Published in final edited form as:

Biochem J. 2016 February 15; 473(4): 411–421. doi:10.1042/BJ20150572.

Kinetic analysis of structural influences on the susceptibility of peroxiredoxins 2 and 3 to hyperoxidation

Rebecca A. Poynton^{*}, Alexander V. Peskin^{*}, Alexina C. Haynes[†], W. Todd Lowther[†], Mark B. Hampton^{*}, and Christine C. Winterbourn^{*,1}

^{*}Centre for Free Radical Research, Department of Pathology, University of Otago Christchurch, Christchurch 8011, New Zealand [†]Department of Biochemistry and Center for Structural Biology, Wake Forest School of Medicine, Winston-Salem, NC 27157, U.S.A.

Abstract

Mammalian 2-cysteine peroxiredoxins (Prxs) are susceptible to hyperoxidation by excess H₂O₂. The cytoplasmic family member Prx2 hyperoxidizes more readily than mitochondrial Prx3 due to slower dimerization of the sulfenic acid (S_pOH) intermediate. Four variant amino acids near the C-terminus have been shown to contribute to this difference. We have performed kinetic analysis of the relationship between hyperoxidation and disulfide formation, using whole-protein MS and comparing wild-type (WT) Prx2 and Prx3 with tail-swap mutants in which the four amino acids were reversed. These changes make Prx3 more sensitive and Prx2 less sensitive to hyperoxidation and accounted for ~70% of the difference between the two proteins. The tail swap mutant of Prx3 was also more susceptible when expressed in the mitochondria of HeLa cells. The hyperoxidized product at lower excesses of H₂O₂ was a semi-hyperoxidized dimer with one active site disulfide and the other a sulfinic acid. For Prx2, increasing the H₂O₂ concentration resulted in complete hyperoxidation. In contrast, only approximately half the Prx3 active sites underwent hyperoxidation and, even with high H₂O₂, the predominant product was the hyperoxidized dimer. Size exclusion chromatography (SEC) showed that the oligomeric forms of all redox states of Prx3 dissociated more readily into dimeric units than their Prx2 counterparts. Notably the species with one disulfide and one hyperoxidized active site was decameric for Prx2 and dimeric for Prx3. Reduction and re-oxidation of the hyperoxidized dimer of Prx3 produced hyperoxidized monomers, implying dissociation and rearrangement of the subunits of the functional homodimer.

Keywords

hydrogen peroxide; kinetics; peroxiredoxin inactivation; thiol antioxidants; thiol oxidation

¹ To whom correspondence should be addressed (christine.winterbourn@otago.ac.nz)..

AUTHOR CONTRIBUTION

Rebecca Poynton, Mark Hampton and Christine Winterbourn designed the study, analysed the data and prepared the manuscript. Rebecca Poynton and Alexander Peskin designed and carried out the experiments. Alexina Haynes and Todd Lowther provided the mutant proteins and all authors advised on the final manuscript.

INTRODUCTION

Peroxisedoxins (Prxs) are a family of antioxidant enzymes that degrade a broad spectrum of hydroperoxides. An interesting feature of eukaryotic Prxs is that their peroxidase function can be blocked by higher concentrations of H_2O_2 in a process called hyperoxidation [1,2]. Prx hyperoxidation has been observed in oxidatively stressed cells [3–8] and in some freshly isolated tissues, e.g. adrenal glands, where it is proposed to show circadian oscillations [9,10]. It can persist for hours after acute oxidant exposure [6] and there is considerable interest in how it modulates the antioxidant and signalling properties of Prxs.

Eukaryotes express six Prx isoforms that are differentially distributed in the cytosol (Prxs 1, 2, 5 and 6), mitochondria (Prx3 and Prx5), endoplasmic reticulum (Prx4) and peroxisomes (Prx5). Eukaryotic Prxs are more sensitive to hyperoxidation than most prokaryotic forms [1], but they also exhibit varying susceptibilities [11]. Both *in vitro* and *in situ*, Prx3 is more resistant than Prxs 1 and 2 [12–15].

Prxs 1–4 are classified as typical 2-cysteine Prxs. They exist as homodimers arranged in a head to tail fashion, with each monomer containing a catalytically-active peroxidatic cysteine (Cys- S_p) and a resolving cysteine (Cys- S_r ; Figure 1) [1]. The dimers further associate to form non-covalent decameric (Prx2) or dodecameric (Prx3) structures [16–18]. In their catalytic cycle, H_2O_2 is reduced to H_2O and the Cys- S_p is oxidized to a sulfenic acid (S_pOH ; Reaction 1). The S_pOH on the opposing Prx subunit condenses with Cys- S_r to form a disulfide (Reaction 3) which can be reduced by the thioredoxin–thioredoxin reductase–NADPH system (Reaction 4). Formation of the disulfide bond requires partial unfolding at the active site, to a conformation referred to as the ‘locally unfolded’ (LU) state. This localized unfolding brings the two cysteines, which in the fully folded (FF) state are separated by a distance of $\sim 13 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$), into closer proximity [17,19]. Hyperoxidation occurs when the S_pOH is oxidized by an additional molecule of H_2O_2 , to form the sulfinate ($S_pO_2^-$, the ionized form of the sulfinic acid). This reaction occurs independently at each active site so it is possible to generate species with one S_p hyperoxidized and one disulfide (hereafter referred to as hyperoxidized dimer; Reactions 2/3) or with both oxidized (referred to as hyperoxidized monomer; Reaction 2). As the reaction of H_2O_2 with S_pOH occurs in competition with disulfide formation, sensitivity to hyperoxidation should reflect the relative rates of Reactions 2 and 3. We have shown that Prxs 2 and 3 differ not in the rate constant for hyperoxidation, but rather in the 10-fold faster rate of unfolding and disulfide formation for Prx3 (Reaction 3) [13]. In a parallel study, the lifetime of the S_pOH intermediate was found to be decreased, allowing less opportunity for hyperoxidation [14]. The rate of disulfide bond formation in the prokaryote Prx, AhpC, has recently been found to be 5-fold faster than for Prx3, consistent with its greater resistance to hyperoxidation [20].

