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Reactive cysteine in proteins: Protein folding, antioxidant defense, redox signaling and more $\stackrel{\sim}{\sim}$

Review

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Abstract

Cysteine plays structural roles in proteins and can also participate in electron transfer reactions, when some structural folds provide appropriated environments for stabilization of its sulfhydryl group in the anionic form, called thiolate (RS⁻). In contrast, sulfhydryl group of free cysteine has a relatively high pK_a (8,5) and as a consequence is relatively inert for redox reaction in physiological conditions. Thiolate is considerable more powerful as nucleophilic agent than its protonated form, therefore, reactive cysteine are present mainly in its anionic form in proteins. In this review, we describe several processes in which reactive cysteine in proteins take part, showing a high degree of redox chemistry versatility. © 2006 Elsevier Inc. All rights reserved.

Keywords: Antioxidant; Cysteine; Disulfide; Peroxide; Electron transfer; Signaling; Thiols; Thiolate

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1. Introduction

Proteins are the final products of gene expression and are responsible to execute the biological information contained in the nucleotide sequences of their respective genes. A classical dogma in biology is that the genetic information presented in the nucleotide sequence of DNA is expressed in a two-stage

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process: transcription and translation. Proteins need to adopt proper tertiary and quaternary structures to perform their biological functions. Because most proteins spontaneously fold into their native conformation under physiological conditions, the central dogma also implies that protein's primary structure dictates its tertiary structure.

Our interest is on proteins that have the ability to participate in electron transfer reactions. Most proteins rely on organic and on inorganic redox cofactors (NAD⁺, FAD, heme, Cu, Fe and other transition metals) for redox activity. In contrast, for other proteins, amino acids, mainly cysteines, are responsible for this property. Free cysteine possesses low reactivity to undergo redox transitions (Wood et al., 2003b). However, protein folding can generate environments in which cysteine residues are reactive. The reactivity of a sulfhydryl group is related to its pK_a , since its deprotonated form (thiolate = RS⁻) is more nucleophilic and reacts faster with oxidants than the protonated form (R-SH). The sulfhydryl groups of most cysteines (linked to a polypeptide backbone or free cysteine) possess low reactivity, which is related to the fact that their pK_a is around 8,5 (Benesch and Benesch, 1955). In contrast, some redox proteins possess a reactive cysteine that is stabilized in the thiolate form by a basic residue, in most cases a lysine or an arginine residue (Copley et al., 2004). In conclusion, reactive cysteines in proteins are kept in a reactive form (thiolate $= RS^{-}$) by structural interactions with other amino acids. These reactive cysteines residues are compounds with very versatile redox chemistry because its sulfur atom can undergo redox transitions into any oxidation state between +6 and -2 (Jacob et al., 2003). Several proteins took advantage of this versatility to perform various biological functions as will be discussed below.

2. Roles of reactive cysteine in biology

2.1. Reduction of disulfide bonds

Many proteins with reactive cysteines are involved in controlling thiol/disulfide exchange reactions (Fig. 1A), a central theme in biology. Thiol/disulfide-exchange reactions are nucleophilic substitutions. A thiol or thiolate (RSH or RS⁻) acts as a nucleophilic agent on a disulfide bond (RS-SR). These reactions are, for example, used to form and reversibly destroy structural disulfides in proteins and peptides, to regulate enzyme activity and to maintain cellular redox balance. The rate of this reaction is dependent on the pKa of the sulfhydryl compound that is the nucleophilic agent. The lower the pKa, higher is the amount of deprotonated form of sulfhydryl group (thiolate) and faster are the reactions at physiological pH. The tri-peptide glutathione (γ -Glu–Cys–Gly, GSH) is the most abundant thiol in cells and is vital for the maintanance of the intracellular redox balance, among other functions (reviewed by Jacob et al., 2003). Glutathione is almost completely protonated at physiological pH because its pK_a is 9,2 (Jung et al., 1972) which disfavor its reaction with disulfides. However, it should be also considered that glutathione levels in cells are very high, which should favor disulfide reduction, since rate of a reaction depend also on the concentration of substrates.

Besides glutathione, thiol proteins such as thioredoxin, glutaredoxin (also known as thioltransferase) and protein disulfide isomerase are also involved in the regulation of the intracellular redox balance and, therefore, they are also known as thiol/disulfide oxido-reductases. Thioredoxin appears to be a very ancient protein since it is widespread among all the living organisms. These small proteins (12-13 kDa) possess disulfide reductase activity endowed by two vicinal cysteines present in a CXXC motif (typically CGPC), which are used to reduce target proteins that are recognized by other domains of thioredoxin polypeptide. The reduction of target proteins results in a disulfide bridge between the two cysteines from the thioredoxin CXXC motif, which is then reduced by thioredoxin reductase that utilizes reducing equivalents from NADPH. Some of the target proteins of thioredoxin include ribonucleotide reductase (important for DNA synthesis), methionine sulfoxide reductase, peroxiredoxins and transcription factors such as p53 and NF-kB (reviewed by Powis and Montfort, 2001).

Since thioredoxin plays multiple roles, it was surprising to observe that deletion of their genes in *Escherichia coli* resulted in a viable bacteria, capable to synthesize desoxyribonucleotides, among other processes. Holmgren (1976) showed that glutaredoxin was the backup for thioredoxin in the reduction of ribonucleotides. Like thioredoxin, glutaredoxin possess a CXXC motif in their active site (typically CPYC) and most of them are low molecular weight proteins (12–13 kDa). Glutaredoxin can also catalyze the reduction of disulfide bond in target proteins like thioredoxin through thiol/disulfide exchange reactions (Fig. 1A).

Furthermore, glutaredoxin also catalyzes the reduction of mixed disulfides with glutathione in a process that only the Nterminal cysteine thiolate participates (reviewed by Fernandes and Holmgren, 2004). Interestingly, some glutaredoxin isoforms possess only the N-terminal cysteine and are only capable to reduce mixed disulfides with glutathione. Thioredoxins reduce a wider range of disulfides in proteins than glutaredoxins, but cannot reduce mixed disulfides with glutathione. The disulfide form of glutaredoxin is reduced by glutathione, which is then reduced by NADPH in a reaction catalyzed by glutathione reductase.

