BJ www.biochemj.org

Biochem. J. (2010) **432**, 313–321 (Printed in Great Britain) doi:10.1042/BJ20101156

online data

Removal of amino acid, peptide and protein hydroperoxides by reaction with peroxiredoxins 2 and 3

Alexander V. PESKIN*[1], Andrew G. COX*, Péter NAGY*, Philip E. MORGAN†, Mark B. HAMPTON*‡, Michael J. DAVIES† and Christine C. WINTERBOURN*‡

*Free Radical Research Group, Department of Pathology, University of Otago, Christchurch, New Zealand, †Free Radical Group, The Heart Research Institute, Sydney, Australia, and ‡National Research Centre for Growth and Development, The Liggins Institute, University of Auckland, Grafton, Auckland, New Zealand

Prxs (peroxiredoxins) are a ubiquitous family of cysteine-dependent peroxidases that react rapidly with H_2O_2 and alkyl hydroperoxides and provide defence against these reactive oxidants. Hydroperoxides are also formed on amino acids and proteins during oxidative stress, and they too are a potential cause of biological damage. We have investigated whether Prxs react with amino acid, peptide and protein hydroperoxides, and whether the reactions are sufficiently rapid for these enzymes to provide antioxidant protection against these oxidants. Isolated Prx2, which is a cytosolic protein, and Prx3, which resides within mitochondria, were reacted with a selection of hydroperoxides generated by γ-radiolysis or singlet oxygen, on free amino acids, peptides and proteins. Reactions were followed by measuring the accumulation of disulfide-linked Prx dimers, via non-reducing SDS/PAGE, or the loss of the corresponding hydroperoxide, using quench-flow and LC (liquid chromatography)/MS. All the hydroperoxides induced rapid oxidation, with little difference in reactivity between Prx2 and Prx3. *N*-acetyl leucine hydroperoxides reacted with Prx2 with a rate constant of 4×10^4 $M^{-1} \cdot s^{-1}$. Hydroperoxides present on leucine, isoleucine or tyrosine reacted at a comparable rate, whereas histidine hydroperoxides were $\sim$10-fold less reactive. Hydroperoxides present on lysozyme and BSA reacted with rate constants of ~ 100 $M^{-1} \cdot s^{-1}$. Addition of an uncharged derivative of leucine hydroperoxide to intact erythrocytes caused Prx2 oxidation with no concomitant loss in GSH, as did BSA hydroperoxide when added to concentrated erythrocyte lysate. Prxs are therefore favoured intracellular targets for peptide/protein hydroperoxides and have the potential to detoxify these species *in vivo*.

Key words: hydroperoxide, oxidative stress, peroxidase, peroxiredoxin (Prx), protein oxidation, thiol.

INTRODUCTION

Proteins represent a major target for reactive oxidants. Oxidation causes a variety of protein modifications including the formation of hydroperoxides. Hydroperoxides can be formed on most amino acid residues and are major products of radical reactions with peptides and proteins [1]. For example, exposure of proteins to hydroxyl and superoxide radicals, can result in a 65% conversion of the radicals into hydroperoxides [2,3]. They have been measured in cells subjected to oxidative stress [4] and the detection of valine and leucine hydroxides (hydroperoxide breakdown products) in a number of pathologies is indirect evidence for their formation *in vivo* [5,6]. Hydroperoxides are most commonly thought of in association with lipid oxidation, but protein hydroperoxide formation may be just as prevalent [1–3].

The susceptibility to peroxidation of different amino acid residues (both free and in peptides/proteins) varies depending on the specific oxidant. Thus singlet oxygen generates hydroperoxides preferentially on tryptophan, tyrosine and histidine, whereas tyrosine can be converted into a hydroperoxide by addition of superoxide to a tyrosine phenoxyl radical, and γ-irradiation generates hydroperoxides on most aliphatic side chains (leucine, isoleucine, valine, proline, lysine and glutamate) [1,2,7]. The formation of protein and peptide hydroperoxides is of biological significance not only because their generation results in modification of the structure and properties of the amino acid residue, but also because hydroperoxide groups are themselves powerful oxidants that can induce secondary damage by either directly inhibiting thiol-dependent proteins [e.g. caspase 3, protein tyrosine phosphatases, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), cathepsins and SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase)] [8–10] or by giving rise to further radical species in the presence of trace transition metal ions [11,12]. Oxidative modification of DNA by protein hydroperoxides and the formation of DNA–protein cross-links [13,14] suggest they could also be mutagenic.

