Chapter 1
Thiol-Based Redox Signaling: Impacts on Molecular Chaperones and Cellular Proteostasis

Amy E. Ford and Kevin A. Morano

Abstract Signaling through protein cysteine residues to regulate diverse biological processes is widely conserved from bacterial to human cells. Differential cysteine reactivity enables cells to sense and respond to perturbations in the cellular redox environment, which may impact protein structure and activity. This chapter will focus on how redox signaling regulates components of the protein quality control network to mitigate proteotoxic stress caused by redox active compounds. While specifics of redox-based activation of the endoplasmic reticulum unfolded protein response and the cytoplasmic heat shock and oxidative stress responses differ, the presence of regulatory proteins containing reactive cysteines is a common feature. Moreover, several protein chaperones are reversibly regulated via cysteine switches that govern their ability to protect or refold damaged polypeptides. These responses are biologically indispensable, given the propensity of dysregulated cells to produce endogenous reactive oxygen species and the prevalence of thiol-reactive xenobiotics in the external environment.

Keywords Chaperone · Oxidative stress · Proteostasis · Reactive oxygen species · Redox · Signaling

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Abbreviations

CRD	cysteine-rich domain
Cys	cysteine
ER	endoplasmic reticulum
HMW	high molecular weight
HS	heat shock
HSP	heat shock protein
HSR	heat shock response
LMW	low molecular weight
NBD	nucleotide binding domain
NEF	nucleotide exchange factor
OS	oxidative stress
OSR	oxidative stress response
PDI	protein disulfide isomerase
PQC	protein quality control
Prx	peroxiredoxin
ROS	reactive oxygen species
SOH	sulfenic acid
TF	transcription factor
Ub	ubiquitin
UPR	unfolded protein response

1.1 Introduction

Cysteine (Cys) is one of the least abundant amino acids, but serves critical and unique roles in protein structure and chemistry due to its irreplaceable functionality as the only amino acid with a readily ionizable thiol group (Marino and Gladyshev 2010). Thiol reactivity depends on its accessibility and protonation state (pK_a), the latter of which is influenced by local protein microenvironment properties such as pH, secondary structure, and hydrogen bonding (Kortemme and Creighton 1995; Ferrer-Sueta et al. 2011). Although methionine also contains a sulfur atom, the thioether is in a relatively less reactive form and is typically not involved in biologically relevant reactions. Cys residues are most often buried within the interior of the protein structure; however, they can also be found exposed to the solvent (Poole 2015). Additionally, cysteines are typically clustered into two or more groups, characteristic of metal binding and redox centers. These chemical and functional properties allow for rapid and reversible redox regulation of protein activity, frequently but not exclusively through the formation of intramolecular disulfide bonds, to sense and control diverse cellular states and processes.

Reactive oxygen species (ROS) produced as a byproduct of aerobic metabolism, oxidative protein folding, and exposure to oxidants and highly toxic xenobiotics

have the potential to modify reactive thiols (Marnett et al. 2003; Tu and Weissman 2004; West et al. 2012). Oxidants such as hydrogen peroxide and diamide can react with protein thiols to form both reversible and irreversible thiolations (Winterbourn and Hampton 2008; Paulsen and Carroll 2010). Following initial formation of sulfenic acid (SOH), the modified thiol can either be further oxidized into sulfinic (SOOH) or sulfonic (SOOOH) acid or form a disulfide bond with a nearby free thiol (e.g. intramolecularly with a proximal Cys residue or with glutathione). These modifications play biological roles in sensing and regulation of activity of redox enzymes and transcriptional programs. The highly toxic heavy metal cadmium and metalloid-anion arsenite can target proteins in multiple ways – covalent binding of free thiols, metal ion displacement, and catalyzing oxidation (Tamás et al. 2014). In addition to oxidants and heavy metals, Cys residues are susceptible to modification by organic electrophiles, which form thiol adducts and may induce intermolecular cross-links between proteins (Zhang et al. 1995; Sánchez-Gómez et al. 2010). While xenobiotics are not involved in normal, steady state redox regulation, exposure to these agents can mimic endogenous modifications and induce similar downstream signaling.

Protein homeostasis (“proteostasis”) is essential for cellular function, and is defined as the status of the protein complement of a cell as determined by protein synthesis, assembly and degradation/turnover. Molecular chaperones assist proteins in their proper folding and prevent non-native conformations that lead to misfolding and aggregation (reviewed by Verghese et al. 2012). Proteins that cannot be folded properly or any non-native confirmations that arise are shuttled to specific protein aggregation sites and/or degradation pathways. These functions are performed by a variety of different chaperone classes and machines that make up the protein quality control (PQC) network. Members of the highly conserved Hsp70 class of chaperones are located in all major subcellular compartments and function in many aspects of proteostasis including native folding, transport, disaggregation, and degradation. Hsp70 performs these functions with the assistance of co-chaperones such as J-domain-containing Hsp40 proteins and nucleotide exchange factors (NEF), including the Hsp110, HspBP1 and Bag protein families (Bracher and Verghese 2015). Unlike Hsp70, the conserved Hsp90 system of chaperones interacts with specific “client” proteins, including kinases, receptors, and transcription factors, to aid in protein maturation and assembly of macromolecular complexes (Röhl et al. 2013). Cells also utilize small heat shock proteins that form multimers to aid in disaggregation (Verghese et al. 2012).

