowledge the involvement of other non-radical oxygen species, like hydrogen peroxide, Harman's theory underwent a final re-definition and is now often referred to as the 'oxidative stress hypothesis of aging' (Yu and Yang 1996).

Since the inception of the free radical theory of aging, numerous studies have been conducted providing convincing evidence that cells constantly produce ROS, not only during mitochondrial respiration but also during host defense, cell signaling and many other physiological and pathological events (Trachootham et al. 2008; Droge 2002). To counteract free oxygen radicals, aerobic organisms have evolved a number of highly efficient antioxidant defense systems, which include ROS detoxifying enzymes, small molecules ROS scavengers and oxidoreductases. These systems appear to work together to maintain a crucial balance of pro-oxidants and antioxidants within cells and sub-cellular compartments, a process commonly referred to as redox homeostasis (Finkel and Holbrook 2000). Shifting the equilibrium towards more oxidizing conditions (i.e., oxidative stress) either by increasing the levels of pro-oxidants or by decreasing the cell's antioxidant capacity,

leads to the toxic accumulation of ROS, which damage cellular macromolecules, including nucleic acids, lipids and proteins. Oxidative damage has been associated with aging as well as many age-related conditions, including cancer, diabetes, artherosclerosis, cardiovascular diseases and a variety of neurodegenerative diseases (Barnham et al. 2004; Ceriello and Motz 2004; Victor et al. 2009; Reuter et al. 2010). Yet, despite the wealth of studies that have been conducted to test the free radical theory of aging, the jury is still out on whether radical formation is the primary cause of aging or represents a secondary effect of aging and age-related diseases. This is in part due to the recent realization that ROS are not toxic *per se*. In fact, it is now clear that cells need to maintain certain levels of oxidants to be able to differentiate, develop and to overall function properly (Finkel and Holbrook 2000). Many physiological processes, including cell signaling (Finkel 2011b; D'Autreaux and Toledano 2007; Ghezzi et al. 2005), protein folding (Kakihana et al. 2012; Margittai and Banhegyi 2010), development (Hernandez-Garcia et al. 2010), and immune response require the presence of certain levels of oxidants (Finkel 2011a). These findings imply that while shifting the redox balance towards pro-oxidants is clearly toxic to the cell, shifting the redox balance towards antioxidants might possible not be beneficial either, as it will interfere with the physiological role that low ROS levels play in cells and organisms. In the following chapter, we will summarize the current view on the role of ROS in aging and age-related diseases, attempting to provide a balanced assessment of the most popular aging theory postulated thus far.

14.2 Interplay of Oxidants and Antioxidants

14.2.1 Physiological Occurrence of Reactive Oxygen Species

14.2.1.1 Oxidant Generation in the Mitochondrial Electron Transport Chain (ETC)

The electron transport chain (ETC) in mitochondria is generally considered to be the major source of ROS in the eukaryotic cell (Cadenas and Davies 2000). Mitochondria produce the energy to oxidatively phosphorylate ADP, utilizing an electrochemical proton gradient, which is generated by a series of redox reactions located in the inner membrane. In a stepwise reaction catalyzed by four enzyme complexes (I–IV), electrons are passed from NADH to the more electronegative electron acceptor oxygen. Three of the complexes (I, III, and IV) also function as proton pumps, which utilize the energy released from the electron transport chain to transfer protons from the matrix into the intermembrane space. The proton gradient is subsequently utilized by complex V (ATP synthase) to drive ATP production. Over 95% of inhaled oxygen is used in this process (Cadenas and Davies 2000). Although very efficient and tightly regulated, the electron transport chain can lead to

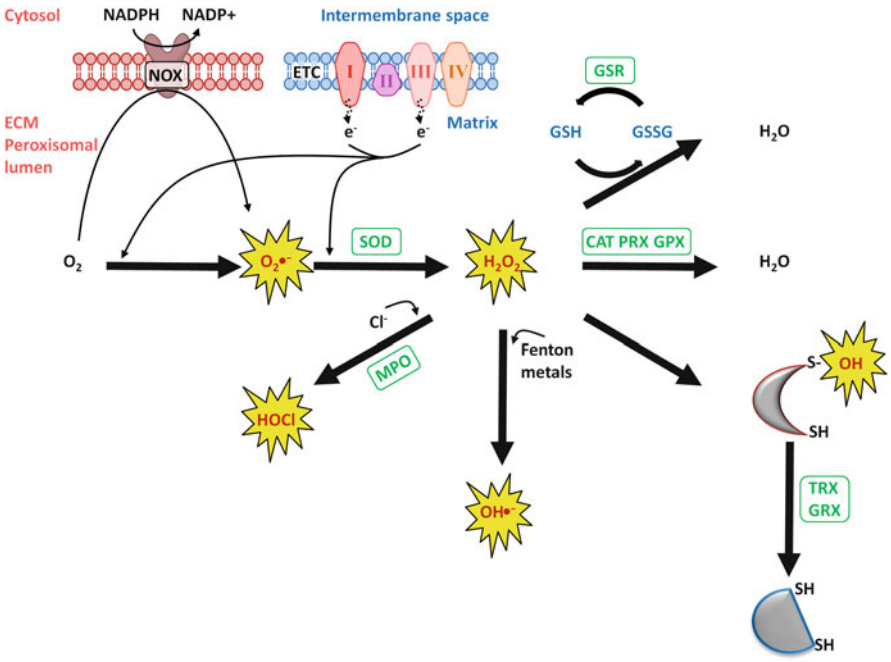

Fig. 14.1 Interplay between oxidants and antioxidants. Reactive oxygen species (ROS) are produced by members of the electron transport chain (ETC), located in the inner membrane of the mitochondria, and by transmembrane NADPH oxidases (NOXs), located in plasma and peroxisomal membranes. Electrons, which constantly leak during the electron transport chain, react with molecular oxygen to form superoxide or hydrogen peroxide (H_2O_2). NADPH oxidases utilize cytosolic NADPH to generate superoxide ($O_2\bullet^-$) either in peroxisomes or the extracellular matrix (ECM). Superoxide is rapidly dismutated to the slow-acting hydrogen peroxide (H_2O_2) in a process that is catalyzed by superoxide dismutase (SOD). H_2O_2 can react with chloride ions to generate the very potent oxidant hypochlorous acid (HOCl), in a process that is catalyzed by myeloperoxidases (MPO) within phagocytes. HOCl is a fast acting oxidant, targeting sulfur-containing amino acids and causing widespread protein aggregation *in vivo*. In the presence of Fenton metals (i.e., iron, copper), peroxide rapidly forms highly reactive hydroxyl radicals ($OH\bullet^-$), which react with and potentially destroy all cellular macromolecules in their vicinity. To detoxify H_2O_2, cells utilize a combination of enzymatic clearance systems, consisting of catalase (CAT), peroxiredoxin (PRX), and glutathione peroxidase (GPX), as well as non-enzymatic small molecule scavengers. One of these scavengers is the small tripeptide glutathione (GSH), which becomes oxidized to GSSG in the process. Regeneration of GSH is achieved by glutathione reductase (GSR). Other peroxide scavengers are surface thiols in proteins, which undergo sulfenic acids (SOH) formation (Hansen et al. 2009; Murphy 2012). Sulfenates are either directly reduced by the thioredoxin (TRX) system or undergo S-glutathionylation, which is reversed by the glutaredoxin (GRX) system

mono- or bivalent reduction of oxygen under physiological conditions, giving rise to superoxide anions and hydrogen peroxide, respectively (Cadenas and Davies 2000; Klotz and Sies 2009) (Fig. 14.1). It is estimated that up to 2% of the molecular oxygen used in mitochondria escapes in form of superoxide anion radicals (Chance and Williams 1956), with complex I and III considered to be the main superoxide

producers (Turrens 1997). Not surprisingly, the generation of superoxide and hydrogen peroxide is thus dependent on the mitochondrial metabolic state. Excess of dietary substrates or decreased ATP production due to lack of ADP will stall the flow of electrons through the ETC, which increases electron leakage and hence ROS formation. In contrast, decreasing the metabolic rate by reducing the amount of substrate intake is thought to reduce electron leakage and thus to minimize superoxide and peroxide generation in mitochondria (Heilbronn and Ravussin 2003).

14.2.1.2 Oxidant Production by NADPH Oxidases and Dual Oxidases

NADPH oxidases (NOX) and dual oxidases (DUOX), which are universally distributed in cells and organisms, generate ROS upon exposure to a variety of stimuli, including growth factors, cytokines or bacterial invasion (Lambeth 2004) (Fig. 14.1). During the innate immune response, for instance, invading microorganisms are engulfed by phagocytes. This process triggers the activation of phagocytic NADPH oxidases, which produce large quantities of superoxide anions by transferring electrons from NADPH to oxygen (i.e., respiratory burst). Superoxide radicals are then converted to hydrogen peroxide, which is either directly released into the phagosome of phagocytes or used by myeloperoxidases in neutrophils (a subgroup of phagocytes) to form the potent antimicrobial hypochlorous acid (the active ingredient of household bleach). Dysfunction of phagocytic NADPH oxidase has been implicated in a number of inheritable immunodeficiency's, such as chronic granulomatous disease (Bedard and Krause 2007), which is characterized by the inability of the innate immune system to kill invading pathogens due to a failure to produce sufficient amounts of ROS (Lambeth 2004).

In addition to NADPH oxidases in phagocytic cells, isoforms of NADPH oxidases are involved in a host of other physiological processes. Growth factors, such as angiotensin II, platelet-derived growth factor (PDGF), or vascular endothelial growth factor (VEGF) utilize NOX-mediated ROS signaling to regulate angiogenesis and blood pressure, among other processes (Lambeth 2004; Ushio-Fukai and Nakamura 2008). Thyroidal NADPH dual oxidases, in contrast, provide hydrogen peroxide for thyroid hormone synthesis (Nauseef 2008; Dupuy et al. 1991). Typically membrane-bound, NADPH oxidases utilize cytosolic NADPH to generate superoxide either in the extracellular matrix or the lumen of intracellular organelles (Fig. 14.1). While superoxide itself is not membrane permeable, it is either transported to other cell compartments by ion channels or converted into the highly diffusible hydrogen peroxide. Many NADPH oxidases are ubiquitously expressed and are thought of being capable of generating higher ROS levels in a regulated manner than those continuously produced during respiration (Krause 2007). It is thus not surprising that increased expression and/or activity of several NOX family members has been implicated to play a key role in a number of age-related diseases, including cancer, cardiovascular diseases and neurodegenerative disorders (Bedard and Krause 2007; Krause 2007).

14.2.1.3 Oxidants as By-Products of Biochemical Reactions

In addition to reactions catalyzed by proteins of the ETC and by NADPH oxidases, many other cellular reactions have been shown to produce ROS. In peroxisomes, for example, electrons generated during the β-oxidation of long fatty acids are transferred onto molecular oxygen instead of components of the ETC, thereby producing hydrogen peroxide. Oxidative deamination of aromatic (dietary) amines and monoamine neurotransmitters, such as serotonin and dopamine, is catalyzed by monoamine oxidases (MAO) in a process that leads to the production of potentially neurotoxic by-products, including ammonia and hydrogen peroxide (Bortolato et al. 2008). Other major endogenous ROS producers belong to the heme-containing cytochrome P450 protein superfamily. Members of this family are involved in oxidizing endogenous substrates as well as a broad range of exogenous compounds, including drugs, carcinogens and other xenobiotics. Since the monoxygenation of these substrates is inefficiently coupled to the electron transfer from NADPH to cytochrome P450, it causes a continuous leakage of electrons, resulting in ROS formation even in the absence of substrates (Zangar et al. 2004). Some xenobiotic compounds such as alcohol or drugs can further increase the P450-uncoupling reaction, thereby increasing ROS generation even more. The need to maintain low intracellular ROS level has apparently resulted in the development of feedback mechanisms as the presence of high ROS levels was recently found to decrease cytochrome P450 levels (Zangar et al. 2004).

14.2.2 Antioxidants – Maintaining the Balance

14.2.2.1 Detoxification Systems and ROS Scavengers

Superoxide anions are known to spontaneously dismutate to hydrogen peroxide (H_2O_2) at a slow rate. *In vivo*, this process is massively accelerated by the presence of superoxide dismutases (SODs), which are located in the cytosol, the mitochondrial intermembrane space and matrix, as well as in the extracellular space (Fridovic 1972; Zelko et al. 2002). While H_2O_2 is less reactive and more stable than other ROS (Giorgio et al. 2007), it reacts rapidly with free ferrous (Fe^{2+}) iron in the Fenton reaction, which generates hydroxyl radicals, one of the most reactive oxygen species known. Hence, removal of peroxide is of utmost importance to avoid widespread oxidative damage. Enzymatic clearance of hydrogen peroxide is performed by catalases, glutathione (GSH) peroxidases, and peroxiredoxins (Fig. 14.1). Catalases are mainly found in the cytosol and peroxisomes, as well as in the mitochondrial matrix of some highly metabolically active tissues, such as heart and liver (Radi et al. 1991; Salvi et al. 2007). They catalyze the decomposition of two hydrogen peroxide molecules to oxygen and two water molecules, using one of the fastest turnover rates known for any enzyme (Nicholls et al. 2001). While catalases contribute to hydrogen peroxide decomposition at high hydrogen peroxide concentrations, selenocysteine-

containing GSH peroxidases work efficiently at low peroxide levels, suggesting that they serve as the predominant peroxide scavengers under physiological H_2O_2 concentrations (Makino et al. 1994). GSH peroxidases catalyze the reduction of hydrogen peroxide to water by utilizing reduced glutathione (GSH) as electron donor. GSH, a highly abundant tripeptide is oxidized in this process to disulfide-bonded GSSG, and subsequently regenerated by GSH reductase, a NADPH-dependent oxidoreductase. The third group of peroxide-detoxifying enzymes is constituted by peroxiredoxins, which compensate for their slow reaction rates with extremely high cellular concentrations, making them one of the most abundant enzymes in many organisms. The structure, function and role of peroxiredoxins in response to oxidative stress is expertly reviewed in Chap. 4 of this book.

In addition to the various antioxidant enzymes that clear reactive oxygen species, organisms have evolved various small molecules such as glutathione, metallothioneins and vitamins, which are capable of scavenging oxygen radicals. While the non-protein thiol γ-L-glutamyl-L-cysteinyl-glycine (glutathione) can act as reductant for peroxide (Fig. 14.1) and free radicals (Orrenius and Moldeus 1984), metallothioneins, which are low molecular weight metal-containing proteins, are capable of scavenging hydroxyl radicals and superoxide (Thornalley and Vasak 1985). The water-soluble ascorbate (vitamin C) scavenges oxygen radicals in aqueous solution whereas α-tocopherol (vitamin E) protects membranes from radical formation (Niki 1987).

14.2.2.2 Maintaining and Restoring Redox Homeostasis

One of the most ROS-sensitive and reactive target in the cell is the sulfur-containing amino acid cysteine. Many cysteine thiols in proteins have been shown to rapidly react with peroxide, HOCl and/or NO, thereby forming a variety of different oxidative modifications, including sulfenic acid and disulfide bond formation, mixed disulfides with glutathione (S-glutathionylation) or S-nitrosylation (Winterbourn and Hampton 2008). Because of their high sensitivity towards oxidation, cysteine thiols are also the amino acids of choice for those proteins, whose function is regulated by the redox conditions of the environment. Redox sensitive proteins are found to play roles in the majority of cellular functions, ranging from signal transduction (e.g., phosphatases and kinases) and gene expression (e.g., p53) to metabolism (e.g., GapDH) and proteostasis (e.g., Cdc-48) (Brandes et al. 2009; Kumsta et al. 2011). Oxidative modification of redox sensitive cysteines leads to the transient activation (e.g., OxyR, Hsp33, Nrf2) or inactivation (e.g., phosphatases) of a protein's function, making thiol-modifications uniquely able to fine-tune cellular pathways and response systems to the cellular redox environment.

Two highly conserved enzymatic systems, the thioredoxin system and the glutaredoxin system are responsible for maintaining redox homeostasis and reducing most forms of oxidative thiol modifications in proteins (expertly reviewed in Chap. 3). The thioredoxin system consists of the small oxidoreductase thioredoxin, which uses direct thiol-disulfide exchange reactions to reduce sulfenic

acids, disulfide bonds or S-nitrosylated cysteines in proteins (Collet and Messens 2010). Thioredoxins are then reduced by thioredoxin reductase, a selenocysteine-containing enzyme in eukaryotes, which utilizes NADPH as the ultimate electron donor. The second redox system consists of the small redox enzymes glutaredoxins, which directly interact with oxidized protein thiols. In contrast to thioredoxins, which are reduced by thioredoxin reductase, glutaredoxins are non-enzymatically reduced by reduced glutathione. Oxidized glutathione is subsequently regenerated by glutathione reductase, which, like thioredoxin reductase, uses NADPH as an electron donor (Holmgren et al. 2005).

As outlined above, both thioredoxin and glutaredoxin systems depend on reduced NADPH as electron source, making both systems and hence the cellular redox status ultimately dependent on the cellular NADPH/$NADP^+$ ratio (Schafer and Buettner 2001). The major source of NADPH within the cell is the pentose phosphate pathway, which generates two molecules of NADPH for every oxidized glucose-6-phosphate molecule. The strict dependence of the cellular redox status on NADPH explains the need for efficient re-routing of glucose from glycolysis to the pentose phosphate pathway under conditions of oxidative stress (Godon et al. 1998; Grant 2008). Oxidative modification and inactivation of key enzymes of glycolysis seem to contribute to these changes in glucose flux, illustrating how redox-sensitive metabolic enzymes play an active part in the oxidative stress defense of organisms (Brandes et al. 2011). Deficiency in glucose-6-phosphate dehydrogenase, which catalyzes the first step of the pentose phosphate pathway, results in lower intracellular NADPH/$NADP^+$ ratios and increased oxidative stress and has been associated with premature cell senescence and a number of different disease conditions (Ho et al. 2007).

14.3 Evidence in Support of the 'Free Radical Theory of Aging'

14.3.1 Oxidative Damage Increases with Age

As outlined above, the free radical theory of aging states that progressive accumulation of ROS during the lifespan of an organism contributes and potentially causes the decline in fitness and might even determine longevity (Finkel and Holbrook 2000). A large amount of correlative evidence has been collected demonstrating a significant increase in oxidative damage to nucleic acids, lipids and proteins as organisms age, using a variety of different model systems ranging from bacteria to human (Golden et al. 2002; Sohal et al. 2002; Muller et al. 2007). By far the most commonly used assay to assess oxidative damage in cells and organisms is the analysis of protein carbonylation. This irreversible oxidative modification occurs primarily on the side chains of prolines, arginines, lysines and threonines in response to oxidative stress (Dalle-Donne et al. 2003). It is commonly assayed by a variety of semi-quantitative techniques, which rely on the detection of carbonylated

side chains upon *ex vivo* derivatization (Moller et al. 2011). Unfortunately, none of the available assays are able to determine what proportion of a protein population is affected to what extent, making the effects of protein carbonylation on the physiology of an organism difficult to assess. No distinction can be drawn whether an increased signal results from an increase in the number of carbonylation sites within a few protein molecules, or from carbonylating an increasing proportion of a protein population at a single, physiologically important site. Nevertheless, it is a convenient method to monitor trends in protein oxidation and has been extensively used in aging research. Tissues of mice fed *ad libitum* showed increasing levels of protein carbonylation with age (Sohal et al. 1994) and tissues derived from individuals with premature aging diseases (e.g., progeria) exhibited higher level of protein carbonyls compared to age-matched healthy humans (Stadtman 1992). The extent of protein carbonylation has also been shown to increase during the last third of the lifespan in tissues from different organisms, including house flies, *C. elegans*, rats and humans, summarized in (Levine 2002).

14.3.2 (Some) Oxidants Accumulate with Age

In addition to evaluating the effects of oxidative stress on proteins, lipids and DNA as a read-out for intracellular oxidative damage, assays have been developed that directly measure the concentration of ROS, such as superoxide or peroxide in aging organisms. The obtained results were not always consistent with the free radical theory of aging as it was shown, for instance, that the concentration of hydrogen peroxide in *Drosophila* homogenates increases during the first trimester of their life but remains stable during the remainder of the lifespan (Sohal et al. 1990). In contrast, mitochondrial matrix hydrogen peroxide was observed to increase during aging in *Drosophila* (Cocheme et al. 2011). Peroxide levels also increased in aging *C. elegans* population as was recently demonstrated by using chromosomally encoded peroxide-specific sensor proteins (Back et al. 2012) whereas microsomal superoxide anion production actually declined from reproductive to senescent age, with long-lived mutant animals (i.e., age-1) exhibiting higher superoxide anion levels than the age-matched control animals (Vanfleteren 1993). In mice, both mitochondrial superoxide and hydrogen peroxide release from heart, brain and kidney tissues increased with the age of the animals (Sohal et al. 1994).

14.3.3 (Some) Antioxidant Levels Decrease with Age

Age-induced oxidative damage can either be caused by increased ROS production, decreased detoxification, or a combination thereof. Many studies have been conducted to assess the activity of ROS-detoxifying enzymes in young and old organisms, with the goal to either confirm or rule out the model that older animals

have lower levels of ROS-detoxifying activity than young animals, hence the accumulation of oxidative damage. As exemplified below, while such correlation does seem to exist for certain antioxidant systems in some tissues and model systems, it does not generally apply to all ROS or model systems, making a more differentiated discussion necessary.

14.3.3.1 Superoxide Dismutases

Comparative analysis of superoxide dismutase activity in kidney, brain and heart tissue of young and old mice did not reveal any significant alteration in total SOD activity (Sohal et al. 1994). Similarly, activity of Cu/ZnSOD in liver homogenates of mice between 4, 12, or 18 months of age appeared unchanged while analysis of MnSOD activity revealed even an increase of SOD activity with age (Andziak et al. 2005). These results suggested that SOD activity levels in mice do not change with age. Cu/ZnSOD activity in brain tissues of aging rats, however, showed a gradual decline in activity, which appeared to be caused by a decrease in SOD expression levels (Semsei et al. 1991).

