Chapter 5
The Peroxiredoxin Family: An Unfolding Story

Zhenbo Cao and John Gordon Lindsay

Abstract Peroxiredoxins (Prxs) are a large and conserved family of peroxidases that are considered to be the primary cellular guardians against oxidative stress in all living organisms. Prxs share a thioredoxin fold and contain a highly-reactive peroxidatic cysteine in a specialised active-site environment that is able to reduce their peroxide substrates. The minimal functional unit for Prxs are either monomers or dimers, but many dimers assemble into decameric rings. Ring structures can further form a variety of high molecular weight complexes. Many eukaryotic Prxs contain a conserved GGLG and C-terminal YF motif that confer sensitivity to elevated levels of peroxide, leading to hyperoxidation and inactivation. Inactive forms of Prxs can be re-reduced by the enzyme sulfiredoxin, in an ATP-dependent reaction. Cycles of hyperoxidation and reactivation are considered to play an integral role in a variety of H_2O_2-mediated cell signalling pathways in both stress and non-stress conditions. Prxs are also considered to exhibit chaperone-like properties when cells are under oxidative or thermal stress. The roles of various types of covalent modifications, e.g. acetylation and phosphorylation are also discussed. The ability of Prxs to assemble into ordered arrays such as nanotubes is currently being exploited in nanotechnology.

Keywords Peroxiredoxin • Redox • Peroxide • Hyperoxidation • Sulfiredoxin • Oxidative stress • Antioxidation • Signalling pathway • Chaperone • Protein complex

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5.1   Peroxiredoxins and Their Classification

Peroxiredoxins (Prxs) were first identified as a new class of peroxidases in 1994 (Chae et al. 1994). Prior to this landmark discovery, catalase and glutathione peroxidase were thought to provide the main line of defence against oxidative stress in living organisms in addition to non-enzymatic reductants and free radical scavengers, such as glutathione, vitamin E and ascorbate. A novel aspect of Prxs was that they did not rely on a heme prosthetic group or an active selenocysteine moiety for activity, instead employing one or two standard cysteine residues in catalysis. In the past 20 years, Prxs have become one of the most intensively studied protein families, owing to their ubiquitous distribution, catalytic efficiency, capacity for reversible and irreversible hyperoxidation and unusual ability to assume multiple oligomeric states with differing properties, e.g. chaperone activity (Wood et al. 2003b; Perkins et al. 2015). More recently, attention has focused on elucidating their emerging roles in vital cell signalling pathways concerned with governing cellular responses to their immediate environment under both stress and non-stress conditions (Latimer and Veal 2016; Netto and Antunes 2016).

Prxs are now considered to be the primary cellular guardians against oxidative stress in all living organisms. As a group, they are responsible for the removal of more than 90% of cellular peroxides based on their relative abundance, catalytic power and presence in multiple intracellular compartments in eukaryotes (Winterbourn 2008). Classification has been problematic, because of the diverse nature of this large family of enzymes. Nelson and co-workers used the Deacon Active Site Profiler (DASP) tool to categorise Prxs in six evolutionary subfamilies, based on sequence similarity, namely: Prx1/AhpC, Prx5, Prx6, Tpx, PrxQ/BCP and AhpE (Nelson et al. 2011). The Prx1 group is commonly referred to as the "typical 2-Cys" Prx subfamily, since its members almost exclusively contain two active-site cysteines and function as a basic homodimeric unit in which a peroxidatic cysteine (C_P) situated in the N-terminal region of one monomer forms an inter-chain disulfide bond with a resolving cysteine (C_R) located in the C-terminal domain of its partner. In mammalian cells, there are four distinct enzymes in this group: PrxI and PrxII are located in the cytosol and nucleus, PrxIII is found in the mitochondrial matrix and PrxIV is confined to the endoplasmic reticulum. PrxV is an "atypical 2-Cys" Prx in which C_P and C_R are on the same polypeptide chain. PrxV is present in mitochondria, peroxisomes and the cytosol, while the "1-Cys" enzyme, PrxVI is also located in the cytosol.

One of the most interesting features of 2-Cys Prxs is their ability to form oligomeric rings (toroids) from their minimal functional dimeric units. Thus, PrxsI, II, IV and VI can exist as stable decamers whereas PrxIII is a dodecamer. Octameric toroids have also been observed in the case of the 1-Cys Prx, AhpE from *Mycobacterium tuberculosis* (Li et al. 2005).

In 1998, sequence alignment and secondary structure predictions revealed that Prxs share a common thioredoxin (Trx) fold, which consists of an N-terminal βαβ motif and a C-terminal ββα motif, linked by a central loop region containing another