The thioredoxin system (NAPDH+thioredoxin reductase+ thioredoxin) and the glutathione system (NADPH+glutathione reductase+glutathione) are the major thiol dependent redox pathways present in the cells. Glutathione systems may or may not contain glutaredoxin, depending on the process considered. Several enzymes and other effectors can be reduced by both systems but many processes are reduced by either thioredoxin or by glutathione system. Interestingly, in platyhelminths, the thioredoxin and glutathione systems are linked in only one pathway. This worm possesses a seleno-cysteine containing enzyme named thioredoxin-glutathione reductase, which possess thioredoxin reductase, glutathione reductase and glutaredoxin activities (Sun et al., 2001).

The two electron redox potential of the cysteine/cystine couple in thiol/disulfide oxido-reductases is influenced by several factors. Thioredoxin and glutaredoxin are strong reducing agents and therefore possess a very negative redox



Fig. 1. Nucleophilic substitutions and reactive cysteines. (A) Thiol-disulfide exchange reaction. This reaction is faster when the sulfhydryl group is deprotonated (thiolate). Therefore, rate of this reaction is given by $V = k[R_n - S^-]$ [RSSR_{1g}]. The same rationale applies for the other reactions depicted here. (B) Peroxide reduction, resulting in a sulfenic acid derivative (Cys-SOH) and an alcohol corresponding to the peroxide. The sulfenic acid derivative can have different outcomes depending on the kind of peroxiredoxin considered (see Fig. 4) and on the environment where it is located. (C) Sulfoxide reduction, resulting in methionine regeneration and sulfenic acid formation in methionine sulfoxide reductase, which is reduced back to its sulfhydryl from by thioredoxin (reviewed by Weissbach et al., 2002). Abbreviations: lg = leaving group; n = nucleophilic agent.

potential. In contrast, protein disulfide isomerases (PDI) and DbsAs (bacterial PDI counterparts) possess much lower negative potentials (Table 1). Therefore reduction potential of the best oxidant (DsbA) is 146 mV higher than the best reductant (thioredoxin), corresponding to a ratio of 105 in thermodynamic stability of the dithiol/disulfide equilibrium (Xiao et al., 2005). This diversity in potentials reflects the biological roles of these thiol proteins. Thioredoxins and glutaredoxins preferentially reduce disulfide bridges, whereas protein disulfide isomerase and DbsAs preferentially generate disulfide bonds in proteins (Jacob et al., 2003).

It is interestingly to observe that in spite of these great differences in redox properties all these oxido-reductases share several similarities: (1) $S\gamma$ atom is mostly deprotonated in N-terminal cysteine residue of the CXXC motif at physiological conditions. Therefore, the most N-terminal cysteine is more

Table 1 Properties of Thiol/disulfide oxido-reductases

Oxido-reductase	Motif in active site	Redox Potential (E ^o , mV)	pK _a		
Thioredoxin Glutaredoxin Tryparedoxin Protein Disulfide Isomerase (PDI)	Cys-Gly-Pro-Cys Cys-Pro-Tyr-Cys Cys-Pro-Pro-Cys Cys-Gly-His-Cys	-270 ^a -198 to -233 ^b -249 ^c -127 ^d	$\begin{array}{r} 6.3{-}7.5^{\rm f} \\ 3.5{-}3.8^{\rm g} \\ 7.2^{\rm h} \\ 3.5{-}6.7^{\rm I} \end{array}$		
DbsA	Cys-Pro-His-Cys	-125 ^e	3.5 ^j		

^a — Miranda-Vizuete et al. (1997), Nishinaka et al. (2001). ^b — Aslund et al. (1997). ^c — Reckenfelderbaumer et al. (2002). ^d — Lundstrom and Holmgren (1993). ^e — Collet and Bardwell (2002). ^f — Holmgren (1972), Kallis and Holmgren (1980), Reutimann et al. (1981), Dyson et al. (1997), Li et al. (1993), Chivers et al. (1997), Dillet et al. (1998), Vohnik et al. (1998). ^g — Gan et al. (1990), Yang and Wells (1991), Mieyal et al. (1991), Jao et al. (2006). ^h — Reckenfelderbaumer and Krauth-Siegel (2002); ⁱ — Darby and Creighton (1995), Hawkins and Freedman (1991); ^j — Nelson and Creighton (1994), Grauschopf et al. (1995).

nucleophilic and more exposed than the second cysteine. The most C-terminal cysteine is usually protonated and more buried in the polypeptide chain; (2) global fold of five stranded β -sheet flanked by four helices, the so-called thioredoxin fold (Fig. 2A); (3) the active site that contains the CXXC motif is located on a surface loop at the end of strand β 2 and followed by a long α -helix (Fig. 2A).

Several reasons have been raised to explain this variation of the reducing capabilities, such as the composition of the amino acids in the CXXC motif (Table 1), network of charged amino acids and structural factors, such as the dipole property of the α -helix where the buried cysteine is located (reviewed by Carvalho et al., 2006). These different redox properties among thiol/disulfide oxido-reductases appear not to be related with the stability of the disulfide bonds, since their lengths are very similar among these proteins (reviewed by Carvalho et al., 2006). In any case, for all of these enzymes, the pK_a of the reactive cysteine is considerably lower than the pK_a of free cysteine (Table 1), but the mechanism by which the thiolate is stabilized varies.

The stabilization of the thiolate anion in thioredoxin is relatively well characterized and was taken as an example for thiol/disulfide oxido-reductases. It depends on: (i) a network of charged residues, especially on specific aspartate and lysine residues (Fig. 2B); (ii) dipole character of the α -helix where the C-terminal cysteine is located and (iii) hydrogen bonding between the reactive and the C-terminal cysteine residues (reviewed by Carvalho et al., 2006).