Cys modification by thiol-reactive compounds (described above) has the potential to alter protein structure and affect protein stability and solubility. Using in vitro folding assays, live cell imaging, and proteomic approaches, thiol stress has been found to induce protein aggregation (Sharma et al. 2008; Jacobson et al. 2012, 2017; Weids et al. 2016). Accumulation of protein aggregates resulting from exposure to these compounds can be toxic to cells as demonstrated by dose-dependent loss of cell viability (West et al. 2011). Protein aggregation is linked to diverse human diseases including diabetes, cancers, and neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Lou Gehrig’s diseases (Valastyan and Lindquist 2014;

Hipp et al. 2014). In addition to protein aggregation, oxidative stress (OS) and metal dyshomeostasis have been implicated, suggesting that disruption of redox balance and, therefore, redox regulation, as a contributing factor to these diseases.

Multiple studies have investigated the in vivo redox state of thiol-containing proteins during steady state conditions, peroxide stress, and changes to redox status due to aging or genetic mutations (Le Moan et al. 2006; Brandes et al. 2011, 2016). A common theme amongst these studies is the diversity of cellular processes that depend on redox-active thiol-containing proteins – redox systems, energy metabolism, translation, and, notably, protein folding. In this chapter, we will discuss the interplay between the PQC network and redox signaling with respect to changes in the protein folding environment.

1.1.1 Regulation of Stress Responses

The ability to respond and adapt to environmental changes through transcriptional reprogramming is essential for survival and proliferation. Bacterial responses to stress are numerous due to the diversity of niches and are regulated by specific or over-lapping stresses (Chalancon and Madan Babu 2011; Helmann 2011). Transcriptional activators, repressors and alternative sigma factors block or recruit RNA polymerase and additional co-regulators to regulate gene expression. Activity of these proteins is often controlled through anti-sigma factors that act as stress sensors and interact with the transcriptional regulator to sequester or facilitate its degradation (Hughes et al. 1998; Zhou et al. 2001; Arsène and Tomoyasu 2000). The major chaperone Hsp70 system composed of DnaK/DnaJ/GrpE (*E. coli*) and the Hsp60 chaperonin (GroEL/ES in *E. coli*) machines protect nascent polypeptides from insults to the folding environment and assist in refolding or degradation of damaged proteins. The two chaperone systems have been implicated in stress response sensing and regulation, most notably the Hsp70 system that regulates stability of the bacterial stress factor σ^{32} (Arsène and Tomoyasu 2000).

A distinguishing feature of eukaryotes is the presence of membrane-bound organelles that allow for the compartmentalization of distinct protein folding environments that differ in redox status: a reducing environment predominates in the cytosol and nucleus, and an oxidizing one is characteristic of the ER and mitochondrial inner membrane space, as well as the extracellular milieu. Changes in the redox balance within these compartments are sensed via protein thiol modifications which lead to activation of transcriptional responses (see Fig. 1.1). Within the ER, the response to redox imbalance is well characterized and is known as the unfolded protein response (UPR). In the cytosol, cells activate a specific transcriptional program to oxidative stress called the oxidative stress response (OSR). On the other hand, the response to misfolded proteins, classically termed the heat shock response (HSR), is primarily modulated by the transcription factor (TF) Hsf1; however, the mechanism of Hsf1 activation by oxidation of the reducing environment is unclear. Within the last 10 years, studies have investigated the connection between OS and

