Chapter 9
The Cellular 'Thiolstat' as an Emerging Potential Target of Some Plant Secondary Metabolites

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1 Introduction: Redox Maintenance and Regulation in Biological Systems

Several biological macromolecules can be reversibly oxidized or reduced and this can affect their properties and thus influence their function either positively or negatively. Indeed, this simple fact is the basis of some complex regulatory machinery in the cell. The redox environment in the cell needs to be closely buffered and monitored so that the multiplicity of the cell's biochemistry runs smoothly in an integrated fashion. Over-reduction, leading for example to misfolding of proteins in the endoplasmic reticulum (ER), and over-oxidation are both harmful to the cell's physiology (Delic et al. 2012; Higa and Chevet 2012). The redox state of particular cysteine thiols in the cell depends upon a number of factors such as their accessibility, specific pK_a, nature of surrounding amino acids and not just the thermodynamics but also the kinetics of possible oxidation/reduction reactions (Dalle-Donne et al. 2009; Nagy 2013; Winterbourn and Hampton 2008). Two paradigms, which are not mutually exclusive but perhaps also are not equally represented in cells, are relevant to the control of thiol-based micro-switches. In the first of these scenarios a particular thiol is in thermodynamic equilibrium within its subcellular environment and the ratio of oxidized to reduced forms is determined by the local redox potential. In the second scenario thermodynamic equilibrium is not assumed and the kinetics of oxidation of a relatively few target protein thiols 'sense' oxidative changes

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in the cell and function as signaling intermediates by relaying information, before being enzymatically reduced back to their basal degree of oxidation (Winterbourn and Hampton 2008). Many experimental data tend to support the second scenario but do not rule out the first for specific instances. It is often stated that the glutathione:glutathione disulfide redox couple (GSH:GSSG) buffers changes in cell redox. However, the situation is complex and although oxidative treatments often lead to a shift in the degree of total cellular glutathione oxidation, GSSG may be largely rapidly removed from the cytosol, for example into the vacuole, thus leaving the local electrochemical cell potential unaltered (Morgan et al. 2013). Thus, caution must be exercised in interpreting whole cell GSH:GSSG ratios in terms of electrochemical potentials in specific cellular compartments. Nevertheless, having stated this, it is a fact of physical chemistry that the redox environment, given the above provisos, will influence the oxidation state of accessible thiols. Therefore, it is important to consider how the redox environment in cells is maintained.

The redox status of the cell is constituted and buffered by a series of redox couples, which are pairs of molecules existing in reduced or oxidized states. For example: $NADH/NAD^+ + H^+$, $NADPH/NADP^+ + H^+$, reduced, and oxidized glutathione (GSH/GSSG) and reduced and oxidized ascorbate (ascorbate/dehydroascorbate) buffer redox systems in the water-soluble cell compartments and reduced and oxidized vitamin E molecules buffer redox changes in the lipophilic cell fractions (Foyer and Noctor 2005). These redox buffers can either directly react with redox active compounds or act as electron donors or reducing equivalents for enzymatic reactions. GSH, for instance, acts as electron donor for glutathione-dependent oxidoreductases ('glutaredoxins') and thioredoxins are reduced via Thioredoxin Reductases (TrxR) with electrons from NADPH. The redox potential (also called the oxidation-reduction potential or midpoint potential) of a redox pair represents the tendency of the oxidized form to acquire electrons and be reduced (and *vice versa*) and is defined by the half-cell electrochemical potential (standard redox potential) of the couple ($E^{0'}$ in mV). The redox buffering capacity of a redox couple is determined by the pool size. The standard-redox potential is empirically defined as the 'dimension' for the relative affinity of an electron-acceptor for electrons and is normalized against the half-cell potential of the standard reaction: $H^+ + e^- \rightarrow ½\ H_2$ (oxidized and reduced forms both at 1 M, 298 K) which is arbitrarily given the value 0 V under standard conditions. In Biochemistry, most of the molecular species met with are not stable under the 'standard' conditions, so the reference potential ($E^{0'}$) for a redox couple is usually quoted at pH 7.0 (see also Chap. 4).

In most cells the $NADH/NAD^+$ and $NADPH/NADP^+ + H^+$ couples have the lowest redox potential ($E^{0'} = -315$ mV). The GSH/GSSG couple exhibits an $E^{0'} = -240$ mV, and the ascorbate/dehydroascorbate couple has a higher half cell potential ($E^{0'} = +54$ mV) (Schafer and Buettner 2001). The direction of electron flow is from lower (more reduced) to higher (more oxidized) redox potential [assuming that such a flow is possible mechanistically and not prohibited for kinetic reasons (see Chap. 4)].

The subcellular compartmentalization of the various redox couples and their *in situ* concentrations are important factors which determine local redox

environments (redox state and buffering capacity) within cells. As defined by Schafer and Buettner "The redox environment of a linked set of redox couples as found in a biological fluid, organelle, cell, or tissue is the summation of the products of the reduction potential and reducing capacity of the linked redox couples present" (Schafer and Buettner 2001). When the oxidized and reduced forms of a redox couple are not present in a 1:1 ratio, as is usually the case in a cellular compartment, the redox potential can be calculated using the Nernst equation. The relative proportions of oxidized/reduced partners in the NAD(P)H/NAD(P)$^+$ and ascorbate/dehydroascorbate couples both determine and reflect the local redox potential (E_{hc}) independently from their overall absolute concentrations, whereas for the GSH/GSSG couple, not only the relative proportions of oxidized and reduced forms, but also their absolute concentrations must be taken into account. A consideration of the Nernst equation makes it clear why not only the proportions but also the concentrations of GSH/GSSG in the pool determine the redox potential (E_{hc}):

The Nernst equation:

$$E_{hc} = E^{0'} - \left[\left(\frac{RT}{nF}\right)\ln Q\right]$$

where E_{hc} = the electrochemical half-cell potential under the prevailing conditions; $E^{0'}$ = the reference half cell potential (pH = 7); R = the universal Gas Constant; T = 298 K or 25 °C; n = the number of electrons exchanged; F = the Faraday Constant; and Q is the mass action term.

Simplifying for the constants and converting from ln to $\log_{10}$ the expression becomes

$$E_{hc} = E^{0'} - \left[\left(\frac{59.1\ \text{mV}}{n}\right)\log Q\right]$$

For example for the NADP$^+$/NADPH half-reaction couple

$$\text{NADP}^+ + 2\text{e}^- + \text{H}^+ \rightarrow \text{NADPH}$$

$$E_{hc} = -320\ \text{mV} - \left[\left(\frac{59.1\ \text{mV}}{2}\right)\log\frac{[NADPH]}{[NADP^+]}\right]$$

at pH 7 and 25 °C.

Thus, irrespective of the absolute concentrations it is sufficient to know the relative proportions of NADP$^+$ and NADPH present in order to calculate the redox potential; whereas for GSH/GSSG:

$$\text{GSSG} + 2\text{e}^- + 2\text{H}^+ \rightarrow 2\text{GSH}$$

$$E_{hc} = -240\ \text{mV} - \left[\left(\frac{59.1\ \text{mV}}{2}\right)\log\frac{[GSH]^2}{[GSSG]}\right]$$

at pH 7 and 25 °C.