Interestingly, mutations of Asp26 and Lys57 of thioredoxin affect only the pK_a of the active site thiol, but not the structure of the protein (Dyson et al., 1997). For the other oxido-reductases the network of charged residues is different and involves Glu 30 for PDI (PDB ID=1MEK), Glu 24 for DsbA (PDB ID=1A23) and Glu30, Asg26 and Lys27 for glutaredoxin (PDB ID=1KTE).

Fig. 2. Structural characteristics of thiol/disulfide oxido-reductases. Thioredoxin from Escherichia coli (PDB ID = 1XOB) was chosen as a model to describe several features common to thiol/disulfide oxido-reductases. (A) General view of thioredoxin fold: B-sheet composed of five strands (yellow) flanked by four α -helixes (red). Both main and side chains of the two cysteine residues belonging to the CXXC motif are showed (gray). The reactive cysteine (Cys 32) is the most exposed one. (B) View of the active site, showing the network of amino acids involved in the stabilization of reactive cysteine in the thiolate form. Residues involved in the network of charged amino acids are represented with colors and with dots representing their electronic densities (Asp 26 - magenta, Cys 32 — green, Cys 35 — cyan, Lys 57 — magenta). Pro76 (yellow) is not involved in the network of charged residues that stabilize the thiolate form of reactive cysteine, but its main and side chains are shown here because this residue plays a central role in the recognition of thioredoxin substrates. Figures were generated by the Pymol software (www.pymol.org). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

We are interested in the functional and structural characterization of proteins belonging to thioredoxin and glutathione systems form the yeast *Saccharomyces cerevisiae*. In this regard, we solved the structure of thioredoxin reductase I (Oliveira et al., 2005) and preliminary data indicated that the two thioredoxin systems are not completely redundant.

2.2. Formation of disulfide bonds

The formation of disulfide bonds stabilizes the most active conformation of proteins that will be secreted. One would

expect that PDI and DsbA would be more suitable to catalyze the formation of disulfide bond given their reduction potential (Table 1). In fact, the search for the enzymatic catalyst of oxidative folding led to isolation of PDI (Goldberger et al., 1963). PDI can catalyze the formation, reduction or isomerization of disulfide bonds depending on the redox conditions of the assay and on the nature of the substrate protein. However, the environment in which PDI is found (endoplasmic reticulum), favors only the formation and isomerization of disulfide bonds. Both, the formation of disulfide bond and isomerase activities occur by thiol/disulfide exchange reactions (Fig. 1A). Like thioredoxins and glutaredoxins, the ability of PDI to catalyze thiol/disulfide exchange reactions is given by a CXXC motif (typically CGHC) among other factors (Aslund et al., 1997). When the cysteines in the active site are present in the disulfide form, PDI can directly oxidize thiol groups of target proteins into disulfide bridges (dithiol oxidase activity). In contrast, the isomerase activity of PDI relies on the dithiol (reduced) configuration state of the active site cysteines, suitable for disulfide reshuffling (reviewed by Frand et al., 2000).

In spite of the different redox properties among all these oxido-reductases, they can catalyze both reduction and formation of disulfide bonds in vitro, depending on the experimental conditions. In fact, Grx1 from E. coli is even more efficient than PDI to catalyze disulfide bond formation (Xiao et al., 2005), indicating that kinetic parameters should also to be taken into account. Therefore, it is important to consider the environment in which the thiol oxido-reductase is located to analyze its function. In eukaryotic cells, protein disulfide bond formation takes place within the lumen of the endoplasmic reticulum. Proteins that will be secreted to the extracellular space are processed inside this organelle. The redox state of the endoplasmic reticulum is more oxidizing than that of cytosol, a difference that favors the formation of disulfide bonds, which is important to maintain the structure of the exported protein in the harsh extracellular environment. The major redox buffer in the cytosol as well as in the lumen of ER is the couple GSH/GSSG. However, GSH/GSSG ratios are quite different: 1:1 to 3:1 for the lumen of endoplasmic reticulum and 30:1 to 100:1 for the cytosol and mitochondrial matrix. Therefore, the ability of thioredoxin and glutaredoxin to catalyze reduction of disulfide bond in protein and of PDI to catalyze the reverse process is consequence of several factors such as redox potentials of vicinal sulfhydryl groups in these proteins and redox balance of the environment.

2.3. Protein S-glutathionylation

Another thiol/disulfide exchange process that deserves special consideration here is *S*-glutathionylanion of cysteine residues in proteins. In resting state, levels of *S*-glutathionylated proteins in cells are around 1%, but upon oxidative stress a significant increase is observed. Therefore, initially, the meaning of the *S*-glutathionylation was thought to be the protection of cysteine residues against overoxidation to sulfinic (RSO₂H) or sulfonic (RSO₃H) acids, which can lead to protein inactivation

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(Thomas et al., 1995). Later, it was shown that for some enzymes, protein S-glutathionylation affects enzyme activities, suggesting a regulatory role for this process (Chrestensen et al., 2000; Davis et al., 1997; Demasi et al., 2003). If S-glutathionylation is in fact a regulatory event, it is expected the occurrence of proteins capable to catalyze the addition and removal of glutathione from target proteins. Glutaredoxins, especially those containing only one cysteine in their active site, have been most frequently implied as dethiolases (Molina et al., 2004). The yeast S. cerevisiae has five glutaredoxins, three monothiolic and two dithiolic, distributed in different compartments and performing similar, but not completely redundant roles (Wheeler and Grant, 2004). We have recently solved the crystal structure of glutaredoxin 2 (Discola et al., 2005) and unpublished results have demonstrated its role on the removal of GSH from S-glutathionylated 20S proteasome extracted from yeast cells. We hope that with the elucidation of glutaredoxin 2 structure it will be possible to obtain insights into the mechanisms by which this thiol/disulfide oxido-reductase act as a dethiolase in the yeast S. cerevisiae.