Fig. 5.1 Decamerisation of human PrxIV. (**a**) B-type dimer: The anti-parallel β-sheet in the core of the structure between monomers creates an extended 14-stranded sheet (β2–β8). The C$_P$ is shown as *yellow* ball. (**b**) A-type dimer: The hydrophobic side chains are highlighted. *Cyan* and *green* represent different monomers. (**c**) Toroidal decamer of human PrxIV: Five B-type dimers (marked by a *box*) form a decamer via A-type interfaces. A and B-type interfaces are marked as *solid lines*
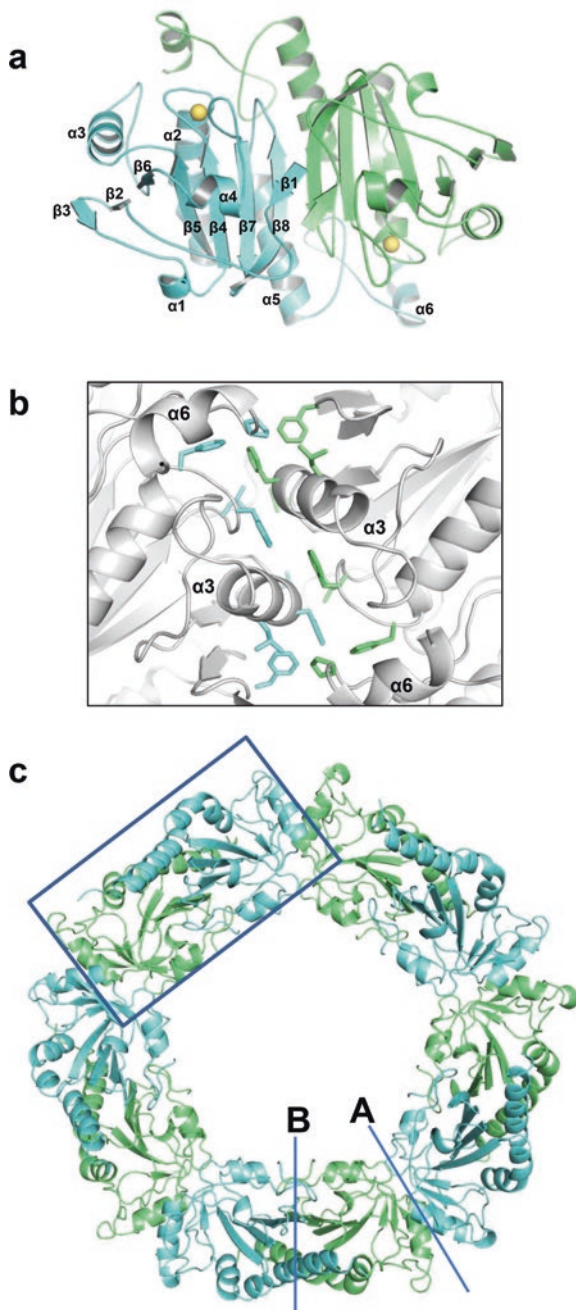
α-helix (Schroder and Ponting 1998). Prxs contain several additions to the basic Trx fold, namely an N-terminal extension, an insertion between the second β-strand and α-helix and in some cases, a C-terminal extension, e.g. in the ubiquitous Prx1/AhpC subfamily. Structural data from all Prx subfamilies have revealed that they share a common core organisation comprising seven β-strands and five α-helices (Fig. 5.1a).

With a few exceptions (e.g. monomeric BCP family members), all Prxs function as dimers or higher-order oligomers. Two different types of subunit interfaces (termed A and B) are employed to form the basic dimeric subunit and then mediate dimer-dimer assembly into toroidal structures (Sarma et al. 2005). Crystal structures of dimeric PrxIV revealed that B-type dimers are organised in a 'head-to-tail' fashion in which the β8-strands of adjacent subunits interact in an anti-parallel manner to generate a stable 14-stranded β-sheet involving the 7 central β-strands of each subunit (Cao et al. 2011). The C-terminal region also extends across the two-fold axis, further stabilising the dimeric interface. In contrast, PrxV forms a dimer via an A-type (alternative) interface, utilising hydrogen bonding and hydrophobic interactions (Declercq et al. 2001).

For PrxsI-IV and some members of the PrxVI family, five or six B-type dimers can further associate through their A-type interfaces to produce decameric or dodecameric toroids with a large central cavity. Higher-order complexes have also been observed. These involve ring stacking, ring catenation or even larger oligomeric assemblies (Cao et al. 2005; Gourlay et al. 2003; Jang et al. 2004). The A-type interface is less extensive and mainly involves residues from the α3 and α6-helices (Fig. 5.1b), the C_P loop, and the loops surrounding the α4-helix. In this group of Prxs, decamers and dodecamers are stabilised in the reduced state and exhibit a pronounced tendency to revert to dimers upon oxidation. This redox dependence in the oligomeric state is accounted for by the associated conformational changes in the C_P loop, which forms an integral part of the dimer-dimer interface.

5.2   The Multifaceted Roles of Prxs

The importance of individual Prxs to normal growth and development has been evaluated in several knockout mouse models. Mice lacking PrxI were viable and fertile but had a shortened lifespan, owing to the development, at about 9 months, of severe hemolytic anemia and several types of malignant tumor. These data suggest that PrxI functions as a tumor suppressor, with an important role in cellular defence against ROS in ageing mice (Neumann et al. 2003). PrxII −/− mice appeared healthy and fertile. However, they also suffered from hemolytic anemia with a decreased hematocrit count and the presence of numerous morphologically abnormal cells. High levels of ROS were detected in the blood and many red blood cell (RBC) proteins were subject to oxidative damage, suggesting that PrxII plays a major role in protecting RBCs from oxidative stress (Lee et al. 2003). Thus, although PrxI and PrxII are both located in the cytosol and share similar anti-oxidant

activities, current evidence indicates that they have overlapping, but distinctive roles in cellular protection in mouse models. In contrast, the main phenotype in PrxIII-deficient mice is increased sensitivity to lipopolysaccharide-induced oxidative stress, whereas PrxIV null mice display reduced sperm viability (Li et al. 2009; Iuchi et al. 2009).

A host of altered cellular responses have now been described to be associated with the deficiency of a specific Prx family member or linked to their overproduction. These range from roles in breast cancer progression and apoptosis, to alterations in inflammatory responses and protection against oxidative, UV or radiation damage (Cha et al. 2009; Klichko et al. 2016; Ito et al. 2014). A plethora of recent publications have also implicated Prxs in many fundamental biological processes, including the control of the cell cycle, the protection of DNA from oxidative damage and an involvement in circadian rhythmicity (Phalen et al. 2006; Lee et al. 2016; Edgar et al. 2012). Thus, while the pleiotropic effects on cells induced by periods of oxidative stress can be linked to specific roles for individual Prx family members in aiding cell survival, Prxs are also increasingly recognised as integral components in major cell signalling pathways, with roles in monitoring and adjusting cell responses to their immediate aerobic environment under normal conditions (Chang et al. 2007; Tavender et al. 2008; Mukhopadhyay et al. 2006; Stresing et al. 2013).