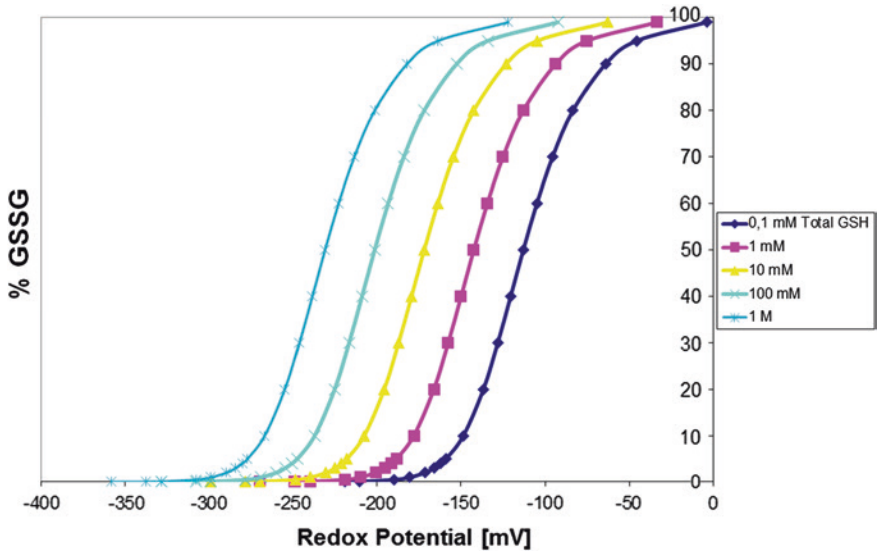

Fig. 1 The redox potential (E_{hc}) for the GSH:GSSG redox couple at varying degrees of oxidation for five GSH concentrations

Note: This form of the equation has the log concentration term for the reductant divided by the log concentration term for the oxidant. Another form of the equation has the log concentration term for the oxidant divided by the log concentration term for the reductant and avoids the use of the 'minus' before the term

$$E_{hc} = -240\ \text{mV} + \left[\left(\frac{59.1\ \text{mV}}{2}\right)\log\frac{[GSSG]}{[GSH]^2}\right]$$

at pH 7 and 25 °C.

Because of the $[GSH]^2$ 'squared term' introduced through the law of mass action, the relative proportion of GSH:GSSG in the pool is not sufficient to calculate the redox potential (E_{hc}); the absolute concentrations must be known and substituted in the equation. This effect is illustrated in Fig. 1. At this point it is pertinent to mention that much work published on GSH reports only changes in the relative amounts of GSH:GSSG. A change in the GSH:GSSG ratio documents a qualitative shift in the redox status of the system but it does not give information as to the degree of redox change or the absolute redox status (Fig. 1).

The GSH concentration in a plant cell is approximately ten times that of NADH and NADPH, respectively, and values in the range from 1 to 10 mM are commonly measured (Noctor 2006). Because of this high intracellular concentration it is generally held that GSH plays an important role as a cellular buffer against redox changes. Interestingly, the size of the ascorbate pool in plant cells can also be relatively large (~10–100 mM), endowing it with a high buffering capacity, but at $E^{0'} = +54$ mV it has a much higher standard half-cell potential than the $NAD(P)^+$/NAD(P)H and GSH/GSSG redox couples (Schafer and Buettner 2001).

Mutants completely unable to synthesize GSH are usually lethal but the traditional view of the GSH pool as a buffer against redox change has recently been challenged, at least for yeast (*Saccharomyces cerevisiae*). Here the major redox buffer role in the cytosol was attributed to Trx with only a back-up role proposed for GSH, which was, however, essential for Fe-S cluster synthesis, making the *gsh*1 deletion lethal. High GSH levels were postulated to be necessary because the pool size reduces under oxidative stress—i.e., the excess GSH was postulated to be simply necessary to ensure adequate supply for iron metabolism whereas Trx protected the redox environment (Kumar et al. 2011). In a further publication from this group the redox control in other yeast cell compartments was also elaborated upon. It was reported that while the cytosol possessed both Trx and GSH pathways in full, of which the Trx pathway was dominant, the mitochondrial matrix also possessed both pathways but here the GSH pathway had the major role in redox control. In both compartments GSH was essential for non-redox functions in Fe-S cluster synthesis. Furthermore, it was reported that the endoplasmic reticulum (ER) and mitochondrial intermembrane space (IMS) were sites of intense thiol oxidation but lacked thiol-reductase pathways except for GSH (Toledano et al. 2013). Furthermore, real-time measurements of the cytosolic redox potential in yeast using a Grx1-roGFP reporter, which is in thermodynamic equilibrium with the GSH/GSSG couple (Meyer and Dick 2010), showed that although under oxidative stress conditions the overall cellular glutathione pool became more oxidized, the cytosolic redox state was little affected (Morgan et al. 2013). The authors suggested that GSSG in the cytosol which was not immediately reduced was transported into the yeast vacuole and that the overall cellular GSH:GSSG ratio was a poor indicator of the actual cytosolic redox potential which tended to be approximately 100 mV lower than would be predicted (*ibid.*).

In cells a kind of 'redox flow' can be envisaged where electrons pass through an open ended system along a gradient from lower (more negative) to higher (more positive) redox potential, and 'new' redox potential is 'created' at the bottom end in plant cells by converting solar energy into electron transport along a series of electron carriers in the thylakoid membranes in chloroplasts to ferredoxin (Fd, $E^{0\prime} = -430$ mV). The enzyme ferredoxin-$NADP^+$ reductase uses reduced Fd to reduce $NADP^+$ to NADPH and these molecules are ultimately the sources of reducing potential at the beginning of the chain to re-reduce oxidized members of downstream redox couples. In animal cells and in non-photosynthetic plant tissues, highly reduced substrates, such as carbohydrates, are oxidized by NAD^+ to release reducing equivalents in the form of NADH. New NAD(P)H must be generated as required to keep the central cellular GSH and ascorbate redox buffers replenished. Ultimately, the majority of electrons flow to oxygen, reducing it to water. On their journey, however, partial one-electron and two-electron reductions of O_2 can occur and give rise to reactive oxygen species (ROS), such as the superoxide radical anion $O_2^{\bullet -}$ and hydrogen peroxide, respectively. Protecting the cell against these potentially damaging products of metabolism is very important. Intermediate on this 'electron highway' are thiol groups and here it must be emphasized that, quantitatively, oxidizable thiols in proteins may exceed the contribution of low

molecular weight thiols (e.g., GSH) by up to five-fold (Dietz 2005; Konig et al. 2012). Furthermore, the thiol-disulfide exchange reactions entered into by thiols under oxidative conditions are perhaps better viewed as nucleophilic substitutions rather than electron transfer reactions, which is why NADPH as a reductant with a lower redox potential than thiols does not protect these from oxidation directly, but only indirectly by serving as a source of reducing power for enzymes such as glutathione reductase (Giles and Jacob 2002; Gruhlke and Slusarenko 2012). Conversely, excess GSH can directly protect protein thiols from oxidation (Foyer and Noctor 2005; Gruhlke and Slusarenko 2012; Noctor et al. 2011).