2.4. Antioxidant defense

Proteins with reactive cysteine considered so far, catalyze thiol/disulfide exchange reactions. In contrast, thiol-dependent peroxidases have evolved the ability to cleave a peroxide bond that is a more difficult process than the reduction of a disulfide bond (Fig. 1B). Copley et al. (2004) elegantly hypothesized that peroxiredoxins, a class of thiol-dependent peroxidases, present



Fig. 3. Structure of peroxiredoxin active site. Reactive cysteine in peroxiredoxins corresponds to the C-terminal cysteine of CXXC motifs in thioredoxins (Copley et al., 2004). Therefore, they are located in an α -helix as the C-terminal cysteine of thioredoxin is. Structure of human peroxiredoxin 5 (PDB ID = 1HD2) is shown as an example. Electronic density of arginine residue (Arg127) involved in the stabilization of the thiolate is represented with dots, as well as reactive cysteine (Cys47). Thiolate function (RS⁻) of Cys 47 is brown. Main and side chains of a threonine residue (Thr44 that corresponds to the N-terminal cysteine in thioredoxin) that plays a role in stabilization of thiolate is shown in green, as well as the chains of Pro 40 (yellow) that is involved in protection of peroxiredoxin from overoxidation. Finally, main and side chains of histidine 51 (purple), forming a salt bridge with Arg 127 (cyan) is also shown here. Figure was generated by the Pymol software (www.pymol.org). (For interpretation of this article.)

several amino acids substitutions from the more ancient thiol/ disulfide oxido-reductases, which make them capable to reduce OO bonds through a reactive cysteine.

Both hydrogen and organic hydroperoxides can be decomposed by peroxiredoxins and in most of cases they utilize reductive equivalents from thioredoxins (Netto et al., 1996). Therefore, the majority of peroxiredoxins are also called thioredoxin peroxidases. Recently, it was shown that some peroxiredoxins can also decompose peroxynitrite (Bryk et al., 2000; Dubuisson et al., 2004; Trujillo et al., 2004; Wong et al., 2002). These reactions catalyzed by peroxiredoxins have been implied in both peroxide detoxification and cellular signaling as will be discussed below. Like other thiol/disulfide oxidoreductases, peroxiredoxins are widespread in nature and are found in several cell compartments such as cytosol, mitochondria, nucleus and chloroplast (Rhee et al., 2005a).

As described for the thiol/disulfide oxido-reductases, the high reactivity of the active site cysteine in peroxiredoxins is related to the fact that the thiol group of this residue possesses very low pK_a . In the case of peroxiredoxins, the presence of a guanidine group from a fully conserved arginine residue (Wood et al., 2003b) is a key factor for the stabilization of the thiolate. Interestingly, the reactive cysteine from peroxiredoxins is homologous to the C-terminal cysteine of the CXXC motif in oxido-reductases, which is not the most nucleophilic. The reactive cysteine (the most N-terminal and most solvent exposed seen in Fig. 2) in oxido-reductases was replaced by a threonine residue in peroxiredoxins and the other cysteine acquired high nucleophilicity due to several structural features and amino acids interactions, such as the hydrogen bonding with an arginine residue mentioned above (Fig. 3).

Other peroxide-removing enzymes evolved other strategies to decompose peroxides. Catalase and mammalian glutathione peroxidase utilize heme or seleno-cysteine to decompose peroxides, whereas peroxiredoxins have a very reactive cysteine in their active site. Initially, these differences in the active sites was thought to reflect the fact that peroxiredoxins would have moderate catalytic efficiency ($\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$), (Hofmann et al., 2002) when compared with catalases ($\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (Hillar et al., 2000) and glutathione peroxidases (~ 10^8 M⁻¹ s⁻¹) (Hofmann et al., 2002). Recently, however, some reports have described higher rate constants $(10^6-10^7 \text{ M}^{-1} \text{ s}^{-1})$ for the reaction of reduced peroxiredoxins with different kinds of peroxides (Akerman and Muller, 2005; Baker and Poole, 2003; Dubuisson et al., 2004; Parsonage et al., 2005). In any case, it is important to emphasize that peroxiredoxins are abundant in aerobic cells. For example: (i) peroxiredoxins are among the ten most abundant proteins in E. coli (Link et al., 1997); (ii) peroxiredoxins are the second or third most abundant protein in erythrocytes (Moore et al., 1991) and (iii) compose 0.1-0.8% of the soluble proteins in other mammalian cells (Chae et al., 1999). Furthermore, it was demonstrated that peroxiredoxin, but not catalase, was responsible for protection of bacteria against endogenously generated hydrogen peroxide (Costa Seaver and Imlay, 2001).

There are several kinds of peroxiredoxins and several classifications were proposed based on different criteria.

Generally, every aerobic cell possesses several different kinds of peroxiredoxins. The most frequently used criteria for classification is the presence or absence of additional conserved cysteines (Wood et al., 2003b). Peroxiredoxins that contain two conserved cysteines are called 2-Cys Prx, whereas those that possess only one conserved cysteine are referred as 1-Cys Prx. In both cases, the reactive cysteine attacks the hydroperoxide and is oxidized to sulfenic acid (Cys-SOH), while the corresponding alcohol is released (Fig. 4). Because the reactive cysteine is the one that directly interact with peroxides it is called peroxidatic cysteine and is located at the N-terminal part of the protein. Three peroxiredoxin classes can be recognized based on the next step of the catalytic cycle (1-Cys Prx; typical 2-Cys Prx and atypical 2-Cys Prx). The 1-Cys Prx presents the simplest mechanism: they are oxidized to a stable sulfenic acid and then reduced back by a reductant. The biological electron donors of most 1-Cys Prx are still unknown. One exception is the 1-Cys Prx from yeast, whose electron donor is mitochondrial thioredoxin (Pedrajas et al., 2000). Furthermore, mammalian 1-Cys Prx can form heterodimer complexes with Glutathione S-transferase π , being capable to accept electrons from glutathione (Ralat et al., 2006).