Cells protect themselves against reactive oxygen species through a combination of specific enzymes and low-molecular-mass antioxidants. However, it is unclear whether they have an effective mechanism for the removal of peptide or protein hydroperoxides. One possibility is that they are substrates for H_2O_2-removing enzymes. Haem-containing peroxidases and catalase react slowly, if at all, with these species [15]. GPx (glutathione peroxidase) reacts with low-molecular-mass hydroperoxides but not with hydroperoxides formed on BSA or lysozyme [15]. A further group of proteins involved in removing H_2O_2 are the Prxs (peroxiredoxins) [16]. This family of ubiquitous cysteine-dependent peroxidases includes Prx2, which is a cytosolic protein, and Prx3, which resides within the mitochondria. Prx2 and Prx3 have second-order rate constants, k, for their reaction with H_2O_2 of $>10^7$ $M^{-1} \cdot s^{-1}$ [17,18], which places them in the 'premier league' of H_2O_2-removing

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; DTT, dithiothreitol; FOX, $FeSO_4$/Xylenol Orange; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; LC, liquid chromatography; MBB, monobromobimane; NEM, *N*-ethylmaleimide; Prx, peroxiredoxin; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; TR, thioredoxin reductase; Trx, thioredoxin.

[1] To whom correspondence should be addressed (email alexander.peskin@otago.ac.nz).

Table 1 Hydroperoxides of amino acids, peptides and proteins employed to react with Prxs

The relative hydroperoxide concentrations were measured as described previously [21] and concentrations were determined as H_2O_2 equivalents. The level of the reduced form of Prxs remaining was determined after incubation for 10–30 s with typically 5 μM Prx2 or Prx3 and 7 μM hydroperoxide and are shown as means $\pm$ S.D. ($n = 2$–5) for pooled Prx2 and Prx3.

Hydroperoxide	Treatment to produce hydroperoxide	Relative concentration of hydroperoxide (mmol per mol of treated compound)	Reduced form of Prxs remaining (%)
Leucine	γ-Irradiation	24	16 ± 1
N-acetyl leucine	γ-Irradiation	8	0
N-acetyl leucine amide	γ-Irradiation	25	0
N-acetyl leucine methyl ester	γ-Irradiation	23	0
Isoleucine	γ-Irradiation	25	0
N-acetyl isoleucine	γ-Irradiation	11	0
N-acetyl isoleucine amide	γ-Irradiation	11	0
Gly-Leu	γ-Irradiation	22	0
Leu-Gly	γ-Irradiation	39	0
Gly-Leu-Leu-Gly	γ-Irradiation	20	0
Tyrosine	Photolysis	62	19 ± 25
Gly-Tyr-Gly	Photolysis	196	33 ± 28
Histidine	Photolysis	16	27 ± 8
Lysozyme	Photolysis	45	55 ± 8
BSA	γ-Irradiation	759	30 ± 1
BSA	Photolysis	583	–

enzymes, along with catalase and GPxs. Prxs also react with organic and lipid hydroperoxides [19]. For example, Parsonage, Karplus and Poole [20] measured second-order rate constants of 10^5–10^7 $M^{-1} \cdot s^{-1}$ for the bimolecular reactions of AhpC, a bacterial analogue of mammalian two-cysteine Prxs, with alkyl hydroperoxides. Catalytic removal of peroxides is achieved through recycling of the Prx disulfides. Prx2 and Prx3 are reduced by Trx (thioredoxin)/TR (thioredoxin reductase), with reducing equivalents supplied by NADPH.