Studies in other model systems were consistent with the results obtained in mice and showed that SOD activity levels either remained unaltered during the lifespan (i.e., *C. elegans*) or linearly increased with age (i.e., *Drosophila* lysates) (Vanfleteren 1993; Sohal et al. 1990). Expression levels of Cu/ZnSOD, as determined by mRNA and steady state SOD analysis, remained relatively constant in aged flies (Radyuk et al. 2004). These results ruled out the possibility that a significant decrease in SOD activity and/or level was directly responsible for the oxidative damage observed in aging organisms.

14.3.3.2 Catalases, Glutathione Peroxidases and Peroxiredoxins

Studies assessing the activity of peroxide-detoxifying enzymes during the lifespan revealed a relatively consistent trend, indicating that the peroxide detoxifying capacity of organisms does indeed decrease with age. Analysis of the catalase activity in liver samples of mice, for example, showed a decline with the age of the animals (Perichon and Bourre 1995). A significantly decreased level of catalase and glutathione peroxidase activity was also observed in liver homogenates of 18 months old mice in comparison to 12 months old animals (Andziak et al. 2005), a finding that was independently confirmed for catalase in heart tissue and for glutathione peroxidase in kidney (Sohal et al. 1994). Brain tissue of aged mice, however, exhibited increased catalase and glutathione peroxidase activity (Sohal et al. 1994). This result was in contrast to brain samples of rats, which exhibited a gradual decline in catalase activity with age that coincided with a decrease in catalase mRNA (Semsei et al. 1991).

Analysis of non-rodent model systems were overall also more consistent, revealing kinetics of catalase activity in *Drosophila* that seem to follow a bell-shaped curve with higher levels of catalase in young animals as compared to older animals

(Sohal et al. 1990; Durusoy et al. 1995). In a subsequent more thorough analysis, catalase expression was shown to be both time- and tissue-specific, coinciding with pulses of ecdysteroid synthesis during development followed by a small decline as flies aged (Klichko et al. 2004). Studies in young *C. elegans* adults revealed a similar initial increase in the catalase activity and a decline as the worms aged (Vanfleteren 1993). Dramatic changes in expression level were also observed for peroxiredoxin 5 in *Drosophila*, which showed the highest expression level during embryogenesis, followed by a decline during aging (Radyuk et al. 2009). These results are largely consistent with the idea that the activity levels of peroxide-detoxifying enzymes decrease as animals age, potentially leading to the accumulation of peroxide in aging tissue.

14.3.3.3 Glutaredoxin, Thioredoxin and NADPH

To assess whether changes in the activity of the cellular redox systems contribute to the oxidative damage observed in aging organisms, expression analyses were conducted to monitor the activity of the thioredoxin and glutaredoxin system. An early study focusing on the thioredoxin system in rat kidneys reported decreasing levels of both thioredoxin and thioredoxin reductase with the age of the animals (Cho et al. 2003). The same study also found decreased levels of reduced glutathione and glutathione reductase activity in older rat kidneys as compared to young animals. These results were independently confirmed in aged rat muscles, where expression levels of both mitochondrial thioredoxin reductase and cytosolic thioredoxin were significantly reduced (Rohrbach et al. 2006). In contrast, however, levels of mitochondrial thioredoxin appeared to increase with age (Rohrbach et al. 2006). Moreover, comparative analysis of the glutathione and thioredoxin system in the heart muscle of young and old rats did not reveal any significant changes but did reveal an increase in oxidized GSSG levels, indicative of a pro-oxidant shift in the glutathione reduction potential (Jacob et al. 2010). A pro-oxidant shift in the glutathione pool has also been reported to occur in multiple other tissues of aging mice and rats, generally caused by an increase in oxidized glutathione and sometimes accompanied by a decrease in reduced glutathione. These changes tend to be most significant in liver tissues (Rebrin and Sohal 2008). Given that both glutaredoxin and thioredoxin systems rely heavily on reduced NADPH to maintain redox homeostasis, this shift in redox potential might be partially explained by a decrease in cellular NADPH levels, which has been observed to occur in the neurons of aging rats (Parihar et al. 2008). Studies in invertebrates confirmed some of the results obtained in rodents. It was shown, for instance, that the reduced glutathione pool sharply declines in older flies (Sohal et al. 1990). Moreover, caloric restriction, one of the few near-universal life prolonging measures (see Sect. 14.5.2) has been shown to partially reverse the detected changes in redox potential (Someya et al. 2010; Cho et al. 2003; Rohrbach et al. 2006). In summary, these studies provide convincing evidence that a combined decline in the cellular antioxidant capacity occurs with the age of the animal, which likely contributes to the accumulation of oxidative damage.

14.4 Do Manipulations of the Cell's Antioxidant Capacity Affect Lifespan?

After years of correlative studies, big hopes were spawned with the development of methods that enable genetic manipulations of model organisms, as they should allow direct and unambiguous testing of the validity of the free radical theory of aging. If correct, modulating endogenous ROS levels either by deleting or overexpressing specific antioxidant systems should have clear effects on lifespan. Below is a summary of the current state of affairs, based on genetic manipulation studies conducted in mice, *Drosophila* and *C. elegans*. An overview of the published results can be found in Tables 14.1 and 14.2.

14.4.1 Effects of Manipulating Superoxide Levels on Lifespan of Model Organisms

14.4.1.1 Effects of SOD Deletion

Deletion of either cytosolic Cu/ZnSOD1 or mitochondrial MnSOD2 in mice was found to significantly reduce their lifespan, illustrating the importance of preventing toxic ROS accumulation in mammals (Elchuri et al. 2005; Li et al. 1995). Deletion of cytosolic Cu/ZnSOD1 was also associated with increased oxidative damage and led to age-related loss of skeletal muscle mass and an increased incidence of liver cancer (Muller et al. 2006; Elchuri et al. 2005). Deletion of mitochondrial MnSOD2 had even more severe effects and led to early neonatal death with many animals dying within the first 16 days after birth (Li et al. 1995; Huang et al. 2001). This mortality rate increased further with higher oxygen concentrations (i.e., hyperoxia) (Asikainen et al. 2002). Yet the severity of the phenotype suggested that the decreased lifespan might be not purely due to accelerated aging. Partially depleting the levels of mitochondrial MnSOD2 by making *sod2*$^{+/-}$ heterozygous mice did not affect their resistance to hyperoxia (Asikainen et al. 2002; Tsan et al. 1998) but did increase their sensitivity towards oxidative stressors (Van Remmen et al. 2003, 2004). These animals showed increased oxidative damage of mitochondrial and nuclear DNA and a significant increase in tumor incidence and age-associated biomarkers, like cataracts and immune response. Yet, their lifespan was unaffected by the mutation (Van Remmen et al. 2003). Homozygous deletion of extracellular SOD (EcSOD) in mice was found to increase their sensitivity to hyperoxia but the lifespan of the animals appeared to not be affected (Carlsson et al. 1995; Sentman et al. 2006). These results provided experimental evidence that deletion of either cytosolic or mitochondrial SOD causes increased oxidative damage and early death in mammals, although it is still inconclusive whether these two phenotypes are directly related.

Table 14.1 Effects of deletion of antioxidant genes on stress resistance and lifespan in model organisms

Antioxidant system	Species	Stress resistance	Lifespan	Refs
Cu/Zn Superoxide dismutase				
Δsod1	Mm		↓	1,2
Δsod1	Dm	↓	↓	3
Δsod1	Ce	↓	↓↔[a]	4–7
Δsod1 + Δsod2; Δsod3; Δsod4; Δsod5	Ce	↓	↓↔	6, 7
ΔECsod	Mm	↓	↔	8, 9
Mn Superoxide dismutase				
Δsod2[Het,b]	Mm	↓	↔	10, 11
Δsod2	Dm	↓ [Het]	↓[b]	12
Δsod2	Ce	↓↔	↑↔	4, 6, 7
Δsod2 + Δsod1; Δsod3; Δsod4; Δsod5	Ce	↓	↑↔	6, 7
Δsod1Δsod2Δsod3Δsod4Δsod5	Ce	↓	↔[c]	13
Catalase				
Δcat	Mm	↔	↔[d]	14
Δcat	Dm	↓	↓	15, 16
Δctl1	Ce		↔	17
Δctl2	Ce		↓	17
Peroxiredoxin				
Δprdx1	Mm		↓	18
Δprdx2[e]	Dm	↓	↓	19
Δprdx2	Ce	↓	↓	20, 21
Δprdx5	Dm	↓	↓[f]	22
Glutathione peroxidase				
Δgpx1	Mm	↓↔	↔	23–25
Δgpx4 [Het,g]	Mm		↑	26
Thioredoxin				
Δtrx1	Ce	↓	↓	27, 28
Δtrx2 [Het,g]	Mm		↔	2
Δtrx2	Dm	↓	↓	29, 30

1 Elchuri et al. 2005, 2 Perez et al. 2009a, 3 Phillips et al. 1989, 4 Yen et al. 2009, 5 Yanase et al. 2009, 6 Doonan et al. 2008, 7 Van Raamsdonk and Hekimi 2009, 8 Carlsson et al. 1995, 9 Sentman et al. 2006, 10 Van Remmen et al. 2003, 11 Van Remmen et al. 2004, 12 Duttaroy et al. 2003, 13 Van Raamsdonk and Hekimi 2012, 14 Ho et al. 2004, 15 Mackay and Bewley 1989, 16 Griswold et al. 1993, 17 Petriv and Rachubinski 2004 18 Neumann et al. 2003, 19 Lee et al. 2009a, 20 Kumsta et al. 2011, 21 Olahova et al. 2008, 22 Radyuk et al. 2009, 23 Ho et al. 1997, 24 Fu et al. 1999, 25 Zhang et al. 2009, 26 Ran et al. 2007, 27 Miranda-Vizuete et al. 2006, 28 Jee et al. 2005, 29 Tsuda et al. 2010, 30 Svensson and Larsson 2007

Mm *M. musculus*, Dm *D. melanogaster*, Ce *C. elegans*, Het heterozygous

[a]Sod1 deletion reduces hypothermia-induced lifespan extension

[b]Homozygous deletion has severe health effects (Li et al. 1995; Huang et al. 2001)

[c]Maximum lifespan extended

[d]Observation up to 1 year of age

[e]Null mutation or RNAi knockdown, stress resistance assessed in RNAi knock-down

[f]Accelerated death during development, maximum lifespan normal

[g]Homozygous deletion embryonic lethal (Yant et al. 2003; Nonn et al. 2003)

Table 14.2 Effects of overexpression of antioxidant genes on stress resistance and lifespan in model organisms

Antioxidant system	Species	Stress resistance	Lifespan	Refs
Cu/Zn SOD overexpression				
sod1 +/− sod2	Mm		↔	1–3
sod1 (motorneurons)	Dm	↑	↑	4
sod1 (ubiquitous)	Dm	↑	↑	5
sod1 +/− sod2	Dm	↔	↔↑[a]	6–8
sod1	Ce	↓	↑	9, 10
Mn SOD overexpression				
sod2 +/− cat	Dm	↑	↑↔↓[b,c]	7, 11–14
sod2	Ce		↑	10
Catalase overexpression				
cat (mitochondria)	Mm		↑	15
cat (peroxisomes)	Mm		↔	2, 15
cat	Dm	↑	↓↔[d]	6, 11, 16
ctl1 + ctl2 + ctl3	Ce		↓[e]	9
Peroxiredoxin overexpression				
prx2 (neurons)	Dm	↑	↑	17
prx5	Dm	↑	↑	18
Thioredoxin overexpression				
trx1	Mm	↑	↑↔[f]	19, 20
trx1(neurons)	Ce		↑	21
trx2	Dm	↑	↑	22, 23

1 Huang et al. 2000, 2 Perez et al. 2009b, 3 Rando et al. 1998, 4 Parkes et al. 1998, 5 Reveillaud et al. 1991, 6 Sun and Tower 1999, 7 Sun et al. 2004, 8 Seto et al. 1990, 9 Doonan et al. 2008, 10 Cabreiro et al. 2011, 11 Mockett et al. 2010, 12 Mockett et al. 1999, 13 Bayne et al. 2005, 14 Sun et al. 2002, 15 Schriner et al. 2005, 16 Griswold et al. 1993, 17 Lee et al. 2009a, 18 Radyuk et al. 2009, 19 Mitsui et al. 2002, 20 Perez et al. 2011, 21 Miranda-Vizuete et al. 2006, 22 Svensson and Larsson 2007, 23 Seong et al. 2001

[a]Simultaneous overexpression of *sod1* and *sod2* increased the lifespan additively

[b]MnSOD has slightly decreased lifespan (4–5%)

[c]Simultaneous overexpression of *sod2* and catalase in mitochondria decreased lifespan by 43%

[d]Two *cat*+ strains were tested; while one strain did not show a change in lifespan, the other strain had a significantly decreased lifespan

[e]Deaths by internal hatching

[f]While overexpression of *trx1* in male mice significantly extended earlier part of life (maximum lifespan was unaffected), female mice showed no significant change in lifespan

Very similar results were obtained in *Drosophila*, where deletion of either cytosolic *sod1* or mitochondrial *sod2* significantly decreased lifespan (Phillips et al. 1989; Duttaroy et al. 2003). Again deletion of the mitochondrial isoform caused more severe defects, as demonstrated by the finding that a large fraction of flies died

within the first 24 h after eclosion (Duttaroy et al. 2003). Genetic manipulation of SOD levels in *C. elegans*, however, yielded a significantly more ambiguous picture, contributing to a substantial amount of controversy in the field (Doonan et al. 2008). At this point, it is unclear to what extent the presence of additional cytoplasmic and mitochondrial SOD homologues (SOD-5; SOD-3) might have affected the outcome of some of these studies. Initially it was reported that deletion of the major cytosolic Cu/ZnSOD-1 increases the sensitivity of *C. elegans* to superoxide-inducing paraquat and shortens *C. elegans'* lifespan while deletion of the mitochondrial MnSOD-2 also increased sensitivity towards oxidative stressors but did not affect lifespan (Yen et al. 2009; Yanase et al. 2009; Doonan et al. 2008). An independent study in which all five SOD isoforms of *C. elegans* were manipulated either individually or in combination also reported increased sensitivity towards oxidative stressors like paraquat and juglone for *sod-1*, *sod-2*, and *sod-3* mutants but did not confirm reduced lifespan for any of the tested mutants (Van Raamsdonk and Hekimi 2009). In fact, deletion of mitochondrial MnSOD-2 (*sod-2*) alone or in combination with other SOD isoforms was found to even extend *C. elegans*' maximum lifespan by 5–19 days (Van Raamsdonk and Hekimi 2009). This result, however, requires further confirmation as an independent study observed an increase in oxidative stress sensitivity for *sod-2* deletion mutants but did not observe any beneficial effects on lifespan (Doonan et al. 2008). In an attempt to ultimately address the question regarding the significance of SOD in the lifespan of *C. elegans*, Van Ramsdoonk and Hekimi went through the effort to construct a quintuple mutant strain, which lacked all five SOD-homologues. Importantly, this mutant strain showed the same mean lifespan as wild type worms but a significantly increased maximum lifespan (Van Raamsdonk and Hekimi 2012). The authors found that the quintuple mutant strain was highly sensitive to superoxide-inducing paraquat, heat shock and osmotic stress. Yet, the strain was more resistant to exogenous peroxide treatment than wild type strains. This increased peroxide resistance was apparently due to the massive upregulation of catalases, PRDX-2 and thioredoxin reductase 1, which seem to serve as compensatory mechanisms to deal with ROS. In summary, these results suggest that deletion of certain superoxide dismutase isoforms affects the capacity of organisms to deal with oxidative stressors, and causes a decline in fitness. It remains to be determined, however, to what extent superoxide-toxicity truly constitutes an aging factor.

14.4.1.2 Effects of SOD Overexpression and SOD Mimetics

Many gene deletions have been shown to affect the health and/or cause early death in organisms without being directly involved in regulating aging. Thus, a by far more desirable and interpretable phenotype is that of lifespan extension, which, if the free radical theory is indeed correct, should be achieved by increasing the ability of organisms to detoxify otherwise harmful ROS. This should reduce oxidative damage and hence extend lifespan. Much has been done to address this question and stimulation of superoxide detoxification can now either

be achieved genetically by overexpressing select SOD isoforms, or chemically by application of SOD mimetics, such as EUK-8 or EUK-134, which detoxify superoxide *in vivo*.

Studies performed in mice showed that the lifespan of mice was unaffected even when both mitochondrial MnSOD and cytosolic Cu/ZnSOD were simultaneously overexpressed (Huang et al. 2000; Perez et al. 2009b). It is of note, however, that Cu/ZnSOD overexpressing mice showed increased lipid peroxidation, suggesting that SOD overexpression might lead to the accumulation of peroxide in cells and tissues, hence potentially off-setting the beneficial effects of enhanced superoxide detoxification (Rando et al. 1998).

Studies in *Drosophila* were highly conflicting with results ranging from (i) 40% lifespan extension upon overexpression of human Cu/ZnSOD in fly motoneurons (Parkes et al. 1998), to (ii) mild lifespan-enhancing effect in flies overexpressing bovine Cu/Zn SOD under the control of the ubiquitous actin 5C gene promoter (Reveillaud et al. 1991), to (iii) no changes in lifespan upon overexpressing of additional copies of either *Drosophila* Cu/ZnSOD or MnSOD (Seto et al. 1990; Mockett et al. 2010), to even (iv) a reduction in lifespan in strains overexpressing mitochondrial MnSOD (Mockett et al. 1999), even when co-overexpressed with catalase (Bayne et al. 2005). It is conceivable that the different effects of SOD overexpression on *Drosophila* lifespan are due to differences in cultivation conditions. Indeed, it has been shown that administration of antioxidants does not cause an extension of mean or maximum lifespan if the control group has an optimal lifespan, while the survival could be extended by antioxidant treatment when the lifespan of the control group was sub-optimal (Kohn 1971). A beneficial effect of Cu/ZnSOD overexpression seems to occur in strains which are rather short-lived, implying that optimal SOD level already exist in wild type strains exhibiting a normal lifespan (Orr and Sohal 2003). It is also possible that expression of antioxidant enzymes affects lifespan in a tissue and/or stage-dependent fashion (Mockett et al. 1999). For instance, overexpression of either cytosolic Cu/ZnSOD or mitochondrial MnSOD initiated during adulthood using a heat shock-inducible expression system extended both mean and maximum lifespan (Sun et al. 2002; Sun and Tower 1999). However, while the inducible expression system enables researchers to work in identical genetic strain backgrounds (Sun and Tower 1999), initiation of transgene expression is based on a heat pulse, which might itself have some negative effects on those strains not overexpressing SOD (Mockett et al. 1999).

Studies in *C. elegans* revealed that despite the fact that overexpression of cytosolic Cu/ZnSOD-1 increases the levels of cellular protein oxidation and enhances the sensitivity of animals towards paraquat-induced oxidative stress, it does increase *C. elegans* lifespan (Doonan et al. 2008; Cabreiro et al. 2011). Overexpression of mitochondrial MnSOD-2 was found to also extend the lifespan of worms by approximately 25% (Cabreiro et al. 2011). A set of independent studies agreed with these results by showing that treatment of worms with the SOD/catalase mimetics EUK-8, EUK-134 or platinum nanoparticles resulted in a significantly increased mean and maximum lifespan of wild type worms, and restored wild type-like lifespan in a short-lived *mev-1* mutant (Kim et al. 2008; Melov et al. 2000). Yet these

studies were questioned by reports that showed that treatment with the same SOD mimetic (i.e., EUK-8) causes a dose-dependent shortening of lifespan, particularly at higher doses, suggesting that the effects of antioxidant interventions might be dependent on culture conditions (Keaney and Gems 2003). Moreover, while EUK-8 treatment protected worms against paraquat-toxicity, it did not extend lifespan in non-paraquat treated worms (Keaney et al. 2004). Note that treatment of worms with lower concentrations of the superoxide-generating compounds paraquat or juglone has recently been found to cause lifespan extension in worms (Heidler et al. 2010; Lee et al. 2010), suggesting that superoxide levels need to be carefully monitored in worms before final conclusions can be drawn about the efficiency of SOD mimetics in worms.

In summary, changing superoxide dismutase activity in mice, *Drosophila* or *C. elegans* either by deletion or overexpression of the respective enzymes did not reveal a clear picture about the effects of superoxide on lifespan. The results reflect a much more complex correlation between the amount of superoxide produced at distinct stages in life, the levels of antioxidants present, and the lifespan. It remains to be determined to what extent superoxide-toxicity might have contributed to the observed lifespan reduction and early deaths observed in mitochondrial MnSOD null mutants in fly and mice, and if this truly constitutes superoxide-mediated aging. Another intriguing question is whether overexpression of superoxide dismutases actually adds to cellular oxidative stress since higher SOD activity inevitably increases the hydrogen peroxide pool in the cells.

14.4.2 Effects of Altering the Hydrogen Peroxide Clearance System on Lifespan of Model Organism

The hydrogen peroxide clearance system typically consists of catalases, peroxiredoxins and glutathione peroxidases. In the following section we will summarize the effects that genetic manipulation of the individual branches of the hydrogen peroxide clearance system has on the lifespan of mice, *Drosophila* and *C. elegans*.

14.4.2.1 Effects of Catalase Deletion and Overexpression

Catalase-deficient mice develop normally and are healthy at least up to 1 year of age. They do not reveal any increased sensitivity towards hyperoxia or photochemically induced oxidative stress even though tissue slices from catalase knockout mice show slower rates in decomposing extracellular H_2O_2 (Ho et al. 2004). Mice expressing human catalase under the control of the endogenous promoter and regulatory elements, which results in peroxisome-specific overexpression of catalase similar to endogenous gene expression (Chen et al. 2003), did not show any lifespan extension even when Cu/ZnSOD was co-overexpressed (Perez et al. 2009b). In contrast, mice overexpressing the human catalase gene targeted to the mitochondria showed an

improved rate of peroxide clearance as well as reduced H_2O_2 production, decreased oxidative damage, and delayed development of cardiac pathology. Most importantly, these mice showed an extension of both median and maximum lifespan by 5 and 5.5 months, respectively (Schriner et al. 2005). These results provide first evidence that increased peroxide clearance from mitochondria might have beneficial effects on mammalian lifespan.