The enzymatic mechanism of 2-Cys Prx differs from the 1-Cys Prx's mechanism because these proteins have a second conserved cysteine, also called resolving cysteine, which is also involved in the catalytic cycle. The sulfenic acid formed in the peroxidatic cysteine reacts with the resolving cysteine of other protein, generating an intermolecular disulfide bridge. In the case of atypical 2-Cys Prx, the resolving cysteine belongs to the same polypeptide backbone of the peroxidatic cysteine, therefore an intramolecular disulfide bond is generated. For the majority of the typical and atypical 2-Cys Prx proteins, disulfide bonds are reduced by thioredoxins (Fig. 4).

Alternatively, peroxiredoxins can be classified according to their amino acid sequence, which is very variable among five different groups (Trivelli et al., 2003). In spite of the fact that peroxiredoxins groups share very low amino acid sequence similarity, they have residues that are very conserved among all members (Wood et al., 2003b): (1) A proline that limits solvent and peroxide access in the active site and therefore probably



Fig. 4. Catalytic mechanism of Prxs. As described in Fig. 1B reduction of peroxides by reactive cysteines generated a sulfenic acid derivative in all kinds of peroxiredoxins. (A) In 1-Cys peroxiredoxins the sulfenic acid derivative is stabilized by the polypeptide backbone and is directly reduced by a thiol reductant. (B) In typical 2-Cys peroxiredoxins, the sulfenic acid interacts with another thiol group from other subunit, generating an intermolecular disulfide bond, which is then reduced by a biological substrate, in most cases thioredoxin. (C) In atypical 2-Cys peroxiredoxins, the catalytical mechanism is very similar to 2-Cys typical, with the exception that an intramolecular disulfide bond is formed.

shields the cysteine sulfenic acid from overoxidation; (2) an arginine residue that is involved in the stabilization of peroxidatic cysteine in the thiolate form and (3) an threonine residue that also interacts with the sulfur atom of peroxidatic cysteine (Fig. 3). Besides these similarities, all peroxiredoxins possess a common structural feature: the thioredoxin fold, which was described before. Interestingly, all other thiol/disulfide oxido-reductases presented here (thioredoxin, glutaredoxin and protein disulfide isomerase) also possess the thioredoxin fold (Fig. 2A). Differently than the thiol/disulfide oxido-reductases, peroxiredoxins contain central insertions, N-terminal and C-terminal expansions to the thioredoxin fold that are different for different groups of these thiol dependent peroxidases. Due to these structural similarities and through a motif analysis, it was proposed that all these thiol proteins might have a common ancestor (Copley et al., 2004).

The yeast S. cerevisiae, which has been used as model for higher eukaryotes, possesses five peroxiredoxins belonging to four different sub-groups (Park et al., 2000). Our studies have demonstrated that although all five yeast peroxiredoxins have the same biochemical activity (thioredoxin dependent peroxidase); their cellular functions are not completely redundant. For example, cytosolic thioredoxin peroxidase I (Tsa1/YML028W) is specifically important for the defense of yeast with dysfunctional mitochondria (Demasi et al., 2001; Demasi et al., 2006), whereas mitochondrial thioredoxin peroxidase I (PrxI/ YBL064C) is more important in conditions where yeast obtain ATP preferentially by respiration (Monteiro et al., 2002; Monteiro and Netto, 2004). Finally, cytosolic thioredoxin peroxidase II (cTPxII/Tsa2/YDR453C) appears to be an important backup for cTPxI for the defense against organic peroxides, independently of the functional state of mitochondria (Munhoz and Netto, 2004). Interestingly, mitochondria are protected not only by the mitochondrial isoform (PrxI/ YBL064C) but also by cytosolic isoforms and in cooperation with mitochondrial pool of glutathione against Ca²⁺ induced stress (Monteiro et al., 2004). This partial redundancy observed among yeast peroxiredoxins probably parallels the roles that these peroxidases play in mammalian cells.

Recently, a new kind of peroxidase that also operates through a reactive cysteine was described (Lesniak et al., 2002; Cussiol et al., 2003). Initially, it was demonstrated that the deletion of genes encoding these peroxidases rendered Xanthomonas campestris specifically sensitive to organic peroxides, but not to hydrogen peroxide (Mongkolsuk et al., 1998). Therefore, this gene was named organic hydroperoxide resistance (Ohr) and was later shown to be exclusively present in bacteria, most of them pathogenic. Interestingly, only dithiols support the peroxidase activity of Ohr and it is considerably more efficient in the removal of organic peroxides than in the decomposition of hydrogen peroxide (Cussiol et al., 2003). It was noteworthy to observe that differently than other thiol-dependent peroxidases (glutathione peroxidases and peroxiredoxins), Ohr does not possess the thioredoxin fold. Instead, Ohr is a dimer composed of two six-strand β -sheet and two central α -helixes (Lesniak et al., 2002; Meunier-Jamin et al., 2004; Oliveira et al., 2006). Contrary to the other thiol/disulfide oxido-reductases



Fig. 5. Ohr structure with hidden cysteines residues. Overall view of *Xylella fastidiosa* quartenary structure (PDB = 1ZB9). Contrary to peroxiredoxins and thiol/disulfide oxido-reductases, reactive cysteine (Cys61 in pink) is buried in the polypeptide backbone (two β -sheet composed of six strands). The side chain of Arg 19 (magenta) that is involved in the stabilization of thiolate form of Cys 61 and Glu51 (red) that forms a salt bridge with Arg19 are also shown in dark color. Cys 125 (in yellow), involved in the formation of an intramolecular disulfide bond, is also represented with black color. Figure was generated by the Pymol software (www.pymol.org). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

and peroxidases described so far, the reactive cysteine is located in a very hydrophobic environment (Fig. 5). Due to these differences and because Ohr are exclusively present in bacteria, these peroxidases might represent interesting targets for drug design.

Finally, antioxidant proteins also make use of reactive cysteine to repair oxidative damage. Methionine sulfoxide reductase has a reactive cysteine capable to cleave an S=O bond, also by a nucleophilic substitution mechanism (Weissbach et al., 2002), (Fig. 1C).