No information is available on the reactivity of Prxs with hydroperoxides on amino acid residues. To assess whether these reactions occur and whether they are fast enough to be a potential detoxification mechanism, we have examined whether Prx2 and Prx3 react with hydroperoxides formed on selected amino acids, peptides and proteins as a result of exposure to either a singlet-oxygen-generating system (photoillumination) or γ-radiolysis. Absolute second-order rate constants have been determined in some cases, allowing a quantitative assessment of the potential importance of these reactions.

EXPERIMENTAL

Hydroperoxide generation and quantification

Hydroperoxides were generated on proteins and amino acids (Sigma–Aldrich) and peptides (Bachem) either by photolysis in the presence of 10 μM Rose Bengal (Sigma) or γ-irradiation (with ^{60}Co, a total dose of 920 Gy) with continuous oxygenation, as described previously [21]. After cessation of illumination/irradiation, 50 μg/ml catalase was added to remove any H_2O_2 generated and hydroperoxides were frozen at $-80\,^{\circ}C$ in aliquots (1 ml). As shown previously [15], the presence of catalase does not affect the stability of amino acid and BSA hydroperoxides. Protein concentrations were 50 mg/ml for photolysis and 10 mg/ml for γ-irradiation; amino acids were at 10 mM and peptides at 2.5 mM. Hydroperoxide concentrations were determined using a modified FOX ($FeSO_4$/Xylenol Orange) assay [22] using H_2O_2 standards, and are expressed as H_2O_2 equivalents. Analyses performed with the FOX assay gave similar values to those measured iodometrically (A. Wright and M.J. Davies, unpublished work). A list of the substances examined, the oxidation method, and yields of hydroperoxide generated is given in Table 1.

Prxs

Prx3 was purchased from Abfrontier. Prx2 was isolated from human erythrocytes as described previously [23]. Membrane-free lysate of washed erythrocytes was treated with 30 % ammonium sulfate and then dialysed. Chromatography was performed first on a Hipper 16/10 DEAE FF column (Amersham Biosciences) followed by a Phenyl Sepharose 6 Fast Flow column (GE Healthcare). Prior to each experiment Prxs were reduced with 20 mM DTT (dithiothreitol) in PBS, pH 7.4, containing 1 mM DTPA (diethylenetriamine-penta-acetic acid) and 10 μg/ml bovine catalase. Reduced Prxs were separated from excess DTT by passing through a Micro Bio-Spin 6 Chromatography column (Bio-Rad), pre-equilibrated with the PBS/DTPA/catalase buffer. Prx concentrations were estimated based on protein concentration as measured with the Bio-Rad Dc Protein Assay, with BSA as a standard. For kinetic analyses, the concentration of reduced Prx was calculated based on the relative intensity of the bands representing the oxidized and reduced forms determined by PAGE.

Reaction of Prxs with hydroperoxides

In most experiments the hydroperoxide was added to the Prx during vigorous vortex-mixing in PBS. The reaction was quenched with 100 mM NEM (*N*-ethylmaleimide) in electrophoresis sample buffer [62.5 mM Tris/HCl, pH 6.8, 4 % (w/v) SDS and 10 % (v/v) glycerol] and analysed by SDS/PAGE (the gel percentage is given in the Figure legends). All reactions were carried out at 25 °C.

Quench-flow experiments

The rate constant for the reaction of Prx2 (typically 4 μM after mixing) with *N*-acetyl leucine hydroperoxide (typically 5 μM total hydroperoxide after mixing) was measured by quench-flow experiments. Reactions were carried out at 25 °C in 10 mM