5.3 The Prx Active Site and Reactivity of the Peroxidatic Cysteine

In both 1-Cys and 2-Cys Prxs, the initiation of the reaction cycle begins with an attack by the peroxidatic cysteine (C_P) thiolate (C_P-S$^-$) on its peroxide substrate, leading to production of a sulfenic acid intermediate (C_P-SOH). At this stage Prxs are in the fully-folded (FF) conformation (see Fig. 5.2 for details) with C_P located towards the end of the α2-helix. In addition, the side chain of C_P is favourably positioned in the active-site pocket, inducing C_P-S$^-$ formation that is required for the nucleophilic displacement (S_N2) reaction, while also providing a suitable environment for optimal orientation and positioning of the substrate (Hall et al. 2010).

Many Prxs display extremely rapid reaction rates, in the range 10^5–10^8 M^{-1} s^{-1} for H_2O_2 for bacterial and mammalian Prxs (Parsonage et al. 2008; Peskin et al. 2007). Three highly-conserved residues, including a proline and threonine/serine in the C_P loop motif, PXXXT(S)C_P and a more-distantly located arginine, are implicated in stabilising/activating the C_P-S$^-$ thiolate and hydrogen bonding with the bound peroxide substrate, ensuring its optimal positioning to achieve these high catalytic rates.

In the FF conformation, the active-site C_P thiolate is located approximately 14 Å from the resolving cysteine (C_R) in the C-terminal extension of the other chain. A major conformational change, involving partial unwinding of the α2-helix in the C_P active-site loop, occurs upon oxidation with the substrate bringing the C_P-SOH
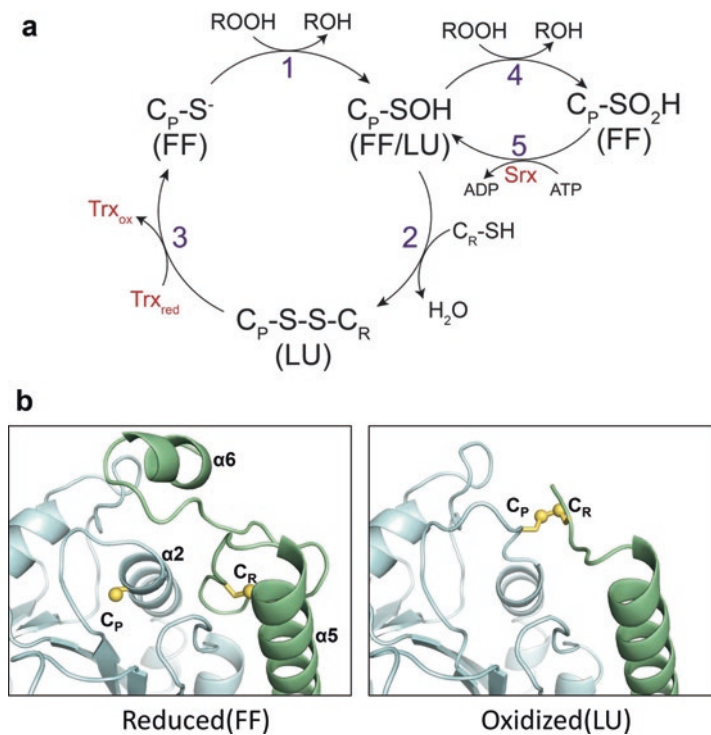
Fig. 5.2 The Prx catalytic cycle. (a) Schematic representation of Prx catalytic cycle. (*1*) *peroxidation*, the peroxidatic cysteine (C_P-S^-) reduces peroxide. (*2*) *resolution*, the resolving cysteine (C_R-SH) reacts with sulfenylated C_P to form an inter-chain disulfide bond. (*3*) *recycling*, reduced thioredoxin (Trx_{red}) is oxidised resulting in the regeneration of C_P. (*4*) *hyperoxidation*, occasionally C_P in the sulfenic acid state can react with a second peroxide and become hyperoxidised to sulfinic acid. This form of Prx is inactive. (*5*) *reactivation*, the inactivated form can be rescued through an ATP-dependent reaction catalysed by Srx. The C_P loop conformation is indicated as fully-folded (FF) or locally-unfolded (LU). **(b)** Structure of active-site in reduced and oxidised PrxIV. Different chains colored *cyan* and *green*. The cysteine side chains are shown as yellow sticks and sulfur atoms are shown as *yellow balls*. Note the C-terminal α6-helix needs to be unwound to permit disulfide formation

intermediate into close proximity (2–3 Å) with C_R (located in the C-terminal domain of its partner subunit) prior to disulfide bond formation.

The C_P-SOH intermediate is proposed to equilibrate between the FF and LU conformations until disulfide bond generation locks the enzyme into the LU state (Cao et al. 2011; Perkins et al. 2013). By using rapid reaction and competitive kinetic approaches, Peskin and co-workers have established a rate constant for disulfide formation at 2/second for PrxII (Peskin et al. 2013). The key conserved Arg residue in the active-site pocket (and a backbone amide group) appears to stabilise and activate the C_P thiolate for nucleophilic attack on its peroxide substrate in the FF conformation (Hall et al. 2010). In a recent crystal structure of the bovine PrxIII F190L mutant, this key Arg has been detected in a novel conformation that

extended away from the C_P thiolate, indicating the dynamic nature of these interactions (Cao et al. 2015).