2.5. Redox signalling

Since reactive cysteines can decompose peroxides yielding products that can be reduced back to the sulfhydryl form, several proteins containing this kind of residues are in principle adapted to participate in redox signaling mediated by hydrogen peroxide. Although hydrogen peroxide has been classically associated with oxidative stress, there is a growing amount of evidences about the role of this mild oxidant as a cell messenger (Rhee et al., 2005b). Hydrogen peroxide can cross membranes and is relatively stable, two features suitable for a cell messenger in analogy to nitric oxide (Stone, 2004). This idea was strengthened by the discovery that non-phagocytic cells also possess NADPH oxidase, a source for hydrogen peroxide (Bokoch and Knaus, 2003). In fact, there are numerous reports about the effect of hydrogen peroxide in terms of both cellular responses and signaling pathways activated (reviewed by Stone, 2004).

The best characterized mediator of peroxide induced stress is OxyR, a transcription activator found only in bacteria. Genes regulated by OxyR includes enzymes involved in peroxide decomposition (catalase, peroxiredoxin, thioredoxin, glutathione reductase and glutaredoxin) and cell signaling (small RNA molecule). The mechanism by which OxyR senses H₂O₂ involves a reactive cysteine that once again is stabilized in the thiolate form by a conserved arginine among other amino acids (Choi et al., 2001). The oxidation of this cysteine generates a disulfide bond that causes a conformational change in the protein. Both the oxidized and reduced forms of OxyR can bind DNA, but only the oxidized form is capable to recognize specific elements in the promoters of target genes and activate their transcription (Fig. 6A). The reactive cysteine of OxyR possesses a relatively high rate constant $(2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1})$, see Aslund et al., 1999) and can activate transcription when intracellular concentrations of hydrogen peroxide are as little as 100 nM (Costa Seaver and Imlay, 2001). The activation of OxyR is reversed by reduction of reactive cysteine by GSH and glutaredoxin 1 (Fig. 6).

Response of bacteria to oxidative stress is mediated by other transcriptional regulators besides OxyR. OhrR is a transcriptional repressor that is also capable to sense peroxides through a reactive cysteine (Mongkolsuk and Helmann, 2002). The only one known target of OhrR so far described is Ohr that is specifically induced by organic peroxides, the preferable substrate of this dithiol-dependent peroxidase. Therefore, Ohr/ OhrR is a pathway specifically involved in the oxidative stress response to organic, but not to hydrogen peroxide (Klomsiri et al., 2005). In vitro, reduced OhrR binds tightly to its target DNA and therefore blocks the transcription of ohr (Fuangthong et al., 2001). Oxidation of a conserved and reactive cysteine in OhrR by peroxides leads to derepression of ohr transcription, which is reversed by a reducing agent such as DTT. Differently than OxyR, OhrR is oxidized to a sulfenic acid (CysSOH) instead of a disulfide (Fuangthong and Helmann, 2002).

Another transcriptional repressor of bacteria that was implied in peroxide sensing in bacteria through reactive cysteines is PerR (Mongkolsuk and Helmann, 2002). PerR belongs to a family of transcriptional regulators that are dimeric proteins and that contain two metal sites per monomer. One binds zinc and appears to play mainly structural roles, whereas the second site can bind both iron and manganese and has a regulatory role. PerR complexed with either Mn⁺² or Fe⁺² can bind DNA and repress transcription of its target genes such as catalase and peroxiredoxin. However, only when PerR is complexed with Fe⁺² there is derepression of gene expression and lack of DNA binding ability (Herbig and Helmann, 2001). Because DNA binding of PerR is restored by thiol reductants and because PerR has a CXXC motif, it was proposed that peroxide sensing might involve a reactive cysteine being oxidized to a disulfide bond. Very recently, however, the same group has shown that PerR senses hydrogen peroxide by a Fenton-like reaction mediated by Fe⁺² complexed with histidines. This process provokes oxidation of histidine residues (His37 and His91) to 2-oxohistidines. This is the first description of a metal catalyzed protein oxidation process involved with redox signaling (Lee and Helmann, 2006).

Besides transcriptional regulators, bacteria also possess a chaperone (Hsp33), whose activity is redox regulated through reduction/oxidation cycles that involve a reactive cysteine (Janda et al., 2004). In this case, cysteines residues in the reduced state can bind zinc but after oxidation to disulfide bonds, Hsp33 loses this ability but acquires high affinity for unfolded proteins (chaperone holdase activity). Thioredoxin (or glutaredoxin) can then reduce the reactive cysteine of Hsp33, restoring its ability to bind zinc. This ensures that proteins with transient exposed hydrophobic surfaces do not form insoluble aggregates. Upon return to non stress conditions other chaperone systems are available to interact with the partially unfolded proteins released by Hsp33 (reviewed by Winter and Jakob, 2004). Interestingly, Hsp33 appears to be active in severe oxidative stress, condition in which other chaperones are inactive (Winter et al., 2005).

Another level of regulation was possible in eukaryotes with the appearance of cellular compartments. In fact, the control of a transcriptional regulator's activity by regulated nuclear



Fig. 6. Redox regulation by OxyR. Each OxyR subunit is represented here by an elliptical symbol. The darker symbols represent the reduced tetramer and the lighter the oxidized (disulfide) tetramer that assumes different conformations. Only the oxidized formed is capable to recognize specific sequences (elements) repeated four times in the promoters of targets genes and as a consequence stimulate their transcription.

accumulation is a common theme in biology. Therefore, the higher the amount of a transcriptional regulator in the nucleus, the higher is its activity (repression or induction of gene expression). The best characterized mechanism of a redox signaling process in an eukaryotic cell through a reactive cysteine is that mediated by Yap1 (reviewed by Paget and Buttner, 2003). Yap1 belongs to the AP-1 family of proteins that includes the proto-oncogenes Jun and Fos, all of them possessing a Leu zipper involved in the dimerization of these proteins (Fig. 7A). Yap1 also possesses a nuclear export signal (NES) that in basal conditions is recognized by Cmr1 that then transport this transcriptional activator from the nucleus to the cytosol (Fig. 7B i). Therefore, in basal conditions Yap1 is preferentially located in the cytosol, does not interact with target promoters and consequently does not induce gene expression. Upon oxidation, Yap1 cysteine residues are oxidized and NES adopt a different conformation, not recognizable by Crm1. Therefore, Yap1 accumulates in the nucleus, being capable to physically interact with target promoters.