Drosophila mutants with catalase activities of less than 2% showed severe viability effects (Mackay and Bewley 1989). An independent study confirmed these findings, and demonstrated that the lack of catalase activity in *Drosophila* results in decreased viability and lifespan (Griswold et al. 1993). Increasing the levels of catalase in *Drosophila,* however, had either no effect on lifespan (Griswold et al. 1993; Mockett et al. 2010; Sun and Tower 1999) or caused a moderately reduced lifespan, depending in part on the insertion site of the transgene and the magnitude of catalase overexpression (Mockett et al. 2010; Sun and Tower 1999). Only when combined with the simultaneous overexpression of Cu/ZnSOD, was overexpression of catalase found to be beneficial for lifespan (Orr and Sohal 1994). This study was, however, contradicted by another group, who reported that the combined overexpression of catalase and Cu/ZnSOD did not result in a benefit beyond the lifespan expanding effect observed for Cu/ZnSOD overexpressing flies (Sun and Tower 1999).

Similar to the results in *Drosophila* where deletion of catalase significantly reduced lifespan, deletion of peroxisomal catalase 2 (*ctl-2*) in *C. elegans* was found to cause progeric phenotypes and a shortened lifespan. In contrast, deletion of the cytosolic *ctl-1* isoform had no effect on the lifespan of worms (Petriv and Rachubinski 2004). To test whether overexpression of catalase increased *C. elegans* lifespan, the *ctl-1*, *ctl-2*, *ctl-3* gene cluster was overexpressed, generating tenfold higher catalase activity (Doonan et al. 2008). Lifespan measurements did not reveal any significant effect of catalase overexpression in *C. elegans*. In fact, it increased mortality rate by internal hatching, an event that could be suppressed by the simultaneous overexpression of Cu/ZnSOD-1.

These studies suggest that animals lacking catalase accumulate high levels of hydrogen peroxide, which might contribute to a decline in fitness and causes early death at least in some model systems. Reducing peroxide levels by overexpressing catalase in mitochondria appears to have beneficial effects on mammalian lifespan but does not seem to enhance the lifespan of invertebrates.

14.4.2.2 Effects of Peroxiredoxin Deletion and Overexpression

Mice lacking cytosolic peroxiredoxin 1 (*prdx1*) exhibit increased DNA damage and a higher incidence of hemolytic anemia and cancer starting at 9 months of age, which effectively reduces their lifespan (Neumann et al. 2003). Deletion of peroxiredoxin 2 (*prdx2*) also results in hemolytic anemia and increased levels of

oxidative stress but the mutant mice seem otherwise healthy and fertile, and their lifespan has not been determined (Lee et al. 2003b). Deletion of the 1-Cys peroxiredoxin 6 (*prdx6*) results in elevated ROS level and increased oxidative damage and heightens the animal's susceptibility towards oxidative stress-mediated damage (Wang et al. 2003). These results confirm the important role that peroxiredoxins play on peroxide detoxification. To our knowledge, only one study presented lifespan data of peroxiredoxin deletion mice so far (Neumann et al. 2003). Adenovirus-mediated overexpression of PRDX6 in the lungs of mice was found to promote resistance to hyperoxia-induced oxidative stress. So far, no lifespan experiments have been reported with these mice.

RNAi knock down of *prx2* in flies resulted in an increased sensitivity to paraquat-mediated oxidative stress. Additionally, flies with a reduction in PRX2 activity, either through RNAi knockdown or a loss of function mutation, exhibited a shortened lifespan (Lee et al. 2009a). The deletion of peroxiredoxin 5 (*Prx5*) in *Drosophila* also leads to a slightly shortened mean lifespan, which appears to be mainly caused by accelerated dying during day 5–10 after eclosion. These results suggest that PRX5 might be important in proliferating tissues and maybe required for embryonic development (Radyuk et al. 2009). After development, the mortality rate of *prx5* null mutants was found to be similar to the mortality rate of wild type flies, resulting in the same maximum lifespan for both wild type and *prx5* mutants (Radyuk et al. 2009). The modest lifespan defect of *prx5* null flies was, however, severely enhanced under oxidative stress conditions (Radyuk et al. 2009). Neuronal expression of either human PRX2 or the *Drosophila* PRX2 homolog as well as ubiquitous expression of PRX5 was found to enhance the resistance of flies to oxidative stress and to extend their lifespan, providing excellent evidence that in flies, detoxification of peroxide through peroxiredoxins prolongs life (Radyuk et al. 2009; Lee et al. 2009a).

Peroxiredoxin 2 (PRDX-2), which is one of the most abundant proteins in *C. elegans*, plays a crucial role for hydrogen peroxide clearance in worms (Kumsta et al. 2011; Olahova et al. 2008). Animals lacking PRDX-2 are much more sensitive to sublethal hydrogen peroxide treatments than wild type *C. elegans,* fail to recover from exogenous peroxide treatment, and exert progeric phenotypes as well as symptoms of chronic oxidative stress (Kumsta et al. 2011; Olahova et al. 2008). Most importantly, *prdx-2* deletion worms show a severe lifespan defect, which seems most pronounced at lower temperatures (Kumsta et al. 2011; Olahova et al. 2008).

These studies show that deletion of peroxiredoxin has some of the most severe effects on the lifespan of model organisms, illustrating its importance in hydrogen peroxide clearance. Importantly, overexpression of peroxiredoxin in *Drosophila* significantly extends lifespan, which is very similar to the effects that overexpression of mitochondrial catalase has in mice. These results suggest that enhanced detoxification of peroxide might indeed be a lifespan-extending factor.

14.4.2.3 Effects of Glutathione Peroxidase Deletion and Overexpression in Mice

In addition to catalases and peroxidases, mice also contain several different glutathione peroxidases, which appear to play a prominent role in detoxifying low levels of peroxide. Mice lacking glutathione peroxidase 1 (*gpx1*) display a normal and healthy development, an unaltered capacity to decompose hydrogen peroxide, a wild type-like protein and lipid oxidation pattern and the same sensitivity to hyperoxia (Ho et al. 1997). While the mutant mice do show an increased sensitivity towards diaquat-induced oxidative stress (Fu et al. 1999) and increased cataract occurrence (Wolf et al. 2005), their lifespan is not affected by the absence of *gpx1* even in combination with a $sod2^{+/-}$ heterozygous mutation (Zhang et al. 2009). In contrast, glutathione peroxidase 4 (*gpx4*) null mutants were found to be embryonic lethal (Yant et al. 2003). Expression of human GPX-4 was able (i) to rescue the embryonic lethality of $gpx4^{-/-}$ mice, (ii) to reduce apoptosis and (iii) to increase cell survival after oxidative insult (Ran et al. 2004). Heterozygous $gpx4^{+/-}$mice showed an increased median lifespan but neither a change in maximum lifespan nor in age-related mortality rate, indicating that aging itself is not slowed in these animals (Ran et al. 2007).

14.4.3 Manipulation of the Thioredoxin/Glutaredoxin System

Genetic manipulations of the thioredoxin system are limited, yet the studies conducted so far all seem to point to the fact that maintainance of cellular redox homeostasis is crucial for development and lifespan. Deletion of either cytosolic thioredoxin 1 or mitochondrial thioredoxin 2 in mice is embryonic lethal (Nonn et al. 2003; Matsui et al. 1996). Heterozygous $trx2^{+/-}$ mice have increased ROS generation and oxidative damage compared to wild type mice but their lifespan is unchanged (Perez et al. 2008, 2009a). Overexpression of TRX-1 was found to extend lifespan of mice (Mitsui et al. 2002). This result was, however, put into question as the lifespan of the control mice was significantly shorter than expected. A more recent study by Perez et al. reported that overexpression of mouse *trx1* causes an increase in the mean lifespan of male mice without affecting their maximal lifespan. These results suggest that the observed decrease in oxidative damage is beneficial to male mice early in life but might not aid in extending lifespan later in life (Perez et al. 2011). Studies in *Drosophila* showed that flies lacking *trx-2* are short-lived while flies overexpressing *trx-2* show enhanced survival upon exposure to hydrogen peroxide or paraquat (Tsuda et al. 2010; Svensson and Larsson 2007). Moreover, a gain-of-function-mutation of *trx* conferred longevity to *Drosophila* (Seong et al. 2001). Very similar results were also obtained in *C. elegans*, where overexpression of *trx-1* was found to extend the mean lifespan of *C. elegans* (Miranda-Vizuete et al. 2006) while deletion of *trx-1* resulted in a reduced resistance to oxidative stress as well as in a shortened mean and maximum lifespan (Miranda-Vizuete et al. 2006; Jee et al. 2005).

14.4.4 Where Are We Now, What Should We Think?

The many conflicting results obtained with genetic manipulations of antioxidant enzymes in a variety of different organisms over the last few years clearly illustrate the complexity of redox homeostasis and its role in lifespan. In general, deletion of antioxidant enzymes appears to have one of two outcomes. One being so serious that the organism is severely affected – thus decreased lifespan may not be a direct result of premature aging. The other one having little to no effect at all, suggesting either significant redundancy with other antioxidant enzymes or implying that their function is so highly specialized as to not affect longevity under "normal" conditions. Hence, it is probably unwise to draw significant conclusions from deletion studies. Overexpression studies could be viewed as a more direct approach to analyze the influence of antioxidant systems on aging. However, confusion arose from studies where the same genetic manipulations revealed different effects in different labs. This suggests that specific growth conditions and/or differences in the strain background might be additional sources that influence lifespan and need to be carefully controlled and monitored.

14.5 Lifespan Extension and the Free Radical Theory of Aging – What Works and Why

14.5.1 Lifespan Extension by Manipulating the Electron Transport Chain

Manipulation of endogenous ROS production has been used as an alternative approach to assess the role of ROS in aging and lifespan. Screens for mutants with increased sensitivity towards the superoxide generator paraquat in *C. elegans* resulted initially in the identification of the mitochondrial *mev-1* mutant (Ishii et al. 1990). *Mev-1* encodes a subunit of succinate dehydrogenase cytochrome b, a component of mitochondrial complex II (Ishii et al. 1998). Mutants in *mev-1* were found to exert elevated levels of superoxide anions (Senoo-Matsuda et al. 2001), reduced Cu/ZnSOD activity levels and a significantly shortened lifespan (Ishii et al. 1990), which could be reversed by treatment with superoxide dismutase/catalase mimetics (Melov et al. 2000). Similarly, Drosophila mutants of succinate dehydrogenase subunit b (complex II) also exhibited increased levels of mitochondrial hydrogen peroxide production, enhanced sensitivity towards hyperoxia and a shorter lifespan (Walker et al. 2006). Furthermore, *C. elegans* mutants of complex I (i.e., *gas-1*) were found to have elevated levels of superoxide anion and also displayed a shortened lifespan (Kondo et al. 2005; Hartman et al. 2001). These findings seemed to support the free radical theory of aging as they suggested that the decrease in lifespan, which is observed in mitochondrial mutants is caused by increased oxidant levels (Senoo-Matsuda et al. 2001; Sedensky and Morgan 2006). More recent reports indicated, however, that the model that a compromised mitochondrial ETC

causes increased oxidant production and hence shorter lifespan is far more complex than previously anticipated. In fact, worms carrying a mutation in *isp-1*, an iron-sulfur protein of complex III, showed increased resistance towards oxidative stress, reduced oxygen consumption, and an extended lifespan, indicating that a reduction in ETC function might also positively affect the lifespan of *C. elegans* (Feng et al. 2001). RNAi-mediated screens for longevity genes further confirmed these results by finding a tenfold overrepresentation of genes involved in mitochondrial function, whose knock-down improved H_2O_2 tolerance (although paraquat tolerance was reduced) and extended lifespan (Lee et al. 2003a). Selective targeting of individual components of the ETC, including proteins of complex I, III, IV and V causes a significant extension in lifespan in *Drosophila* and *C.elegans* (Dillin et al. 2002; Copeland et al. 2009). It is of note that reducing ETC function had to be initiated during *C. elegans* development to achieve lifespan extension whereas reduction of ETC components during adulthood of *C. elegans* resulted in lowered ATP level but no changes in lifespan (Dillin et al. 2002). These results imply that the rate of mitochondrial respiration during development is at least partly responsible for adjusting *C. elegans'* growth rate, development, and adult lifespan. The developmental window during which the intervention seems to be successful ends by the third or early fourth larval stage of *C. elegans*, indicating that an event occurring during larval development might set the clock for lifespan (Rea et al. 2007). One mechanism, which seems to play a role in the lifespan extension mediated by ETC reduction is the mitochondria-specific unfolded protein response (UPR), which can be induced in a cell-non-autonomous way, meaning that signals from one tissue can trigger or control processes in other tissues (Durieux et al. 2011) (Fig. 14.2).

It is of note that many long-lived strains with mutations in the ETC, such as *nuo-6, isp-1* and *clk-1* showed elevated ROS level, which speaks clearly against the original idea that accumulation of ROS is toxic *per se* (Yang and Hekimi 2010; Lee et al. 2010). In fact, treatment of *nuo-6* and *isp-1* mutants with the antioxidant N-acetylcysteine (NAC) abolished the observed lifespan extension, suggesting that (transient) accumulation of oxidants might actually be required for the lifespan-prolonging phenotype of those mutants (Yang and Hekimi 2010). In a similar way, Yang and co-workers showed that treatment with low concentrations of the superoxide generator paraquat caused a transient superoxide boost, which significantly increased the lifespan in *C. elegans* despite an apparent increase in oxidative damage as assessed by protein carbonylation (Yang and Hekimi 2010). These results are in good agreement with the recently postulated mitohormesis theory, which will be discussed in detail later in this chapter.

Deletion of *clk-1* has been shown to increase ROS levels (Lee et al. 2010), UV-resistance (Murakami and Johnson 1996) and lifespan in worms (Wong et al. 1995). A heterozygous deletion mutant of the *clk-1* homologue in mice (*mclk1*) was found to also accumulate higher mitochondrial oxidative protein damage and, most importantly, to extend lifespan in mice (Liu et al. 2005; Lapointe and Hekimi 2008). What appears to contribute to this lifespan extending effect is the mitochondrial ROS-mediated activation of the transcription factor HIF-1α, which in

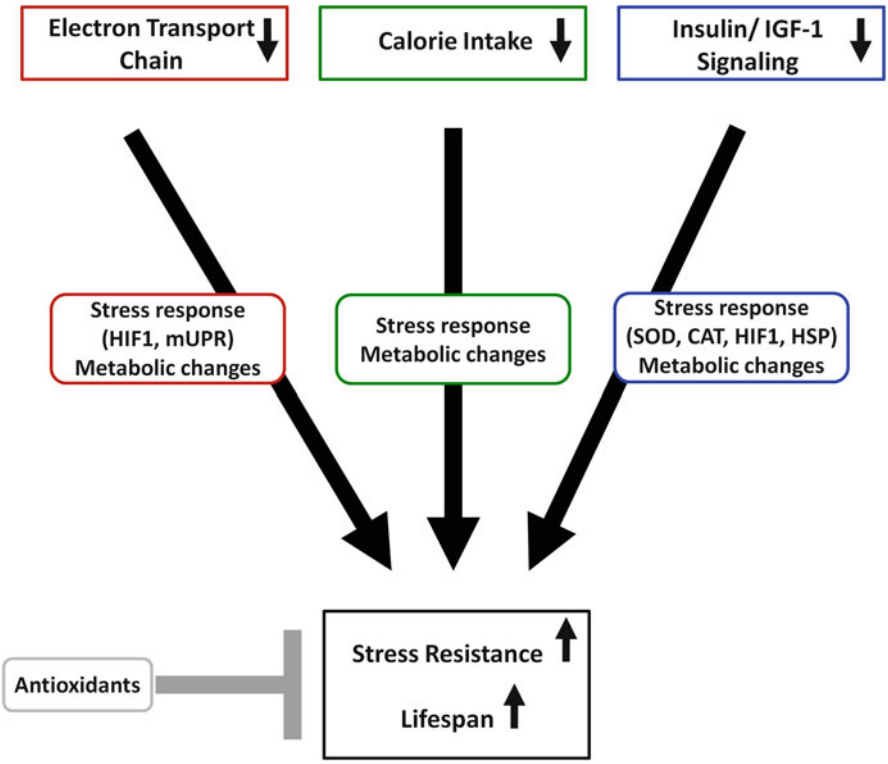

Fig. 14.2 Interventions that extend lifespan. Reduction of components of the electron transport chain (ETC), a decrease in the caloric intake, or a decline in Insulin/IGF-1 signaling extends lifespan in a variety of different organisms. One factor that might contribute to the observed lifespan extension is an increased stress resistance. The role that oxidants play in these lifespan-extending interventions is not fully elucidated yet. However, it has been demonstrated that antioxidants typically interfere with the observed lifespan extensions. Note: Some ETC mutants can also be short-lived

mice is involved in regulating the inflammatory immune response (Wang et al. 2010) (Fig. 14.2). This result led to speculations that the increased lifespan of *mclk1*$^{+/-}$ mice results from an elevated resistance to pathogenic infection especially in aging mice (Wang et al. 2010). Observations in *C. elegans* suggested that compromised respiration could be involved in the ROS-mediated activation of HIF-1, which is indeed responsible for the lifespan extension observed in *C. elegans clk-1* and *isp-1* mutants (Lee et al. 2010). Similarly, the lifespan enhancing effect observed in *C. elegans* upon administering low levels of the superoxide-generator paraquat might also work through the ROS-induced activation of HIF-1, suggesting that the HIF-1 mediated lifespan extension might be evolutionarily conserved (Lee et al. 2010).

Taken together, these studies suggest that lifespan extension achieved by reducing mitochondrial respiration is not simply caused by minimizing the output of

harmful reactive oxygen species and decreasing oxidative damage. It rather seems to involve the activation of complex pathways, including the unfolded protein response (UPR) (Durieux et al. 2011), cell-cycle checkpoint control (Rea et al. 2007), changes in HIF-1-mediated gene expression (Lee et al. 2010) and possibly a switch in energy metabolism (Rea and Johnson 2003). That respiratory mutants do not mediate their longevity by one unifying feature was also suggested by the finding that reduction of HIF-1 (either by mutation or RNAi) significantly reduced the extended lifespan of *isp-1* and *clk-1* mutants but only partially affected the long-lived phenotype of other mitochondrial mutants, such as *cyc-1* or *cco-1* (subunits of complex III and IV) (Lee et al. 2010). This finding might also explain the apparently controversial results concerning the role of ROS in lifespan extension of mitochondrial mutants. While antioxidant treatment was found to not affect lifespan extension of some mitochondrial RNAi mutants (Durieux et al. 2011), other studies reported that superoxide is in fact required to mediate lifespan extension (Yang and Hekimi 2010).

14.5.2 Lifespan Extension by Caloric Restriction

Reduction of the daily caloric intake by 30% (dietary or caloric restriction) routinely extends lifespan up to 50% in a variety of different model organisms, including yeast, flies, *C. elegans*, mice and primates (McCay et al. 1935; Fontana et al. 2010). Calorically restricted mice have been found to have lower mitochondrial generation of superoxide and hydrogen peroxide than animals fed *ad libitum* (Sohal et al. 1994; Lambert and Merry 2004; Bevilacqua et al. 2004). Similarly, animals with reduced caloric intake show lower levels of age-accompanying oxidative DNA damage and oxidative protein damage as measured by protein carbonylation, and reveal higher levels of NADPH, specifically in the mitochondria of brain, liver, heart, kidney, ear and eyes of mice (Dubey et al. 1996; Sohal et al. 1994; Someya et al. 2010). Hence, caloric restriction appears to decrease oxidative damage by shifting the glutathione pool to a more reducing redox potential relative to age-matched controls. Whereas earlier findings seemed to indicate that caloric restriction resulted in a lowered metabolic rate, hence the decrease in oxidative damage, more recent studies that were corrected for body mass suggested quite the opposite (Houthoofd et al. 2002). In fact, caloric restriction appears to enhance mitochondria biogenesis (Lopez-Lluch et al. 2006) and to increase the rate of respiration (Lin et al. 2002; Nisoli et al. 2005) (Fig. 14.2). These results, although initially counterintuitive, are fully consistent with recent studies in *C. elegans*, which showed that 2-deoxyglucose (DOG)-mediated glucose restriction during adulthood increased mitochondrial respiration and ROS production, and significantly extended the lifespan (Schulz et al. 2007). Interestingly, pre-treatment of these animals with antioxidants, such as NAC or vitamin E abolished the beneficial effect of glucose restriction on lifespan (Schulz et al. 2007). These findings led to the model of mitohormesis, in which generation of slightly elevated levels of oxidants through increased respiration during a

defined time in life enhances expression of antioxidant genes and with that the capacity of organisms to detoxify ROS. Contrary to glucose-restriction, glucose-supplementation of *C. elegans'* diet prevented longevity of *daf-2* worms, a mutant of the Insulin signaling pathway (described in 14.5.3), and shortened the lifespan of wild type animals by inhibiting the transcription factor DAF-16 (Lee et al. 2009b; Schlotterer et al. 2009). Interestingly, increased ROS generation was found at day 15 in worms fed on high-glucose-diet, suggesting that the duration of increased ROS level (or the magnitude) might make a difference in lifespan determination (Schlotterer et al. 2009).

Although it is now generally accepted that caloric restriction increases respiratory rates, the jury is still out whether ROS levels increase, stay unchanged or decrease. Several studies in calorically restricted yeast, flies or rodents observed either no change or even a decrease in ROS production accompanying increased cellular respiration (Barros et al. 2004; Bevilacqua et al. 2004; Miwa et al. 2004; Cocheme et al. 2011; Ash and Merry 2011). So while caloric restriction clearly increases respiratory rates, it might also lead to more efficient respiration possibly through mild uncoupling between electron transport and oxidative phosphorylation (Barros et al. 2004; Bevilacqua et al. 2004; Lopez-Lluch et al. 2006). A mild uncoupling of the two processes could result in an increase of ETC function, which could lead to less opportunity for electron leakage, and hence more efficient mitochondria function and less ROS formation. This conclusion of course argues against a hormetic effect in lifespan extension. However, since CR can be considered as a mild stress condition, it could lead to the observed increase in antioxidant defenses by other means.