In the cell, it is often assumed, although this may not be the case, that redox couple partners tend to be in equilibrium between their reduced and oxidized forms and the global redox environment. Perhaps paradoxically, the position of the equilibrium for a given redox couple both depends upon and helps to determine the global cellular redox environment, which includes all thiols of a particular cellular compartment, including protein cysteine residues, free cysteine/cystine, and the tripeptide glutathione. The reduction of a disulfide by a thiol resulting in a mixed disulfide is known as thiol/disulfide exchange reaction and establishes the redox equilibrium of all thiol and disulfide groups (Jocelyn 1972). The nucleophilic exchange occurs best with the thiolate ion ($R-S^-$) rather than the thiol group and due to the high pK_a of most –SH groups (pK_a for free cysteine = 8.3) this means that below pH 7.0 the reaction will not proceed 'unaided' (Hofmann et al. 2002). As pointed out, however, earlier the redox state of a particular cysteine thiol depends on its accessibility, the specific pK_a, whether there are nearby basic amino acids and not just on the thermodynamics but also, very importantly, the kinetics of possible oxidation/reduction reactions (Dalle-Donne et al. 2009; Nagy 2013; Winterbourn and Hampton 2008). The prevailing opinion is perhaps that many protein thiols in the cell are not in thermodynamic equilibrium with the global redox environment and that oxidative stress signaling occurs predominantly upon the triggering of specific sensor trigger proteins which are direct targets for oxidants (Winterbourn and Hampton 2008). Many experimental data tend to support this scenario but do not rule out the influence of the thermodynamic equilibrium mechanism for specific instances.

The alternative view assumes a greater role for the global redox environment of the subcellular compartments and presumes that this is largely governed by the GSH/GSSG redox couple. The cysteine-containing tripeptide glutathione, the thiol species with the highest cellular concentration, can act as electron-donor for different redox reactions either directly (by reduction of different oxidants) or in an enzyme-catalyzed manner (e.g., enzymes that reduce protein-disulfides in a glutathione-dependent manner [glutaredoxins] or reduce peroxides using glutathione as electron source [glutathione-peroxidases]) and for the regeneration of other redox buffering systems like ascorbate/dehydroascorbate). Because of the central redox buffer position that GSH holds, and because of the likely equilibrium between GSH and at least some other cellular thiols, one can conceive of a cellular 'thiolstat' (Gruhlke and Slusarenko 2012; Jacob 2011) in analogy to a 'rheostat' as a 'variable resistor' or an 'instrument

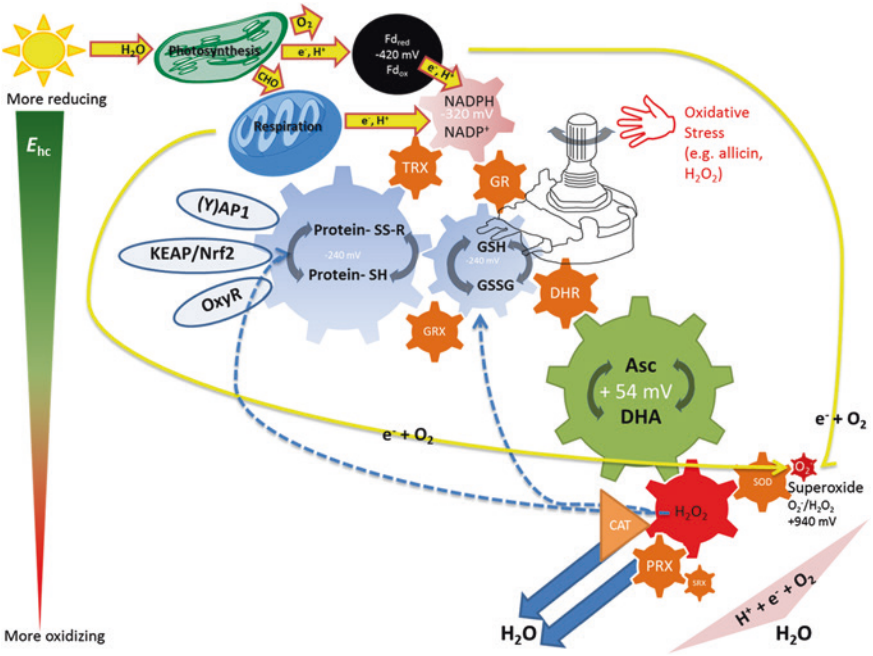

Fig. 2 Reductant–antioxidant–oxidant interactions in redox homeostasis and signaling in a typical plant cell. The flow of electrons through the thermodynamic 'open system' from photosynthetically produced reduction potential (reduced ferredoxin, NADPH) to oxygen along the electrochemical potential gradient is illustrated. *Asc* ascorbate, *CAT* catalase, *CHO* carbohydrate, *DHA* dehydroascorbate, *DHR* dehydroascorbate reductase, e^- electron, *Fdred/Fdox* reduced/oxidized ferredoxin, *GR* glutathione reductase, *Grx* glutaredoxin, *Prx* peroxiredoxin, *SOD* superoxide dismutase, *Srx* sulfiredoxin, *Trx* thioredoxin; KEAP/Nrf2, OxyR, and Y(AP1) are transcription factors involved in oxidative stress responses (see text)

for controlling and varying within limits the value of resistance in an (electrical) circuit' (Anon 1968). Thus, on the supposition that there is potentially an equilibrium between cellular thiols (glutathione and protein thiols) and other redox systems with either higher (e.g., ascorbate or tocopherol) or lower (e.g., $NAD(P)^+/NAD(P)H$) redox potentials, glutathione because of its high cellular abundance (around 5 mM in animal cells, which is around 500-fold higher than the NADPH/thioredoxin system) (Filomeni et al. 2002; Schafer and Buettner 2001) is often considered to be a central regulon of the redox state in the cell. Hence, the 'thiolstat' can be defined as the overall redox status, which is in equilibrium with the glutathione status in particular and so allows using the GSH/GSSG-redox couple as an indicator for all cellular thiols and thus cellular redox in general. Viewed simply, the cellular 'thiolstat' reflects the proportion of oxidized:reduced thiol groups in the cell (Fig. 2).

An excellent example of the thiolstat in action is the observation that seed viability can be predicted from knowing the redox potential of the GSH/GSSG

redox couple (Kranner et al. 2006). The authors studied seeds from several plant families and species and showed that when under stress conditions, $E_{GSSG/2GSH}$ increased to −180 mV that seed germination rate decreased by ~50 % and at a potential higher than −160 mV seeds lost viability completely. The authors concluded that $E_{GSSG/2GSH}$ was a universal marker of plant cell viability (*ibid*.). However, the situation is complex and although oxidative treatments often lead to a shift in the degree of total cellular glutathione oxidation, GSSG may be largely rapidly removed from the cytosol, for example into the vacuole, thus leaving the local electrochemical cell potential relatively unaltered (Morgan et al. 2013). Thus, caution must be exercised in interpreting whole cell GSH:GSSG ratios in terms of electrochemical potentials in specific cellular compartments. Nevertheless, having stated this, it is a fact of physical chemistry that the redox environment, given the above provisos, will influence the oxidation state of accessible thiols.