5.4 Hyperoxidation of Prxs and Sulfiredoxin Mediated Reactivation

Shortly after the discovery of the Prx family in 1994, it became evident that several of its members could be inactivated by their common substrate H_2O_2, even at physiological (submicromolar) concentrations (Yang et al. 2002; Wagner et al. 2002; Konig et al. 2002). Initially, these hyperoxidised states in which the catalytic, peroxidatic cysteine C_P was further oxidised beyond its normal transition state of the sulfenic acid (Cys-OH) intermediate, were thought to be dead-end products with hyperoxidation being seen as a limitation in the catalytic properties of Prxs. However, Wood and co-workers noted that hyperoxidation was an exclusive feature of eukaryotic peroxiredoxins, whereas their prokaryotic counterparts were not subject to inactivation under similar conditions (Wood et al. 2003a). It was further suggested that the susceptibility to hyperoxidation to their sulfinylated (C_P-O_2H) or sulfonylated (C_P-O_3H) states had been selected for during evolution and that inactivated (hyperoxidised) peroxiredoxins may act as key mediators in evoking cell signalling responses to oxidative stress. The extent of hyperoxidation *in vivo* or *in vitro* can be monitored as these inactivated species carry a higher net negative charge, allowing for their separation on 2-D gels (Mitsumoto et al. 2001) or 1-D isoelectric focusing gels (Cao et al. 2014). Antibodies specific for the hyperoxidised states of all typical 2-Cys Prxs are now widely employed to quantitate their susceptibility to inactivation or the extent of hyperoxidation occurring in living cells subjected to various treatment regimens (Woo et al. 2003).

Two specific motifs that were exclusively present in the sensitive members of eukaryotic 2-cys Prxs were also identified by Wood and co-workers, namely an invariant GGLG sequence residing in the loop region following the α3-helix and a YF motif in the C-terminal α-helical domain (Wood et al. 2003a). As seen in Fig. 5.3, these two motifs are predicted to stabilise the FF state by packing against the C_P loop. Indeed, the YF motif would interfere with the locally-unfolded conformation of the C_P loop if it did not also move away during the reaction cycle (Wang et al. 2012). Recent evidence from a crystal structure of the TSAII C48S mutant indicates that an unfolded C-terminus is needed to permit unfolding of the C_P loop due to steric hindrance effects (Nielsen et al. 2016). Thus, the conformational stability of the C-terminus is likely to be a sensitive regulator of Prx function.

These initial studies indicated that the balance between FF and LU states is crucial to determining the rate of reaction with H_2O_2. Thus, stabilisation of the FF was anticipated to slow the transition to the LU state, thereby favoring hyperoxidation by allowing time for a reaction with a second or third molecule of substrate. As expected, no similar stabilisation occurs in robust members of the same Prx family,
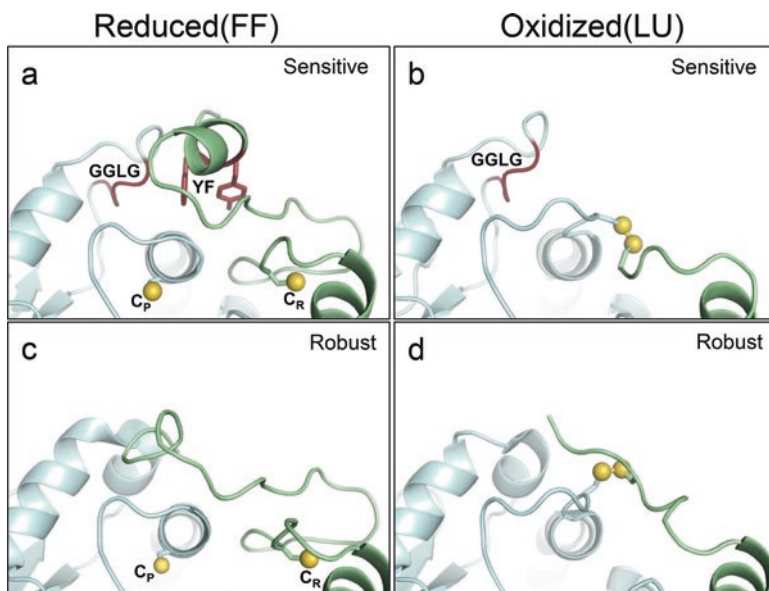
Fig. 5.3 Structural differences between sensitive (eukaryotic) and robust (prokaryotic) Prx1s. Comparison of active-site region of human PrxIV (**a** and **b**) and AhpC from *S. typhimurium* (S.t.) (**c** and **d**). Different chains in the homodimer are colored in *cyan* and *green*. C_P and C_R are shown as *yellow balls*. The conserved GGLG and YF motifs in sensitive Prx1s are colored in *red*. The reduced (FF) conformation is on the *left* side of the panel and the oxidised (LU) conformation is on the *right*. Comparison of **a** and **c** clearly show that the GGLG and YF motifs are blocking the unwinding of C_P from its FF conformation

which lack these motifs (Fig. 5.3). Subsequently, interchanging C-terminals between different Prxs has also established that other C-terminal residues (apart from YF) are instrumental in fine-tuning the precise sensitivity of individual Prxs to the prevailing conditions in the appropriate intracellular compartment. For example, the mitochondrial enzyme, PrxIII is more resistant than PrxII located in the cytosol, although both possess identical GGLG and YF motifs. Exchanging their C-terminal domains, however, reverses this sensitivity (Haynes et al. 2013). The reaction of the C_P-SOH intermediate with C_R in PrxII is 11-times slower than in PrxIII, which is in agreement with the hypothesis that the rate of transition between FF and LU conformations is the major determinant of sensitivity to hyperoxidation (Peskin et al. 2013).