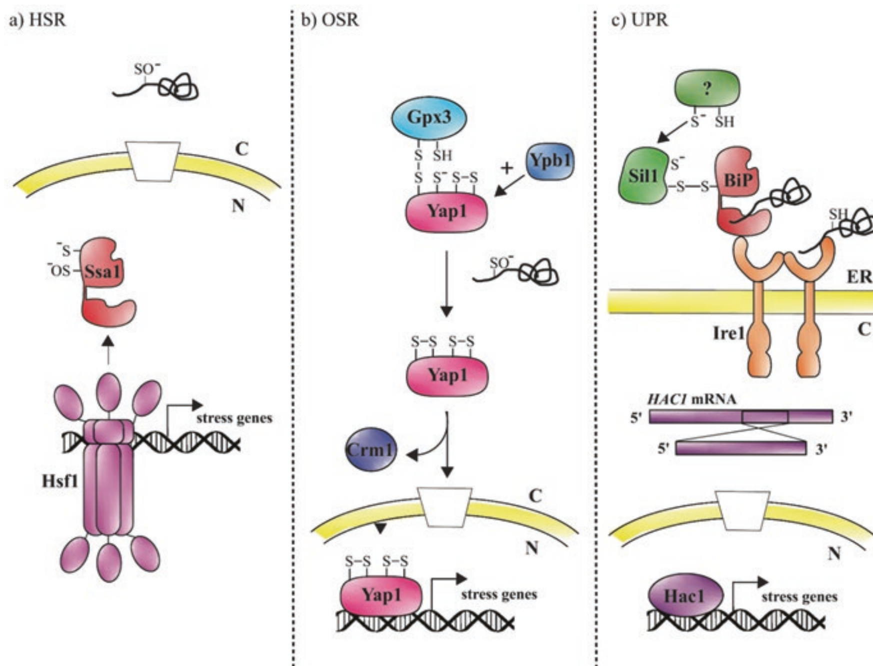

Fig. 1.1 Redox regulation of stress responses in the eukaryotic model budding yeast. (**a**) Proposed mechanism of redox regulation of the Heat Shock Response (HSR) following oxidative or thiol-reactive stress. Modification of the Hsp70 Ssa1 cysteines in the nucleotide-binding domain (NBD) decreases Ssa1 interaction with Hsf1 activating domains leading to de-repression of the HSR and protection of redox sensitive substrates. (**b**) Yap1-mediated redox regulation of the Oxidative Stress Response (OSR). Following oxidative or thiol-reactive stress, Yap1 undergoes oxidative re-arrangement through interactions with Gpx3 and Ypb1 allowing translocation into the nucleus and transcription of stress-protective genes. (**c**) Redox regulation of the ER Unfolded Protein Response (UPR). Redox sensing through the Hsp70 BiP and its co-chaperone Sil1 as well as unfolded peptides triggers the UPR through Ire1-mediated activation of Hac1

protein folding in the cytosol in the budding yeast *Saccharomyces cerevisiae*. Specifically, transcriptional profiling experiments demonstrated that thiol-reactive compounds induce both the HSR and OSR, implying evolutionary pressure to mobilize the PQC network in response to both environmental insults (Trott et al. 2008; Wang et al. 2012). Although these compartmentalized responses control distinct transcriptional programs, the different stress response pathways (UPR, HSR) occasionally converge to co-regulate expression of the same stress genes, indicating crosstalk between different cellular stresses and the responses they elicit (Liu and Thiele 1996; Sugiyama et al. 2000; Solis et al. 2016; Morano et al. 2012). This is further shown in how reductive stress in one compartment such as the ER can alter redox state of the cytosol (Delic et al. 2012). This section will discuss how chaperones play a role in regulation of each of these stress responses by either direct interaction with TFs or upstream effectors.

1.1.1.1 Heat Shock Response

Bacterial regulation of the HSR is mediated through negative autoregulation of the transcriptional activator σ^{32} (reviewed by Arsene et al. 2000). Under normal growth conditions, direct binding of the DnaK chaperone system facilitates σ^{32} rapid degradation via the FtsH mediated protease. Cellular stress such as HS induces dissociation of this complex and subsequent activation of stress defense genes. Glutathione modification of DnaK following dual HS-OS results in loss of chaperone activity, suggesting a potential mechanism of dissociation and subsequent σ^{32} activation (Winter et al. 2005). This notion is further supported by the finding that glutathione-modified DnaK is impaired in peptide binding and its interaction with σ^{32} (Zhang et al. 2016).

In yeast, the HSR activator Hsf1 exists as a stable trimer constitutively bound to high affinity HSEs located upstream of heat shock induced genes (Hahn et al. 2004). The prevailing model for Hsf1 activation stipulated that protein misfolding due to heat shock is sensed by chaperones such as Hsp70 being titrated away from Hsf1, allowing productive recruitment of Mediator and RNA polymerase complexes, thereby resulting in transcription of heat shock genes. However, definitive experimental support for this mechanism was lacking. Recent work has shed considerable light on the precise mechanism of how the Hsp70 yeast homolog Ssa1 regulates the HSR, with the discovery of reversible binding to sites identified in the N- and C-terminal transcriptional activation domains of Hsf1 (Peffer and Morano, unpublished results, Zheng et al. 2016). These findings are consistent with an earlier study that established a role for Ssa1 in regulation of Hsf1 in response to thiol stress. Two Cys residues (C264, C303) in the Ssa1 ATPase domain were found to be required for activation of the HSR in response to multiple thiol-reactive compounds ranging from H_2O_2 to organic electrophiles (Wang et al. 2012) (see Fig. 1.1a). Interestingly, the cysteines are not required for HSR activation in response to HS, suggesting distinct mechanisms regulate the HSR in response to different types of stress. The precise role the cysteines play in modulating the Ssa1-Hsf1 regulatory circuit remains to be established.