2 The Redox Switch Concept

Cysteine residues are particularly sensitive to redox changes. The redox couple cysteine/cystine has a standard half-cell potential of around −220 mV (Jocelyn 1967). Since the half-cell potential describes the equilibrium conditions of the redox system, this means that at higher reduction potentials the equilibrium is shifted to a more oxidized, at lower potentials to a more reduced state. Nonetheless, especially for protein-thiols the equilibrium does not exclusively depend upon the half-cell potential, but also depends on the pK_a of the surrounding functional groups, which affect the degree of dissociation of the protein-thiol (Nagy 2013).

The translation of changes in the cellular thiol status to changes in the oxidation state of a single protein-thiol has been called a ‘nano-switch’ (Paget and Buttner 2003; Schafer and Buettner 2001). Since the catalytic or regulatory function of proteins is often dependent on the oxidation state of particular cysteine residues, a shift of the overall cellular thiolstat can change the position of many switches in a single sweep, and not necessarily only regulate a few specific targets in a conventionally ‘linear’ signaling cascade (Jacob et al. 2006; Jensen et al. 2009; Jones 2008; Kamata and Hirata 1999; Winterbourn and Hampton 2008).

It was shown empirically in cell cultures that physiological states like proliferation, differentiation, apoptosis, or necrosis correlated with the calculated cellular redox potential using the Nernst equation and the absolute glutathione concentrations (Cai and Jones 1998; Cai et al. 2000; Gruhlke et al. 2010; Hutter et al. 1997; Hwang et al. 1992; Jones et al. 1995; Kirlin et al. 1999).

Although these concrete correlations between redox and physiological status were shown in animal and human cell lines, the inference that the findings are applicable to other cell types, such as fungal, bacterial, or plant cells, seems likely,

since the mechanism of influencing protein function by alteration of the thiol status is transferable. Indeed, this assumption has been shown to be valid at least for seed germination potential by Kranner et al. (2006). Hence, attempts to intentionally influence the thiolstat, based on the redox switch concept, need not be limited to animal systems (e.g., in cancer treatment), but would also represent a valid strategy in agricultural plant protection.

The change in a protein's redox status needs to be transduced into a physiological response, i.e., by affecting the cell's metabolism or altering gene transcription. An important point in this respect is how global redox change is translated into specific signaling events regulating specific responses. Well-known examples of transcription factors which transduce the redox switch into a physiological response are the OxyR and SoxS transcription factors from *Escherichia coli* and members of the mammalian AP1 family (see also Chap. 5). The latter have been well studied for the yeast AP-1 homolog (YAP1). A comparison of these two redox switch models is given in Choi et al. (2001).

Bacteria have developed a broad range of different redox sensors and resistance mechanisms to adapt to and to defend against oxidative stress conditions (Green and Paget 2004). OxyR regulates the response to H_2O_2 and the SoxRS system is important for resistance against redox active natural products with antibiotic activity (Chater 2006; Dietrich et al. 2008; Mavrodi et al. 2006). The SoxS transcription factor regulates the superoxide response (Demple 1991; Green and Paget 2004). Mechanistically the change in redox status is transduced to changes in the cysteine residues of the transcription factor that becomes activated and subsequently facilitates the transcription of stress-related and antioxidative enzymes in a coordinated way.

How the thiol microswitches in Yap1 are differently regulated by oxidants such as H_2O_2 and diamide is relatively well understood (Azevedo et al. 2003, 2007; Delaunay et al. 2000) and specific switching by H_2O_2 or diamide leads to largely oxidant-specific protection responses via the activation of characteristic sets of defense genes (Morano et al. 2012; Ouyang et al. 2011). The Yap1 transcription factor has N- and C-terminal cysteine rich domains (n-CRD and c-CRD) and a nuclear export protein (Crm1) binding domain. In the absence of oxidative stress Yap1 in the nucleus is bound by Crm1 and rapidly exported into the oxidative stress cytosol. In conjunction with a glutathione peroxidase (Gpx3) and the Yap1-binding protein, H_2O_2 leads to an oxidative intramolecular folding of Yap1 involving both n-CRD and c-CRD domains. The intramolecular folding of Yap1 masks the Crm1 binding site and allows Yap1 to accumulate in the nucleus. Thiol reagents such as NEM (*N*-ethylmaleimide), diamide and others, however, have been shown to form adducts to cysteines in the c-CRD and thus block access of Crm1 to the Crm1-binding site (Morano et al. 2012). Accumulation of Yap1 in the nucleus leads to the transcription of sets of oxidative stress induced genes which can be different, depending on whether H_2O_2 or NEM/diamide activated Yap1. Oxidized Yap1 is reduced back to its initial state by the thioredoxin Trx2 (Delaunay et al. 2000, 2002; Meyer et al. 2009; Wood et al. 2004).

3 Examples of How Physiologically Active Secondary Metabolites Can Affect the Overall Thiol Status of the Cell: Plant Defense Compounds

Many metabolites, e.g., glucose or alanine, are present in all living organisms and are thus called 'primary metabolites'. Some metabolites in contrast have a limited taxonomic distribution and are often produced in specific organs or at particular developmental stages; these are called 'secondary metabolites'. Secondary metabolites often accumulate to very large amounts. A variety of natural products of so-called 'secondary metabolism', especially in plants, play important roles in defense against pathogens, predators, and competitors (see Chap. 1). It might be mentioned here that organisms other than plants produce secondary metabolites, for example fungi and bacteria, and here crucial roles are often to be found in ecological relationships such as competitor suppression. Plants produce a large variety of chemically diverse bioactive molecules. Plant defense substances are classified into two broad groups as either 'phytoanticipins' or 'phytoalexins'. Preformed substances which are present before the plant is attacked, or which are produced rapidly and spontaneously from a preformed substrate by simple chemical or enzymatic modification via a pre-existing enzyme, are called 'phytoanticipins' (Van Etten et al. 1994). They build a first line of chemical defense which can be likened to a booby trap which, when triggered, has immediate unpleasant consequences for any attacker. Allicin from garlic, synthesized when cell damage results in the mixing of the preformed alliin substrate with the preformed alliinase enzyme, is a good example of a phytoanticipin. The second group of chemical defense substances, phytoalexins, are synthesized from distant precursors after pathogen attack and require *de novo* gene expression and the production of enzymes leading to the installation of new biosynthetic pathways not usually present in the unchallenged plant. Thus, in the biological sense, phytoanticipin and phytoalexin production can be viewed as components of 'passive' and 'active' defense strategies, respectively (see also Chap. 2) (Mansfield 2000).

The spectrum of organisms that attack plants is broad and from an evolutionary point of view it is advantageous to produce defense molecules with a global mode of action that is not restricted to a narrow class of organisms attacking the plant. Therefore, plant defense substances, far from being 'magic bullets', often have rather general mechanisms of action. Thus, many destroy the selective permeability of membranes by creating channels, e.g., the steroidal glycoside and phytoanticipin α-tomatine from tomato (*Lycopersicon esculentum*), and the isoflavonoid phytoalexins from the *Leguminosae*. Some defense compounds work by inducing oxidative stress, for example α-tomatine and allicin (Ito et al. 2007). Thus, α-tomatine is an example of a defense compound with a dual mode of action. In this context cellular redox homoeostasis can be viewed similarly to cell membrane integrity, in that both are essential for normal cell function and viability (homeostasis) and make good general targets for broadly acting defense compounds (Dubreuil-Maurizi and Poinssot 2012; Kerchev et al. 2012).