In this context, as discussed previously, the highly-conserved YF motif is a major determinant in ensuring the susceptibility to hyperoxidation in eukaryotic Prxs. Interestingly, a similar YL motif is found in many prokaryotes; however, a mutant bovine PrxIII containing the YL motif is fully active, but also insensitive to H_2O_2-induced inactivation (Cao et al. 2015). Moreover, crystal structures of both the oxidised and the reduced forms of this PrxIII mutant confirm the presence of a disordered C-terminal region, which is consistent with a role for YF in stabilising

this domain (Cao et al. 2015). These observations are in agreement with the current hypothesis that the rate of transition from the FF state to the LU state, which is necessary for disulfide bond formation, determines the extent of the resistance to hyperoxidation. Partial unwinding of the α2-helix and the C-terminal helix are required to bring the active C_P and C_R cysteine closer together, from 14 Å apart in the reduced state to within 2–3 Å when facilitating disulfide bond formation. Thus, the slower this transition, the more opportunity for the Cys-OH intermediate to react with a second or third molecule of hydrogen peroxide, i.e. to attain its hyperoxidised sulfinylated or sulfonylated states.

In general, it now appears that eukaryotic Prxs are more sensitive to hyperoxidation than their prokaryotic counterparts as a result of the slower transition from FF to LU states during the reaction cycle (Wood et al. 2003a), which is governed by the stability of the C-terminal α-helix and its conserved YF motif in particular. Surprisingly, although PrxIV is as sensitive to hyperoxidation as PrxII *in vitro*, it is protected from hyperoxidation in the oxidising environment of the endoplasmic reticulum (ER), even upon exposure to high levels of H_2O_2 (Cao et al. 2014). This appears to reflect the lack of an efficient ER recycling system, resulting in the low turnover of PrxIV. Variation in H_2O_2 concentration *in vivo*, for example by localised H_2O_2 accumulation, could also be a factor influencing the extent of Prx hyperoxidation (Woo et al. 2010).

Hyperoxidised forms of Prxs have also been detected in cells that are not subjected to oxidative stress (Schroder et al. 2000; Musicco et al. 2009). Interestingly, regular cycles of reduced and hyperoxidised Prx accumulation have been observed in several cell types, indicating that they are intimately linked to the control of circadian rhythms (Edgar et al. 2012).

Recognition of the biological significance of hyperoxidation in eukaryotic systems was quickly reinforced by the discovery of sulfiredoxin (Srx), a cytoplasmic enzyme that was initially shown to re-reduce PrxI in a Mg^{2+} and ATP-dependent manner (Biteau et al. 2003). *In vitro* studies of the mechanism of Prx reactivation have demonstrated that its action is confined to the 2-Cys Prxs and that Srx has been reported to enter the mitochondrial compartment where it promotes reactivation of hyperoxidised PrxIII, in addition to its cytoplasmic role in the maintenance and restoration of PrxI and II function (Noh et al. 2009). Detailed descriptions of the enzymatic mechanism of the re-reduction of sulfinylated (Cys-SO_2H) active-site cysteines have been published, although it appears that further oxidation to the sulfonylated state (Cys-SO_3H) represents an irreversible step in the process (Jonsson et al. 2008).

5.5 Role of Hyperoxidised Prxs in H_2O_2-Mediated Signalling

The inherent susceptibility of eukaryotic Prxs to inactivation in non-stress conditions initially gave rise to the 'floodgate' model, in which transient local increases in H_2O_2 were proposed to result in temporary Prx inhibition and the resultant

oxidation of downstream targets. To date the best documented example of a 'floodgate-type' mechanism is represented by the regulation of corticosteroid production from cholesterol in adrenal mitochondria (Kil et al. 2012). Steroid synthesis is subject to negative feedback in which H_2O_2 is formed indirectly from the inadvertent leakage of electrons onto molecular oxygen during the NADPH-dependent cytochrome P450-catalysed step in their production. Thus, the local elevation of H_2O_2 is potentiated by transient PrxIII inactivation during times of increased steroid production, enabling its increased access to the cytoplasm, where it stimulates p38 mitogen-activated protein kinase and thereby inhibits cholesterol import into mitochondria and the suppression of glucocorticoid biosynthesis. A variation on the floodgate theme has been reported in which growth factor signalling leads to the stimulation of an NADPH oxidase (NOX1) and hence increased H_2O_2 production. Src kinase is also activated under these conditions, catalysing the phosphorylation/inactivation of PrxI on Tyr194 and allowing local increases in H_2O_2 (Woo et al. 2010). Elevated peroxide levels also mediate the inactivation of Protein Tyrosine Phosphatases and influence a host of downstream signalling events (Chiarugi and Cirri 2003). Importantly, phosphorylated PrxI is confined exclusively to the vicinity of NOX1-associated lipid rafts, ensuring local control of regulation.

The interplay between phosphorylation state, hydrogen peroxide production and growth factor regulated signalling in this case indicates similar control mechanisms may exist for a variety of cell signalling pathways. In this respect, it is also of interest that a number of covalent modifications of Prxs have been reported that modulate their activity and susceptibility to inactivation. These include Ser and Tyr phosphorylation, Tyr nitration, S-nitrosylation, glutathionylation of active-site and non-catalytic cysteines, N- or C-terminal acetylation and protein truncation. Many of these post-translational modifications have been shown to enhance or inhibit Prx function. In several cases, these are reported to influence Prx activity by affecting the balance and rate of transition between the fully-folded (FF) and locally-unfolded (LU) conformations during oxidation-reduction. For example, phosphorylation of Thr90, which is located close to the conserved GGLG motif in human PrxI, causes inactivation, as does phosphorylation of Tyr194 (Jang et al. 2006; Woo et al. 2010). In both cases, introduction of a negatively-charged phosphate in the vicinity of the active-site is predicted to destabilise the FF conformation, causing a loss of activity. Similarly nitration of the conserved Tyr in the C-terminal YF motif of human PrxII stimulated activity and protected against hyperoxidation, possibly by increasing the rate of disulfide formation via destabilisation of the C-terminal α-helix (Romero-Puertas et al. 2007). In addition, N-terminal acetylation of PrxII has been reported to prevent its irreversible hyperoxidation, without altering its affinity for H_2O_2 (Seo et al. 2009). In contrast, acetylation of Lys197 or 196 in the C-terminal domains of PrxI and PrxII, respectively, leads to enhanced peroxidase activity and protection against H_2O_2-induced inactivation (Parmigiani et al. 2008). Finally, glutathionylation of the active-site cysteine (Cys83) in human PrxI also appears to influence the decamer-dimer transition, stabilising the dimeric state and abolishing chaperone activity (Park et al. 2011).