Some of the structural features that regulate sensitivity to hyperoxidation have been characterized. The greater sensitivity of the eukaryotic over the prokaryotic Prxs is due to an extension at the C-terminus that restricts unfolding of the protein [21]. GGLG and YF motifs near the active site, as shown in Figure 2(A), are critical [19,22]. On this basis, it was proposed that other regions in the C-terminus could be responsible for the differing

sensitivities of eukaryotic Prxs [12]. A sequence alignment identified a possible site downstream of Cys-S_r that could affect unfolding where four amino acids differ between Prx3 (Asn²³², Thr²³⁴, Asp²³⁶ and Pro²³⁸) and Prxs 1 and 2 (Gly¹⁷⁵, Lys¹⁷⁷, Gly¹⁷⁹ and Asp¹⁸¹ in Prx2; Figure 2B). Haynes et al. [14] constructed tail-swap mutants in which the Prx3 amino acids were substituted into Prx2 and vice versa [designated CT* (four C-terminal residues swapped) Prx2 and CT* Prx3 respectively] and analysed their response to H₂O₂ using chemical quenching and MS. They found that changing these four residues did make Prx2 more resistant and Prx3 less resistant to hyperoxidation.

In the present study, we have further characterized features of Prxs 2 and 3 that influence their facility to undergo hyperoxidation and examined the susceptibility of CT* Prx3 to hyperoxidation when it is expressed in cells. We have carried out kinetic studies on the CT* mutants to show that these amino acid differences can account for a large part of the difference in susceptibility. We have also found that in contrast with Prx2, only approximately 50% of the active site cysteines in Prx3 can be converted into the S_pO₂⁻ by excess H₂O₂ and the product is predominantly the hyperoxidized dimer (Reaction 3; Figure 1). Using size exclusion chromatography (SEC) of the wild-type (WT) proteins we also show that oligomers of Prx3 are not as strong as for Prx2 and that the hyperoxidized dimer of Prx3 does not readily oligomerize.

EXPERIMENTAL

Preparation of recombinant Prx proteins

Full length recombinant human WT and CT* Prx2 and Prx3 (mutations as in Figure 2B) were generated with no extra N-terminal residues and without the mitochondrial targeting sequence (i.e., starting as residue 62) as described [14]. In the final column steps, the proteins were purified in the absence of reductant in order to stabilize the protein in the oxidized form. As such, they ran as dimers on non-reducing SDS/PAGE.

The reduced proteins were generated just before use by treating for 1 h with 10 mM DTT. The excess DTT was removed using a micro BioSpin 6 column (BioRad), which was pre-equilibrated with deionized H₂O, followed by 100 μ l of a 10 mg/ml bovine catalase (Sigma–Aldrich), then 5 ml of 50 mM phosphate buffer, pH 7.4, containing 0.1 mM diethylenetriamine penta-acetic acid. The phosphate buffer was pre-treated with 10 μ g/ml catalase which was removed by an Amicon Ultra-15 10 kDa filter. These procedures were important for minimizing re-oxidation of the Prx. After reduction, concentrations were determined from 280 nm absorbance and the theoretical absorption coefficients of the individual proteins (WT Prx2, 20,460 M⁻¹·cm⁻¹, CT* Prx2, 21555 M⁻¹·cm⁻¹, WT Prx3, 20065 M⁻¹·cm⁻¹, CT* Prx3, 20065 M⁻¹·cm⁻¹) [14].

Reactions of Prxs with H₂O₂ and preparation of samples for MS and SDS/PAGE

The reduced Prxs (5 μ M in 20 μ l) were rapidly mixed with a range of H₂O₂ concentrations (1 μ l volumes) as in the study by Peskin et al. [13]. After 5 min at 20°C, reactions were stopped with 1 μ l of catalase (0.5 mg/ml). For SDS/PAGE, the samples were alkylated with 25 mM *N*-ethylmaleimide (NEM) before the addition of sample buffer (final concentration:

2% SDS, 10% glycerol and 62.5 mM Tris, pH 6.8). For whole protein analysis by LC/MS the samples were reduced (10 mM DTT for 30 min) then alkylated (25 mM NEM for 7 min at room temperature). To prevent subsequent non-specific alkylation, 50 mM DTT was added to react with excess NEM. For LC/MS analysis under non-reducing conditions, reactions were stopped by adding catalase and the proteins were treated with 25 mM NEM without prior addition of DTT. To prevent non-specific alkylation, 1 μ l of concentrated formic acid was added to lower the pH.