Yap1 can be oxidized into two products: (1) a disulfide between cysteines residues of the C-terminal cysteine rich domain (Fig. 7B ii) or (2) a disulfide between one cysteine of the N-terminal and the other of the C-terminal rich domain (Fig. 7B v). Mode (1) of Yap1 oxidation is the simplest and is mediated by thiol oxidizing agents such as diamide (Fig. 7B ii). The mode (2) is a pathway that involves other proteins besides Yap1. In this case, the oxidant is a peroxide molecule that is sensed by a protein, homologous to the seleniumdependent glutathione peroxidase (Gpx3/Orp1) from mammalian cells (Delaunay et al., 2002). Gpx3/Orp1 is oxidized to a sulfenic acid derivative (Fig. 7B iii), which condenses with a reactive cysteine of Yap1, generating a mixed disulfide bond (Fig. 7B iv). Finally a thiolate group from the N-terminal cysteine rich domain attacks the mixed disulfide, generating an intra-molecular disulfide bond in Yap1, which is not recognized by Crm1 and accumulates in the nucleus (Fig. 7B v). Besides Yap1, other transcriptional regulators are involved in the response of yeast to oxidative stress which is a very complex phenomenon. As an example, the regulation of mitochondrial thioredoxin peroxidase I involves Hap1 (YLR256W), Msn2/4 (YMR037C/YKL062W) and Yap1 among other regulators (Monteiro et al., 2002; Monteiro and Netto, 2004).

The mechanisms by which hydrogen peroxide is sensed in mammalian cells are much more controversial. Much attention



Fig. 7. Yap1 activation by nuclear accumulation dependent on oxidation. (A) Yap1 domains. Leu-ZIP = leucine rich domain; N-CRD = N-terminal cysteine rich domain; C-CRD = C-terminal cysteine rich domain. NES= Nuclear Export Signal. (B) The names of cellular compartments with capitol letters indicate the location where Yap1 accumulates. The arrow represents exportation of Yap1 out of the nucleus and the symbols of arrows with an axis represent inhibition of this process by oxidation of Yap1 cysteines. (i) Yap1 in the ground state is reduced and, therefore, its NES is recognized by Cmr1, leading to its exportation out of the nucleus. (ii) Thiols oxidizing agents, such as diamide, oxidize thiolate groups of the C-CRD, provoking inhibition of its exportation. After consumption of the oxidant, thioredoxin can reduce Yap1 back to the reduced state (i). (iii) Gpx3/Orp1 is oxidized by peroxide, generating a sulfenic acid derivative. (iv) Sulfenic acid form of Gpx3/Orp1 condenses with a thiolate group from C-CRD generating a mixed disulfide bridge, which is attacked by a thiolate from N-CRD, generating an intra-molecular disulfide bridge between cysteines of different domains (v). After consumption of the peroxide this disulfide can be reduced back to the ground state (i).



Fig. 8. Nrf2 activation by nuclear accumulation dependent on oxidation of Keap1. (A) Under basal conditions, Keap1 is in the reduced state and sequester Nrf2 in the cytoplasm. Keap1 is also connected to the cell cytoskeleton. In this condition, Keap1 also induces ubiquitination of Nfr2 that is then degraded by proteasome. (B) Under oxidative stress, thiolate groups of keap1 are oxidized, leading to Nrf2 release that can then accumulate in the nucleus and activate transcription in the target genes.

is given to protein tyrosine phosphatases (PTP) as biological sensors of hydrogen peroxide. Hydrogen peroxide can react with cysteines from the active site of PTP, generating sulfenic acids, which was proposed to be a redox regulatory event (Lee et al., 1998). Later, two groups have shown independently that sulfenic acids in PTP are converted to sulfenyl-amide by reaction of sulfenic acids with backbone amide of a serine residue (Salmeen et al., 2003; Van Montfort et al., 2003). The sulfenyl-amide form of PTP is inactive; therefore this process should provoke an increase in the levels of tyrosine phosphorvlation. As a consequence, PTP targets such as MAP kinases should be phosphorylated in higher levels. Besides sulfenylamides, reactive cysteines were also found in the sulfinate (RSO_2^-) and sulfonate (RSO_3^-) forms in the crystal structure of PTP, when these proteins were treated with large excess of hydrogen peroxide (Van Montfort et al., 2003). Contrary to the sulfinate and sulfonate forms, sulfenyl-amides can be reduced back by classical reductants such as DTT and thioredoxin (Salmeen et al., 2003; Van Montfort et al., 2003). Therefore, because their formation is reversible, sulfenyl-amides were proposed as an important step in the redox signaling by PTPs.

However, redox regulation by PTP is controversial, mainly because the reaction of these phosphatases with hydrogen peroxide is slow (reaction constant is around $10 \text{ M}^{-1} \text{ s}^{-1}$), (Stone, 2004). Considering that intracellular concentration of hydrogen peroxide is in between 1 to 700 nM and that the levels of glutathione are around 1-10 mM, a target for redox regulation should react faster with this mild oxidant than PTP does. In fact, as mentioned before, one biological sensor of hydrogen peroxide in bacteria, the transcriptional factor OxyR

possesses a reaction constant of $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Aslund et al., 1999). OxyR, like other thiol proteins mentioned here, possesses a very reactive cysteine, which is deprotonated at physiological pH. Therefore, the hydrogen peroxide sensor in mammalian cells should be in principle a protein that possesses a reaction constant with hydrogen peroxide in this range.