In addition to the extensive range of modifications described above, the ability of PrxI to interact with signalling molecules, such as PTEN phosphatase and mammalian ste-20-like kinase-1 (MST1), which are implicated in cell cycle regulation and apoptosis, provide new insights into the complex network of regulatory pathways controlling cell and tissue responses in normal and oxidative stress conditions (Morinaka et al. 2011). Hyperoxidation of 2-Cys Prxs, linked to alterations in their oligomeric state, have also been associated with alterations in the cell cycle, an indication that the oxidation status and quaternary structure of Prxs may act as reporters, monitoring local peroxide levels and generating the appropriate cellular responses (Phalen et al. 2006). In similar fashion, Prxs have also been shown to act as intermediates in so-called redox relays, by transferring oxidising equivalents directly or indirectly to key regulatory components, e.g. transcription factors or signalling kinases/phosphatases. In one example, PrxII was shown to form a mixed disulfide with STAT3, giving rise to disulfide-bonded dimers/tetramers of this transcription factor that could be re-reduced by thioredoxin (Sobotta et al. 2015). Importantly, depletion of PrxII enhanced the rate of STAT3-mediated transcription, suggesting that PrxII-induced oligomerisation of STAT3 inhibits transcription.

Another well-documented case is found in *Schizosaccharomyces pombe,* where the thiol peroxidase (Tpx1) oxidises the AP1-like transcription factor (Pap1) by disulfide exchange, concealing a nuclear export signal for Pap1 (Calvo et al. 2013). The resultant nuclear accumulation of Pap1 leads to upregulation of genes involved in antioxidant defence. Interestingly, at higher H_2O_2 levels, Tpx1 becomes hyperoxidised, rendering it unable to activate Pap1. At this stage, a mitogen activated signalling pathway, employing the protein kinase sty-1, is recruited to perform a similar role. This secondary pathway is also dependent on Tpx1, but operates independently of its state of hyperoxidation.

Recent studies by Day and co-workers have revealed that Tpx1 is reduced by thioredoxin at low H_2O_2 concentrations, however, following Tpx1 hyperoxidation, Trx is available to reduce Pap1 and the disulfides of antioxidant defence proteins (Day et al. 2012). In this regard, the redox status of Tpx1 determines whether it acts in an adaptive mode at low H_2O_2, ensuring upregulation of antioxidant defence genes. After its inactivation, at elevated H_2O_2 levels, Trx can focus on the repair of cellular damage when cell survival is the priority.

5.6 Alterations in Oligomeric State and Chaperone-Like Functions

As mentioned previously, 2-cys Prxs contain a basic functional dimeric unit in which the N-terminal active site, C_P, of one subunit forms a transient disulfide bond with the C-terminal resolving cysteine, C_R, of its partner. Although functional as dimers, 2-cys Prxs generally exist as higher-order oligomers, specifically as octameric, decameric and dodecameric toroids. As seen in Fig. 5.4, small stacks of two
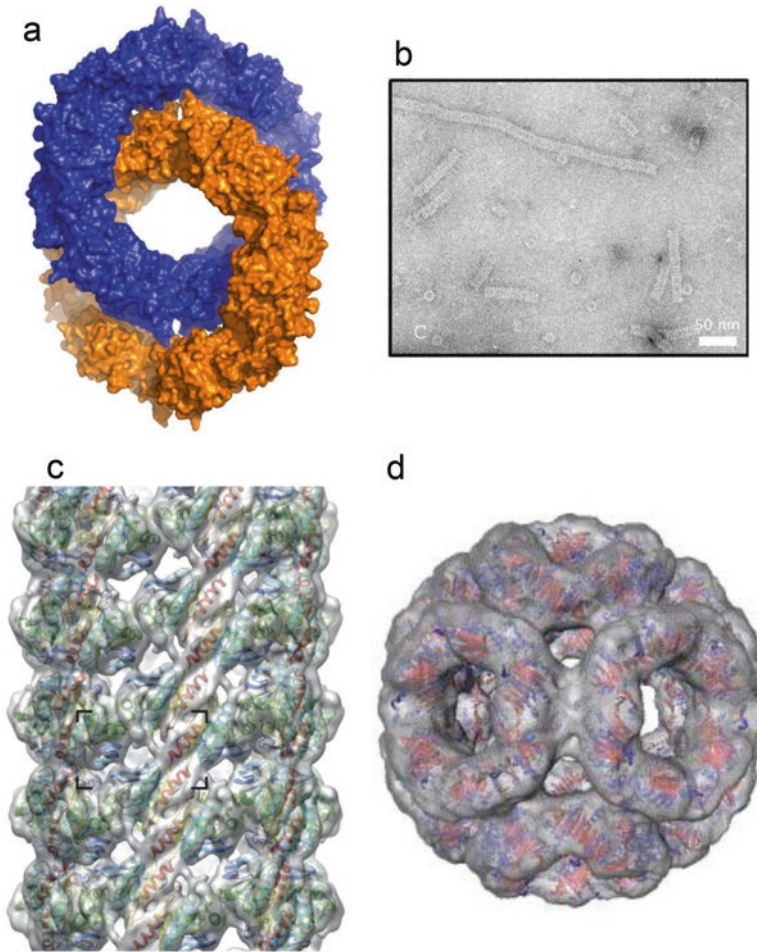
Fig. 5.4 Oligomerisation of the Prx toroid. (**a**) Crystal structure of bovine PrxIII F190L show-
ing two interlocking dodecameric rings forming a catenane (Adapted from Cao et al. 2015). (**b**)
Transmission electron microscopy showing stacked dodecameric rings of bovine PrxIII C47S
(Adapted from Gourlay et al. 2003). (**c**) Cryo-EM 3D-reconstruction of human PrxIII. Bovine
PrxIII crystal structure was docked into the electron density (Adapted from Radjainia et al. 2015).
(**d**) The 16 Å 3D-reconstruction of human PrxII dodecahedron (Adapted from Meissner et al.
2007)