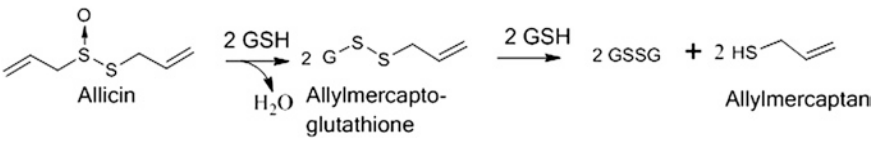

Fig. 3 The reaction of one mol of allicin with in total four mol GSH to yield two mol GSSG

Theoretical considerations suggest at least four ways in which redox active secondary metabolites might affect the cellular redox homoeostasis, none of which are mutually exclusive. Indeed quite the opposite is true, and effects might be expected to occur concomitantly:

1. *Function* via *direct interaction with redox sensitive proteins*

Secondary metabolites might affect cellular redox homeostasis by reacting directly with redox sensitive proteins. Since protein thiols make up the largest group of thiol compounds in the cell, the direct quantitative effect of 'titrating out' protein thiol groups on redox homoeostasis cannot be neglected, i.e., protein thiols themselves constitute part of the cellular thiolstat machinery. Furthermore, oxidation of sensitive regulatory proteins which coordinate cellular oxidative stress adaptations will lead to a cascade effect on the redox environment (see Sect. 3.4).

2. *Function* via *GSH:GSSG causing direct redox shift*

Secondary metabolites can affect the cellular redox potential by direct oxidation of GSH to GSSG. This can occur in a direct chemical manner as is the case with some natural compounds containing oxidized sulfur, like allicin (Gruhlke and Slusarenko 2012) resulting in intermediate mixed-disulfide adducts but ultimately in the formation of GSSG (Fig. 3). Similarly, the reaction of a substance like allicin with other cellular thiol groups, e.g., protein-thiols, affects the glutathione pool since glutathione is potentially in equilibrium with other cellular thiol groups via the thiol disulfide exchange reaction (TDER) and thus such compounds can oxidize GSH indirectly.

3. *Function* via *GSH-depletion*

Besides a direct reaction mechanism, a glutathione *S*-transferase (GST)-mediated reaction with a secondary cassette metabolite, e.g., in the course of detoxification, can lead to a depletion of the glutathione pool because glutathione-adducts are often exported out of the cell or, in the case of plants and fungi, into the vacuole. Multidrug resistant pumps (ATP-Binding Cassette, ABC-Transporters) often play an important role in this form of detoxification (Franco and Cidlowski 2012). Depletion of the GSH pool reduces the redox buffering capacity of the cell. As a consequence, even minor oxidative stress could then lead to strong influences on the thiolstat.

4. *Function* via *ROS* (*or RNS*) *induction*

Secondary metabolites can be sources of ROS or RNS which can act as strong oxidants for thiol groups thus shifting the thiolstat to a more oxidized state.

3.1 Throwing a Nano-Switch: Might Allicin Lead to Apoptosis by Affecting Tubulin Polymerization?

It has been shown that cytoskeletal disruption can lead into apoptosis in yeast (Leadsham and Gourlay 2008). Furthermore, it was shown that allicin destroys the tubulin cytoskeleton and this effect is abolished by treatment with reducing agents such as DTT or β-mercaptoethanol in mouse fibroblasts (Prager-Khoutorsky et al. 2007). Allicin induces apoptosis in yeast cells by shifting the cellular redox state and the concentration of GSH and GSSG. Allicin treatment showed both a loss in total glutathione and an increase in GSSG concentration (Gruhlke et al. 2010). Induction of apoptosis by allicin was also demonstrated in cancer cell lines (Miron et al. 2008). These observations suggest an influence of allicin on thiol groups of the tubulin protein. It is not yet clear, however, whether allicin acts directly by forming mixed disulfides with cysteine thiols in tubulin, which it can do *in vitro*, or indirectly via the oxidation of glutathione, hence affecting the thiolstat and leading to cysteine-based mixed disulfide formation in tubulin (e.g., *S*-glutathiolation). Furthermore, tubulin has been shown to be an important target of protein-glutathiolation and the formation of mixed disulfides affects the polymerization of the tubulin monomers (Landino et al. 2010).

3.2 Secondary Metabolites Depleting Cellular GSH

Isothiocyanates are well-studied bioactive reactive sulfur species (RSS) widely distributed in members of the *Brassicaceae*. Isothiocyanates are produced by myrosinase enzymes from glucosinolates (see also Chap. 10 in this book) (Nwachukwu et al. 2012).

Isothiocyanates can react directly with thiol groups to produce a dithiocarbamate that cannot be reduced by regenerating enzymes like thioredoxins, glutaredoxins, or glutathione reductases; thus, these compounds take glutathione out of the pool and consequently affect the cellular thiolstat. The benzoxazinoid DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one), a phytoanticipin from maize and some other cereals also removes glutathione from the thiolstat pool by forming a spirocyclic adduct (Dixon et al. 2012). Furthermore, the authors showed that DIMBOA reacted with exposed cysteine thiols in proteins, producing adducts that could not be re-reduced with DTT (*ibid.*).

3.3 Secondary Metabolites Inducing ROS

Some redox active natural compounds affect the cellular thiolstat indirectly by induction of ROS that in turn leads to oxidation of components in the cell.

There are many instances of ROS induction in cells by natural compounds; some organic polysulfanes, for instance, are known to induce ROS, because they undergo complex redox reactions with thiol groups, forming products that can generate ROS in the presence of glutathione and Fe^{2+} (see also Chap. 10) (Munday et al. 2003; Nwachukwu et al. 2012; Schneider et al. 2011).

Organic polysulfane treatment can lead to the generation of O_2^- which can react with thiol groups and produce GSSG and glutathione sulfonic acid (Winterbourn and Metodiewa 1994). Furthermore, H_2O_2 can be generated from O_2^- by the activity of superoxide-dismutase and can directly oxidize GSH to GSSG.

Some natural products produce ROS on exposure to light via a photodynamic effect, e.g., the accumulation of singlet oxygen ($^1O_2$) on light exposure of some furanocoumarins in the *Apiaceae* (Bode and Hansel 2005) or by the sulfur-containing thiophenes (Champagne et al. 1986; Hudson et al. 1993; Nwachukwu et al. 2012). Although lipophilic redox buffer systems are efficient at scavenging singlet oxygen, the latter can react directly with glutathione or, more importantly, can be quenched by ascorbate (Devasagayam et al. 1991; Triantaphylides and Havaux 2009). Nevertheless, dehydroascorbate is recycled by GSH in the Halliwell-Asada-Cycle, resulting in glutathione oxidation, which could also affect the thiolstat. Furthermore, methionine seems to be able to form methionine sulfoxide via a photodynamic effect involving $^1O_2$ (Devasagayam et al. 1991; Triantaphylides and Havaux 2009).

Thiophenes have been shown to inhibit superoxide dismutases, which lowers the effectivity of ROS detoxification mechanisms and thus results in greater accumulation of these reactive species (Nivsarkar et al. 1991). Thus, it is not always the natural compound itself which alters the thiol status but sometimes the ROS which are produced as result of the natural compound.