LC/MS analysis

The redox states of the Prxs were analysed by LC/MS using a Thermo Scientific Velos Pro ion trap mass spectrometer with an ESI source, coupled to a Thermo Finnigan Surveyor HPLC system (Thermo Finnigan) as described previously [13]. Samples (20 μ l) containing 2 μ g of proteins were loaded on to a Jupiter C18 HPLC column (150 $\times$ 2 mm, 5 μ m, 100 Å, Phenomenex). The different redox forms were not separated and spectra over the full-length of the total protein peak were deconvoluted to yield the molecular masses and relative intensities using ProMass for Xcalibur (Version 2.8, Novatia LLC). The accuracy of the deconvoluted masses was $\pm$ 7 Da when compared with the theoretical masses. A small proportion of the Prx protein (~5%–10%) represented species in which one or both of the reactive cysteines were not alkylated or there was an additional NEM bound due to non-specific alkylation. These under- and over-alkylated species were included in the calculations of the proportions of the different redox species detected. Species that represented <5% of the total signal were not included in the analysis. The masses of the different Prx redox forms are shown in Table 1. Standard curves generated for reduced monomers, dimers and hyperoxidized monomers were linear for 0–1 μ g injected into the MS. The responses for the monomeric species were comparable but on a per weight basis monomers gave a greater response than dimer species. Results, which are expressed as percentages of total peak intensity, should therefore give a reasonable representation of absolute proportions for the reduced samples but be less accurate and underestimate dimeric species when present.

Analysis of oligomeric structure by size exclusion chromatography

Prx2 and Prx3 were treated to generate reduced, oxidized (dimer) and hyperoxidized species (confirmed by LC/MS). Reduced Prxs were generated by adding 25 mM DTT to the recombinant proteins. For the oxidized Prxs, equimolar H₂O₂ was added in the absence of DTT. Hyperoxidized Prx monomer was generated by treating the protein with 10 mM H₂O₂ in the presence of 25 mM DTT for 30 min. Preparations in which a major proportion of the protein was hyperoxidized dimer were generated by treating reduced Prx3 with 5 mM H₂O₂ and Prx2 with 0.25 mM H₂O₂ in the absence of DTT. Each protein preparation (50 μ l at 10 μ M) was separated on a BioSep-SEC-S3000 300 $\times$ 7.80 mm 5 micron column (Phenomenex) equilibrated with 50 mM sodium phosphate buffer (pH 7.0 or 7.5). Absorbance at 280 nm was monitored.

Peroxidase activity and reduction–oxidation properties of hyperoxidized dimer of Prx3

The peroxidase activity of the hyperoxidized dimer of Prx3 was compared with that of the active protein. Hyperoxidized dimer (confirmed by LC/MS) was prepared by treating 10 μ M

reduced protein (prepared as above) with 1 mM H₂O₂. NADPH oxidation was monitored at 340 nm in a plate reader, with 1.5 μ M thioredoxin, 0.5 μ M thioredoxin reductase, 200 μ M NADPH, 200 μ M H₂O₂ and either 0.3 μ M the double disulfide or 0.6 μ M hyperoxidized dimer of Prx3 (thus giving an equal concentration of active sites), in 200 μ l 50 mM phosphate buffer, pH 7.4, with 0.1 mM diethylenetriaminepenta-acetic acid.

Cell culture

HeLa cells (directly sourced from the A.T.C.C.) were cultured in Dulbecco's minimum essential medium (DMEM; Gibco) containing 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37°C in 95% humidified air with 5% CO₂.

Cloning and cellular expression of streptavidin-binding protein-tagged WT and CT* Prx3

WT streptavidin-binding protein (SBP)-Prx3 in pcDNA3 (obtained from Dr Andrea Betz, University of Otago, Christchurch) was mutated using the Gene Tailor™ Site Directed mutagenesis system (Invitrogen). The mutations N232G, T234K, D236G and P238D were introduced using the appropriate primers (forward -5'GAAGTCTGCCCAGCGGGCTGGAAACCGGGTTCTGATACGATCAAGCC3' and reverse - 5'CGCTGGGCAGACTTCTCCATGTGTTTC3'). WT and CT* Prx3 sequences were confirmed by DNA sequencing and used for the transient transfection of HeLa cells. HeLa cells were seeded in six-well plates at 8×10^5 cells, to reach ~80% confluency by the following day. The cells were then transfected with 4 μ g of DNA encoding either SBP-tagged WT or CT* Prx3 with a mitochondrial localization sequence, using Lipofectamine LTx according to the manufacturer's instructions (Invitrogen). The cells were incubated for a further 48 h to allow for expression of the target protein, before treatments with H₂O₂.

H₂O₂ treatment of cells and mitochondria isolation

HeLa cells (10^6 per ml in fresh media) were treated with various concentrations of H₂O₂ for 10 min in medium at 37°C, then washed with ice-cold PBS, trypsinized and resuspended in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 300 μ g/ml digitonin, 5 mM phosphate buffer, pH 7.4, and Complete™ protease inhibitors; Roche). Samples were centrifuged at 1000 *g* for 10 min and supernatants collected as the cytosol-enriched fraction. Pellets were resuspended in mitochondrial lysis buffer (150 mM NaCl, 20 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, 0.5% NP-40, 50 mM Tris/HCl, pH 7.4, and Complete™ protease inhibitors) and supernatants collected after centrifugation at 12000 *g* as the mitochondria-enriched fraction. The protein concentration of both fractions was measured using a detergent compatible protein assay (BioRad).

SDS/PAGE and Western blotting

Cell lysates and recombinant protein were separated by SDS/PAGE on 12% gels under reducing or non-reducing conditions [23]. Separated proteins were Western blotted using the appropriate antibody against Prx2 (Sigma), Prx3 (AbFrontier), hyperoxidized Prx (AbFrontier), β -actin or α -ketoglutarate dehydrogenase (Santa Cruz Biotechnology). Bands were visualized by ECL (BioRad ECL reagents) using the UVitech gel documentation system (UVitech) and quantified using ImageJ freeware (NIH, <http://imagej.nih.gov/ij/>).