How the models of HSR redox sensing through Hsp70 in bacteria and yeast might apply to mammalian biology is unclear. For example, Hsf1 activation and repression domains are not precisely conserved from yeast to humans. Although all Hsf1 homologs possess a C-terminal activation domain that appears to include a putative Hsp70 binding site similar to that identified in yeast, human and other metazoan HSF1 homologs lack an N-terminal activation domain. Furthermore, human and murine HSF1 contain two cysteines in the DNA binding domain that form a reversible disulfide bond upon exposure to HS and OS (Ahn and Thiele 2003). Disulfide bond formation between C35 and C105 activates Hsf1 trimerization, nuclear localization, and target gene expression, indicating a direct sensing mechanism. This redox regulation of nuclear import is similar to what has been shown for the OSR TF Yap1 (described below). However, yeast Hsf1 lacks any cysteines, necessitating a partner protein(s) capable of sensing OS, possibly Ssa1. In additional contrast to yeast, mammalian Hsf1 exists primarily as an Hsp90-repressed

monomer in the cytoplasm that trimerizes and localizes to the nucleus to activate stress genes (Bharadwaj et al. 1999; Abravaya et al. 1992; Grunwald et al. 2014). Following oxidative stress produced by xenobiotics, Hsp90 is cleaved resulting in loss of chaperone activity indicating redox inactivation of Hsp90 and a potential role in Hsf1 regulation in response to OS (Shen et al. 2008; Beck et al. 2009). Collectively, evidence supports thiol-mediated loss of chaperone function as a likely mechanism of HSR redox regulation.

1.1.1.2 Oxidative Stress Response

In the yeast cytosol, OS is sensed primarily through the Yap1 TF (see Fig. 1.1b) (Harshman et al. 1988; Kuge and Jones 1994). Yap1 contains six Cys residues located in both N- and C-terminal cysteine-rich domains (CRD). Under normal growth conditions, these residues exist in a reduced state which allows binding of the *trans*-regulator Crm1 to the C-terminal CRD and nuclear export (Kuge et al. 1998; Yan et al. 1998). In oxidizing conditions, Yap1 undergoes oxidative protein folding with the help of glutathione peroxidase Gpx3 and Ypb1, the Yap1 binding protein. Gpx3 induces disulfide bond formation of Cys pairs between the CRD domains through a transient mixed disulfide mechanism (Delauney et al. 2002), while Ybp1 is thought to enhance oxidative folding efficiency. This alternative conformation blocks Crm1 binding and allows nuclear import (Delauney et al. 2002; Veal et al. 2003; Wood et al. 2004). Yap1 can then activate the OSR by inducing transcription of antioxidant defense genes.

1.1.1.3 Unfolded Protein Response

As proteins destined for secretion are synthesized and translocated across the ER membrane, the oxidative environment along with chaperones enable the formation of disulfide bonds necessary for proper protein folding. A switch to either a reducing or hyperoxidative environment prevents proper disulfide bond formation resulting in protein misfolding and activation of the UPR (Braakman et al. 1992; Haynes et al. 2004; Merksamer et al. 2008; Hetz et al. 2011). In yeast, protein misfolding is sensed through the ER resident Hsp70 chaperone BiP via binding of exposed hydrophobic patches of unfolded polypeptides (Wei and Hendershot 1995; Knittler and Haas 1992). Substrate-bound BiP and/or misfolded substrates directly interact with the ER lumenal domain of the transmembrane endonuclease Ire1 inducing dimerization and cytosolic intron splicing of the UPR TF *HAC1* precursor mRNA (Ng et al. 1992; Gardner and Walter 2011; Mori et al. 2000). Splicing of *HAC1* mRNA allows translation and downstream expression of ER stress genes such chaperones, disulfide isomerases, and those involved in ER-associated degradation (Rüegsegger et al. 2001; Travers et al. 2000). In mammals, ER stress is sensed through the Ire1 mechanism as well as other pathways (Haze et al. 1999; Yoshida et al. 2001; Harding et al. 2003) Each of these pathways utilizes BiP to some degree to regulate UPR activation and attenuation.

Oxidative protein folding produces peroxides that contribute to the oxidative environment and have the potential to tip the redox balance to unfavorable folding conditions (Tu and Weissman 2004). Recently, studies have elucidated the role of BiP and its co-chaperone Sil1 in the response to OS using budding yeast. Following exposure to OS, hyperoxidation of the ER lumen triggers the UPR via BiP/Sil1 redox sensing (see Fig. 1.1c). Oxidation of BiP leads to inactivation of its ATPase activity similar to the mechanism described for bacterial Hsp70 DnaK (Winter et al. 2005). However, unlike DnaK, loss of ATPase function in BiP leads to enhancement of the holdase activity and prevention of protein aggregation (Xu et al. 2016). Sil1 acts as a reductant to reduce BiP following return to optimal conditions (Seigenthaler et al. 2017). Together they help to integrate redox balance with proteostasis in the ER. BiP/Sil1 redox sensing may enable fine tuning of UPR regulation.

1.1.2 Regulation of Cytosolic Hsp70

The Hsp70 chaperone functions in all aspects of a protein's life span including proper native protein folding, aggregation prevention, and protein degradation. The protein structure and "cradle to grave" function is highly conserved from bacteria to humans (Verghese et al. 2012). The Hsp70 structure contains an N-terminal nucleotide binding domain (NBD) in which co-chaperones interact to enhance ATP hydrolysis and nucleotide exchange, and a C-terminal substrate binding domain (SBD) that cooperates allosterically with the NBD to bind and release peptides.