14.5.3 Manipulation of the Insulin/IGF-1 Signaling (ILS) Pathway

Genetic manipulation of the ILS pathway has been shown to reproducibly modulate lifespan in *Drosophila*, *C. elegans* and mice (Longo and Finch 2003). As outlined below, interference with the insulin signaling pathway appears to affect levels of both oxidants and antioxidants in organisms, providing additional support for a role of ROS in lifespan determination. Insulin/IGF-1 signaling is a highly conserved pathway, which has been implicated in a multitude of physiological processes, including stress response, diapause, reproduction, metabolism, growth, and aging (Tatar et al. 2003). Signaling through the Insulin/IGF-1 receptor occurs via a phosphorylation cascade, which ultimately causes phosphorylation of the forkhead transcription factor FOXO, and prevents FOXO from its translocation into the nucleus. Conversely, lack of the IGF-1 receptor or disruption of the kinase cascade promotes FOXO's translocation into the nucleus and allows the transcriptional regulator to induce the expression of a variety of stress-related genes (Kenyon 2005). FOXO-controlled genes encode for heat shock proteins, for proteins involved in pathogen resistance, metabolism (e.g. β-oxidation of fatty acids and

gluconeogenesis), transcriptional repression and protein degradation as well as for antioxidant enzymes, such as superoxide dismutase, catalase and glutathione S-transferases (reviewed by Murphy 2006) (Fig. 14.2). These findings suggested that the lifespan extension observed in mutants with compromised insulin signaling might be, at least in part, due to increased oxidative stress protection.

Studies in *C. elegans* also revealed that mutants lacking the insulin/IGF-1 receptor *daf-2* or the phosphoinositide kinase PI3K (*age-1*) show increased level of SOD and catalase activity, significantly increased oxidative stress resistance and exhibit very extended lifespans (Honda and Honda 1999; Johnson 1990; Kenyon et al. 1993; Brys et al. 2007). Similar results were observed in *Drosophila*, where deletion of either the insulin receptor or CHICO, the fly insulin receptor substrate, increases both oxidative stress resistance and lifespan (reviewed by (Giannakou and Partridge 2007)). The lifespan-extending features were found to strictly depend on the presence of the FOXO transcription factor *daf-16* as *C. elegans* mutants lacking DAF-16 show increased sensitivity towards paraquat-mediated oxidative stress and are severely short-lived (Yanase et al. 2002; Lin et al. 2001). *Daf-16* mutant worms also show significantly increased protein damage as measured by protein carbonylation, providing further confirmation that these animals experience increased levels of oxidative stress (Yanase et al. 2002).

That the insulin signaling pathway not only influences the lifespan of invertebrates but also of vertebrates became clear when female mice carrying a heterozygous mutation of the insulin-like growth factor type 1 receptor gene *igf-1r*$^{+/-}$ (homozygous null mutation of IGF-1R has been found to be embryonic lethal) were found to be more resistant to oxidative stress and were long-lived (Holzenberger et al. 2003). These results were consistent with earlier studies on the IGF-1R substrate p66^{Shc}, whose mutation was found to increase oxidative stress resistance and extend lifespan (Migliaccio et al. 1999). Subsequent studies with tissue-specific knock-outs of the IGF-1R in the adipose tissue of mice further confirmed these results and showed that these mice have an extended lifespan (Bluher et al. 2003). At this point it is still unclear which of the many FOXO-regulated genes are ultimately responsible for the observed lifespan extension in worms and other organisms, and what exact role(s) ROS play in the ILS-mediated lifespan regulation. A RNAi-mediated knock-down of FOXO-target genes, including glutathione transferase, cytosolic *ctl-1*, peroxisomal *ctl-2*, or mitochondrial superoxide dismutase *sod-3* was found to individually reduce the long lifespan of *daf-2* mutants while deletion of the cytosolic superoxide dismutase *sod-1* had no effect on the lifespan of *daf-2* mutants. Moreover, loss of the extracellular superoxide dismutase *sod-4* further extended *daf-2*-mediated lifespan (Murphy et al. 2003; Ayyadevara et al. 2005; Doonan et al. 2008). That lifespan-extension by compromised Insulin/IGF-1-signaling is not due to a reduction of oxidants and decreased oxidative damage became obvious when long-lived *daf-2* mutants were found to have higher respiratory rates, and exhibit increased levels of mitochondrial ROS level (Houthoofd et al. 2005; Zarse et al. 2012). Moreover, their lifespan was slightly reduced upon treatment with antioxidants (Yang and Hekimi 2010; Brys et al. 2007; Zarse et al. 2012). These results were highly reminiscent of studies conducted with some long-lived

ETC mutants, which also showed increased ROS level that seemed necessary for lifespan extension (see Sect. 14.5.1). When Insulin/IGF-1 signaling was reduced during adulthood of worms, an increase in respiration was observed that caused a transient increase in ROS, which eventually led to increased activity of catalase and SOD (Zarse et al. 2012). The observed lifespan extension was diminished when antioxidants were supplemented (Zarse et al. 2012). The observation that treatment of worms with the superoxide generator juglone caused nuclear translocation (i.e., activation) of DAF-16 while exposure to hydrogen peroxide lead to phosphorylation (i.e., inactivation) of DAF-16 (Weinkove et al. 2006; Nemoto and Finkel 2002) suggested that the type of oxidant and possibly its sub-cellular accumulation might affect signaling processes, oxidative stress resistance and ultimately the lifespan of organisms.

14.6 Is the Free Radical Theory of Aging Still Valid?

Taken all the published studies together, it is now clear that both the expression levels of antioxidants and the production of oxidants are optimized for individual organisms and finely balanced. Any drastic change in oxidant or antioxidant level brings about the possibility to negatively affect tightly regulated processes, such as development, differentiation, signaling, host defense, metabolism, and ultimately lifespan (Fig. 14.3). Future studies aimed to further manipulate the interplay between endogenous oxidants and antioxidants will provide, at most, more correlative evidence. However, to ultimately prove or disprove the free radical theory of aging, we need to temporally and spatially dissect both positive and negative effects of distinct ROS-levels on individual processes during the lifetime of an organism to define which levels, timings and kinetics are required for proper functioning of the organism. Only when we understand the effects of ROS *in vivo* can we begin to manipulate their levels with a predictable outcome. The development of quantitative *in vivo* ROS sensors and read-outs of cellular targets of distinct ROS levels provides us now with the opportunity to make clear distinctions between onset, duration and magnitude of oxidant formation, and to assign the role of ROS in development, proliferation, host defense, aging and disease.

Over the past decades, our view on reactive oxygen species has changed from branding them as undesired, potentially harmful by-products of aerobic life to acknowledging their importance in many physiological processes. The necessity of certain levels of oxidants became even more obvious by the observations that antioxidants were capable of reversing beneficial effects on physiology and lifespan, and by the discovery that many transcription factors and signaling pathways can be influenced by reactive oxygen species (Hamanaka and Chandel 2010; Trachootham et al. 2008). Hence, we need to now move beyond the free radical theory of aging to acknowledge the fact that ROS are major players in lifespan and aging but likely not (only) in their function as cytotoxic agents but as regulators of essential physiological processes in the cell.

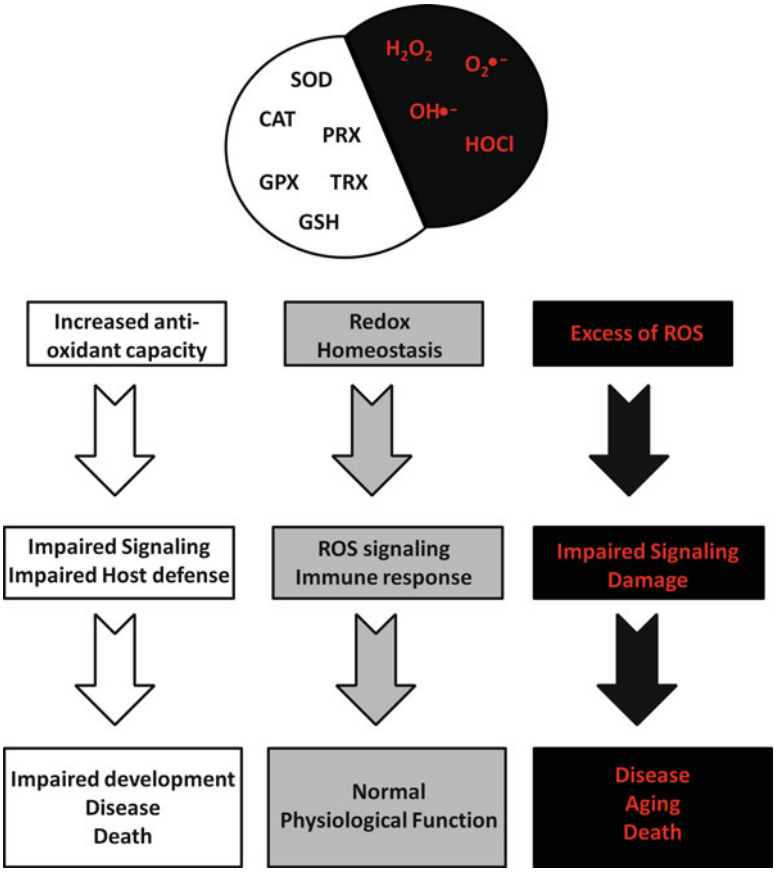

Fig. 14.3 Maintaining redox homeostasis – Essential for the physiology of organisms. Maintaining the proper redox balance between oxidants, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$), hypochlorous acid (HOCl) and antioxidants, like catalase (CAT), peroxiredoxin (PRX), glutathione peroxidase (GPX), superoxide dismutase (SOD), thioredoxin (TRX), glutathione (GSH) is essential for correct physiological processes. Shift towards more oxidizing conditions (i.e., oxidative stress) or increase in the antioxidant capacity of the organism will result in pathophysiological conditions

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Chapter 15
Oxidative Stress in Cancer

Peter Storz

Abstract Increased oxidative stress is a common feature observed in many different types of cancer. Depending on the radical formed, its concentration, and cellular location where its generation occurs, reactive oxygen species (ROS) have multiple functions within tumor cells. ROS-induced macromolecule damage can contribute to tumor initiation. Low levels of ROS can initiate cellular signaling pathways that mediate tumor cell proliferation, survival and tumor progression to a metastatic phenotype. High levels of ROS initiate signaling pathways that mediate tumor cell death, but also contribute to formation of cancer stem cells that induce tumor recurrence. Understanding the multitude and complexity of ROS-regulated pathways in cancer cells and targeted modulation of intracellular ROS levels using antioxidants or chemotherapy at different stages of tumor progression may be an effective strategy for combination therapy.

Keywords Oxidative stress • Reactive oxygen species • Antioxidants • Tumor • Cancer

Abbreviations

2DG	2-deoxyglucose
4-HNE	4-Hydroxy-noneal
Ask1	apoptosis signal-regulating kinase-1
ATM	ataxia telangiectasia mutated
BSO	L-S, R-buthionine sulphoximine
CSC	cancer stem cells

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DR	death receptor
DRL	death receptor ligand
EGF	epidermal growth factor
EGF-R	epidermal growth factor-receptor
Erk1/2	extracellular-regulated kinases 1/2
FOXO	forkhead homeobox type O
GF	growth factor
GF-R	growth factor receptor
GSH	glutathione
GSSG	glutathione disulphide
GPX	glutathione peroxidase
GST	Glutathione S-transferase
H_2O_2	hydrogen peroxide
HIF-1	hypoxia-inducible factor-1
JNK	c-Jun N-terminal Kinase
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MPTP	mitochondrial permeability transition pore
mROS	mitochondrial ROS
NF-κB	nuclear factor κ-B
NOX	NADPH oxidase
PDK1	phosphoinositide-dependent kinase 1
PDGF	platelet-derived growth factor
PDGF-R	platelet-derived growth factor-receptor
PI3-K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PKD	protein kinase D
ROS	reactive oxygen species
Prdx	peroxiredoxin
PTEN	phosphatase and tensin homologue
SOD	superoxide dismutase
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinases
TNFα	tumor necrosis factor α
VEGF	vascular epithelial growth factor.

15.1 Oxidative Stress in Cancer Cells

Oxidative stress occurs when an imbalance between pro-oxidant and anti-oxidant molecules alters the redox potential in a cell with a net effect of increasing ROS levels. Accumulation of intracellular oxidative stress can lead to the conversion of normal cells to cancer cells, and increased levels of oxidative stress are hallmarks of many cancers (Storz 2005; Trachootham et al. 2006). Oxidative stress-induced

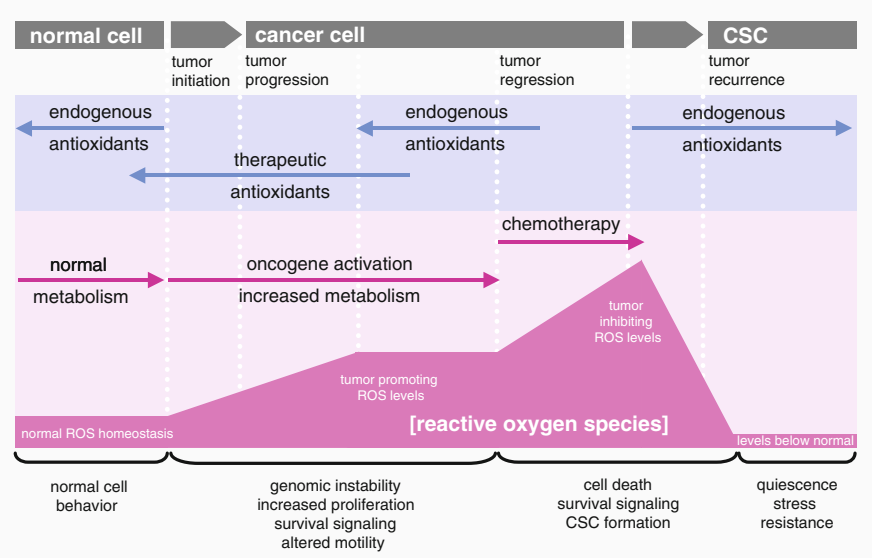

Fig. 15.1 Relations between cellular or therapy-induced oxidative stress and cellular or therapeutic antioxidants at different stages of tumor biology. While in normal cells antioxidants and ROS generation are in balance (normal homeostasis), increased ROS levels are hallmarks of tumor cells. In tumors increased ROS generation can lead to genomic instability, increased cell proliferation, survival signaling and increased cell motility. At this stage, lowering intracellular ROS levels by therapeutic antioxidants may have regulatory effects on tumor progression. Exuberant increase in ROS can be achieved by chemotherapy and can mediate tumor regression by inducing irreparable cell damage and tumor cell death. However, some tumor cells can develop stress resistance by upregulating antioxidant systems. Such cancer stem cells (*CSCs*) are highly resistant to stresses and after clonal expansion can be responsible for tumor recurrence

macromolecule damage and ROS-regulated signaling events have been reported to affect all aspects of tumor formation and progression, including genomic instability and mutagenesis, energy production, cell proliferation, survival and chemoresistance, increased cell motility and metastasis, stemness and recurrence, and angiogenesis (Fig. 15.1).

However, when produced in excess or when cancer cell antioxidant systems fail, they can contribute to tumor cell death. Tumor cells react to increases in oxidative stress by upregulating antioxidant systems, with the outcome that a balance is established that allows beneficiary effects of ROS, but avoids damaging effects that induce cell death. The cellular signaling pathways, by which such fine tuning is accomplished are little known, and some of the ROS-sensing proteins or molecules that contribute to such signaling are discussed in this chapter. Moreover, ROS-sensitive signaling pathways are constitutively switched on in many cancer cells and they participate in all aspects of tumor biology.

The sources for intracellular oxidative stress in cancer cells are manifold and different reactive oxygen species are generated. These include radicals such as

superoxide ($O_2 \cdot^-$), nitric oxide (NO·), or hydroxyl radicals (·OH), as well as non-radicals such as hydrogen peroxide (H_2O_2). Among the variety of cellular ROS that can play roles in cancer, hydrogen peroxide, superoxide and hydroxyl radicals are the best studied in cancer cells (Liou and Storz 2010). Hydroxyl ions are macromolecule damaging molecules and can contribute to DNA damage and genomic instability. Superoxide and hydrogen peroxide have roles in cell signaling (Finkel 2000; Sundaresan et al. 1995).

15.1.1 Sources for ROS in Cancer Cells

In cancer cells, several sources can be responsible for increased oxidative stress levels. Conditions leading to increased ROS production include increased oncogenic signaling, increased activity of mitochondria or peroxisomes, or increased metabolic and enzymatic activity (Liou and Storz 2010; Babior 1999; Szatrowski and Nathan 1991). Additional inducers of oxidative stress in tumor tissue are infiltrating immune cells such as macrophages (Storz 2005).

Growth factors and cytokines such as tumor necrosis factor α (TNFα), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor β (TGFβ), or insulin can induce intracellular ROS production in cancer cells. Most of them induce the formation of superoxide, which then is converted to hydrogen peroxide (Storz 2005; Sundaresan et al. 1995; Bae et al. 2000; Lo and Cruz 1995; Meier et al. 1989; Ohba et al. 1994; Tiku et al. 1990). Additionally, oncogenic mutations of molecules downstream of growth factor receptors such as K-ras lead to increased superoxide production (Minamoto et al. 2000, 2002). Another downstream effector of many growth factor receptors, including epidermal growth factor-receptor (EGF-R) and c-Met, is the small RhoGTPase Rac-1 (Ferraro et al. 2006). Both, oncogenic K-ras and Rac-1 have been shown to induce superoxide production either via NADPH oxidases (NOX) or at the mitochondria (Liou and Storz 2010; Chiarugi and Fiaschi 2007).

Increased superoxide generation at the mitochondria is also induced by mitochondrial dysfunction (Cadenas 2004; Dayal et al. 2009; Pelicano et al. 2009). Mitochondrial respiration produces superoxide as a byproduct of oxidative phosphorylation at complexes I (NADH-ubiquinone oxidoreductase) and III (ubiquinol-cytochrome c oxidoreductase). Superoxide is released into the mitochondrial matrix and the inter-membrane space, but can be released into the cytoplasm via the mitochondrial permeability transition pore (MPTP) (Crompton 1999; Storz 2006). Superoxide usually has a very short half-life and rapidly is dismutated into hydrogen peroxide. This step is mediated by superoxide dismutase (SOD) enzymes. Its breakdown product hydrogen peroxide is a *bona fide* second messenger since it is highly diffusible and can regulate the activity of signaling molecules by direct and reversible oxidation (Liou and Storz 2010; Chiarugi and Fiaschi 2007; Rhee et al. 2000).

Other respiratory organelles that can contribute to increased superoxide and hydrogen peroxide levels in cancer cells are the peroxisomes (Dansen and Wirtz 2001). Peroxisomes generate ROS through acyl-CoA oxidase and xanthine oxidase (del Rio et al. 1992; Singh 1996). Additionally contributing to increased intracellular ROS levels in cancer cells are increased activities of metabolic enzymes such as oxidases, cyclo-oxygenases, lipoxygenases, or thymidine phosphorylase (reviewed in Liou and Storz 2010).

Macrophages or other inflammatory cells (i.e. neutrophils, eosinophils) infiltrating the tumor tissue can be a source of external oxidative stress. Macrophages infiltration into normal tissues may contribute to tumor initiation since infections or inflammation (i.e. pancreatitis) have been identified as a critical risk factor for tumor development (Balkwill 2009). Macrophages can produce and release reactive oxygen species such a superoxide, nitric oxide or hydrogen peroxide (respiratory burst), and released components such as nitric oxide and superoxide can react with each other to generate the highly toxic peroxinitrite radicals (Babior 1999; Cui et al. 1994). Moreover, macrophages can secrete cytokines such as TNFα to induce ROS production in target cells (Babior 1999; Balkwill 2009).

15.1.2 Endogenous Antioxidants in Cancer Cells

Increases in oxidative stress in normal cells can lead to mutations and initiate cancer. In proliferating cancer cells increased ROS levels that are induced by above described mechanisms need to be kept in check to facilitate optimal growth conditions. Cancer stem cells (CSC) often have upregulated antioxidant systems that make them unresponsive to chemotherapy. The detoxification from ROS in cancer cells can be mediated by several antioxidant enzymes.

Depletion of cancer cells from superoxide is occurs through upregulation of superoxide dismutase enzymes. These include MnSOD, Cu/ZnSOD and FeSOD. All are metallo-enzymes that utilize divalent metal ions as cofactors to dismutate superoxide to hydrogen peroxide. Different isoforms of SODs are located at distinct compartments in the cell. For example, MnSOD is located in the mitochondria matrix and Cu/ZnSOD in the cytosol (Liou and Storz 2010). SOD enzymes are encoded by nuclear genes and signaling pathways exist by which increases in mitochondrial oxidative stress are sensed and translated to nuclear gene induction (Storz 2006, 2007; Storz et al. 2005a). The breakdown product of superoxide in SOD-catalyzed dismutase reactions is hydrogen peroxide.

Multiple enzymes detoxify cells from hydrogen peroxide by generating water and oxygen. Most prominent are catalase enzymes located in the cytosol and peroxisomes (Bendayan and Reddy 1982; Hashimoto and Hayashi 1990; Litwin et al. 1987). Peroxiredoxins (Prdx) are thioredoxin peroxidases that catalyze the reduction of hydrogen peroxide, organic hydroperoxides and peroxynitrite (Hofmann et al. 2002; Rhee et al. 1999; Wood et al. 2003). Disregulation of enzymes that detoxify

cells from hydrogen peroxide can have dramatic effects on cancer development or progression. For example, the knockout of the Prdx1 gene in mice leads to dramatically increased levels of oxidative stress and animals prematurely die of cancer (Neumann et al. 2003).