Peroxiredoxins are good candidates as biological redox sensors in mammalian cell, since their reaction constants with hydrogen peroxide are around 10⁵ M⁻¹ s⁻¹ or even higher (Akerman and Muller, 2005; Baker and Poole, 2003; Parsonage et al., 2005). In fact, there are many suggestions that peroxiredoxins could be the biological sensors of hydrogen peroxide (Wood et al., 2003a,b). In this regard, it was shown that bacterial 2-Cys Prx are one hundred times more resistant to hydrogen peroxide inactivation than some of their counterparts in eukaryotic cells (Wood et al., 2003a). In both cases, the inactivation by hydrogen peroxide occurs due to oxidation of sulfenic acid (Cys-SOH) in the reactive cysteine to sulfinic acid (Cys-SO₂H). Interestingly, the all 2-Cys Prx that are sensitive to inactivation possess two common motifs: GGLG and YF (Wood et al., 2003a). Therefore, it seems very probable that the high sensitivity of these peroxiredoxins to peroxide inactivation it is not a limitation in the mechanism of catalysis, but instead a property that was selected during evolution of eukaryotes (Wood et al., 2003a).

In support to this hypothesis, it was shown that sulfinic acids in 2-Cys Prx are reduced in vivo in sensitive 2-Cys Prx (Woo et al., 2003). This was a quite surprising result, since it is well established that sulfinic acids in peroxiredoxins and in any other protein are not reducible in vitro by classical reducing agents, such as DTT and thioredoxin. The enzymatic system responsible to regenerate sulfhydryl groups from sulfinic acids in 2-Cys Prx was first identified in the yeast S. cerevisiae and was named sulfiredoxin (Biteau et al., 2003). Sulfiredoxin is a low molecular weight protein (13 kDa) that possesses homologues in higher eukaryotes including human, but its physiological role was unknown. The proposed mechanism of catalysis involves phosphotransferase and thiol transferase activities through a reactive cysteine and it is dependent on ATP. The basis of this mechanism was confirmed by biochemical and crystallographic studies (Jonsson et al., 2005). Biteau et al. (2003) suggested that sulfinic acid formation in 2-Cys Prx could represent an additional level of redox regulation for peroxiredoxins. The sulfiredoxin homologue in mammalian cells was also identified and in this case it was shown that sulfinic acid regeneration is exclusive for 2-Cys Prx (Chang et al., 2004; Woo et al., 2005). Recently, Budanov et al. (2004) have shown that another class of proteins can also reduce sulfinic acids specifically of mammalian peroxiredoxins. Like sulfiredoxins, sestrin possesses a conserved cysteine that is responsible for the catalytic mechanism. Moreover, the reduction is also dependent on ATP. However, sestrins do not share homology with sulfiredoxins. Sestrin expression is regulated by p53 indicating that this process possesses high physiological relevance.

The importance of sulfinic acids generated in the peroxidatic cysteines of 2-Cys Prx was further strengthened by the observation that peroxiredoxins from yeast also possess chaperone activity. Interestingly, the chaperone activity is independent of peroxidatic and resolving cysteines. Both peroxides and high temperatures induce chaperone activity, which is dependent on the oligomerization of peroxiredoxin polypeptides. Remarkably, under oxidative and thermal stresses these protein form very high molecular weight complexes that can be visualized by electron microscopy. Therefore, peroxidatic cysteines of these yeast peroxiredoxins are important not only for the decomposition of peroxides but also to induce protein oligomerization and consequently chaperone activity. In fact, sulfinic acid formation was suggested as a trigger event for the formation of a superchaperone that possesses a molecular weight of more than 1000 kDa (Jang et al., 2004). This dual chaperone/ peroxidase activities of yeast 2-Cys Prx was implied with the observation that it specifically protects cells with dysfunctional mitochondria from peroxide insult (Demasi et al., 2006). Recently, the chaperone activity was also described for peroxiredoxins from mammals and bacteria (Moon et al., 2005; Chuang et al., 2006).

The versatility of peroxiredoxin function can be further demonstrated by the observation that addition of single amino acid (Phe) close to the reactive cysteine converts a bacterial peroxiredoxin into a disulfide reductase (Ritz et al., 2001). This appears to be a relevant phenomenon, since bacteria lacking both thioredoxin reductase and glutathione reductase are viable only if cells possess peroxiredoxin with disulfide reductase activity (Ritz et al., 2001).

Recently, a novel redox mechanism for regulation of gene expression was demonstrated in mammals (Venugopal and Jaiswal, 1996; Itoh et al., 1997). As the redox regulation of Yap1 activity, the regulation of Nfr2, also a leucine zipper transcriptional activator, involves control of its nuclear localization. However, differently than Yap1, Nfr2 is not directly redox regulated, but instead reactive cysteines of a cytoplasmatic anchor (Keap1) are susceptible to oxidation by peroxides and electrophiles. Under basal conditions, Nfr2 is sequestered from the nuclei by Keap1 through non-covalent interactions (Itoh et al., 1999). Because Keap1 is bound to actin, Nrf2 is also connected to the cell cytoskeleton (reviewed by Motohashi and Yamamoto, 2004). These protein-protein interactions also induced ubiquitination of Nfr2 and consequently proteolytic digestion by proteasome (Fig. 8A). When mammalian cells are exposed to peroxides and electrophiles, reactive cysteines of Keap1 are oxidized to disulfide bonds, it suffers a conformational change and consequently Nfr2 is released and accumulates in the nucleus, being capable to recognize its target promoters (Fig. 8B).

3. Conclusions

The majority of the cysteine residues in proteins play no role in electron transfer reaction, because their pK_a make them appear mainly in the protonated form in physiological conditions. In contrast, some protein foldings create environments in which the deprotonated form of cysteine (RS⁻ = thiolate) is stabilized, being susceptible to oxidation. Thiol/ disulfide reactions are the most frequently considered, but thiol/ sulfenic acid reactions have also been implicated in some biological processes a long time ago. Recently, thiol/sulfinic redox chemistry has received attention in terms of redox signaling. Therefore, the versatile redox chemistry of thiolate in proteins has served to various biological roles as described in this review and should be a promising research field.

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