The bacterial Hsp70 DnaK functions with the co-chaperones DnaJ and GrpE forming a protein folding machine that protects substrates from aggregation during cellular stress such as HS (Szabo et al. 1994; Arsene et al. 2000). However, during OS or dual OS-HS, DnaK loses this protective property (see Fig. 1.2a) (Winter et al. 2005; Zhang et al. 2016). OS results in a drop in ATP levels due to oxidation of metabolic enzymes such as Gap1 that are involved in glycolytic and mitochondrial ADP phosphorylation pathways (Hyslop et al. 1988). When depleted of ATP, ATP-free DnaK is unstable and undergoes domain unfolding exposing its single cysteine to the oxidative environment. Oxidation-induced glutathionylation of C15 leads to inactivation of DnaK, which can be reversed upon return to normal, reducing conditions (Winter et al. 2005).

Hsp70 redox regulation in yeast is poorly understood. As mentioned in the previous section, Cys 264 and Cys 303 in the yeast Hsp70 homolog Ssa1 can be modified in vitro and in vivo by thiol-reactive compounds, specifically the formation of covalent adducts with organic electrophiles (see Fig. 1.2b). The two redox-active residues are located within the NBD and Cys 303 is analogous to Cys 306 in mammalian Hsp72, suggesting that thiol modification may lead to loss of ATPase activity in the yeast isoform as it does for Hsp72 (see below). Although the precise mechanisms of thiol-based regulation of Hsp70 may differ between prokaryotes and eukaryotes, it is striking that this chaperone exhibits conserved redox regulation across such a striking evolutionary distance.

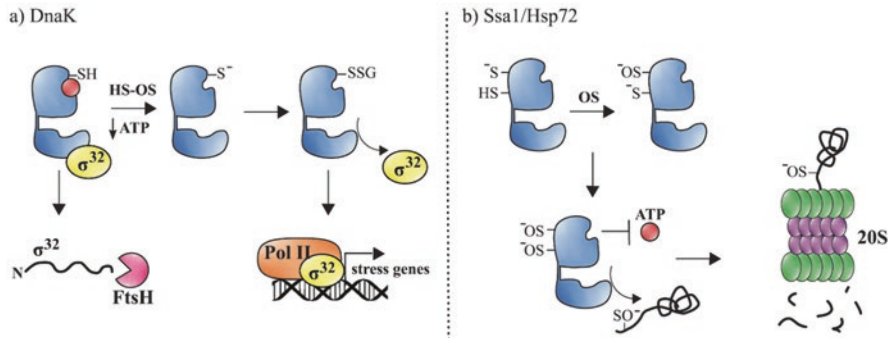

Fig. 1.2 Redox regulation of the cytosolic Hsp70 chaperone. (**a**) Modification of the bacterial DnaK following dual heat shock and oxidative stress (HS-OS). In optimal conditions, DnaK is active as a holdase and facilitates degradation of the σ^{32} transcriptional activator by the protease FtsH. Following dual HS-OS, ATP levels drop resulting in instability of nucleotide-binding domain (NBD). This leads to cysteine oxidation and inactivation of DnaK. Inactive DnaK releases σ^{32} and allows activation of stress genes. (**b**) Potential mechanism of yeast Ssa1 and mammalian Hsp72 cysteine modification during oxidative or thiol-reactive stress (OS). Following redox stress, modification of cysteine(s) leads to conformational changes in the NBD, which may prevent ATP-binding and chaperone "holdase" function leading to degradation of damaged proteins

Multiple isoforms of Hsp70 are expressed in mammalian cells including the constitutively expressed Hsc70 and stress-inducible Hsp72 (Hageman et al. 2011). Through chemical screening, the thiol oxidant methylene blue was identified to inhibit activity of only the inducible isoform Hsp72 (Kelner and Alexander 1985; Wang et al. 2010; Miyata et al. 2012). Inhibition was mediated through oxidation of C306 resulting in conformational changes in the NBD that may block ATP binding (see Fig. 1.2c). Additionally, peroxide was found to inactivate Hsp72 by a similar mechanism. Hsp72 has been implicated in modulation of stability of the Alzheimer's disease-linked protein tau, and inactivation of Hsp72 led to decreased levels of tau (Petrucelli et al. 2004). This suggests that redox modulation of Hsp72 may result in loss of stability for specific clients that require the chaperone at one or more points during their lifetime.