Glutathione peroxidases (GPX) mediate the breakdown of hydrogen peroxide and organic hydroperoxides (Brigelius-Flohe 1999). Glutathione (GSH) protects cellular proteins from oxidative stress by reducing disulphide bonds to cysteins. During this process GSH gets oxidized to glutathione disulphide (GSSG). Glutathione reductase enzymes then recycle GSSG to GSH (Beutler 1969). These enzymes are constitutively active in many tumor cells. Glutathione S-transferases (GST) are overexpressed in a variety of tumors and catalyze the conjunction of GSH to electrophilic compounds (Sharma et al. 2004; Townsend and Tew 2003). Glutathione S-transferases have been shown to contribute to chemoresistance, as well as to regulate cellular signaling pathways (Townsend and Tew 2003).

15.2 Altered ROS Levels – Consequences for Cancer Cells

15.2.1 ROS in Macromolecule Damage and Tumor Initiation

Increased oxidative stress in cells can induce the damage of macromolecules including DNA, proteins and lipids. This can have a role in tumor initiation (DNA damage and mutation). Additionally, altered proteins and lipids could serve as biomarkers.

DNA damaging effects of ROS are mostly induced by hydroxyl ions since hydrogen peroxide for example is not very reactive towards DNA. Hydroxyl ions are radicals that are highly diffusible. They can initiate the formation of DNA lesions through mediating single-strand or double-strand breaks or by oxidation of DNA bases and formation of DNA adducts (Maynard et al. 2009; Wiseman and Halliwell 1996). If cells harboring such DNA modifications escape programmed cell death they can continue to proliferate, raising the likelihood for cancerous growth.

Oxidation of proteins can alter their function, including inactivation or constitutive activation, which can contribute to oncogenic growth. Examples are direct oxidation of signaling molecules that lead to their activation (i.e. K-ras, Src) (Lander et al. 1997; Nakashima et al. 2002; Sun and Kemble 2009) or their inactivation (i.e. phosphatases, DNA repair enzymes) (Meng et al. 2002). In contrast to such specific ROS-regulated mechanisms, oxidative stress can induce less-specific modifications including formation of crosslinked and glycated proteins, increased protein carbonylation, nitration of amino-acid residues and protein degradation (Levine 2002; Squier and Bigelow 2000; Wells-Knecht et al. 1997).

Finally, by reacting with polyunsatureated or polydesaturated fatty acids ROS can induce lipid peroxidation (Gardner 1989; North et al. 1994). Lipid peroxidation generates several genotoxic molecules such as reactive aldehydes that can

modify proteins and DNA. As an example, 4-Hydroxy-noneal (4-HNE) is the most cytotoxic lipid peroxidation product. 4-HNE can diffuse away from its membrane productions site and covalently modify proteins to alter their function. Clinical studies show that lipid peroxidation events induced by ROS can be used as tumor markers in the serum of patients (Lauschke et al. 2002).

15.2.2 ROS in Tumor Cell Proliferation

Tumor cell proliferation can be induced by increased production of superoxide at the mitochondria (Burdon 1995; Burdon et al. 1990; Parkash et al. 2006). The increased expression of MnSOD depletes superoxide levels and mediates quiescence (Wang et al. 2005). For example, stable expression of MnSOD in pancreatic cancer cells, which are known for high intracellular oxidative stress levels, reduced their proliferation rate (Cullen et al. 2003). This led to the hypothesis that mitochondrial superoxide drives proliferation of cells, whereas SOD-induced conversion into hydrogen peroxide drives quiescence, identifying MnSOD as a mitochondrial ROS switch that determines cell fate (Sarsour et al. 2008).

Tumor cell proliferation is regulated by ROS-mediated alterations in cell cycle control and induction of signaling pathways. Cellular signaling mechanisms that are used by superoxide to induce cell proliferation are not well defined. One *bona fide* signaling pathway that can regulate tumor cell proliferation and may be activated by superoxide, but also its breakdown product hydrogen peroxide, is the mitogen-activated protein kinase (MAPK) cascade (Irani et al. 1997; Kumar et al. 2008; McCubrey et al. 2007; Roberts and Der 2007). Extracellular-regulated kinases 1/2 (Erk1/2) signaling is activated by growth factors and K-ras activating mutations (Roberts and Der 2007; Khavari and Rinn 2007). Mechanisms of how Erk1/2 is activated by oxidative stress are either direct oxidation and activation of Ras (Lander et al. 1997), ROS-induced inactivation of negatively-regulatory phosphatases (Chan et al. 2008), or activation of other upstream kinases such as $p90^{RSK}$ (McCubrey et al. 2007). Other ROS-sensing signaling proteins are Akt, protein kinase C (PKC) or protein kinase D (PKD) enzymes, and the phosphatidylinositol 3-kinase (PI3-K)/Akt as well as the PKC-PKD pathways can induce cell proliferation in various cancer cell lines (Storz 2005; Rozengurt 2011). However, above signaling molecules were shown to be activated by hydrogen peroxide rather than superoxide (Liou and Storz 2010; Song et al. 2009; Storz and Toker 2003; Prasad et al. 2000).

Loss of redox control or increased oxidative stress can also mediate cell cycle progression by upregulating gene transcription of cyclins (Felty et al. 2005; Menon et al. 2005; Ruiz-Ramos et al. 2009). Another cell cycle-related protein activated by ROS is ataxia telangiectasia mutated (ATM) (Ditch and Paull 2012). Animal models lacking ATM have high levels of ROS and increased oxidative stress-induced damage (Browne et al. 2004). Similar is found in patients lacking ATM (Reichenbach et al. 2002).

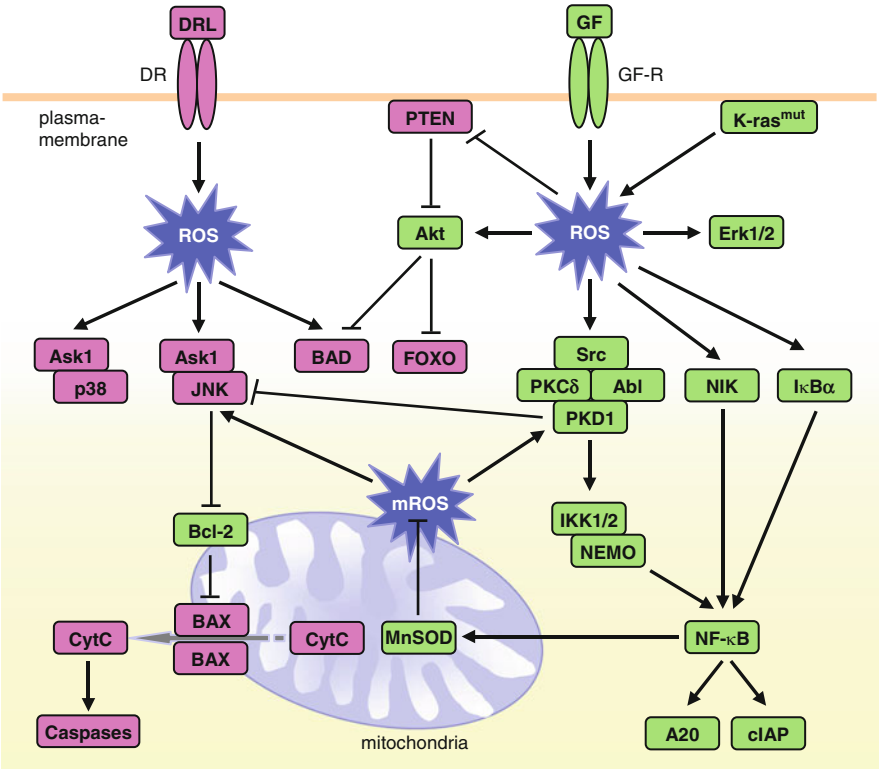

Fig. 15.2 ROS signaling in tumor cell survival and apoptosis. Reactive oxygen species (*ROS*) either generated at the plasma membrane through growth factor receptors (*GF-R*) or death receptors (*DR*) or at the mitochondria (*mROS*) can initiate both protective and cell death signaling pathways. The activation of these pathways is also dependent on radical formed and its concentration. Shown in *green* are protective signaling molecules and their pathways of activation. Shown in *red* are signaling molecules that contribute to cell death in response to ROS

15.2.3 ROS in Tumor Cell Survival and Apoptosis

Dependent on the radical formed, cellular location where this occurs, as well as its concentration, reactive oxygen species can have divergent effects on cell survival and cell death pathways (Fig. 15.2).

Mitochondria-generated oxidative stress and treatment of cells with hydrogen peroxide can lead to the recruitment and activation of a signaling complex consisting of Src, Abl, PKCδ, and PKD1 that initiates activation of the transcription factor nuclear factor-κB (NF-κB) via the canonical IKK complex and phosphorylation and degradation of IκBα, an inhibitory protein for NF-κB (Storz 2006; Storz et al. 2004a, b, 2005a; Song et al. 2009; Storz and Toker 2003; Chiu et al. 2007; Mihailovic et al. 2004). Target genes for NF-κB in this signaling pathway encode

anti-apoptotic proteins such as cIAP and A20, as well as antioxidant proteins including MnSOD (Storz 2007; Storz et al. 2005a, b). Inhibition of this pathway in tumor cells renders them susceptible to ROS-mediated cell death (Storz and Toker 2003). This survival pathway is also activated when cells loose their anchorage (Cowell et al. 2009). Loss of anchorage in non-transformed cells induces an apoptotic cell death program called anoikis (Chiarugi and Fiaschi 2007). In tumor cells survival to this mechanism allows tumor metastasis to distant sites.

NF-κB in cancer cells is a redox sensor and many cancers show increased expression and activity of NF-κB (Rayet and Gelinas 1999; Li and Karin 1999; Schreck et al. 1992). Besides the PKC/PKD pathway, its activation by ROS can be caused through several other signaling mechanisms. These include hydrogen peroxide-mediated activation of NIK (Wang et al. 2007) through oxidative inhibition of NIK-regulating phosphatases, resulting in downstream signaling of NIK to the IKK complex (Li and Engelhardt 2006). Finally, NF-κB activation in response to oxidative stress can occur in an IKK-independent fashion. For example, it was shown that ROS can lead to tyrosine phosphorylation of IκBα. Tyrosine phosphorylation of IκBα can lead to its release from NF-κB, without inducing its degradation (Imbert et al. 1996). Besides mediating tumor cell survival, NF-κB was implicated in tumor cell proliferation, increased tumor cell motility, and development of drug resistance during therapy (chemoresistance) (Ahmed et al. 2006).

Mitochondrially-generated ROS are increased by the tumor suppressor protein p53 and $p66^{shc}$ through various pathways (reviewed in Pani and Galeotti 2011). p53-induced ROS production and activation of p53 target genes participate in the induction of cell growth arrest, apoptosis and senescence (Johnson et al. 1996). Dependent on its acetylation status, p53 also promotes the expression of antioxidant genes, which decreases its effects on senescence (Lu and Finkel 2008).

Growth factor receptors such as EGF-R or platelet-derived growth factor-receptor (PDGF-R) as well as activating mutations of K-ras can lead to oxidative stress-mediated activation of Akt. Akt is activated by hydrogen peroxide, either directly or through ROS-induced activation of its upstream kinase phosphoinositide-dependent kinase 1 (PDK1) (Prasad et al. 2000; Higaki et al. 2008). Moreover, the tumor suppressor phosphatase and tensin homologue (PTEN), a negative regulator of Akt signaling, can be inactivated by hydrogen peroxide, further potentiating Akt functions (Lee et al. 2002). Inactivation of PTEN additionally-increases cellular ROS levels due to deregulation of antioxidant enzymes (Huo et al. 2008). Akt can increase tumor cell survival through phosphorylation and inactivation of pro-apoptotic proteins including forkhead homeobox type O (FOXO) transcription factors, Bad, BimEL and Bax (Pastorino et al. 1999; Qi et al. 2006; Xin and Deng 2005). FOXO transcription factors are redox-sensing proteins and oxidative stress can mediate their activation (Storz 2011). This leads to induction of genes that mediate cell cycle arrest, induce apoptosis (i.e. FasL), or encode antioxidant genes (Storz 2011).

Another kinase that was implicated in ROS-induced cell survival and resistance to oxidative stress is Erk1/2, but in some cancers Erk1/2 seems to sensitize to ROS-induced apoptosis (Chan et al. 2008; Lee et al. 2005; Ostrakhovitch and

Cherian 2005). Besides Erk1/2, other members of the MAPK family such as p38 and c-Jun N-terminal Kinase (JNK), when activated by oxidative stress, are pro-apoptotic. Both p38 and JNK are activated by apoptosis signal-regulating kinase-1 (Ask1). Ask1 is regulated in its activity by interacting with thioredoxin, a redox-regulated protein (Saitoh et al. 1998; Takeda et al. 2003). JNK activation, for example, can occur in response to mitochondrial, as well as NADPH oxidase-induced oxidative stress (Storz 2005, 2006, 2007; Simon et al. 2000; Xu et al. 2002). When activated by ROS, JNK mediates phosphorylation and downregulation of Bcl-XL and Bcl-2, two proteins that protect from ROS-mediated apoptosis (Cadenas 2004; Gottlieb et al. 2000). JNK also increases the expression of pro-apoptotic proteins such as Bax to facilitate mitochondrial cytochrome c release (Shim et al. 2007; Zhang et al. 2008).

15.2.4 ROS in Tumor Cell Motility

The intracellular ROS state in cells governs all crucial mechanisms contributing to tumor cell metastasis, including degradation of extracellular matrix, increased potential to migrate and invade, intravasation and protection from anoikis.

Increased oxidative stress can contribute to increased cell motility, resulting in tumor expansion and metastasis. For example, subpopulations of breast cancer cell lines that show higher levels of endogenous ROS production than their origin counterpart, showed increased motility. Orthotopic tumors generated with these cells were metastatic, whereas parental cells were not (Pelicano et al. 2009). Another example is that carcinoma cells, when treated with hydrogen peroxide, prior to intravenous injection into mice show enhanced metastasis (Kundu et al. 1995). On the cellular levels it was shown that highly invasive tumor cell lines express rather high levels of intracellular ROS, probably due to downregulation of MnSOD (Hitchler et al. 2006, 2008).

Above we discussed the importance of the PI3-K/Akt pathway for tumor cell survival and the transcription factor FOXO3a as one of the primary targets of Akt to mediate this. Dependent on the tumor cell type and isoform expressed Akt also can decrease cell motility (Yoeli-Lerner and Toker 2006; Yoeli-Lerner et al. 2005). It was shown that stress conditions such as increased oxidative stress or nutrient depletion can activate the transcription factor FOXO3a (Storz et al. 2009). When activated in cells that grow under suboptimal conditions (where Akt is not active), FOXO3a induces an increase in their motility, potentially allowing their escape from stress or nutrient depletion (Storz et al. 2009). This is facilitated by upregulation of the matrix metalloproteinases (MMP) MMP-9 and MMP-13 (Storz et al. 2009). MMPs reorganize the extracellular matrix, degrade proteins that compose the basal membrane and have been implicated in many aspects of tumor progression (Radisky et al. 2005). Besides FOXO3a other ROS-induced transcription factors such as NF-κB have been implicated as regulators of MMP expression (Brenneisen et al. 2002; Kheradmand et al. 1998). Moreover, it was shown that hydrogen peroxide

and not superoxide is responsible for MMP induction (Wenk et al. 1999). Hydrogen peroxide induces MMP expression via activation of the MAP kinases Erk1/2, p38 and JNK (Nelson and Melendez 2004). Increases in ROS can also lead to activation of MMPs by direct oxidation or downregulate tissue inhibitor of metalloproteinases (TIMP), proteins that inactivate MMPs (Rajagopalan et al. 1996). Treatment of tumor cells with MMP-3 can induce mitochondrial ROS production, initiating epithelial-to-mesenchymal transition (EMT) (Radisky et al. 2005). One of the drivers of this process is the RhoGTPase Rac1b (Radisky et al. 2005).

Another member of the Rac family, Rac-1, activates the NADPH oxidase NOX1 and increases superoxide production at cellular membranes (Werner and Werb 2002). NOX1 expression not only can lead to cell transformation, it also can maintain the transformed state (Mochizuki et al. 2006; Tobar et al. 2008). Further it is necessary for formation of invadopodia, which are actin-based structures needed for tumor cells to invade tissue and blood vessels (Diaz et al. 2009). In this context Rac-1/NOX signaling can mediate actin cytoskeleton rearrangements. For example, Rac-1 can activate the actin severing enzyme cofilin to increase cell migration (Kim et al. 2009; Sundaresan et al. 1996). Similar to Rac-1, Src can regulate NOX1-mediated induction of ROS to mediate cell migration (Gianni et al. 2008). Moreover, ROS can regulate the transcriptional repressor Snail to decrease expression of E-cadherin, contributing to EMT and increased cell motility (Wu 2006).

The Rac-1/Nox pathway can act through NF-κB to modulate cell adhesion (Tobar et al. 2008). In endothelial cells this can lead to a loss of cell-cell adhesions, loosen the integrity of the endothelium, and result in increased vascular permeability and intravasation of cancer cells (Cheng et al. 2004; van Wetering et al. 2002). Besides NOX-induced ROS, mitochondrially-induced ROS have been shown to regulate cell adhesion (Chiarugi and Fiaschi 2007). This is probably mediated through Src and FAK, both kinases located to focal contacts, actin-based structures that contribute to cell spreading, cell migration and prevention of anoikis. Interestingly, Rac-1 may be involved in this process too, since growth factors and integrins can induce a Rac-1-caused increase in mitochondrial oxidative stress, leading to Src activation, cell adhesion and cell spreading (Bae et al. 2000; Werner and Werb 2002).

15.2.5 ROS in Cancer Stem Cells

Following radiotherapy or chemotherapy, recurrence of tumors can be initiated by a small subpopulation of surviving cancer cells. These cells are highly stress- and drug-resistant, express markers of stem cells, and therefore were named cancer stem cells (Diehn et al. 2009). CSCs are capable of self-renewal and differentiation and therefore have features to initiate tumors. Their adaption to increased ROS and promotion of cell survival and stress resistance is mediated by an increase in their antioxidant capacity (Tanno and Matsui 2011). For example, mammary epithelial CSCs contain low levels of superoxide when compared to the more mature progeny or even normal epithelia cells (Diehn et al. 2009). This is caused by increased

expression of a variety of genes encoding ROS scavenging enzymes or regulating glutathione synthesis (Diehn et al. 2009; Trachootham et al. 2009). Further, keeping ROS at such low levels is critical for maintaining their stem cell phenotype.

Increased levels of ROS are critical mediators of many chemotherapeutics and ionizing radiation therapy leading to DNA damage and tumor cell death (Shackleton et al. 2006; Ward 1985). The expression of antioxidants in CSCs prevents oxidative damage of DNA and cell death, and CSC-enriched tumor cell populations accumulate fewer DNA strand breaks or mutations after irradiation.

Important regulators of stress resistance in CSCs are FOXO transcription factors (Storz 2011). These transcription factors in cancer have been implicated in regulation of migration, proliferation, DNA repair, cell cycle arrest and cell death. They are less expressed in the bulk of tumor cells, but are critical for the maintenance of leukemic stem cells. For example FOXO1 confers stress resistance by upregulating catalase and superoxide dismutase genes (SOD) (Tothova et al. 2007). Consequently, treatment of CSCs with L-S, R-buthionine sulphoximine (BSO), a compound that pharmacologically depletes the ROS scavenger glutathione significantly sensitizes them to radiotherapy (Diehn et al. 2009).

15.2.6 Hypoxia, Angiogenesis and HIF-1

In primary tumors an increasing tumor mass faces cycles of oxygen depletion (hypoxia) and reoxygenation (Dewhirst et al. 2008; Hockel and Vaupel 2001). If prolonged, such limitations in oxygen supply can be damaging to the tumor cells. An escape mechanism for tumor cells to such conditions is their metabolic switch to anaerobic glycolysis (Pani et al. 2009). While normal cells only switch to glycolysis when adequate oxygen supply is not ensured or when mitochondria function is suppressed (Ganapathy et al. 2009), tumor cells can constantly use glycolysis as an energy source under normoxia (Warburg effect) to become independent of such conditions (Dang and Semenza 1999). This switch can be induced by mitochondrial dysfunction, oncogenic transformation or loss of tumor suppressor genes (Hsu and Sabatini 2008). The transcription factor hypoxia-inducible factor-1 (HIF-1) regulates glycolysis-related genes and inhibits mitochondrial respiration (Pani et al. 2009), which in sum results in adaption of tumor cells to hypoxic conditions (Pouyssegur et al. 2006) and the development of an aggressive tumor phenotype (Harris 2002).

With increased tumor mass, nutrient and oxygen support is limited to cells in the tumor center. Therefore, more nascent blood vessels are required to ensure supply to the tumor center (Claffey et al. 1996; Senger et al. 1994). Hypoxic conditions stimulate blood vessel development (angiogenesis) through induction of intracellular oxidative stress. For example, an increase in intracellular oxidative stress levels can be mediated by hypoxia-induced expression of VEGF (Jo et al. 2011). Antioxidants effectively inhibit or decrease angiogenesis since they can modify densitometry of microvessels and proliferation of endothelial cells (Rabbani

et al. 2009). Blood flow in newly developed vessels often is instable, leading to periods of high oxygen and hypoxia, and causing additional oxidative stress (Brown and Bicknell 2001). Nutrient deprivation and hypoxia both can increase expression of vascular epithelial growth factor (VEGF) (Spitz et al. 2000). This is mediated through HIF-1 and its co-factor p300 (Liou and Storz 2010).