RESULTS

Hyperoxidation of WT and C-terminal mutants of Prxs 2 and 3

We previously showed that Prx3 is more resistant than Prx2 to hyperoxidation due to a shorter lifetime of the SpOH moiety and faster condensation with the Cys-Sr residue to form the disulfide [13,14]. In extending these observations, we have identified another difference in the response of the two proteins to H₂O₂ (Figure 3). As shown by non-reducing SDS/PAGE, both Prxs were converted fully into the disulfide-containing dimer at the lower H₂O₂ concentrations. Higher concentrations progressively converted Prx2 back into the monomer (Figure 3A) but this occurred to a lesser extent with Prx3 (Figure 3B). Accumulation of the monomer band at high H₂O₂ has been shown to be due to hyperoxidation [23] and this was confirmed by blotting for the hyperoxidized protein. Hyperoxidized protein was also detected in the dimer position. The relative signal intensity was higher at lower H₂O₂ concentrations with Prx2 than Prx3, which is consistent with Prx2 being more sensitive to hyperoxidation. However, a more striking difference was that hyperoxidized Prx2 converted from being dimeric into monomeric, but the majority of hyperoxidized Prx3 remained dimeric even at the highest H₂O₂ concentrations.

To quantify these effects and investigate how the four variant C-terminal amino acids influence hyperoxidation, the CT* mutants and their WT counterparts were treated with H₂O₂, then the disulfides were reduced with DTT, alkylated and analysed by LC/MS. With this procedure, the oxidation products of the oxidized dimer, hyperoxidized monomer and hyperoxidized dimer (Figure 1) were respectively analysed as reduced monomer, hyperoxidized monomer and an equimolar mixture of the two. As expected, increasing concentrations of H₂O₂ gave increased hyperoxidation of all the WT and CT* Prxs (Figure 4). Consistent with Haynes et al. [14], treatment with the lower H₂O₂ concentrations showed that the CT* mutations protected Prx2 from hyperoxidation and increased the sensitivity of Prx3 (Figure 4A). Interestingly, at the higher concentrations of H₂O₂, hyperoxidation reached a maximum that differed for Prxs 2 and 3 but was apparently independent of the C-terminal mutations (Figure 4B). Maximal hyperoxidation approached 80% for Prx2 and 40%–50% for Prx3. The presence of a small fraction of each protein in the disulfide before treatment (see figure legend) could account for the incomplete hyperoxidation of the Prx2 species but not Prx3. Therefore, these results indicate that half of the active sites of Prx3 are highly resistant to hyperoxidation.

These differences were further investigated by analysing for the monomeric and dimeric oxidation products by LC/MS under non-reducing conditions. The percentages give only a semi-quantitative picture as they are based on peak area and underestimate the dimeric species (see Experimental). Nevertheless, the distributions of products (Figure 5) are consistent with the Western blotting and reducing LC/MS results. In each case, the disulfide-linked dimer was prominent at low H₂O₂ concentrations and decreased as the concentration increased. There was a corresponding increase in hyperoxidized species that was first evident as hyperoxidized dimer. This species declined for Prx2 and CT* Prx2 as the H₂O₂ concentration increased, with concomitant formation of hyperoxidized monomer as the major product. For Prx3 and CT* Prx3, the hyperoxidized dimer remained the major

hydroperoxidized species up to the highest H₂O₂ concentration. From this we can conclude that Prx3 can form hydroperoxidized monomer but much less readily. This suggests a difference in kinetics at the two active sites of the protein.

Kinetic analysis

We carried out kinetic analysis on the MS data presented in Figure 4, using a similar approach to that used with WT Prx2 and Prx3 [13]. Based on competition between reactions 2 and 3 (Figure 1) and considering a single active site, the ratio of hydroperoxidized monomer to disulfide can be expressed as:

$$\text{Hydroperoxidized/disulfide} = k_2 \cdot [\text{H}_2\text{O}_2] / k_3$$

The slope of the H₂O₂-dependence curve gives the ratio of the two rate constants. Because some of the starting material was already dimerized and, as discussed below and half the active sites of Prx3 were resistant to hydroperoxidation, hydroperoxidation could not compete with all the disulfide formation. Therefore, when calculating the ratio of the two species, we took into account the maximal amount of hydroperoxidation that could be achieved, approximately 80% with the Prx2 proteins but only ~50% for Prx3. In each case, there was good linearity with H₂O₂ concentration (Figure 6). The rate constant ratios are given in Table 2. The value for Prx2 is comparable to that previously obtained with a different recombinant preparation [13] and is substantially higher than for Prx3. The difference between Prx3 and Prx2 (~6-fold higher for Prx3) is less than the 12-fold calculated previously [13]. This is likely to be due to our considering hydroperoxidation as competitive for only half of the active sites, which was not apparent at the lower H₂O₂ concentrations used in the previous study. Comparison of the rate constants for the mutants indicates that the tail swaps on Prx2 and Prx3 account for 77% and 61% respectively of the difference between Prx2 and Prx3.

Oligomeric structure analysis of WT Prxs 2 and 3

To probe whether the differences in behaviour of Prxs 2 and 3 might relate to their oligomeric structure, SEC was performed on the WT proteins in different redox states. The catalytic units of 2-cysteine Prxs function as stable homodimers, which associate to form non-covalent oligomers [24,25]. Studies of the relationship between redox state and oligomerization have shown that for various Prxs including Prx2 [26,27], the reduced form is most stable as a decamer, the disulfide more readily dissociates into dimers and the decameric state of fully hydroperoxidized protein is even more stable. There is less known for Prx3 [18,28] and the quaternary structure of the hydroperoxidized dimers has not been investigated. When 10 μ M Prx2 was separated at pH 7.5, we observed the expected pattern of the reduced and fully hydroperoxidized forms both eluting as a single high-molecular-mass (M_r) peak in a position expected for a decamer and the disulfide eluting as two approximately equal decamer and dimer peaks (Figure 7A). Reduced and fully hydroperoxidized Prx3 eluted at a slightly earlier high M_r position, as expected for a dodecameric structure (Figure 7B). But in contrast with Prx2, the disulfide of Prx3 was fully dissociated into dimeric units. Furthermore, at 1 μ M reduced Prx2 still eluted as a decamer whereas Prx3 was fully dissociated (result not shown).