1.1.3 Redox Sensing in the ER

The ER is a specialized compartment for oxidative protein folding of secretory, membrane, and organelle-targeted proteins, many of which require glycosylation and formation of disulfide bonds between Cys residues for proper folding; thus, necessitating an oxidative environment. Consequently, redox characteristics of the ER have been extensively studied using both yeast and mammalian systems. Folding of proteins is aided by oxidoreductases that catalyze and isomerize disulfide bonds using molecular oxygen and the glutathione pool. This leads to accumulation of

glutathione disulfide and peroxide within the ER lumen, which is potentially detrimental at high levels (Tu and Weissman 2004). Therefore, eukaryotic cells have evolved mechanisms to sense and respond to changes in ER redox status (discussed above). Additionally, the resident ER Hsp70 folding system of chaperones (BiP and co-factors) functions along with the oxidoreductases to assist in protein maturation. Here, we focus on how these two types of folding chaperones switch roles during ER stress to promote proteostasis.

1.1.3.1 PDI, the Classic Redox Chaperone

Protein disulfide isomerase, or PDI, is located within the ER lumen and assists in proper protein folding by catalyzing disulfide bond formation, reduction and isomerization of protein thiols (Givol et al. 1964; Laboissière et al. 1995; Freedman et al. 1994). Following translocation across the ER membrane, oxidized PDI will catalyze formation of disulfide bonds between reduced cysteines to promote protein folding and stability. Reduced PDI can also isomerize non-native disulfides that arise and recognizes polypeptide regions that are misfolded due to improper disulfide linkages via a chaperone-like substrate recognition domain. PDI is itself converted between oxidized and reduced states by the oxidoreductase Ero1, which uses molecular oxygen to form the catalytic disulfide, and the glutathione pool, respectively (Frand and Kaiser 1998; Tu et al. 2000; Molteni et al. 2004). PDI has low peptide binding specificity and was shown to prevent protein aggregation of a non-disulfide bond containing protein indicating its chaperone function is independent of its disulfide isomerase activity (Wang and Tsou 1993; Cai et al. 1994; Gillece et al. 1999). In support of this ability, PDI interacts with ER peptides through an acidic amino acid-rich region at the C-terminus and not its redox catalytic sites (Noiva et al. 1993; Klappa et al. 1995). Following ER stress, PDI enzymatic activity is diminished coinciding with oxidization of Ero1, suggesting the inability of Ero1 to regenerate PDI to a reduced state (Nardai et al. 2005). Additionally, PDI expression is induced at the transcriptional level, likely promoting protein oxidation and preventing protein aggregation (Delic et al. 2002). PDI may functionally switch from an oxidoreductase to a molecular chaperone under ER stress conditions that result in modification of its Cys residues. This scenario is similar to what has been shown for other redox-regulated chaperones (see below) (Jang et al. 2004; Zhou et al. 2006).

1.1.3.2 BiP, the Resident ER Hsp70

The Hsp70 family chaperone BiP/GRP78 is the most abundant and best characterized ER-resident chaperone. Like other Hsp70s, BiP binds hydrophobic peptide stretches and facilitates protein folding through the binding and hydrolysis of ATP, which is aided by nucleotide exchange factors and Hsp40 co-chaperones (Knittler

and Haas 1992; Wei and Hendershot 1995; Meunier et al. 2002). Recently, BiP has been shown to be a sensor for redox changes in the ER lumen. The Sevier group found that the conserved cysteine residue C63 in the ATP binding pocket can be oxidized into a SOH and further glutathionylated, leading to decreased ATPase activity (Wang et al. 2014; Xu et al. 2016; Wang and Sevier 2016). As demonstrated using in vitro assays, oxidized BiP retains the capacity to prevent protein aggregation. Passive substrate holdase activity may even be enhanced in the absence of ATP-driven substrate binding and release. Oxidation of BiP may additionally reduce translocation of polypeptides into the ER lumen until redox balance is restored, limiting synthetic flux to promote ER proteostasis.

1.1.3.3 Sil1 Is a BiP Reductase

As an Hsp70-family chaperone, BiP utilizes an ATP hydrolysis cycle to efficiently assist in proper protein folding. Within the yeast ER, the NEFs Sil1 and Lhs1 exchange ADP for ATP to promote BiP ATPase activity, and thereby accelerate the folding cycle. As previously discussed, the redox status of BiP may transmit the redox state of the ER lumen to trigger a response to OS (Wang and Sevier 2016). Redox status of C63 within the yeast BiP Kar2 was found to be reversible and governed by Sil1 (Seigenthaler et al. 2017). Within its N-terminal domain, Sil1 contains a pair of cysteines (C42 and C57) that resemble a thioredoxin oxidoreductase motif characteristic of proteins such as PDI and the peroxiredoxin Tsa1. Seigenthaler et al. found that both cysteines can act as either the attacking nucleophile or resolving cysteine to reduce oxidized BiP. Sil1 specifically interacts with BiP to form a mixed disulfide-bonded intermediate, reducing the protein thiols to restore BiP ATPase activity. Loss of Sil1-BiP interaction prevents recycling of BiP and attenuation of the cellular response to OS shown by increased resistance to the thiol oxidant diamide. This redox regulated pair therefore allowed cells to rapidly sense, respond, and recover from changes in the ER redox state relevant to proteostasis.