HIF-1 is composed of the two subunits HIF-1α and HIF-1β (Harris 2002). Under normoxia conditions HIF-1α is proteasomally degraded (Kaelin and Ratcliffe 2008), whereas under conditions of hypoxia, formation of superoxide and hydrogen peroxide lead to accumulation of HIF-1α (Wang et al. 2005). HIF can be induced by increased production of both reactive oxygen species in response to oncogenic signaling through the PTEN/PI3-K/Akt signaling pathway, or to mitochondria-generated oxidative stress (Huo et al. 2008; Liu et al. 2006; Xia et al. 2007). Due to its function as an inducer of VEGF signaling and contribution to angiogenesis, increased HIF-1 expression was shown to correlate with poor prognosis and increased metastasis. HIF-1 also impacts tumor cell metastasis by impacting cell motility and EMT (Erler et al. 2006). This is mediated through HIF-1α regulation of metastasis-related genes including lysyl oxidase and MMPs. For example, MMP-9 and MMP-13 can directly mediate tumor cell invasion and metastasis when expressed by tumor cells (Storz et al. 2009). As a consequence of its effects on preventing cellular acidification, HIF-1 can contribute to cell invasion by increasing the formation of lactate and CO_2, both of which favor the degradation of extracellular matrix (Pouyssegur et al. 2006; Rofstad et al. 2006). MMPs are important mediators of vessel growth within the tumor microenvironment and MMP-induced formation of capillary-like structures occurs through upregulation of ROS.

15.3 Modulation of ROS Levels as Therapeutic Strategy

ROS can be beneficial for tumor cells allowing acquisition and maintenance of tumorigenic characteristics, but can be damaging when in excess. Many tumor tissues have elevated levels of oxidative stress, which in most normal tissues would induce cell death (Storz 2005). The upregulation of intracellular antioxidant systems allows tumor cells not only to keep ROS levels in check, but also to keep them at levels where they contribute to beneficiary signaling. Therefore, tumor cells can thrive under oxidative stress, inducing increased tumor cell proliferation, anti-apoptotic signaling and increasing cell motility (Liou and Storz 2010).

There are several therapeutic strategies aiming to increase intracellular ROS levels in cancer cells, with the goal that oxidative stress reaches a level that induces senescence or even cell death. This may be achieved in cancer cells by compounds that inhibit antioxidant systems or downregulate cellular pathways that mediate expression of antioxidants. Decreasing their antioxidant capacity can dramatically increase intracellular ROS levels in cancer cells, leading to irreparable damage. Normal cells may not be affected by such a strategy since they generally have lower

basal ROS levels than cancer cells and are less dependent on antioxidant systems. A caveat with this approach is that a threshold of cytotoxicity may not be reached in cancer cells. An advantage is that cancer stem cells may also be targeted by this approach, since they show increased expression of antioxidant systems to keep their intracellular ROS levels low (Trachootham et al. 2009).

Another strategy is to exuberantly-increase oxidative stress in tumor cells to levels at which they induce cell death. This can be achieved by radiation or chemotherapy (Trachootham et al. 2009). Increased ROS levels in tumor cells can be obtained by blocking glucose metabolism, which is increased in tumor cells and compensates excess metabolic production of ROS. It was suggested that inhibition of glucose metabolism may provide a mechanism by which cancer cells can be specifically targeted. This can be achieved with glucose analogues that can not be metabolized such as 2-deoxyglucose (2DG) and is even more pronounced when combined with mitochondrial electron chain blockers that mediate additional induction of ROS (Aykin-Burns et al. 2009; Coleman et al. 2008). Strategies to increase intracellular ROS generation in tumor cells may be even more effective when antioxidant systems are depleted at the same time (Trachootham et al. 2009). However, high levels of ROS can drive tumor cells into a stem cell-like phenotype, which is stress resistant and responsible for recurrence of tumors. Thus, therapies that increase intracellular ROS in cancer cells may represent a good strategy to target the bulk of proliferating tumor cells, but may drive a subpopulation of tumor cells into a dormant state or a CSC phenotype. These cells then need to be targeted with a separate strategy that considers their unique redox status.

Depleting ROS levels with therapeutic antioxidants may have tumor preventive functions. For example, oncogenic mutations in K-ras have been linked to ROS formation leading to premalignant lesions in pancreatic cancer (Li et al. 2002). Moreover, ROS generated by macrophages have been implicated in the initiation and progression of many tumor types (Keibel et al. 2009; Qian and Pollard 2010). Depleting oxidative stress at early stages therefore may be effective in prevention or delaying tumor development. Examples of antioxidants that are tested for tumor therapy are NOV-002, a mimetic of glutathione disulfide, or EUK-134, a mimetic of superoxide dismutase (SOD) (Townsend and Tew 2003; Bechtel and Bauer 2009). It becomes obvious that depending on the strategy used for therapy, the use of antioxidants in combination therapy could have adverse effects on chemotherapeutic drugs that kill cells by increasing intracellular ROS levels. Therefore, combination therapy with antioxidants and apoptosis-inducing agents may only be effective when these compounds can mediate cell death via pathways independent of ROS. Another explanation of why many tumor therapies in which antioxidants were used were quite ineffective is that at treatment the tumor cells may already have acquired a mutational state in which they are independent of ROS as a tumorigenic factor.

In summary, specific modulation of oxidative stress levels or regulation of specific production of certain reactive oxygen species in tumor cells could be a powerful tool to enhance therapeutic outcomes. However, it may require combination therapy with inhibitors of other pathways, specifically tailored to the signaling pathway or tumor type that needs to be targeted. One example where this type of therapy may be

effective is the use of chemotherapy in combination with a specific strategy to target antioxidant systems of CSCs, with the goal to kill the bulk of tumor and additionally prevent recurrence.

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Chapter 16
Redox Pathways as a Platform in Drug Development

Danyelle M. Townsend and Kenneth D. Tew

Abstract Redox homeostasis is frequently aberrantly regulated in human diseases such as cancer and neurological disorders. Partly as a consequence, there is optimism in validating and extending redox controlling pathways as a platform for the discovery/development of drugs, particularly in cancer. As the primary redox buffer, cellular thiols have been variously therapeutically targeted. N-acetylcysteine is the simplest pharmaceutical version of a bioavailable redox equivalent. It has uses in a number of disparate human pathologies. Other agents have redox active centers primarily as a function of nucleophilic centers associated with the variable valence states of sulfur. Redox homeostasis is aberrantly regulated in cancer cells and this has provided an opportunity to advance treatment concepts that attempt to produce a beneficial therapeutic index. A component of the approaches to prevent cancer is based upon the possibility that thiols provide a way of detoxifying environmental electrophiles prior to enacting damage to DNA that could progress a cell towards a cancerous phenotype. Further, therapies designed to enhance myeloproliferation, hematopoietic progenitor cell mobilization and immune response also have a foundation in modulation of redox pathways within the bone marrow compartment. As a consequence of these principles, a number of "redox modulating" drugs are under development and progressing towards FDA review.

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16.1 Introduction

Imbalance of redox homeostasis is a characteristic of a number of human disease pathologies. In particular, cancer and neurological disorders are characterized by alterations in a variety of pathways involving reduced glutathione (GSH). GSH (γ-glutamyl-cysteinyl-glycine) is the most abundant non-protein thiol in biological systems. GSH participates as a redox donor in many aspects of metabolism, gene expression, signal transduction, proliferation and apoptosis and as a cofactor for redox modulating enzymes such as glutathione S-transferases (GSTs), peroxidases, peroxiredoxins and thiol reductases (Tew 1994). Aberrant redox homeostasis may be cause: effect related to altered regulation of signal transduction pathways that lead to uncontrolled cell growth (cancer) and/or cell death (neurodegenerative disorders; Tew et al. 2011). At least one classical view of redox status is exemplified by the dynamic equilibrium between the production of reactive oxygen or nitrogen species (ROS; RNS) and the detoxification of these species by a variety of antioxidants. Under ROS/RNS accumulating conditions, the reversible formation of mixed disulfides between redox-sensitive protein thiol groups and Cys (S-thiolation) and/or GSH (S-glutathionylation) can occur. These represent reversible redox regulation events important in many aspects of cell signaling and protein function. Cell redox status may be defined by thiol balance and the equilibrium of free thiols:disulfides. The ratio of GSH and its disulfide GSSG are primary variables used to estimate the reduction equivalents of a cell (sometimes expressed as the Nernst potential) and redox dysregulation is linked with initiation and progression of cancer. Constitutively elevated intrinsic oxidative stress in cancer tissues has been linked to increases in ROS generation and accumulation (Szatrowski and Nathan 1991), disruption of thiol and non-radical circuits and expression of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase, glutathione S-transferases (GST) and peroxiredoxins (Tew 1994; Li and Oberley 1997). Chronic oxidative stress can activate redox sensitive transcription factors such as nuclear factor κB (NF-κB) and Nrf2, oncogenes and signaling pathways. Downstream of these events, expression of survival factors, such as BCL2 and MCL1 can influence expression of pro-apoptotic pathways mediated by caspases. Cancer progression can occur via regulation of redox dependent gene expression in pathways involving cell proliferation, senescence, metastasis, and angiogenesis.

Expression of a number of oncogenes and tumor suppressors is also impacted by redox conditions. For example, constitutive up-regulation of Ras is associated with elevated cellular oxidative stress, as is the chimeric BCR-ABL tyrosine kinase (Trachootham et al. 2008). p53 has a critical role in regulating a number of genes associated with oxidative stress, including SOD and glutathione peroxidases (Drane et al. 2001). Its inactivation increases oxidative stress and enhances tumor growth, both of which could be suppressed via antioxidants (Sablina et al. 2005). During cancer initiation the S-glutathionylation of p53 prevents DNA binding (Velu et al. 2007), exemplifying the importance of redox sensitive cysteines in these pathways. In many instances, these pathways provide a fertile platform for the discovery

and development of novel therapeutic agents. A particularly fertile platform for drug discovery in cancer has come from the observations that GST (especially GSTP) is expressed at high levels in many tumors (e.g. ovarian, non-small cell lung, breast, colon, pancreas and lymphomas) and in a wide range of drug resistant cell lines and tumors (Tew 1994). Mechanistically, structural properties of GSTP are critical in facilitating GSH dependent catalysis. Its N-terminal domain adopts a topology similar to several proteins from a thioredoxin fold super-family, which binds GSH with high affinity. This glutathione binding site (often referred to as the G-site; Mannervik 1985) is situated in a cleft formed between intra-subunit domains. Domain 1 is connected to domain 2 by a short linker sequence at the C-terminus of the linker sequence. The C-terminal domain together with a loop from the N-terminal domain forms the substrate-binding site (H-site) that is hydrophobic and is adjacent to the G-site, permitting proper orientation of the bound reactants. Different amino acids in the H site of GST isozymes account for distinct substrate specificities. The homodimeric structure is common for proteins containing thioredoxin folds and structural interactions at the inter-subunit interface are crucial for complex assembly and stability. The domains at the inter-subunit surface of the GSTP homodimer are dominated by hydrophobic interactions between residues from domain 1 of one subunit and domain 2 of the other. Aromatic residues play major roles in these interactions. The interface is approximately 25–35 Å and at a height of 25 Å, diverges to create a V-shaped crevice that is solvent-accessible (Sinning et al. 1993). In addition, the active site cleft in GSTP is shallow, while in other GST isozymes it is larger and more open (Sheehan et al. 2001).

There are drugs that have been developed for cancer that impact specific redox pathways and this review will summarize such examples.

16.1.1 Sulfur as a Nucleophile

Maintenance of sulfur homeostasis is crucial in determining cellular redox homeostasis and response to a variety of chemical and physical stimuli/stresses. Sulfur has unique variable valence states, which in cysteines can empower biological flexibility permitting certain proteins to participate in sensing and/or signaling reactions that can influence cell fate. There are >200,000 cysteines encoded by the human genome (Jones 2008), implying restricted incorporation into proteins. There is perhaps some link with selective evolutionary status, since organism cysteine content appears to correlate with degree of biological complexity (Miseta and Csutora 2000). The major non-protein thiol in cells is glutathione (GSH), where the cysteine can exist as a thiolate with a pKa of 9.65, accounting for its nucleophilic reactivity. GSH homeostasis is maintained in cells by a complex series of balanced pathways including the γ-glutamyl cycle and recycling through the cleavage activity of membrane-associated γ-glutamyl transpeptidase. A characteristic of the transformed phenotype is that cancer cells have millimolar concentrations of GSH combined with aberrant redox homeostasis. Balanced oxidation and reduction

reactions determine cellular redox homeostasis and play essential roles in regulating signaling cascades, including those associated with proliferation, inflammatory responses, apoptosis, and senescence.

16.1.2 Selenium vs. Sulfur

Selenium (Se) is a rare element found in the form of selenocysteine (the 21st amino acid) coded for by the UGA codon in 25 proteins (Kryukov et al. 2003). The absence of Se from the mammalian diet leads to death, and indeed, there have been preclinical studies and extensive clinical trials with Se supplements in a chemopreventive setting, particularly in high-risk prostate cancer patients. Since earlier clinically positive results in the mid 1990s, (Clark et al. 1998) there has been much debate as to the value of Se as a preventive supplement (Marshall 2001). There is also debate over which chemical form of selenium is most bioavailable and whether this influences outcomes of selenium based trials. While the therapeutic efficacy of Se will await final scrutiny when many large-scale trials reach completion in the next 5 years, there is value in analyzing how the chemistry/biochemistry of Se complements and/or differs from that of sulfur. Moreover, from an evolutionary standpoint, recoding of the UGA stop codon into selenocysteine sense codon involves a significant expenditure of biological energy (Forchhammer et al. 1989), implying that the amino acid fulfills some critically important biological role that sulfur containing cysteines cannot. Such a conclusion has initiated a variety of theories as to what properties of selenocysteine facilitate its maintenance in the genome/proteome. It has been argued that the enhanced nucleophilicity of Se (with a pKa of 5.2 (Wessjohann et al. 2007)) compared to S and the lower Pka of selenol compared to thiol may convey enhanced catalytic functionality. However, the enhanced nucleophilicity is a factor of <10 (Songstad and Pearson 1967, 1968), quite modest by the rate constant changes that would be imbued. In an eminently readable article, Hondal et al. (Hondal and Ruggles 2011) discuss three chemico-biological properties (with particular reference to thioredoxin reductase (Lothrop et al. 2009)) that may be involved in selenocysteine functionality. By considering three contingencies, (i) selenium is a better nucleophile than sulfur; (ii) selenium acts as a better leaving group; (iii) selenium has better electrophilic characteristics; they reach the conclusion that electrophilicity rather than nucleophilicity is the advantage maintained by natural selection. Se, they argue, is needed for its superior electrophilicity since this property is shared with all small molecule substrates that are reduced by the N-terminal redox center. To account for the selective pressure on the genome that is needed to maintain the insertion machinery Se also has the property of being highly resistant to irreversible oxidation, making Se-containing enzymes resistant to inactivation by oxidation. The other physicochemical properties of Se and S are quite similar, but the chemical rate of recycling of the oxidized selenocysteine residue back to its parent form is very large compared to

the same cycling of an oxidized Cys residue (Cys-SO_2– to Cys-SH). This ability to resist irreversible oxidation can explain the biological pressure to maintain the selenocysteine-insertion machinery (Hondal and Ruggles 2011).

16.2 Redox, Thiols and the Bone Marrow Environment: Myeloproliferative Drugs

In humans, hematopoietic stem cells (HSCs) in the bone marrow give rise to all differentiated hematopoietic cell lineages, including erythroid, myeloid, lymphoid, and megakaryocytes. Functionally and spatially characterized niches within the marrow cavity are determinant factors in stem cell fate (Schofield 1978). The osteoblastic niche maintains HSC quiescence while the vascular niche microenvironment promotes HSC proliferation and differentiation (Wilson et al. 2008; Calvi et al. 2003). Adhesion proteins, cytokine and chemokine signaling molecules direct cells to their respective niches. Ca2+ gradients in the endosteum enable calcium-sensitive HSCs to sense and migrate appropriately (Adams et al. 2006). The chemokine CXCL12 and its receptor CXCR4 are regulators of HSC quiescence and niche retention (Nie et al. 2008). Metallo- and/or serine proteases are involved in CXCL12 and CXCR4 inactivation and mobilization (Christopherson et al. 2003). The balance between proteases and their inhibitors is critical in the steps involved in HSC mobilization. Given that a number of proteases and their inhibitors contain Cys residues that are subject to oxidative regulation, redox conditions will regulate protease activity. In addition, serine protease inhibitors are also regulated by S-glutathionylation, a property that conveys a further level of regulatory control on this process (Fig. 16.1).

Hematopoiesis operates optimally when the marrow compartment is between 1 and 3% oxygen (Cipolleschi et al. 1993), but hypoxic niches and gradients are required for HSC maintenance. A hypoxic environment in the osteoblastic niche encourages quiescence in HSCs and movement to the more oxygenated vascular niche promotes HSC differentiation (Iwasaki and Suda 2009). Regions of low oxygen may protect quiescent HSCs from toxic oxygen free radicals and a ROS gradient may regulate HSC-niche interactions (Parmar et al. 2007). N-acetyl cysteine (NAC) reverses ROS suppression of N-cadherin-mediated HSC adhesion to osteoblasts, inducing cell migration (Hosokawa et al. 2007). Gradients of ROS may affect elements of the niche structure and serve to regulate HSC within the marrow microenvironment. Redox modulation is a regulator of cell self-renewal and differentiation in glial cells, where cysteine pro-drugs alter extracellular redox and enhance self-renewal of progenitor cells while buthionine sulfoximine (BSO), by depleting GSH, promotes differentiation (Smith et al. 2000). These types of observations imply that redox gradients and their pharmacological manipulation have a role in influencing HSC migration and differentiation and myeloproliferation.

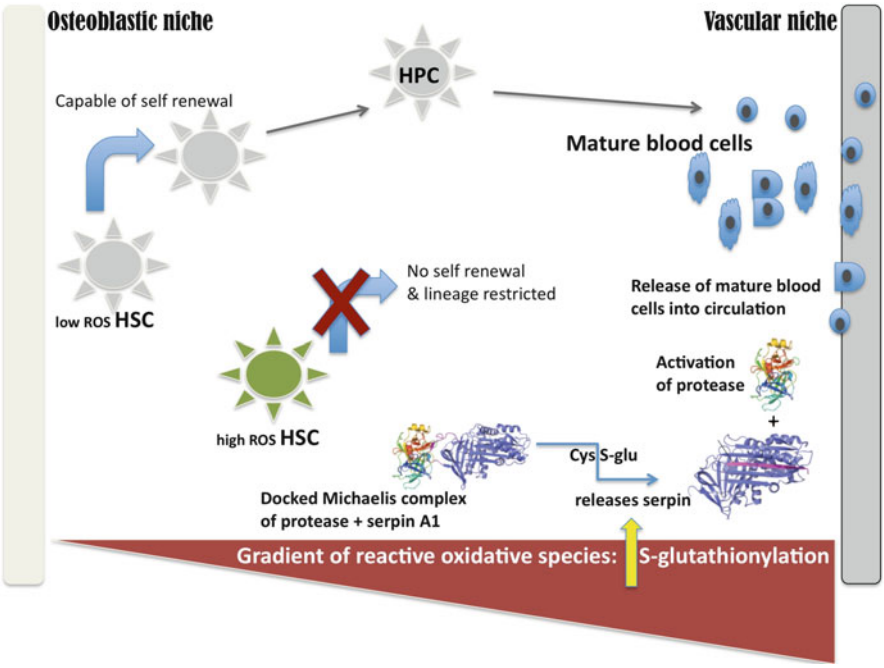


Fig. 16.1 Representation of how S-glutathionylation of a critical cysteine residue in serpin A1 may influence release of mature blood cells from the bone marrow compartment. Various gradients help to define the osteoblastic and vascular niches of the marrow compartment. Hematopoietic stem (*HSC*) and progenitor (*HPC*) cells traverse this space and mature towards the variety of blood cells that present the circulating blood lineages. Two types of HSC exist. Depicted are low and high ROS populations. The former are characterised by cell surface markers that are consistent with their existence in the osteoblastic niche. High ROS HSC lack the capacity to self-renew and are lineage restricted, although treatment with agents such as NAC can reverse this (Jang and Sharkis 2007). Eventual release of mature cells from the marrow involves some form(s) of proteolytic action that result in their entry into the vascular niche and general circulation

Over a half-century ago cystine and cysteine were shown to be important for maintaining the required balance between HSC quiescence, self-renewal, and lineage commitment in human bone marrow (Baldini and Sacchetti 1953). Subsequently it became clear that thiol conditions inversely correlated with the capacity of ROS to attenuate HSC self-renewal differentiation. Bone marrow has two HSC populations defined by low and high intracellular ROS. Low ROS populations have markers (N-cadherin, Notch1, p21, and calcium-sensing receptor) indicating osteoblast association and retained self-renewal capacity in serial transplantation. High ROS cells lacked this repopulating capacity and were lineage restricted (Fig. 16.1). NAC treatment restores functional activity to this high ROS population. (Jang and Sharkis 2007). Such information would suggest that therapeutic strategies that aim to convert high ROS cells into low may be useful in treatments involving HSC transplantation and engraftment.

Excess accumulation of ROS can occur as a consequence of cancer chemotherapy and this can cause phosphorylation of p38 MAPK in HSC contributing to a failure to maintain quiescence and self-renewal capabilities (Ito et al. 2006). The forkhead box-O (FoxO) family of redox-sensitive transcription factors up-regulates ROS detoxifying genes such as MnSOD, catalase, and GADD45 in HSC. Triple FoxO knockout animals develop a fatal bone marrow defect characterized by loss of quiescence, increased apoptosis and elevated intracellular ROS in HSC. Supplementation of thiol pools by NAC rescues these HSC, but increased ROS levels can trigger differentiation of HSCs to myeloid progenitors (Tothova et al. 2007). NAC treatment during granulocyte colony-stimulating factor (G-CSF) induced mobilization suppresses HPC mobilization (Tesio et al. 2011). NAC induced changes in thiols and redox state increases transcription of cytokines such as IL-1, IL-4, IL-6, IL-7, IL-8 and TNF-α (Rovin et al. 1997; Gosset et al. 1999) and directional migration of HSC in response to stromal cell-derived factor-1 (SDF-1) define a requirement for GSH. These studies emphasize how pharmacological manipulation by agents like NAC can control fluctuations of ROS and thiol balance and maintain HSC self-renewal, proliferative capacity and lineage fate.