3.4 Increasing the Cellular GSH Pool by Activating γ-Glutamylcysteine Ligase, the First Enzyme in the Biosynthetic Pathway

The examples discussed so far are of natural compounds that shift the thiol status to a more oxidized state. The converse, however, is also possible. Thus, some natural compounds can act to increase the glutathione pool and reinforce the buffering capacity of the thiolstat, and could therefore be expected to increase resistance against oxidative stress and its consequences.

The rate-limiting step in glutathione biosynthesis is the ligation of glutamate and cysteine to γ-glutamylcysteine, a reaction catalyzed by the enzyme γ-glutamylcysteine ligase (GSH1, GCL, and GSHA in plants and fungi, animals and bacteria,

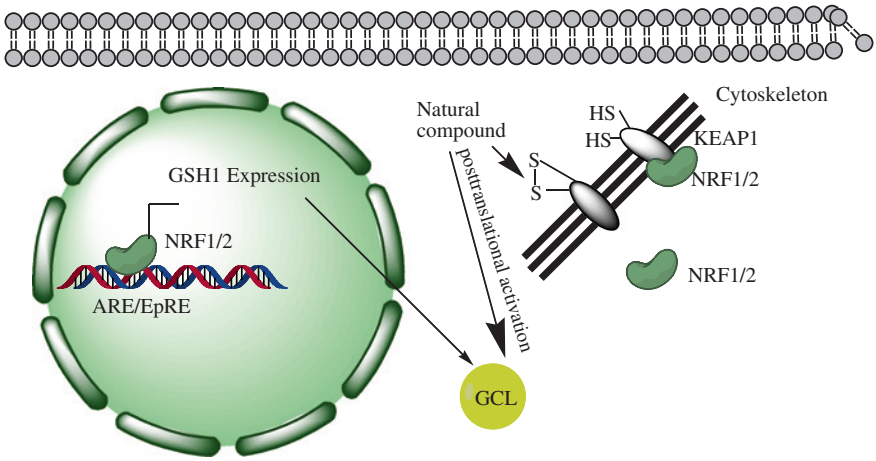

Fig. 4 Natural products can stimulate glutathione biosynthesis via two avenues. First, by post-translational activation of regulatory cysteines in GCL, and second, by releasing the Nrf1/2 transcription factor from its actin-bound complex with KEAP1 and its subsequent migration to the nucleus where GCL gene expression is stimulated by Nrf1/2 binding to ARE elements in the GCL promoter (Moskaug et al. 2005; Surh et al. 2008)

respectively), and both transcriptional and posttranscriptional regulation has been documented. Activation of γ-glutamyl-cysteine ligase by oxidation of specific cysteine thiols has been shown in the model plant *Arabidopsis* and in mammals such as rat and human (Hicks et al. 2007; Huang et al. 1993; Krejsa et al. 2010).

It was shown in oxidatively stressed yeast that *GSH*1 expression was regulated by the oxidation-sensitive transcription factor Yap1 (see Sect. 2) (Dormer et al. 2002). In humans GCL has regulatory (GCSI) and catalytic (GCSh) subunits. The expression of both subunits is controlled by the Antioxidant Response Element (ARE) motifs in the promoter sequence interacting with the Nrf1/2 transcription factor. The localization of the Nrf1/2-proteins depends on the Kelch like-ECH-associated protein 1 (KEAP1). KEAP1 binds to Nrf1/2 and this complex is normally associated with the actin cytoskeleton (Kang et al. 2004). Under the influence of oxidative stress, the Nrf1/2-KEAP1 complex dissociates and Nrf1/2 migrates into the nucleus where it can bind to ARE elements in the promoters of genes coding for antioxidative enzymes (Fig. 4).

It has been shown that certain plant phenolics, for example naringin (a flavone in grapefruit), can stimulate the expression of the genes coding for both the regulatory and catalytic subunits of GCL in humans leading to increased glutathione synthesis (Gopinath and Sudhandiran 2012; Moskaug et al. 2005; Surh et al. 2008).

Although allicin has been shown to rapidly deplete cells and blood of glutathione (Gruhlke et al. 2010; Rabinkov et al. 2000) at low concentrations and in the long term allicin up-regulates the intracellular glutathione concentration by affecting the GCL enzyme, as shown in vascular endothelial cells (Horev-Azaria et al. 2009). This effect is tied together with the observation that although allicin

clearly has oxidative properties, physiologically at low concentrations it works as an 'antioxidant' by inducing protection mechanisms (Munday et al. 2003). Because this process invigorates the 'antioxidative shield' of a cell, induction of glutathione biosynthesis by natural compounds, e.g., from nutrition, might be of great interest for health care (Masella et al. 2005). In this way *oxidatively* active substances can, paradoxically, be useful indirectly as 'antioxidants' in a physiological sense (see also Chap. 5) (Jung and Kwak 2010).

4 The Biological Consequences of Altering the Thiolstat: Targets and Signaling Pathways

4.1 Endogenous Disulfide Formation

Upon shifting the cellular thiolstat and thus the cellular redox environment to a more oxidized position, the formation of disulfides will be triggered. Two principle options exist. If two thiol groups in the same molecule are positioned such that they can react with each other, an internal disulfide bridge can form, whereas in the case that no other thiol group in the same molecule is available, the reaction might occur with thiol groups in other molecules. Glutathiolation (discussed in Sect. 4.2) is a special case of this more general scheme. Overproduction of disulfides in cells leads to the condition known as 'disulfide stress' (Aslund and Beckwith 1999). A consequence of disulfide stress can be further oxidation of protein thiols with serious implications on the catalytic function of enzymes affected. Furthermore, protein-disulfide isomerases (PDIs), which catalyze disulfide bond formation between protein cysteines, can be important targets of natural compounds. Inhibition of the ER-localized PDIs leads to denaturation and misfolding of proteins in the ER. In turn this leads to the ubiquitous 'unfolded protein response' (UPR) which aims to restore normal cell function by stopping mRNA translation and activating pathways leading to the production of molecular chaperones to restore proper protein folding. If normalization is not achieved rapidly enough, the UPR pushes cells into apoptosis (Walter and Ron 2011). It has been shown that the natural sesquilactone juniferidin from *Ferula malacophylla* (Sagitdinova et al. 1978) inhibits PDI and induces apoptosis via the UPR (Khan et al. 2011).