The hyperoxidized dimers of Prxs 2 and 3 each gave a different elution profile. These samples were prepared by adding H_2O_2 at concentrations that would be expected from the gel and MS results (Figures 3 and 4) to give a predominance of hyperoxidized dimer with lesser amounts of other oxidation products. This preparation of Prx2 behaved like the fully hyperoxidized protein and eluted in the decamer position (Figure 7A). However, hyperoxidized Prx3 with one disulfide and one hyperoxidized active site was dimeric (Figure 7B). Lowering the pH to 7 stabilizes the decameric structure of Prx2 [27,29]. This pH effect was also observed with Prx3, with the disulfide and the hyperoxidized dimers associating to dodecamers at pH 7 (result not shown).

The higher order structures formed when reduced Prx2 and Prx3 were treated with sub-equimolar concentrations of H_2O_2 (which would progressively convert the protein into dimers with one and then two inter-subunit disulfides) were also characterized. Prx2 remained predominantly as a decamer at H_2O_2 /Prx monomer mole ratios of 0.25 and 0.5 and progressively converted into the mixed dimer–decamer profile observed for the disulfide with equimolar H_2O_2 (Figure 7C). In contrast, as little as 0.25 moles of H_2O_2 caused dissociation of a large proportion of Prx3 dodecamer, and with 0.5 moles, where half the active sites would be disulfides, all of the protein was dissociated (Figure 7D). Together these results show that at all stages of oxidation, the Prx3 dodecamer is less stable than the Prx2 decamer.

Properties of hyperoxidized dimer of Prx3

Having demonstrated that hyperoxidized dimer is the major hyperoxidized species formed with Prx3, we investigated some of its redox properties. We first compared its activity with the disulfide (at an equivalent concentration of active sites) in a peroxidase assay with H_2O_2 , plus thioredoxin and thioredoxin reductase to reduce the Prx. Both catalysed H_2O_2 -dependent oxidation of NADPH in this system at the same rate (A_{340}/min of 0.026 and 0.025 respectively).

As the rate of recycling of the Prx is the limiting step in the peroxidase assay, it may not detect a difference in reactivity of the reduced Prx with H_2O_2 . Therefore, we attempted to reduce the hyperoxidized dimer and perform a competition assay with catalase or horseradish peroxidase to measure the rate of this reaction directly. Although this was unsuccessful, mainly because of major losses of the hyperoxidized protein during the required purification steps, our attempts led to an interesting and novel observation. When the hyperoxidized dimer was reduced with DTT then re-oxidized with 2-fold excess of H_2O_2 , we expected that the association between monomers would be tight enough to be retained following reduction and it would all be re-oxidized to the same hyperoxidized dimeric species. However, only approximately half the protein dimerized on re-oxidation and hyperoxidized protein was seen in the monomeric position on a non-reducing gel (Figure 8). This could be explained by dissociation and re-association of the monomers, giving rise to homodimers that cannot form disulfides because they contain two hyperoxidized active sites.

Effect of mutations at the C-terminus of Prx3 on sensitivity to hyperoxidation in cells

To establish whether mutations in Prx3 affect its susceptibility to hyperoxidation in cells, WT and CT* Prx3 were transiently expressed in the mitochondria of HeLa cells. The proteins were constructed with the endogenous Prx3 mitochondrial targeting sequence that would be removed once the protein was transported to the mitochondria and a 4 kDa N-terminal SBP-tag directly after the translocation sequence, to increase the molecular mass and make them distinguishable from endogenous Prx3. Fractionation of the cells (Figure 9A) showed that the tagged Prx3 co-localized with the endogenous protein and was expressed at a similar or somewhat higher level (Figure 10A). Prx3 was not detectable in the cytosolic fraction, indicating no mitochondrial contamination. However, the Prx2 blot shows a small amount of cytosolic contamination in the mitochondrial fraction. As both proteins co-migrate, hyperoxidation of endogenous Prx3 may be overestimated in blots probed with the antibody against hyperoxidized Prxs.

It was first investigated whether the N-terminal SBP-tag affected sensitivity to hyperoxidation (Figure 9B). When the reduced protein was treated with lower concentrations of H_2O_2 , hyperoxidation was comparable to that with untagged Prx3. At higher concentrations, a slight increase in susceptibility was apparent.

The transiently transfected HeLa cells were treated for 10 min with a bolus of H_2O_2 at increasing concentrations. In contrast with endogenous Prx3, some hyperoxidation of the SBP-tagged proteins in untreated cells was evident (Figure 10A). This implies that the N-terminal-tag affects the intracellular behaviour of the expressed proteins, independently of the C-terminal tail swap. Both SBP-tagged proteins showed greater sensitivity to H_2O_2 than endogenous Prx3, but over and above this, there was more hyperoxidation of SBP-CT*Prx3 than the SBP-WT Prx3 at all H_2O_2 concentrations (Figures 10A and 10B). The results are therefore consistent with the increased sensitivity of CT* Prx3 in the mitochondrial environment.