1.1.4 Atypical Heat Shock Proteins

Several unrelated and atypical HSP respond to OS by acquiring or activating chaperone activity normally quiescent in the absence of redox stress. Unlike small HSP that form large oligomeric structures and aid in protein disaggregation via passive chaperone “holdase” activity, these chaperones frequently act as dimers to prevent protein aggregation (Jakob et al. 1993). Furthermore, regulation of chaperone activity is governed via distinct mechanistic routes with the common theme of generating robust protein holdase capacity in the presence of thiol-reactive stress.

1.1.4.1 Bacterial Hsp33

The bacterial chaperone Hsp33 is a novel family of ATP-independent chaperones conserved among prokaryotes (Jakob et al. 1999). Hsp33 is regulated by the redox potential of the cellular environment to protect proteins from stress-induced misfolding and aggregation. In optimal, reducing conditions, Hsp33 is maintained as an inactive monomer through C-terminal stabilization of a cluster of four Cys residues by zinc coordination – C232, C234, C265, and C268 (Graumann et al. 2001; Graf et al. 2004). In this conformation, both the substrate binding and dimerization domains are masked. Exposure to OS induces dissociation of zinc from the Cys cluster resulting in a partially unfolded conformation that enables accessibility of the cysteines and substrate binding domain. Subsequent disulfide bond formation between the two Cys pairs induces further conformational changes and dimerization (Barbirz et al. 2000; Graf et al. 2004). These active, oxidized dimers act as passive chaperones with substrate holdase ability (Graumann et al. 2001). Following return to reducing conditions, the oxidized dimer will reduce, release substrate, and disassociate with the combined aid of the thioredoxin and DnaK folding systems (Hoffmann et al. 2004). Deletion of Hsp33 results in an increase in sensitivity to OS and severe dual OS-HS. Conversely, overexpression enhances survival when cells are exposed to severe stress (Jakob et al. 1999; Winter et al. 2005). Furthermore, redox activation of Hsp33, perhaps, functionally replaces OS-inactivated DnaK as described in a previous section.

1.1.4.2 The DJ-1 Family of HSP

The DJ-1/ThiJ/Pfp1 superfamily of HSP is present in bacteria, yeast, and humans (Lee et al. 2003; Wilson et al. 2004; Skoneczna et al. 2007). All contain a conserved nucleophile elbow Cys-containing motif and protease-like catalytic triad, suggestive of peptidase activity; however, structural differences indicate they may have distinct functions. Here we specifically focus on Hsp31 and its role in oxidative stress protection.

The bacterial Hsp31 (bHsp31), was first identified in *E. coli* to be transcriptionally induced by HS via the general stress response regulator σ^S (Richmond et al. 1999; Mujacic and Baneyx 2006). Studies have found bHsp31 functions dually as a glyoxylase and molecular chaperone to prevent protein aggregation in cooperation with the DnaK/DnaJ/GrpE protein folding system (Malki et al. 2003; Mujacic et al. 2004; Subedi et al. 2011). In optimal growth conditions, Hsp31 eliminates toxic metabolic products such as methylglyoxal and acetic acid. Upon exposure to severe HS, bHsp31 undergoes heat-induced conformational changes to expose the conserved hydrophobic patch as well as the catalytic site allowing enhanced peptide binding (Quigley et al. 2004; Sastry et al. 2004). In yeast, deletion of Hsp31 (yHsp31) renders cells sensitive to thiol-reactive stresses including peroxide and cadmium, due to the intracellular accumulation of ROS (Skoneczna et al. 2007). yHsp31 expression is induced by Yap1 indicating a role in protection against

OS. More recent studies have shown yHsp31 to act as a homodimer to eliminate toxic ROS and prevent protein aggregation (Bankapalli et al. 2015; Tsai et al. 2015). How the two homologs functionally switch and whether this activity is redox regulated similarly to human Hsp31 (described below) is yet to be determined. The domain unfolding mechanism of inducing chaperone activity is similar to what has been shown for Hsp33 (described above). Furthermore, heat-induced unfolding exposes the Cys-containing catalytic triad, which may be susceptible to modification in dual HS-OS resulting in deactivation of glyoxylase activity and activation of chaperone activity.

The human Hsp31 homolog DJ-1 has been implicated in oxidative stress resistance as DJ-1 deficient human cells exhibit increased sensitivity to peroxide ultimately leading to cell death (Martinat et al. 2004). In contrast to bacteria and yeast, regulation of its chaperone activity is better understood and more developed. Like the bacterial Hsp33 (described above), DJ-1 chaperone activity is regulated by the redox environment as shown by in vitro “holdase” assays in the presence of the reducing agent DTT and oxidizing peroxide (Shendelman et al. 2004). DJ-1 contains three Cys residues that are all susceptible to oxidation: C106, C53, and C46 (Zhou et al. 2006). Oxidized DJ-1 has been shown to inhibit α-synuclein fibril formation and aggregation, a hallmark of Parkinson’s disease, both in vitro and mammalian cell culture, albeit with conflicting results on whether oxidation of C106 or C53 plays the primary role in mediating chaperone activation (Shendelman et al. 2004; Zhou et al. 2006). Overall, oxidation of DJ-1 activates chaperone activity to block protein aggregation and allow cell survival in response to changes in redox status or a disease state.