to three rings are also readily observed in negatively-stained EM images, while
extensive stacking has been reported on occasions, e.g. with PrxI and III in which
50 or more rings coalesce to produce long nanotubes (Gourlay et al. 2003; Phillips
et al. 2014). Human PrxII has also been reported to assemble into a novel dodeca-
hedral structure after incubation in 1% PEG at pH 6.5 and similar 'spherical' struc-
tures have been observed for yeast TSAI and II (Meissner et al. 2007; Jang et al.
2004).

While ATP-dependent molecular chaperones, such as Hsp-70 and Hsp-40 classes of heat shock proteins, are necessary to direct the ordered folding of nascent polypeptide chains as they emerge from the ribosome, ATP-independent chaperones, generally referred to as 'holdases', are crucial for cellular protection under stress conditions, which favor protein unfolding and the formation of harmful aggregates. The *in vitro* switch from peroxidase to chaperone function amongst the Prxs is usually induced by thermal, oxidative stress or low pH conditions (Jang et al. 2004; Teixeira et al. 2015; Saccoccia et al. 2012). This can also be achieved by phosphorylation or hyperoxidation in some cases (Jang et al. 2004, 2006). For example, for yeast TSAI and human PrxII, treatment with thioredoxin and H_2O_2 to induce the rapid recycling that is required for extensive hyperoxidation, promotes the formation of high molecular weight complexes with chaperone activity (Jang et al. 2004; Moon et al. 2005). The chaperone action of Prxs appears to require the presence of two or more stacked rings, although in several instances, including PrxI and PrxIII, long nanotubes are formed that are composed of 50 or more stacked toroids. The novel dodecahedral assemblies that have been observed in 3D reconstructions of negative stain EM images of yeast TsaI and human PrxII, are also reported to exert chaperone activity (Meissner et al. 2007). Hanzen and co-workers have reported an interaction between Tsa I and Hsp 70, showing that hyperoxidation of Tsa I acts as a redox switch to recruit Hsp70 and Hsp104 during aging. This provides an alternative explanation to account for the chaperone function triggered by peroxiredoxin hyperoxidation (Hanzen et al. 2016).

A considerable amount of data exists on the fine structure of nanotubes and in particular on how mutations, truncations, post-translation modifications or hyperoxidation can affect their appearance, physical properties and putative chaperone activity (Angelucci et al. 2013; Gourlay et al. 2003; Jang et al. 2004). In many cases, the degree of exposure of hydrophobic surfaces in these assemblies correlates well with their ability to act as molecular chaperones. In our studies, the C47S active-site mutant of PrxIII has a pronounced tendency to form long nanotubes devoid of chaperone activity (Gourlay et al. 2003). Thus, it appears that hyperoxidation, or general perturbation of the active-site region, can induce the formation of large high molecular weight complexes. In one case involving the mitochondrial enzyme tryparedoxin peroxidase (mTXNPx) from a Leishmania parasite, the reduced decameric form was shown to exhibit chaperone-like activity above 35 °C (Teixeira et al. 2015). Thermal stress promoted conformational changes and exposure of hydrophobic surfaces as assessed by circular dichroism and enhanced bis-ANS fluorescence. Negative-stain EM images of mTXNPx with an unfolded client protein, luciferase, showed that luciferase was located in the central cavity of the decamer, implicating the inwardly-facing N-terminal regions of the peroxidase in this interaction. These authors also demonstrated that mTXNPx maintained unfolded polypeptides in an assembly-competent state, which required mediation of ATP-dependent chaperones, e.g. members of the Hsp 70/40 family, to complete the renaturation process. Such a model is consistent with reports that the unfolded proteins also bind internally to hollow nanotubular forms of Prxs at their extremities and that

N-terminal modifications, e.g. His tags, can interfere with chaperone function (Teixeira et al. 2015).

The toroidal structure of many 2-Cys Prxs, which enclose a large central cavity, is reminiscent of the Hsp60/Hsp10 chaperone family, where unfolded polypeptides can undergo multiple rounds of ATP-dependent refolding in an isolated environment. In addition, their ability to switch between an array of oligomeric states with differing physical and functional properties, at least *in vitro*, has prompted the idea that most Prxs can block protein aggregation and, in concert with ATP-requiring chaperones, initiate the ordered refolding of aggregated/denatured polypeptide chains. However, while there is increasing evidence for a key *in vivo* role for Prxs in protein renaturation as described above, the physiological relevance of many of the widely-reported chaperone-like actions of high molecular weight Prx complexes is still an open question in our view.

It is well documented that for most 2-Cys peroxiredoxins, the switch between dimers and decamers/ dodecamers is redox-dependent with the higher oligomeric state stabilised in the reduced FF conformation. Since unfolding of the α_2-helix in the active-site region is essential for disulfide bond formation during oxidation by H_2O_2 and as this region also forms part of the A-type interface involved in dimer-dimer interactions, it is unsurprising that dimer-dimer affinity is altered during the redox cycle that leads to dimer-decamer/dodecamer conversion *in vitro* (Cao et al. 2007). However, in the crowded environment of the cell, where protein concentrations are in the range 100–200 mg/ml, although subunit affinities will undoubtedly be altered, the overall oligomeric state is unlikely to be affected by the redox conditions. A cryo-EM and crystallography study of human PrxIII found that both FF and LU states show stacked ring formation, also suggesting that this formation could be favored in the crowded environment inside the mitochondrion (Yewdall et al. 2016).