DISCUSSION

Even though reduced Prx2 and Prx3 react with H_2O_2 at similar rates, Prx3 is more resistant to hyperoxidation [12,13]. It was proposed that differences in four C-terminal residues near the Cys- S_r could contribute to this effect [12] and results obtained when these residues were transposed [14] support this proposal. Our study has extended these observations with analysis of the relative rates of disulfide bond formation and hyperoxidation for the WT and mutants proteins. We found that they accounted for much but not all of the difference between the two Prxs. The difference between the two proteins lies in the rate of condensation of the S_pOH intermediate with Cys- S_r (Reaction 3; Figure 1) which is faster for Prx3 [13]. The variant sites (between positions 175 and 181 in Prx2; Figure 2), are located in a region that is present only in eukaryotic Prxs and is considered to account for their greater susceptibility to hyperoxidation compared with their prokaryotic counterparts [19,22]. This loop influences transformation from the reduced FF form to the LU state of the disulfide, thus allowing the S_pOH more time for hyperoxidation. Our results indicate that this unfolding and structural reorganization is slower for Prx2, with the identified amino acids accounting for much of this effect.

CT* Prx3 also showed increased sensitivity to hyperoxidation compared with WT Prx3 when the proteins were expressed (with N-terminal SBP-tags) in mitochondria of HeLa cells. The differential when the cells were exposed to H_2O_2 was perhaps less than expected from the results with the isolated proteins, but there may have been some masking by the SBP-tag. Both tagged proteins were already slightly hyperoxidized in untreated cells and appeared to respond more to H_2O_2 than endogenous Prx3. It is possible that the slight enhancing effect of the tag on hyperoxidation observed with isolated Prx3 could contribute to the cellular effect or the tag could influence other processes such as reduction by sulfiredoxin or degradation. Others have noted that addition of a tag to a Prx can affect peroxide reactivity and oligomeric state [28,30,31].

Our study has also identified a striking difference in the extent to which Prx2 and Prx3 can be hyperoxidized. With Prx2, both active sites of the functional dimer were progressively modified with increasing H_2O_2 concentration and almost complete hyperoxidation was achieved. In contrast, only approximately half the Cys- S_p residues of Prx3 were hyperoxidized, even at high H_2O_2 concentrations and the product was predominantly the hyperoxidized dimer with one disulfide bond and one $\text{S}_\text{p}\text{O}_2^-$. This difference in behaviour was not due to the C-terminal amino acid variations, as the CT* mutants were hyperoxidized to a similar extent to their WT counterparts. We do not have a definitive mechanistic interpretation, but these findings are compatible with the two active sites in the dimeric unit of Prx3 being asymmetric. Support for this notion comes from other biochemical and modelling studies [14] and the observation that hyperoxidation can occur with substoichiometric amounts of H_2O_2 [32,33]. It is also consistent with observations that Prx3 is preferentially inhibited by thiostrepton, a compound that covalently links the monomers and reacts preferentially with the single disulfide dimer [34]. A possible explanation is that formation of a $\text{S}_\text{p}\text{O}_2^-$ at one active site increases the rate of disulfide formation at the other (Reaction 3; Figure 1). An effect on Reaction 2 cannot be excluded but is perhaps less likely as its rate does not appear to vary between the two Prxs.

We identified a difference in oligomerization properties between Prx2 and Prx3 that could be relevant when considering hyperoxidation. For all redox states, the dissociation of oligomers into dimeric units occurred much more readily for Prx3 than Prx2. As expected [18,27,28], the reduced forms of both proteins were oligomeric under our SEC conditions. However, in contrast with Prx2, the oxidized (disulfide) form of Prx3 and the reduced protein at a lower concentration were fully dissociated. Furthermore, Prx3 dissociated when only half its Cys- S_p residues were oxidized to disulfides whereas Prx2 remained fully decameric. The partially hyperoxidized forms of the Prxs also differed in their oligomeric states: Prx2 was decameric like the fully hyperoxidized species whereas Prx3 was dimeric like the disulfide. Therefore, under all redox conditions the dimeric form is likely to have more of an influence for Prx3 than Prx2.

The different oligomerization properties of the Prxs could potentially give rise to other functional differences such as in their ability to act as chaperones or to interact with binding partners that may be involved in transmission of redox signals. A chaperone function for Prxs was first identified in yeast [35] and is now well accepted [26,36–38]. Although initially linked to hyperoxidation, it has recently been demonstrated that the reduced form of

Prx3 is an effective chaperone [39] and it is emerging that oligomeric state may be more relevant. More cases of chaperone activity have been reported for Prx1 and Prx2 than Prx3 [15], perhaps because of their greater decamer stability. There are several instances where binding of Prxs to target proteins requires oxidation and formation of higher order structures. For example, interactions of Prx1 with components of phosphorylation pathways have been seen in association with H₂O₂-mediated cell senescence [40] and p53-mediated cell death after treatment with anti-cancer drugs [41]. Hyperoxidized Prx2 in its high-molecular-mass structure was also found to interact noncovalently with the protein disulfide isomerase ERp46 [42]. The decameric state has also been proposed for barley Prx to associate with thylakoids [43,44]. The propensity for Prx3 to dissociate may make it less likely to undergo such interactions.