1.1.4.3 Peroxiredoxins

The yeast thioredoxin-dependent peroxidases, or peroxiredoxin (Prx), are part of the Trx-fold superfamily of antioxidant proteins, which include thioredoxin, PDI, and Dsb proteins (Schröder and Ponting 1998; Jang et al. 2004). These proteins contain a dithiol CxxC motif that functions in protein folding, and enzymatic detoxification of ROS and xenobiotics. Prxs are classified into two groups based on Cys conservation and mechanism of multimerization: 1-Cys and 2-Cys. Specifically, the yeast 2-Cys Prx, Tsa1, forms homomultimers in sizes ranging from low molecular weight (LMW) dimers to >1000 kDa high molecular weight (HMW) structures and is abundant in the cytosol. The multimeric state or size is associated with a specific function: peroxidase or molecular chaperone. As a homodimer or LMW tetramer, Tsa1 functions as a peroxidase to serve as an antioxidant and reduce protein thiols in coordination with thioredoxin. During hyperoxidative conditions, Tsa1 undergoes a thiol-dependent functional switch. Oxidation of the peroxidase active site Cys47 results in inactivation of peroxidase activity and multimerization. These HMW structures are associated with chaperone activity and have been shown to prevent protein aggregation. These chaperones typically form multimeric complexes and are thought to intercalate within protein aggregates as they are found in

isolated insoluble protein fractions (de Jong et al. 1993; Wallace et al. 2015). Furthermore, it has been shown that Tsa1 will localize to sites of aggregation in vivo in response to both HS and OS conditions (Weids and Grant 2014). Although similar to small HSP such as Hsp42 that normally form multimeric complexes and function in protein disaggregation regardless of redox status, Tsa1 is unique in that both multimerization and function are redox regulated.

1.1.5 Regulation of the Ubiquitin-Proteasome System

The eukaryotic proteasome is a specialized protein degradation machine that has both ubiquitin (Ub) dependent and independent conformations (reviewed by Bhattacharyya et al. 2014). Both conformations function as part of the protein quality control network to maintain proteostasis by degrading normal and aberrant proteins. The Ub-dependent 26S proteasome contains the evolutionarily conserved 20S proteolytic core and a 19S regulatory subunit that utilizes ATP and the Ub-machinery to degrade protein substrates. In contrast, the Ub-independent 20S proteasome lacks both the 19S regulatory subunit and requirement for ATP. The core particle consists of two outer alpha rings that regulate proteasome activity and two inner beta rings that utilize caspase-like, trypsin-like, and chymotrypsin-like activity to degrade substrates (Coux, et al. 1996: Groll et al. 1997). The regulatory particle acts an ATPase that recognizes, unfolds and translocates ubiquitinated substrates into the core for degradation.

During OS, there is a preferential switch to primarily utilize Ub-independent proteasomal degradation of damaged proteins (Pajares et al. 2015). Evidence suggests this is due to the increased sensitivity of the 19S regulatory subunit and Ub machinery to OS-induced modifications, such as glutathionylation, as compared to the 20S core (Jahngen-Hodge et al. 1997; Reinheckel et al. 1998). The majority of modifications lead to decreased activity. For example, the regulatory subunits Rpn1 and Rpn2, which allow translocation of Ub-substrates into the 20S core, undergo glutathionylation following exposure to OS resulting in inhibition of 26S proteasome function (Zmijewski et al. 2009). On the other hand, a study has shown that glutathionylation of C76 and C221 of the 20S α5 subunit increased proteasomal activity by triggering and maintaining the core in an open conformation (Silva et al. 2012). Again, this suggests that the Ub-independent machine is preferentially activated for degradation of oxidized proteins whereas the Ub-dependent iteration is inactivated. Furthermore, in human cells, the majority of oxidized proteins are not ubiquitinated with the exception of the molecular chaperones Hsp70, Hsp90, and Hsp60, which is a late consequence of OS; while the purpose of chaperone ubiquitination is unclear, it may help maintain appropriate chaperone levels and facilitate recovery from OS (Kästle et al. 2012).

1.2 Conclusions

The intersection of redox regulation and protein homeostasis is a burgeoning area of investigation that has enabled identification of novel redox sensors and atypical chaperones. These and other known redox PQC components are regulated through cysteine modifications that alter protein activity to control stress responses and promote protein stability and cell viability. Notably, many aspects of integrated redox and protein quality control biology are conserved from bacteria to humans. It is becoming increasingly clear how perturbations in the redox environment are sensed in each compartment. A key goal of future research is to better understand connections between sensing mechanisms and cellular responses. Additionally, while crosstalk at the gene expression level is apparent, how this is achieved locally is unclear. Because many human diseases are now recognized to be fundamentally linked to defects in proteostasis and dysregulation of ROS metabolism, it is expected that insights into how these two phenomena are linked will be of significant future therapeutic benefit.

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