4.2 Protein-Glutathiolation as a Consequence of a Thiolstat Shift

Under oxidizing conditions, glutathione readily form mixed disulfides with protein thiols; this process is called *protein-glutathiolation* (more precisely: protein-*S*-gluta-thiolation) (Dalle-Donne et al. 2009). A list of proteins found to be glutathiolated under oxidative stress conditions is documented in Table 1 (modified from Michelet et al. 2006). The extent to which this reaction occurs

Table 1 A selection of proteins that have been shown to become glutathiolated under oxidative conditions, together with the effect of glutathiolation on protein function (modified from Michelet et al. 2006)

Protein	Impact of glutathiolation on protein activity	References
Actin	Glutathiolation of actin leads to reduced polymerization of G-actin and thus regulates turnover between F- and G-actin	Dalle-Donne et al. (2003, 2005), Eaton and Shattock (2002), Fratelli et al. (2003, 2004), Lind et al. (2002), Wang et al. (2001, 2003)
AP-1 (C-subunit)	Reduces DNA-binding affinity of AP-1 protein	Klatt et al. (1999a, b, 2000)
Caspase-3	Glutathiolation inhibits proteolytic activation and enzyme activity of caspase-3 in HL-60 cells	Huang et al. (2008), Klatt et al. (2000)
Cofilin	? (Most likely reduced actin-depolymerization)	Fratelli et al. (2002, 2004)
HSP90	Possibly, in analogy to HSP90 nitrosation, inhibition of its ATPase activity	Lind et al. (2002), Martinez-Ruiz et al. (2005)
JNK	Inhibition of JNK-signaling	Adler et al. (1999)
Myosin	Lower ATPase activity and higher sensitivity to proteolytic cleavage	Fratelli et al. (2002)
NFκB (p50 subunit)	Glutathiolation of p50 inhibits NFκB-ediated gene expression	Ji et al. (1999), Klatt et al. (2000), Pineda-Molina et al. (2001)
Protein phosphatase (1B and 2A)	Glutathiolation inhibits protein phosphatases	Rao and Clayton (2002)
Protein kinase A	The catalytic subunit of protein-kinase A in humans is inhibited by glutathiolation	Humphries et al. (2002)
Protein kinase C	Inactivation	Chu et al. (2003), Eaton and Shattock (2002), Ward et al. (2000, 2002)
h-Ras	Glutathiolation of Cys^{118} in Ras2 enhances the activity of RAS2	Adachi et al. (2004), Eaton and Shattock (2002), Ji et al. (1999), Mallis et al. (2001)
Tubulin $\beta 1$	?	Landino et al. (2004), Lind et al. (2002)

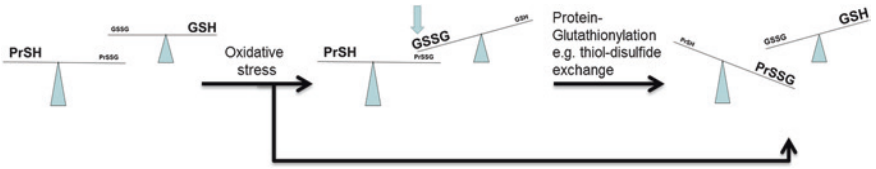

Fig. 5 The redox equilibrium is comparable to an asymmetric balance. In an unstressed cell the majority of the glutathione pool is in the reduced GSH form and protein 'switch' thiols (*PrSH*) are largely reduced. Upon oxidative stress there is not only direct glutathiolation of protein thiols, but the increased GSSG pool leads indirectly also to enhanced glutathiolation of protein thiols via a spontaneous enzyme catalyzed TDER. Please note that PrSSPr may be found in addition to PrSSG

as a result of oxidative stress depends on a number of factors, e.g., the local redox potential, the absolute GSH concentration (law of mass action) and the pK_a of the protein thiol in question (Dalle-Donne et al. 2009). The lower the pK_a of a particular cysteine, and thus the greater its tendency to dissociate at physiological pH, the higher is usually its reactivity with GSSG to build a disulfide. Depending on the redox environment it is also possible for a protein-cystine to become glutathiolated by reacting with a molecule of GSH in thiol-disulfide exchange reaction (Fig. 5).

Protein glutathiolation is central to the concept of the thiolstat and redox switch-regulation of cell metabolism. The pi class of human glutathione-*S*-transferases (GST-P) can catalyze this reaction (Tew et al. 2011) and several secondary metabolites stimulate (e.g., isothiocyanates) GST activity via the already mentioned Nrf2/ARE pathway (Andorfer et al. 2004; Xu et al. 2006). Furthermore, for several sulfur-containing garlic metabolites a direct, non-enzyme-catalyzed stimulation of protein *S*-glutathiolation has been documented (Pinto et al. 2006).

Elements of the cytoskeleton are targets for glutathiolation, regulating actin polymerization via the proportion of filamentous (F-) to monomeric (G-) actin (Sakai et al. 2012; Wang et al. 2001). It has been shown in yeast that the degree of actin polymerization correlates with the mitochondrial membrane potential ($\Delta\psi_m$). A similar situation can be expected in mammalian cells (for an appropriate 'intracellular diagnostics' of such phenomena see Explanatory Box 1) (Dalle-Donne et al. 2003).

Explanatory Box 1: Intracellular Diagnostics

Several chapters of this book discuss intracellular processes, for instance in the context of redox modifications and signaling cascades. Interestingly, many of these processes are rather subtle and their investigation requires sophisticated tools. Some of these cell bioanalytical methods have already been discussed in Chap. 3. Here, we will briefly consider some aspects of 'intracellular diagnostics'. During this discussion, it is important to bear in mind that most cellular events can only be measured reliably in living

cells, and that fixed or even lysed cells only provide a static picture of real events. This picture is often also marred by artifacts due to the fixing, staining, and lysis techniques, and due to the fact that the normal processes have come to a standstill (and decay may have set in) and that cells (or parts thereof) become exposed to dioxygen. It is therefore not surprising that techniques involving living—or at least intact—cells have recently gained considerable prominence. Here, we find some of the most cutting-edge techniques of "Live Cell Imaging". Western blots cannot be performed on living or intact cells and I have never heard of chromogenomics. As redox active agents often cause disturbance in the intracellular redox balance, several fluorescent dyes have been developed which enable researchers to stain cells in order to subsequently quantify oxidative stress (OS) in general or certain Reactive Oxygen Species in particular. Here, one may consider the OS-sensitive 2′,7′–dichlorofluorescein diacetate (DCFDA), the superoxide radical anion-sensitive hydroethidium bromide or the singlet oxygen-sensitive *meso*-tetraphenylporphyrin (H_2TTPS). At the same time, stains are available to quantify intracellular thiols. A combination of fluorescent staining and microscopy and/or a plate reader can also be employed to analyze for disruption of cellular organelles and their function. Dyes such as MitoTracker® and ER-Tracker® can be used to stain mitochondria and the Endoplasmatic Reticulum, respectively. In contrast, the MitoSOX™ dye (JA-1), does not simply stain the mitochondria but itself is redox sensitive and changes fluorescence according to the mitochondrial membrane potential $\Delta\Psi_M$. Fluorescent dyes can also be used to visualize specific intracellular proteins under the microscope. Here, larger protein aggregates, such as tubulin and actin networks, which form the cytoskeleton, can be stained by fluorescent dyes attached to specific antibodies which recognize and hence attach to these proteins. Smaller aggregates or single proteins stained with fluorescent antibodies cannot be spotted individually under a simple inverted light microscope as they are too small to be seen at the magnification available. However, their presence in the cell can be quantified by using such dyes and an appropriate plate reader. At the same time, the activity of such enzymes can also be measured using specific substrates which become fluorescent or change their emission wavelength or intensity once they are turned over.

These whole-cell-based techniques can be supplemented by a wide spectrum of more invasive techniques. For instance, the concentration of a specific protein in the cell can also be determined fairly adequately using Western Blotting techniques. More recently, methods to measure mRNA levels as indicators of gene expression in the cell, such as real-time PCR, have also become available. Indeed, there are many proteomic and genomic methods now available to map out specific gene expression patterns, protein levels (and changes thereof), posttranslational modifications in proteins (such as

sulfenic acid formation), and the activity of certain transcription factors. Such techniques are often associated with genomics, proteomics, transcriptomics, or redoxomics, and share the desire to analyze the entire cellular network of entities or process in one go, and to compare its appearance to similar maps of sick or healthy cells, or cells responding to a specific external stimulus, such as an administered drug.