Our findings that Prx2 and Prx3 readily form hyperoxidized dimers are relevant to understanding how Prxs function in cells. They imply that Prx2 will form this species with mild to moderate excess of H₂O₂ whereas it is likely to be the major Prx3 hyperoxidation product under most conditions. Additionally, where hyperoxidation has been detected in cells exposed to physiologically realistic concentrations of H₂O₂, it has generally represented only a fraction of the Prx [3–6,12] and as observed in some instances [45,46], the hyperoxidized product was predominantly dimeric. It is important, therefore, to understand the properties of this species with one hyperoxidized active site. Our results with Prx3 show that it will not readily form oligomers, as is normally assumed for hyperoxidized Prxs. It can still exhibit peroxidase activity at its second active site and we saw no change in activity in a recycling assay with thioredoxin/thioredoxin reductase. However, the reaction of the thiolate with H₂O₂ would not have been limiting in this assay and we cannot say whether it was affected. Although we were unable to generate reduced protein to measure the rate of this reaction, our attempts revealed the interesting observation that on reduction and re-oxidation, Prx3 redistributed from being fully hyperoxidized dimers to a mixture of hyperoxidized dimers and monomers on non-reducing SDS/PAGE. Although this is a preliminary result, the most obvious explanation is that on reduction, the monomers dissociate and re-associate to form some homodimers with two hyperoxidized active sites that cannot be re-oxidized. This is a surprising finding as the homodimeric unit of 2-cysteine Prxs is assumed to be very tight and its implications warrant further investigation. One consequence could be that even if the hyperoxidized dimer is formed in cells under oxidative stress, recycling could lead to gradual rearrangement. Follow-up molecular and cellular studies are therefore required.

ACKNOWLEDGEMENTS

We are grateful to Dr Andrea Betz for providing the cell expression system, Dr Louise Paton for assistance with the MS and Jill Clodfelter for assistance with protein purification.

FUNDING

This work was supported by the Health Research Council of New Zealand (to M.B.H. and C.C.W.); the Gravida National Centre for Growth and Development (to R.A.P.); the National Institutes of Health [grant number WTL, GM072866]; and the Comprehensive Cancer Center of Wake Forest University [grant number NCI CCSG P30 CA012197].

Abbreviations

CT*	four C-terminal residues swapped
Cys-S_p	peroxidatic cysteine
Cys-S_r	resolving cysteine
LU	locally unfolded
NEM	<i>N</i> -ethylmaleimide
Prx	peroxiredoxin
SBP	streptavidin-binding protein
SEC	size exclusion chromatography
WT	wild-type

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Figure 1. Catalytic cycle of typical 2-cysteine Prxs

The Cys- S_pH reacts with H_2O_2 to form a S_pOH (Cys- S_pOH (Reaction 1). The S_pOH can then condense with the Cys- S_rH on the opposing Prx subunit to form intermolecular disulfides (Reaction 3) which is reduced by thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH (Reaction 5). Alternatively, the S_pOH can react with an additional molecule of H_2O_2 to become hyperoxidized to the $S_pO_2^-$ form (Cys- $S_pO_2^-$). As each active site reacts independently, it is possible to generate a fully hyperoxidized monomer (Reaction 2) and a hyperoxidized dimer containing one disulfide and one $S_pO_2^-$ (Reaction 2/3). Note that although the catalytic unit is a dimer as shown, 5 or 6 of these associate to form decamers (Prx2) or dodecamers (Prx3).

Figure 2. Structure of the C-terminus of Prx2 (hyperoxidized)

(A) Crystal structure showing the proximity of the Cys-S_p residue and its interaction with a conserved arginine residue, the Cys-S_r residue and the GGLG motif in one monomer (blue) to the C-terminus of the adjacent monomer (grey). The C-terminus includes the four highlighted amino acid differences between Prx 2 and Prx3 (cyan) and the YF motif; PDB code 1QMV. (B) Sequence alignments of human Prxs 1, 2 and 3 at the C-terminus. Gly¹⁷⁵, Lys¹⁷⁷, Gly¹⁷⁹ and Asp¹⁸¹ of Prx2 (cyan) are replaced with Asn²³², Thr²³⁴, Asp²³⁶ and Pro²³⁸ of Prx3. Conserved sequence is boxed and the Cys-S_r residue is highlighted in yellow.

Figure 3. Non-reducing gels showing oxidation and hyperoxidation of (A) Prx2 and (B) Prx3 WT proteins (5 μ M) were reduced then treated with increasing concentrations of H_2O_2 . After 5 min, the reaction was quenched by adding catalase (0.25 μ g). The samples were then alkylated with NEM, resolved by non-reducing SDS/PAGE and Coomassie stained or immunoblotted for hyperoxidized Prx (Prx- $\text{SO}_{2/3}$).

Figure 4. Hyperoxidation of WT and CT* mutants of Prx2 and Prx3 at lower (A) and higher (B) concentrations of H_2O_2

Reduced Prxs ($5 \mu M$) were treated with H_2O_2 . After 5 min, reactions were stopped by adding catalase ($25 \mu g/ml$), disulfides were reduced with DTT and free thiols were derivatized by NEM. Whole protein LC/MS was performed. The signals corresponding to reduced (derived from the disulfide) and hyperoxidized monomers were determined from the deconvoluted spectra (Table 1) and the relative peak intensities were used to calculate the percent hyperoxidation. Analysis of the Prxs before treatment showed mean disulfide levels of 10% (Prx2), 7% (Prx3), 8% (CT*Prx2) and 11% (CT*Prx3). Data points represent mean $\pm$ S.E.M. ($n = 3$ for CT* Prx2, WT Prx2, $n = 4$ for WT Prx3 and $n = 7$ CT* Prx3) and double exponential curves have been fitted. WT Prx3 (▲); CT* Prx3 (◆); WT Prx2 (□); CT* Prx2 (○).