16.3 GSH and GSTP as Drug Platforms

16.3.1 Direct Manipulation of GSH

In the context of drugs that impact redox pathways there are examples of agents that achieve this through direct targeting of thiol maintenance pathways and there are drugs that have indirect effects, frequently as a consequence of their capacities to produce electrophilic centers. A number of standard anticancer drugs are known to influence redox homeostasis in both cancer and normal cells. Even drugs that do not have an apparently obvious electrophilic character will cause chain reactions that alter intracellular ROS levels. Metabolism in cardiac sarcoplasmic reticulum and mitochondria of quinone based anticancer drugs such as the anthracycline antibiotics is linked to cardiac toxicity (Doroshow 1983). The efficacy/toxicity of a number of alkylating agents and platinum drugs with established electrophilic centers is also linked with ROS induced damage (Wondrak 2009). Others, such as paclitaxel, cytarabine, vinblastine, campotothecin, or histone deacetylase inhibitors result in production of ROS that is unrelated to direct drug properties (Rahmani et al. 2005; Fawcett et al. 2005). Arsenic trioxide has found a niche in the treatment of promyelocytic leukemia and its therapeutic activity stems in part from the enhanced sensitivity of the cancer cells to the thiol active components of arsenic (Pelicano et al. 2003) and other metabolites that regulate components of the NADPH oxidase complex (Chou et al. 2004). Combining arsenic trioxide with the superoxide dismutase inhibitor 2-methoxyestradiol has shown promise in targeting chronic lymphocytic leukemia cells (CLL) (Zhou et al. 2003). Motexafin gadolinium is

a thioredoxin (Trx) reductase inhibitor that causes apoptosis in malignant cells through induction of ROS (Evens et al. 2005). Agents that cause a reduction in glutathione levels, such as phenyl-isothiocyanate can also effectively kill resistant CLL and chronic myeloid leukemia (CML) cells (Trachootham et al. 2008). Use of thiol modulating agents has also been successfully adapted as a modality for the prevention of drug toxicities (Wondrak 2009). As an example, TDZD-8 (4-benzyl-2-methyl-1,2,4-thiazoline-3,5-dione) selectively induces apoptosis through the depletion of free thiols of leukemic stem cells (LSC), but not hematopoietic stem and progenitor cells (Guzman et al. 2007). Inhibition of NF-κB with niclosamide produces elevation of ROS in AML cells without targeting normal bone marrow (Jin et al. 2010). Nevertheless, many drugs have toxic effects through their impact on myeloid progenitor stem cells. It is clear that the bone marrow compartment is a tissue with complex associations with thiols and redox balance.

16.3.2 Biologically Available Cysteine: NAC, GSH Ethyl Ester

The simplest form of "bioavailable sulfur reducing equivalents" is the n-acetylated derivative of L-cysteine (NAC) that serves as a precursor for cysteine, enhances the de novo synthesis of GSH and acts as a scavenger of ROS such as OH· and H_2O_2 (Aruoma et al. 1989). As a pharmaceutical, NAC has a broad usage in a number of unrelated pathologies. In each case, the delivery of thiol equivalents is linked with the beneficial impact of the drug. A major therapeutic usage is in the reversal of the potentially lethal liver damage associate with acetaminophen overdose (Kanter 2006). However, the agent has utility in each of the following conditions (for review see Kelly 1998): reversal of carbon monoxide poisoning; unstable angina; amyotrophic lateral sclerosis (ALS); bile duct blockage in infants; Alzheimer's disease; allergic reactions to the anti-seizure drug phenytoin, amelioration of symptoms from ocular infection (keratoconjunctivitis). It is also used for reducing levels of a certain type of cholesterol and homocysteine thereby influencing risk factors for heart disease in patients with serious kidney disease. Additional therapeutic uses include: the agent has mucolytic activity by reducing mucus viscosity by splitting disulfide bonds in various mucoproteins. Inhaled NAC is indicated as an adjuvant in other respiratory pathologies with excessive and/or thick mucus production (e.g. emphysema, bronchitis, chronic obstructive pulmonary disease). Although there is some debate, high doses of oral NAC have been found to modulate inflammation in cystic fibrosis patients (Tirouvanziam et al. 2006) Mechanistically, the data indicate that NAC provides thiol equivalents that compensate redox imbalance that is implicated in inflammatory imbalances in cystic fibrosis. In a similar vein there are studies that support the utility of NAC in the prevention of radiocontrast induced nephropathy, however this indication is not entirely supported by other clinical trial testing studies (Marenzi et al. 2006).

At a cellular level, NAC can impact cell survival and apoptosis through the activation of cell surface signal-regulated kinase pathways. NAC increases mitogenesis and suppresses apoptosis of B-lymphocytes in p53-deficient mice (Martin et al. 2000). NAC prevents activation of c-Jun N-terminal kinase, p38 MAP kinase and redox-sensitive AP-1 and NFκB transcription factor activities, all of which have roles in T cell proliferation (Zafarullah et al. 2003). Administration of NAC in HIV patients restores GSH levels and ameliorates viral replication, diminishing infection and disease progression (Nakamura et al. 2002), enhances the antibody-dependent cellular toxicity of neutrophils (Roberts et al. 1995) and attenuates CD4+ T lymphocyte decline (Akerlund et al. 1996). In cancer patients, NAC has been used both as a chemoprotectant and in the setting of chemoprevention. NAC mediated GSH restoration has been used to ameliorate chemotherapy-induced immunity disorders and to prevent cancer development in ROS-susceptible Atm- and p53-deficent mice (Reliene and Schiestl 2006). In a rat brain tumor model, NAC treatment prevents myelosuppression without compromising treatment efficacy (Neuwelt et al. 2004). With the chloroethylnitrosourea BCNU, pretreatment with NAC prevents drug associated redox dysregulation and myelotoxicity. Through carbamoylation of active cite cysteines, BCNU can inactivate glutathione reductase and other enzymes linked with GSH homeostasis (Tew et al. 1985). In this regard, the drug's myeloprotective activity may be mediated through the protection of some or all of these pathways enabling normal GSH synthesis and maintenance of intracellular GSH balance.

Although considered quite benign from a pharmaceutical perspective, adverse effects have been reported for NAC. In a mouse model extremely high doses can cause cardiac and pulmonary damage. Primarily in the liver, NAC is metabolized to *S*-nitroso-*N*-acetylcysteine that can create a hypoxia like response and lead to pulmonary artery hypertension (Palmer et al. 2007). Even though the mouse studies used doses of NAC that were significantly higher than those used in therapeutic applications in man, these toxicities are relevant.

Other bioavailable GSH precursors include glutathione monoesters in which the carboxyl group of the glycine residue is esterified. Unlike glutathione itself, such esters are effectively transported into various types of cells and can be converted intracellularly into glutathione (Levy et al. 1993). Glutathione diethyl ester is rapidly split to the glutathione monoethyl ester by mouse plasma glutathione diester α-esterase. Both the glutathione mono- and diesters have similar effects on cellular glutathione levels in mice. However, human plasma lacks glutathione diester α-esterase and as a consequence human cells transport glutathione diethyl ester much more effectively than the corresponding monoethyl (glycyl) ester. Human cells rapidly convert glutathione diethyl ester to the monoester and can provide the cells with a means of producing glutathione over a protracted period of time. Thus the glutathione diethyl ester is an effective delivery agent for glutathione monoester and thus for glutathione.

16.3.3 NOV-002

Viral diseases frequently cause depletion of GSH and treatment of HIV patients with precursors of GSH such as N-acetylcysteine have a positive impact upon survival (Herzenberg et al. 1997). GSH levels in antigen presenting cells can influence their T helper1 and Th2 cytokine response patterns in immune response and explains for the differences in immune responses in "Th1" and "Th2" mouse strains (Peterson et al. 1998). These effects may be mediated by extracellular redox changes that can also impact intracellular redox signaling and proliferation in peripheral blood mononuclear cells (Sahaf et al. 2005). Such factors are consistent with some aspects of the pharmacology of NOV-002, an agent that has myeloproliferative properties and causes transient oxidation of cell surface proteins, reflected by time sensitive alterations in intracellular redox homeostasis. NOV-002 (Fig. 16.2, panel A) is a formulation of glutathione disulfide (GSSG), containing a trace amount of cisplatin, (1,000:1molar ratio). The cisplatin component does not contribute to efficacy via cytotoxicity. A 60 mg/day dose of NOV-002 administered over 6 months would result in a cumulative total dose of cisplatin equivalent to <2% of a typical chemotherapeutic single dose of cisplatin. Animal pharmacokinetic studies suggest that the addition of cisplatin serves to increase the C_{max} and $t_{1/2}$ for GSSG bioavailability (Townsend et al. 2008).

Unusually for a drug in preclinical and clinical development, the availability of clinical data preceded full preclinical evaluation of NOV-002. A significant number of clinical trials were generated from earlier Russian investigations indicating an unusual pharmacological profile for oncology indications. The data suggested that for lung cancer patients addition of NOV-002 to existing chemotherapeutic regimens produced higher patient survival statistics and improved quality of life parameters. Unfortunately, further Phase III clinical trials carried out under the auspices of the US Food and Drug Administration failed to confirm these positive efficacy results. As a consequence, further development of the drug is now on hold. Nevertheless, a number of interesting features related to the mechanism of action of the drug did emerge from these studies. For example, NOV-002 has little or no cytotoxicity even at high doses, but has the capacity to modulate redox conditions at the cell surface with subsequent intracellular changes in the redox status and cell signaling pathways. Certain cell surface protein thiols can act as sensors of extracellular redox stress and their oxidative modification has been linked to regulation of cell signaling and other functions (Dominici et al. 1999). NOV-002 treatment of cells serves to oxidatively modify cell surface proteins with commensurate changes in a number of characteristics of membrane structure. Subsequent to these changes hydrogen peroxide levels increase resulting in a process that was temporally consistent with the time and concentration dependent increase in the phosphorylation of three kinases, JNK, p38 and ERK. In bone marrow these kinases can play direct roles in the regulation of cell proliferation. Moreover AKT, a kinase also implicated in marrow proliferation acting in concert with JAK2 and STAT5 was also impacted by NOV-002. The JAK-STAT signaling pathway is intimately involved in governing the

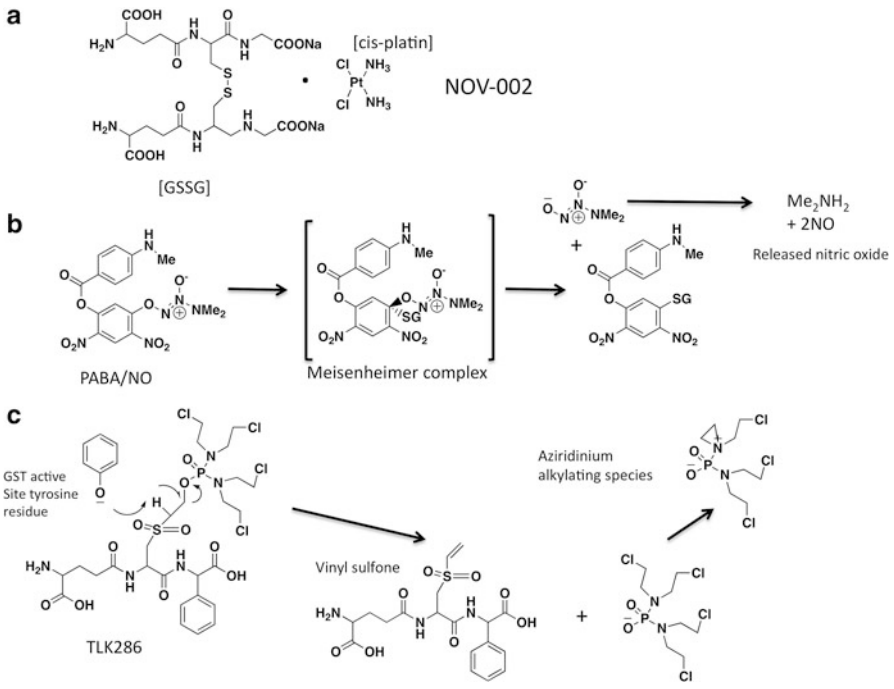


Fig. 16.2 Structures and metabolism of the diglutathione mimetic NOV-002 (*panel A*) and two GSTP activated pro-drugs. For PABA/NO (*panel B*), release of nitric oxide is achieved through an intermediate Meisenheimer complex formed through GSH complex formation under the catalytic activity of GST. *Panel C* shows TLK286 metabolism where proton abstraction can activate the pro-drug to release a vinyl sulfone and tetra-functional alkylating species that become aziridinium species after the chlorines act as leaving groups

response of cells to cytokines and growth factors (O'Shea et al. 2002) and activation of this pathway by NOV-002 indicates that the drug is affecting those pathways that control hematopoiesis and myeloproliferation.

16.3.4 Mesna

Mesna (Mesnex) is the sodium salt of 2-mercaptoethane sulfonate. It is a synthetic sulfhydryl compound used as an adjuvant to reduce the complications arising from hemorrhagic cystitis and hematuria as a consequence of treatment with alkylating agents such as cyclophosphamide or ifosfamide. Each of these cancer drugs is metabolized through the liver cytochrome p450 system to release acrolein that has an electrophilic vinyl group but can be detoxified by interaction with cysteine and GSH. Mesna provides the biologically available thiol that can carry out this reaction in the bladder. In some countries, Mesna is also used as a mucolytic agent, with indications and properties characteristic of NAC (Brock et al. 1984).

16.3.5 Tavocept

Tavocept is 2,2′-dithio-bis-ethane sulfonate a water-soluble disulfide with some characteristics similar to NOV-002. Cellular reducing equivalents provided by glutathione and/or the Grx/Trx systems can reduce Tavocept into the free thiol form, 2-mercaptoethane sulfonate mesna (Verschraagen et al. 2004), the disulfide remains predominant in the oxidative environment of the plasma (Shanmugarajah et al. 2009). Preferential uptake of Tavocept in the kidneys leads to reduction to mesna and results in protection against cisplatinum-induced nephrotoxicity. Tavocept has also been shown to protect against chemotherapy-induced myelosuppression (Hausheer et al. 1998). Mechanisms of action may include pharmacological modulation of physiological thiol and disulfide levels (Pendyala et al. 2003), or the direct conjugation of mesna with platinum species. The γGGT-mediated formation of platinum-SG adducts is prevented by the premature formation of mesna-platinum adducts (Hausheer et al. 1998). Mesna conjugation with GSH also inhibits γGGT and prevents further activation of GS-platinum species. Unreduced Tavocept does provide disulfide equivalents that can influence the Trx and Grx systems and such features do seem to be important in the therapeutic utility of Tavocept in treating lung cancer patients. A drug-induced shift towards oxidized Trx and Grx may impact the signaling pathways that are mediated by these proteins. With most of the thiol:disulfide drug entities, their interface with the Trx system is a critical aspect of their mechanism of action. Trx in particular is involved directly in mediating a series of events critical to cell proliferation or apoptosis. For example, loss of Trx blocks T lymphocyte activation and proliferation and circulating Trx-1 can act like a cytokine as a chemo-attractant for monocytes, neutrophils and lymphocytes in addition to inhibiting neutrophil migration into inflammatory sites (Nakamura et al. 2001; Bertini et al. 1999). Tavocept may impact Trx activity in a manner similar to thioredoxin-interacting protein (TXNIP) that is involved in regulation of HSC quiescence and mobilization (Jeong et al. 2009). Tavocept mediated modulation of the ratio of oxidized and reduced Trx may be important to the myeloproliferative activities associated with the drug. Other redox-cycling antioxidants such as sodium thiosulfate, diethyldithiocarbonate and tempol have some actions that overlap with Tavocept.

16.3.6 Amifostine (WR-2721; Ethyol)

Amifostine (WR-2721) is an agent that emerged from early research by scientists at Walter Reed Army Medical Center in the 1970s. It is an thiophosphate pro-drug subject to activation by blood and tissue alkaline phosphatase to the thiol WR-1065 (Korst et al. 1997). WR-1065 may function mechanistically through the prevention and/or repair of DNA damage induced by ROS, induction of cellular hypoxia, inhibition of apoptosis, as well as the direct covalent binding and inactivation of

cytotoxic drugs. More recent studies indicate additional roles in the regulation of transcription factors and involvement in apoptosis, cell cycle regulation and cell differentiation (Khodarev et al. 2004). The rationale for this lies in the fact that normal tissues have relatively high levels of alkaline phosphatase creating the expectation that more of the prodrug will be activated and protect normal tissues compared to tumor. The therapeutic utility of amifostine is in combination with chemo- or radiation therapies and in 1999 US Bioscience received full FDA approval for Ethyol for the indication of reduction of moderate-to-severe, post-operative radiation-induced xerostomia (dry mouth) in patients undergoing radiation treatment for head and neck cancer.

Amifostine also protects HPCs during chemotherapy and reduces engraftment periods after autologous bone marrow transplantation and stimulates the formation of multipotent and erythroid bone marrow progenitor cell lineages (List et al. 1997) and is used in patients receiving bone marrow transplants, as well as in the treatment of a range of pathologies characterized by deficient hematopoiesis resulting from increased cell death of bone marrow precursors. In MDS, amifostine stimulates hematopoietic recovery (Schanz et al. 2009), selectively induces apoptosis in MDS cell lines but protects non-malignant HPC from cytotoxic drugs (Ribizzi et al. 2000). Mechanistically, such protection has been attributed to inhibition of stress-induced apoptosis in normal HPCs via activation of NF-κB/Rel transcription factors (Romano et al. 1999).

Another organic thiol, vitalethine (Knight et al. 1994) also abrogates apoptotic signals and potentiates hematopoiesis. Aminothiols essentially increase levels of intracellular thiols and thereby abrogate apoptotic signaling initiation, alter DNA replication and cell cycle progression and influence the binding of redox-sensitive transcription factors to target DNA sequences. As with other thiols, amifostine binding to NF-κB, AP-1, and p53 enhances the affinity of these proteins to target regulatory sequences with subsequent impact on the transactivation of downstream genes (Shen et al. 2001). Within this mechanism of action, there is commonality with how thioredoxins influence the binding of the redox-transcription factors, *C-fos* and *C-jun* to DNA. There is a common potentiation of responses to proliferative signals and activation of hematopoietic growth factors, such as the stem cell factor kit ligand (KL) (Abate et al. 1990). The myeloid growth promoting property of amifostine may coincide or even compete with that of KL (List et al. 1998). Furthermore, amifostine can regulate cell cycle progression and apoptosis through the induction of p53 through a JNK-dependent signaling pathway and subsequent expression of the cyclin-dependent kinase inhibitor p21 (Pluquet et al. 2003). Amifostine has recently been identified as a potent hypoxia-mimetic that induces HIF-1 expression and accumulation (Koukourakis et al. 2004). Because the bone marrow niches are defined by gradients of oxygen tension activation of hypoxia-sensitive genes may be required to promote events such as HSC mobilization and differentiation. In all, the thiol delivery system serves a number of distinct and overlapping functions in influencing normal tissue proliferative pathways.