In natural products research, these techniques are complemented by a range of methods used to validate the intracellular targets of such compounds. Here, we find sophisticated mass spectrometric methods to identify small modifications on proteins and enzymes, such as the oxidation of cysteine residues to sulfenic acids (i.e., to map out the cellular 'sulfenome'). Chemogenomics based on an extensive depository of yeast mutants enables researchers to identify mutants, and hence proteins involved in the action of a specific substance. Here, mutants particularly sensitive or resistant to a specific compound direct the researcher to the cellular pathway(s) most likely to be involved or affected. Another comparably new method worth mentioning in this context is "drug affinity responsive target stability" (DARTS), which again is particularly suited to identify intracellular targets of redox active substances. Here, the compound in question is applied to the cell, which is subsequently lysed and its protein content digested by a mixture of powerful proteases. As such proteases cannot digest chemically modified proteins, however, the latter survive and can be identified as potential targets using a combination of Western blots and mass spectrometry.

This list of modern and emerging techniques, of course, is far from complete and still expanding rapidly. It is therefore worthwhile to keep a close eye on the progress in this field of intracellular diagnostics. During the next decade or two, such emerging methods will ultimately be able to address many rather difficult questions in the field and also resolve a number of riddles, puzzles, and apparent contradictions currently challenging the research community.

4.3 Effects on Metalloproteins or How to Affect Electron Transport and DNA Transcription

Thiol groups are able to bind to Lewis acids, for example to Zn^{2+} ions. Thus, cysteine-coordinated zinc-centers can also act as redox switches (Ilbert et al. 2006). Zinc coordination is of great importance for catalytic activity and protein structure in a variety of proteins and perturbation of zinc coordination can lead to inactivation of proteins. Hence, changes in the cellular thiolstat can be transduced into physiological responses via the effect of redox changes on cysteine-coordinated zinc-clusters.

So-called 'zinc-finger' motifs are well-known zinc-chelating elements and the binding of a Zn^{2+} ion leads to the formation of a ribbon, which allows the peptide chain to interact with nucleic acids. Thus, zinc-fingers are common motifs in transcription factors and perturbation of the structure, which depends on the zinc-center, results in loss of DNA-binding activity. This highlights a further possible way in which changes in the cellular thiolstat might be transduced to changes in gene expression.

In murine macrophages, for example, it was shown that allicin is able to trigger the release of zinc from proteins in a concentration-dependent manner, leading to an inhibition of phosphatase activity and subsequently to enhanced ERK1/2 phosphorylation (Haase et al. 2012). This example illustrates that metalloproteins can also be important 'transducers' of changes in the cellular thiolstat.

Metalloproteins with iron-sulfur clusters play important roles in numerous electron transfer redox reactions in cells. These proteins complex two to four iron atoms via cysteine-sulfur atoms or through complex formation with histidine residues. Changes in such Fe-S clusters resulting from changes in the thiolstat can have significant consequences for cell metabolism and this turns Fe-S clusters into targets for oxidative stress (Gruhlke and Slusarenko 2012).

5 Redox Activity as the Heart of Antibiotic Activity

Up to now this chapter has dealt with the influence of the thiolstat on eukaryotic systems and in particular on mammalian cells. Nevertheless, shifting the cellular thiolstat can cause cell death in prokaryotes and in non-mammalian eukaroytes (e.g., in fungi). The induction of ROS was shown to contribute to the antibiotic activity of compounds toward bacteria. Thus, three major classes of bactericidal antibiotics, regardless of drug–target interaction, stimulate the production of highly deleterious hydroxyl radicals in Gram-negative and Gram-positive bacteria, which ultimately contribute to cell death (Kohanski et al. 2007). Elucidating how changing the cellular thiolstat correlates with the antibiotic properties of compounds is of great interest for developing antibiotic therapies and in plant protection. Targets of antibiotic compounds are diverse. Thus, commercial antibiotics like ampicillin (which targets cell wall biosynthesis of bacteria) or kanamycin (which inhibits protein biosynthesis at the 30S ribosomal subunit) have specific targets that are necessary for the cells to survive or to proliferate. As discussed before, targeting the thiolstat of a cell is a potential mechanism for antibiotic action which may affect *several* cellular functions simultaneously (and hence may also avoid the development of resistance). Thus, substances that specifically change the cellular redox environment could be promising antibiotics.

Interesting examples of bacterially produced antibiotics which are redox active are pyocyanin from *Pseudomonas aeruginosa* and actinorhodin from *Streptomyces*

coelicolor. Both are able to cause oxidative stress in bacteria, presumably via O_2^- production and cause activation of the O_2^--regulated SOXR-regulon (Dietrich et al. 2008). Another interesting example is the phytoanticipin 'tomatine' from the *Solanaceae* that was thought to target exclusively the fungal membrane (Roddick and Drysdale 1984). However, recent studies imply that tomatine causes oxidative stress, as shown in the fungus *Fusarium oxysporum* and induces apoptosis via changing the cellular redox state in the fungus (Ito et al. 2007).

6 Conclusions and Outlook

As part of this chapter we have described and discussed some examples as to how a change in the cellular redox environment, or thiolstat, can be transduced to physiological effects. In mammals and fungi the induction of apoptosis is an important consequence of redox perturbation. Some studies with sulfur-containing molecules like polysulfanes and allicin in yeast (*Candida albicans* or *Saccharomyces cerevisiae*) demonstrated that a redox shift is responsible for the fungicidal activity of these compounds (Gruhlke et al. 2010; Lemar et al. 2005, 2007).

While the biochemical consequences of shifting the thiolstat (e.g., protein *S*-glutathiolation, effects on Fe-S clusters) are conserved between prokaryotes and eukaryotes, the consequences are thought to be different. For example, programmed cell death (PCD), one form of which is apoptosis, is held to be a solely eukaryotic phenomenon. Indeed, until a few years ago it was widely believed that apoptosis occurred only in multicellular eukaryotes. The overwhelming body of evidence for apoptosis in the unicellular model microorganism *Saccharomyces cereviseae* (Baker's yeast) led to a shift in the paradigm. In this sense it is important to take notice of recent reports of PCD in prokaryotes, and that this is a possible mechanism for the action of antibiotics (Engelberg-Kulka et al. 2004). Thus, some typical markers of apoptosis have been observed in bacteria (Dwyer et al. 2012; Kohanski et al. 2007, 2010). Nevertheless, how far the principle concept of redox dependence of PCD in the sense of Schafer and Buettner's model (2001) can be transferred to bacteria is, to our knowledge, not yet known. Since a shift in redox state in general affects a plethora of different cellular targets (in contrast to a concept of 'one compound-one target'), it is likely that the bacterial PCD might also be redox dependent and by this provides an avenue for antibiotics to act via redox modification(s) (Schafer and Buettner 2001).

Ultimately, a deeper understanding of how natural products can influence the thiolstat may allow us a targeted approach for designing new uses as nutriceuticals and in plant protection.

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