Figure 5. LC/MS analysis of Prxs treated with H_2O_2 under non-reducing conditions
 Reduced WT and CT* Prxs 2 and 3 proteins (5 μ M) were treated with H_2O_2 for 5 min. Reactions were stopped with the addition of catalase (25 μ g/ml) followed by 25 mM NEM. Samples were analysed by LC/MS and the deconvoluted signals quantified for dimer (two disulfides), ○; semi-hyperoxidized dimer (with 1 disulfide), ■; and hyperoxidized monomer, ▲. Results are expressed as percent of total peak area without correcting for variations in machine response. In these experiments, WT Prx3 with eight additional amino acids [42] was used instead of WT Prx3 with no additional N-terminal amino acids as the presence in the latter of approximately 15% of a contaminant protein 615 Da smaller than the theoretical mass impacted on the accuracy of deconvolution of the increased number of peaks.

Figure 6. Competitive kinetic analysis of the data in Figure 4

Relative yield of the disulfide and hyperoxidized products have been calculated based on 80% hyperoxidation being maximal for Prx2 and 50% for Prx3 (see text). Slopes of the plots give k_2/k_3 ratios (Figure 1), given in Table 2.

Figure 7. SEC elution profiles of Prxs 2 and 3 in different redox states

(A) Prx2 and (B) Prx3 (10 μ M in 50 μ l) were applied to the column and eluted at pH 7.5. Reduced (solid black line), disulfide (dotted line), fully hyperoxidized (dashed line) and partially hyperoxidized (grey line). See 'Experimental' for details of sample preparation. (C) Reduced Prx2 and (D) reduced Prx3 (10 μ M) untreated (black line) or treated with 0.25:1 (grey line), 0.5:1 (dashed line) or 1:1 (dotted line) mole ratio of H_2O_2 . Standards used for calibration were ferritin (Fer: 400 kDa), catalase (Cat: 250 kDa), serum albumin (BSA: 66 kDa) and cytochrome *c* (Cyt *c*: 12.5 kDa).

Figure 8. Altered mobility of hyperoxidized Prx3 on non-reducing SDS/PAGE following reduction and re-oxidation of the hyperoxidized dimer

Hyperoxidized dimer of Prx3 was generated by treating reduced protein (100 $\mu\text{g/ml}$; 4 μM) with 1 mM H_2O_2 for 5 min (lane 1). This protein was treated for 1 h with an amount of catalase (1 $\mu\text{g/ml}$) sufficient to remove the H_2O_2 but not compete with reduced Prx3 when more was added, then for a further 1 h with 10 mM DTT to reduce the single interchain disulfide (lane 2). The reduced protein was then treated with 10 μM H_2O_2 and blocked with 50 mM NEM within 30 s (a time that was independently shown to allow the DTT to reduce very little of the newly formed disulfides). Samples were separated by non-reducing SDS-PAGE and blotted for Prx3 (LH panel) or hyperoxidized protein.

Figure 9. (A) Expression and localization of SBP-tagged Prx3 and (B) effect of an N-terminal SBP tag on the sensitivity of WT Prx3 to hyperoxidation

(A) HeLa cells were extracted and separated into mitochondrial (MP) and cytosolic (CP) fractions. Samples were subjected to Western blotting probing with antibodies against Prx2 or Prx3. Each lane was loaded with 20 μ g of total mitochondrial or cytosolic protein under reducing conditions. Typical result from 1 of 4 experiments. (B) Recombinant purified reduced untagged (■) or SBP-tagged (●) WT Prx3 (5 μ M) was treated and analysed as in Figure 4. Data points represent mean $\pm$ S.E.M. ($n = 3$).

Figure 10. Prx hyperoxidation in HeLa cells expressing WT or CT* SBP-Prx3 after treatment with H₂O₂

(A) Representative blots of mitochondrial fraction prepared after 10 min treatment with H₂O₂. Reducing gels were probed for hyperoxidation (anti-Prx SO_{2/3}H; upper panel); Prx3 as an expression control (middle panel); and the mitochondrial matrix protein, α -ketoglutarate dehydrogenase (α -KGD) as a loading control (lower panel). (B) Combined densitometry showing relative changes to Prx hyperoxidation normalized against α -KGD. Data are mean $\pm$ S.E.M. of four separate experiments; * P < 0.05 determined using paired sample t tests. White and light grey bars represent endogenous Prx3 from each expression system; dark grey bars SBP-WT Prx3; black bars SBP-CT* Prx3.

Table 1
Theoretical molecular masses (Da) of the redox states of the Prxs as analysed by LC/MS

Minor amounts of under- and over-alkylated forms of the proteins (± 125 mass units per NEM) were also detected and included in the analysis. With the alkylation conditions used, the third, non-active site cysteine remained largely underivatized. The Prx2 mass corresponds to the protein without an N-terminal methionine. The Prx3 mass corresponds to the protein beginning at Ala⁶².

	WT Prx2	WT Prx3	CT* Prx2	CT* Prx3
Reduced (+ 2NEM)	22142	21789	22080	21719
Hyperoxidized (+ NEM)	22049	21696	21987	21626
Dimer (no NEM)	43780	43074	43656	42934
Hyperoxidized dimer (+ NEM)	43937	43231	43813	43091

Table 2
Ratio of rate constants for hyperoxidation and disulfide formation determined from the slopes of the plots in Figure 6

For comparison, values determined previously for different preparations of recombinant Prx2 (with an additional N-terminal methionine) and Prx3 (with eight additional N-terminal amino acids) are 6600 and 540 M⁻¹ respectively [13].

Species	$k_2/k_3 \cdot \text{M}^{-1}$
WT Prx2	4700
WT Prx3	800
CT* Prx2	1700
CT* Prx3	3200