16.3.7 TLK199 (Telintra)

In the mid 1990s, because increased expression of GST isozymes was frequently associated with drug resistant cells, their inhibition was considered a rationale approach to improve therapeutic response. For example, ethacrynic acid, although an effective inhibitor of various GST isozymes was not successful in the clinical management of cancer patients. The dose-limiting toxicity of fluid imbalance was triggered by the diuretic properties of the drug (O'Dwyer et al. 1991). One consequence of this platform was the conceptual design of a peptidomimetic inhibitor of GSTP, TLK199 (γ–glutamyl–S–(benzyl)cysteinyl R(−)phenyl glycine diethyl ester) now called Telintra or ezatiostat. While designed to sensitize drug-resistant tumors that overexpress GSTP (Morgan et al. 1996), preclinical mechanism of action studies with this agent revealed an unanticipated myeloproliferative activity in animals (Gate et al. 2004; Ruscoe et al. 2001). Given the link between GSTP and the kinase pathways, a model for how TLK199 can produce proliferative effects in the marrow compartment was proposed (Fig. 16.3). Increased myeloproliferation in GSTP deficient mice compared with wild type animals (Ruscoe et al. 2001) was characterized by higher levels of white blood cells in GSTP knock out mice. Spleen cell counts were higher in knock out animals, and this was associated with a twofold increase in B-lymphocytes, whereas T lymphocytes and NK cell counts were similar in both strains. In contrast, no difference in thymocyte counts and thymus subset composition was observed. Red blood cell and platelet counts were also higher in GSTP null mice. As a whole, these data inferred that the absence of GSTP expression potentiates hematopoiesis by influencing the proliferation and/or the differentiation of hematopoietic progenitor cells. TLK199 stimulated colony formation (i.e. proliferation) of most major progenitor cells in wild type, but not knock out animals. The JNK inhibitor SP600125 decreased marrow colonies produced by cytokine treatment of knock out animals and JNK phosphorylation was endogenously elevated in bone marrow cells from GSTP null animals. Such data are consistent with GSTP acting as a physiological inhibitor of JNK, where TLK199 disassociates GSTP from JNK allowing kinase phosphorylation and subsequent activation of the kinase cascade (Fig. 16.3; Adler et al. 1999). Figure 16.3 also stylizes the formation of GSTP oligomeric structures. Such structures are frequently observed during gel separation of proteins, even in the presence of reducing conditions and imply that disulfide bonds can inactivate GSTP in either a reversible or irreversible manner (Adler et al. 1999; Townsend et al. 2006) Inhibition of JNK abrogates the increased phosphorylation of this kinase observed in the presence of TLK199 and consequently reduces the myeloproliferative effects suggesting that JNK plays a role in both the genetic and pharmaceutical enhanced myeloproliferation. This is consistent with reports indicating a possible role for JNK in control of proliferation. Discrimination between the survival and apoptotic functions of JNK seems to correlate with the level and duration of the enzyme activation. A strong and sustained activation is associated with apoptosis, whereas a weaker and transient phosphorylation is correlated with proliferation (Shaulian and Karin 2001). For example, in mouse hematopoietic BaF3 cells, JNK activity was

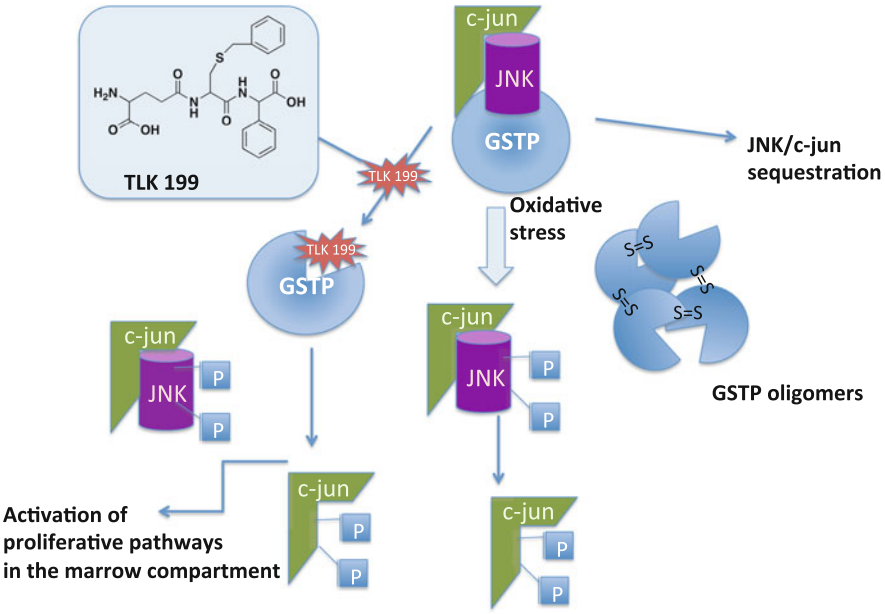


Fig. 16.3 Representative model of signaling cascade regulated by the complex formed between GSTP and proteins of c-jun NH2-terminal kinase. The cartoon illustrates how TLK199 can cause myeloproliferation by activation of JNK mediated proliferative pathways leading to phosphorylation of both JNK and c-jun in the bone marrow compartment. A common theme in regulation of myeloproliferation is the activation potential of ROS and this is mimicked by TLK199. Subsequent drug induced dissolution of the complex can lead to the formation of higher order GSTP structures such as the oligomers shown

three times lower when cells were exposed to mitogenic concentrations of IL–3 than to cytotoxic concentrations of anisomycin (Terada et al. 1997). From such reports, regulation of JNK activity by GSTP should be a viable target for drug intervention. Consequently, Telik Inc. has underwritten a number of clinical trials to test the drug in myelodysplastic syndrome (MDS) patients. A liposomal formulation was developed for intravenous administration and used in Phase 1–2A studies in MDS patients (Raza et al. 2009a). An oral dosage formulation (tablet) was subsequently developed and a Phase 1 trial completed suggesting that the drug is well-tolerated and showed clinical activity (Raza et al. 2009b). Thereafter, the tablet formulation was studied in a Phase II clinical trial setting (Raza et al. 2012). Results from all *Telintra* clinical trials suggested that the agent has a favorable safety profile and provided clinical benefit to some patients and while clinical testing of *Telintra* is still ongoing, a recent case report on a patient with idiopathic chronic neutropenia (ICN; is a heterogeneous group of hematologic diseases characterized by low circulating neutrophil levels often associated with recurrent fevers, chronic mucosal inflammation, and severe systemic infections) was encouraging (Lyons et al. 2011). A 64-year-old female with longstanding rheumatoid arthritis developed ICN with

frequent episodes of sepsis over a 4 year period was treated with granulocyte colony stimulating factors (G-CSF) but had a delayed, highly variable and volatile response. She was enrolled in a clinical trial evaluating the oral Telintra (ezatiostat). She responded by the end of the first month of treatment with stabilization of her ANC (despite tapering and then stopping G-CSF), clearing of fever, and healing of areas of infection. This response was sustained for over 8 months and continues.

16.4 GST Activated Prodrugs

16.4.1 TLK286 (Telcyta)

An improvement of the therapeutic index has always been a major goal in the design and development of new anticancer drugs. Trends towards targeted therapies have resulted in new drugs with particular specificities (e.g., geftinib, erlotinib and imatinib) and prior to the development of drug resistance, improved treatment successes. The first GST activated prodrug to emerge from preclinical studies was TLK-286, now called Telcyta. Its design strategy was based on the principle that proton-abstracting sites at the active site of GST could initiate a cleavage reaction converting an inactive prodrug into a cytotoxic species (Lyttle et al. 1994). The presence of a histidine residue in proximity to the G binding site of GSTP was integral to the removal of the sulfhydryl proton from the GSH co-substrate, generating a nucleophilic sulfide anion. This is more reactive with electrophiles in the absence of GSH and crystallographic analysis of GSTP assigns the tyrosine hydroxyl residue as the proton abstraction moiety (Reinemer et al. 1992). For the scheme shown in Fig. 16.2 (panel B), the proton abstraction deprotonates the α-carbon to yield the sulfone, that subsequently undergoes β -elimination to give the active alkylating species. Unlike other standard anticancer nitrogen mustard drugs (e.g. melphalan, chlorambucil) TLK-286 contains a tetrakis (chloroethyl) phosphorodiamidate moiety. Other compounds with this structure are more cytotoxic than a similar structure with a single bis-(chloroethyl) amine group. As with other mustards, the chlorines can act as leaving groups, creating aziridinium ions with electrophilic characteristics. Although the precise sequential formation of the four possible chlorine leaving events is not known, these species presumably possess the capacity to alkylate target nucleophiles such as DNA bases. The four chlorines could provide the reactivity to form crosslinks with bonding distances greater than those for bifunctional agents. However, the absence of some form of myelosuppression indicates that organ toxicities are not similar to those standard bifunctional alkylating agents studied to date. In Phase I and II trials, Telcyta did not show any significant hematological or cumulative toxicities despite drug treatment periods that were >1 year (Rosen et al. 2004). Furthermore, despite being a micromolar inhibitor of DNA-dependent protein kinase, TLK-286 causes minimal direct damage to DNA (Townsend et al. 2002). For TLK-286, activation to the alkylating species also results in the production of a vinyl sulfone derivative of the

GSH backbone (Fig. 16.2, panel C). Although the biological importance of this species to the pharmacology of the drug is not clear, it could be a factor in the chain reactions leading to lipid peroxidation and even production of hydrogen peroxide. Of relevance to this observation, catalase is over-expressed in cells selected for resistance to TLK-286 (Rosario et al. 2000). In terms of human pharmacology, an optimized two-compartment model describes the pharmacokinetic characteristics of TLK-286 in humans. This model only applies when structural parameters are not normalized for size, but instead for body surface area and body weight. In this model, the clearance and volume of distribution at steady state decrease with increasing dose. The decrease in blood clearance with increasing drug dose caused the dose-normalized area under the blood concentration versus time curve to increase with dose. The combined effect of dose-related decreases in both the clearance and volume of distribution is that increasing doses also increase the distribution half-life but decrease the elimination half-life (Rosen et al. 2004).

The drug's favorable therapeutic index is gained from selective activation by GSTP, an enzyme that is widely over-expressed in tumors compared with normal tissues and in various drug-resistant diseases. The preclinical data support the prediction of the mechanism of activation. Published Phase I and Phase II clinical results were encouraging from the standpoint of tolerance and toxicity. Toxicities were mild, non-cumulative and well tolerated, especially in older patient populations. However, Phase III clinical studies in platinum-refractory or -resistant ovarian cancer have not been conclusive. At this time, the company has somewhat diffuse plans to have further Phase III studies, particularly in NSCLC and ovarian cancer. Development of the drug towards FDA registration will by necessity await further indications of positive enhancements in disease response and/or survival.

16.4.2 PABA/NO

NO is involved in a diverse number of physiological processes characterized in many previous publications, but can also have cytotoxic consequences. As a consequence, directed delivery (tumor cells with high GSTP) of a therapeutic concentration of nitric oxide is a relevant approach to drug design. A strategy to derivatize the 0_2 position of a diazeniumdiolate with protective groups has been used to convert them into substrates for GST (Saavedra et al. 2006). The resulting inactive prodrug can become cytotoxic when localized in a cell that has high GSTP concentrations. PABA/NO (O_2-[2,4-dinitro-5-[4- (N-methylamino)benzoyloxy]phenyl]1-(N,N-dimethylamino)diazen-1-ium-1,2-diolate) has N-methyl-p-aminobenzoic acid bound via its carboxyl oxygen as a 5-substituent on the 2,4-dinitrophenyl ring (Saavedra et al. 2001). PABA/NO belongs to the O_2-aryl diazeniumdiolates, electrophiles shown to transfer their aryl groups to attacking nucleophiles with simultaneous production of ions that release NO at physiological pH (Xinhua 2008). In the presence of GSH PABA/NO is activated by GSTP (Saavedra et al. 2006).

This reaction results in the formation of a Meisenheimer-complex intermediate, and subsequently the leaving group of the reaction generates two moles of NO according to the scheme:

$$\mathrm{PABA/NO + GSH \rightarrow \underset{\text{Meisenheimer complex}}{[PABA/NO\text{-}SG]} \rightarrow PABA\text{-}SG + 2NO + HNMe_2.}$$

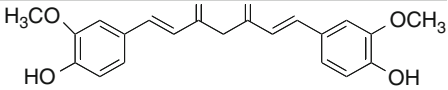
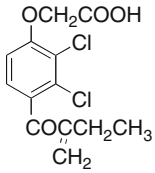
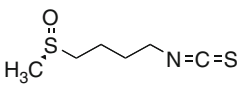
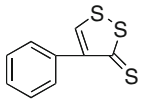
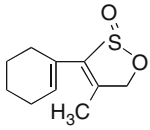
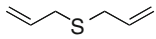
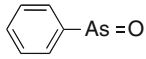
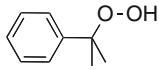
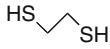
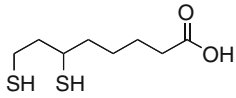
Elevated NO levels lead to cytotoxic effects by forming RNS/ROS intermediates. PABA/NO-induced nitrosative stress results in limited levels of protein nitrosylation but high levels of S-glutathionylation and activates endoplasmic reticulum stress mediated cell death pathways (Findlay et al. 2004). PABA/NO is presently in preclinical development, however, early stage clinical trials with agents of this class are under consideration.

16.5 Sulfur Based Agents in Cancer Chemoprevention

For rational reasons and perhaps because pharmacological cures have been slow to emerge, general approaches to cancer prevention have gained mainstream status over the last few decades. While there are many principles underlying these efforts, improving the detoxification environment by administration of reasonably non-toxic compounds has been a major focus. Connections with redox can be classified into two domains. First, many inductive chemicals influence Phase II metabolic enzyme expression with the outcome that levels of GSH and associated enzyme systems are increased with commensurate protective effects. Second, many small molecules that induce protection have active sulfur moieties that influence the process. Common to each is the indication that the small molecule will have one or more electrophilic centers capable of reacting by one of two basic mechanisms. Bifunctional inducers are large planar or polycyclic aromatics that elevate both Phase II and Phase I (mostly cytochromes p450) genes or monofunctional inducers (a wide diversity of chemical structures) that elevate Phase II enzymes selectively (Spencer et al. 1990). An in depth analysis of those chemical characteristics that contribute to protective enzyme induction can be found elsewhere (Dinkova-Kostova et al. 2004), however, some common characteristics do apply, their reactivity with thiol groups and their impact upon the Nrf2 transcription complex.

Although a wide array of chemical structures have the capacity to induce Phase II detoxification enzymes, close scrutiny of the chemical classes reveals that these compounds have characteristics that place them into approximately ten classes. Further breakdown identifies commonalities with respect to thiols and to induction of genes through the Nrf2 transcription factor complex. For example, Table 16.1 shows some of the chemical structures of small molecules that either have active sulfur centers or impact through thiol-mediated routes on downstream gene expression pathways. These agents can include, curcumin, ethacrynic acid, cinnamates, crotonates, acrylates and a variety of indole agents that have Michael reaction acceptor characteristics. Reactivity of Michael acceptors with nucleophiles

Table 16.1 Examples of agents with chemopreventive properties

Drug name	Type of agent	Structure
Curcumin	Double Michael addition	
Ethacrynic acid	Michael addition	
Sulforaphane	Isothiocyanate	
4 phenyl-1, 2-dithiole-3-thione	Dithiol thione	
3-cyclohexenyl-4-methyl-1,2 oxathiol-3-ene-2-oxide	Oxathiolene oxide	
Diallyl sulfide	Alkyl sulfide	
Phenylarsine oxide	Trivalent arsenical	
Cumene hydroperoxide	Organic peroxide	
1,2 ethanedithiol	Vicinal mercaptans	
Lipoic acid	Vicinal dithiol	

is contingent on the strength of the electron-withdrawing function and the potency of induction of Phase II enzymes in a prevention setting correlates with the strength of the electron withdrawing group and subsequently the nucleophilicity of the electrophilic carbon center.

These react quite readily with thiol nucleophiles particularly GSH and the thioether conjugates can have biological impact on a number of functional processes. For example, for many years ethacrynic acid has been used as a diuretic and as a reversible and irreversible inhibitor of GST isozymes. Michael addition reactions tend to be quite non-specific and this equates to a broad spectrum of "off-target" effects that complicate the use of this type of drug in a prevention setting

and as mentioned earlier, its therapeutic activity in cancer treatment was restricted by the diuretic properties.

The N=C=S functional group of all isothiocyanates reacts at its highly electrophilic central carbon atom with biological thiols to give dithiocarbamates. Those isothiocyanates, thio-, dithiocarbamates and related sulfur compounds shown in Table 16.1 all induce expression of detoxification enzymes. Some isothiocyanates are found in cruciferous vegetables (e.g. sulforaphane is in broccoli) as glucoside precursors. The consumption of these vegetables exposes them to myrosinase that catalyzes their conversion to the reactive isothiocyanates. The accumulation of sulforaphane in various tissues contributes to its high potency as an inductive agent and intracellular accumulation can occur following conjugation with cellular glutathione under catalysis by GST's. In mice, 1,2-dithiole-3-thione derivatives can protect against toxicity and carcinogenicity. The anti-schistosomal agent oltipraz has been developed as a chemopreventive agent against liver cancer in China, where aflatoxin contamination of stored food can produce DNA adducts. More recently, a variety of polysulfides from Allium plants have been developed as preventive agents, with diallyl disulfide being the most potent. Hydroperoxides, including hydrogen and cumene hydroperoxide produce species that react rapidly with non-protein thiols such as GSH. Trivalent arsenicals, particularly those with vicinal thiols, for example phenylarsine oxide, are potent inducers of Phase II enzymes. In a similar manner, vicinal dimercaptans and dihydrolipoic acid also have closely spaced thiol groups.

Analysis of the common aspects of the chemistry of these inducers points to the critical nature of the thiol reactive centers. In general, each inducer has the potential to interact with the Nrf2:Keap1 transcription machinery in a manner that results in widespread over-expression of Phase II metabolism genes. Whether the events are likely to have long-term value as a cancer prevention strategy remains to be established, but the mechanism of this process is discussed in more detail in the next section.

16.5.1 Transcriptional Regulation and Drug Response – Nrf2 and ARE's

Reactive oxygen and nitrogen species (ROS; RNS) are byproducts of aerobic metabolism and contributors to cellular redox balance. Upstream of the many components of GSH homeostasis, the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) is one master switch that regulates the expression of stress response genes critical in mounting a cellular defense against electrophiles or ROS/RNS. Nrf2 can impact expression of genes that have one or more antioxidant response elements (ARE) in their promoter regions. One domain of NRF2 negatively regulates its activity through protein–protein interactions with Kelch-like ECH-associated protein 1 (KEAP1), a member of a metalloprotein family with

between 25 and 27 conserved cysteine residues, essentially half of which are in basic regions, and maintain the thiolate state under physiological conditions. Such cysteines can fulfill the criteria required of redox sensors. They also provide a platform for cancer chemoprevention by which long-term exposure to low doses of inductive natural product (dietary) electrophiles can induce protective phase II enzyme systems.

There are complications associated with the approach of enhancing Nrf2 as a mechanism of chemoprevention (Hayes and McMahon 2006, 2009). For any of the (primarily) electrophilic agents discussed in the preceding section, their activities are contingent upon long term, low dose regimens that constitute chronic "low key" stress. Over a period, these chemical events could prove to be problematic. For example, somatic mutations that alter Nrf2/Keap1 interactions can cause constitutive activation of the Nrf2 transcription complex with potentially oncogenic consequences. This concept has been addressed by a report (DeNicola et al. 2011) using primary murine cells, where expression of endogenous oncogenic alleles such as *Kras, Braf* and *Myc* has an impact upon Nrf2 and the downstream gene clusters that regulate cellular redox balance. In cancer cells, constitutively elevated levels of Nrf2 can occur through both diminished Nrf2 turnover and increased levels of Nrf2 mRNA. Conditional endogenous oncogenes K-Ras^{G12D}, B-Raf^{V619E} and Myc^{ERT2} can each stably elevate basal Nrf2 through enhanced transcription, activating the cluster of genes involved in ROS response and lowering intracellular levels of ROS (a more reduced intracellular environment). Oncogene-directed, enhanced expression of Nrf2 may be a mechanism for the activation of the Nrf2 antioxidant program and genetic targeting of the Nrf2 pathway may impair K-Ras^{G12D}-induced proliferation and tumorigenesis *in vivo*. In such circumstances, the Nrf2 antioxidant and cellular detoxification program may be a (Hayes and McMahon 2006) mediator of oncogenesis. This interpretation may be contextual since oncogenic signaling mediated Nrf2 activation stimulates not only the antioxidant battery but also transporters, proteasome subunits, heat shock proteins, growth factors and other Phase II metabolizing gene products (Hayes and McMahon 2009). Moreover, glutathione S-transferases are among the genes regulated by NRF2 and the importance of the link with S-glutathionylation may prove important (Townsend et al. 2009). Alterations in Nrf2 activity could shift the balance of GST involvement in the forward S-glutathionylation reaction, perhaps producing changes in post-translational patterns that in turn promote proteins that regulate oncogenesis. In the end, these results do not directly suggest that chemoprevention is a flawed strategy, since dietary supplements would need to be viewed through a requirement for existing predisposing genetic abnormalities, at least one of which would involve Nrf2. Nevertheless, they do support the principles that tumor cells have aberrant methods for equilibrating ROS and these pathways could prove to be viable therapeutic targets. A schematic representation of the impact of oncogene signaling on Nrf2 functions is shown in Fig. 16.4.

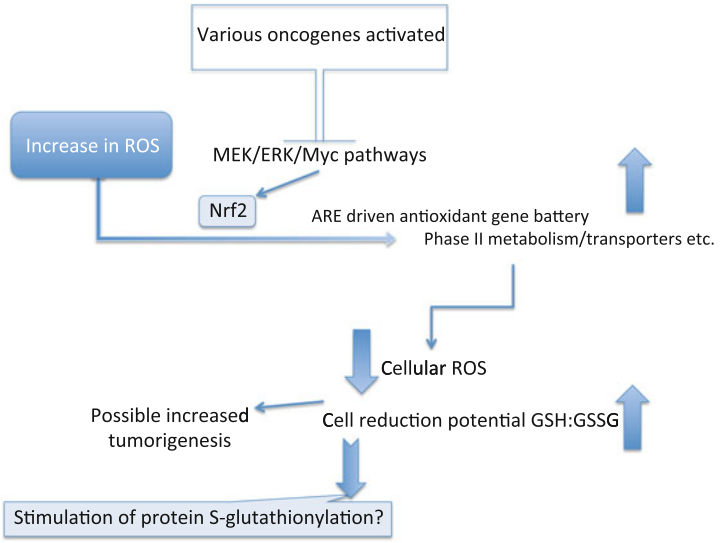


Fig. 16.4 Nrf2 is one of the primary transcriptional controls governing antioxidant response to stress. Its activation by electrophilic natural products is one approach to the therapeutic approaches involved in chemoprevention. As discussed in the narrative, there are possible inherent complications to this as a chronic or long-term strategy to enhancing cellular protective enzymes. The scheme summarizes the influence of Nrf2 and oncogene stimulation on downstream redox pathways

16.6 Summary and Outlook

Evolution has acted over many millennia to fine tune adaptations to an oxidative environment. As a consequence, cellular pathways to handle ROS and RNS are complex, redundant and multi-layered. Perhaps because of their inherent genetic plasticity and instability, many cancer cells have abnormal redox balance. Consequently, those pathways that regulate redox homeostasis have been a focus for therapeutic intervention. Along the way, it has become apparent that the regulation of bone marrow cell proliferation is also sensitive to redox changes and a number of small molecule myeloproliferative drugs are at various stages of preclinical and clinical development. Success to date in gaining FDA approval for such agents has been limited (see summary in Table 16.2), but the pipeline for redox targeted drug discovery and development remains robust. Moreover, the growing interest in the importance of manipulating redox in other human pathologies, particularly neurodegenerative, traumatic brain injury and cardiovascular diseases provides further opportunities to design and test small molecule agents that may gain therapeutic advantage.

Table 16.2 Redox active drugs at various stages of development

Drug name	Target and/or mechanism	Development status
N-acetyl cysteine (NAC)	Cysteine precursor. Enhances GSH synthesis	Many clinical applications (see text) particularly rescue from acetaminophen toxicity
Mesna	Provides available thiol equivalents	Used to prevent side effects of alkylating agent therapies and as mucolytic agent
Tavocept	Can be reduced to provide thiol equivalents	Pharmacology similar to sodium thiosulfate, diethyldithiocarbonate and tempol
Amifostine (WR-2721; Ethyol)	Thiophosphate pro-drug	1999 FDA approval for Ethyol for reduction of radiation-induced xerostomia (dry mouth)
NOV-002	Glutathione disulfide mimetic alters cellular redox	Phase III trial in combination with cancer drugs in lung cancer failed 2010. Some clinical trials continue
Telintra	Peptidomimetic inhibitor of GSTP	Ongoing Phase II trials in myelodysplastic syndrome
Telcyta	GSTP activated pro-drug	Phase III trials in ovarian cancer were negative, but other Phase II/III trials continue
PABA/NO	GST activated pro-drug releasing NO	Preclinical development but some early Phase I studies with similar prodrugs are beginning

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