Similarly, any exposure of hydrophobic surfaces in recombinant, wild-type or mutant Prxs will contribute to apparent chaperone activity in *in vitro* assays involving protection against thermal aggregation of citrate synthase or insulin. BSA, for example, can act as an effective chaperone or refolding agent in such *in vitro* assays thanks to its ability to bind hydrophobic patches on the surfaces of unfolded proteins (Lindsay et al. 2000).

Interestingly, in the case of the yeast cytoplasmic 2-Cys Prxs TSAI and TSAII, there is evidence to suggest that their reported chaperone functions are of physiological relevance (MacDiarmid et al. 2013). TSAI *null* mutants are sensitive to Zn^{2+} deficiency, a condition that leads to accumulation of intracellular protein aggregates. However, expression of an inactive TSAI mutant lacking the resolving cys, is able to rescue this phenotype and prevent protein aggregation, as is the overexpression of known ATP-independent chaperones, e.g. Hsp26 and Hsp42. TSAII can also substitute for its cytoplasmic partner, provided it is upregulated as it is normally present in much lower levels. TSAII has also been reported to protect cells against ribosomal protein aggregate induced by exposure to oxidative stress (Rand and Grant 2006).

One of the most fascinating aspects of chaperone structure and function is their unique ability to form a variety of structures and oligomeric states *in vitro* and the

possible implications for function. However, in many instances, the *in vivo* significance and indeed existence of these high molecular weight complexes in an intracellular environment remains to be established, as they are usually generated *in vitro* under stress conditions, such as thermal/oxidative shock or low pH, often using mutant Prxs. For example, the ability of PrxIII to form long nanotubes was confined to an active-site C47S mutant, whereas the wild-type and hyperoxidation resistant (YL) mutants are present as dodecameric toroids (Gourlay et al. 2003; Cao et al. 2015). Interestingly, crystal structures of oxidised and reduced PrxIII at 2.4 and 2.2 Å respectively, show that that both are present as two physically-interlinked dodecamers inclined at an angle of 55° to each other in a so-called 2-ring catenane (Cao et al. 2015). Inter-ring contacts are maintained by an extensive network of hydrogen bonds. At present, it is unclear whether the physically-interlinked 2-ring toroidal form exists *in vivo* or if it represents an artefact of crystal packing, made possible by the presence of the increased diameter of the central cavity (70 Å) in this unusual dodecameric toroid. This novel structure is also favored because inter-ring contacts are maintained by multiple hydrogen bonds involving amino acid residues that are poorly conserved in other members of the Prx family. EM analysis of PrxIII oligomers after crystal dissolution indicates that they have largely reverted to their dodecameric state in dilute solution, indicating that catenane assembly/disassembly is a dynamic process (Cao et al. 2007). However, as catenane formation is concentration-dependent, it is probable that PrxIII exists largely in this conformation in the crowded environment of the mitochondrial matrix. Interestingly, the novel properties of a newly-discovered enzyme catalysing CS_2 conversion to H_2S and CO_2 in the acidothermophile Acidianus A1-3, have been attributed to its unusual structure, namely a catenated, hexadecameric oligomer (Smeulders et al. 2011; van Eldijk et al. 2013).

5.7   Concluding Remarks

The dramatic increase in research on peroxiredoxins in the past 20 years was driven initially by the realisation that these ubiquitous and versatile enzymes constitute the main line of defence against ROS and Reactive Nitrogen Species (RNS) in the vast majority of living systems. ROS and RNS are generated intracellularly as natural by-products of metabolism and from exogenous sources including xenobiotics, UV and ionising radiation. Since the majority of ROS are rapidly converted to H_2O_2, its main route of removal in all intracellular compartments is via the various Prx family members that operate as efficient catalytic machines and are normally present in high abundance.

Subsequently, the landmark discovery that eukaryotic Prxs were amenable to temporary inactivation via hyperoxidation of their conserved active-site cysteines during the catalytic cycle under both non-stress and stress conditions coupled with the realisation that these modified Prxs could be re-reduced/reactivated in an ATP-dependent reaction by Srx, added impetus to this already vibrant research field.

Further recognition that Prxs could exist in a range of oligomeric states with differing enzymatic and chaperone-like properties that could be fine-tuned by a series of post-translation modifications confirmed their central status in eliciting appropriate cellular responses to the fluctuations in the aerobic environment under normal or extreme conditions. In this context, a huge volume of current data is focused on the interaction of Prxs with key signalling molecules, e.g. transcription factors, members of the Tyr/Ser kinase and phosphatase families that are implicated in fundamental cell processes, such as cell cycle regulation, circadian rhythmicity, DNA repair and induction of apoptosis. Research in this emerging field continues at an impressive rate. Undoubtedly, research over the next few years will provide detailed insights into the exact nature of the molecular mechanisms by which Prxs interact with and modulate a host of cellular activities concerned with adaption to changes in their immediate environment.

While Prxs are currently the subject of intensive investigation by cell and molecular biologists, their ability to form large supramolecular assemblies, such as nanotubes, is now being exploited in nanofabrication. Recently a PrxI mutant was employed as a molecular template to promote metal-induced self-assembly of 1-D nanoscopic structures housing linear arrays of Ni^{2+}-functionalised gold nanoparticles in their central cavities (Ardini et al. 2014). Formation of protein-metal complexes is increasingly being exploited in the assembly of electronic nano-devices for a variety of technological applications.

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