phosphate buffer (pH 7.3) in the presence of 50 μM DTPA and 10 μg/ml bovine catalase, and were quenched with a receiving solution of *t*-butyl-hydroperoxide (500 μM starting concentration, 167 μM at completion) under vigorous stirring to completely oxidize Prx and stop further hydroperoxide consumption. The necessary minimum concentration of the quenching solution (to have no more than 4 % error) was calculated with the aid of kinetic simulations using the Euler–Cauchy method (program written in Mathematica 5.2). Using the assumption that the second-order rate constants of the reactions of *t*-butyl-hydroperoxide and *N*-acetyl leucine hydroperoxide with Prx2 are similar, the model calculates >60 μM final *t*-butyl-hydroperoxide for efficient quenching under the conditions of these experiments. Hydroperoxide consumption was quantified by LC (liquid chromatography)/tandem MS with electrospray ionization, which was performed with a Thermo Finnigan LCQ Deca XP Plus ion-trap mass spectrometer coupled to a Surveyor HPLC system and photo diode array detector, using positive-ion mode. Chromatographic separation was achieved by a Thermo Hypercarb (100 mm × 2.1 mm) column using a linear gradient (aqueous, 0.1 % formic acid as Solvent A, and 0.1 % formic acid in methanol as Solvent B). The consumption of *N*-acetyl-leucine hydroperoxides was followed by singular ion monitoring.

The quench-flow system was comprised of two syringes (SGE) and a T-shaped mixer. The syringes were driven simultaneously by a stepper motor at constant speed (typically 100 μl/min) to ensure efficient mixing. Chemically inert tubing with a 0.01-inch diameter was used. Delay times (representing the time of the reaction) were varied by changing the length of the tube between the mixer and the quenching solution. The volumes of the tubes were calculated based on their diameter and length, and corroborated by measuring the mass of water contained within. Delay times were calculated based on these volumes and the calibrated speed of the stepper motor. The quench-flow device was tested by following the well-characterized loss of TNB (5-thio-2-nitrobenzoic acid) on reaction with taurine chloramine [24] under pseudo first-order conditions, using methionine to quench the reaction. Calculated and measured delay times were similar and overlapped with the simulated kinetic trace (Supplementary Figure S1 at http://www.BiochemJ.org/bj/432/bj4320313add.htm). This gave us confidence that the quenching solution (which was determined by kinetic simulations) was efficient.

Analysis of Prx oxidation state

Reduced monomers and disulfide-bonded dimers were separated by non-reducing SDS/PAGE (10, 12 or 15 % gels). Gels were either silver-stained or transferred on to a PVDF membrane and blotted with an antibody against either Prx2 or Prx3 (Abfrontier). A horseradish peroxidase-conjugated secondary antibody was used to visualize the immunoblots through enhanced chemiluminescence. Stained gels and immunoblots were scanned using a Fluor-S® MultiImager or ChemiDoc® XRS (Bio-Rad) respectively. Bands were quantified using Quantity One® software from Bio-Rad.

Treatment of intact erythrocytes

Human blood was obtained from healthy volunteers with informed consent. Erythrocytes were isolated, washed, diluted to 10^8 cells/ml in PBS containing 10 μg/ml catalase, and mixed with an equal volume of *N*-acetyl leucine methyl ester or *N*-acetyl leucine methyl ester hydroperoxide in PBS. After a 10 min incubation at 20 °C, the samples were either quickly washed with 20 volumes of PBS, followed by the addition of MBB (monobromobimane) in PBS, pH 8.0, for GSH detection, or were incubated for a further 20 min in the presence of 20 mM NEM, for Prx2 detection. In the latter case erythrocytes were washed twice with 20 volumes of PBS containing 20 mM NEM, and were then added to electrophoresis loading buffer with 100 mM NEM.

Treatment of erythrocyte lysates

Packed erythrocytes were mixed with 2 volumes of distilled water followed by two cycles of freeze-thaw. The cell lysate was incubated for 15 min at room temperature (20 °C) with or without BSA hydroperoxide in an equal volume of PBS. Samples were treated with NEM or MBB as above and analysed for Prx2 oxidation and GSH levels.

GSH detection

GSH was measured by HPLC with fluorescence detection after derivatization with MBB [25].

RESULTS

Reaction of Prx2 and Prx3 with amino acid and peptide hydroperoxides

Oxidation of typical two-cysteine Prxs can be followed by non-reducing SDS/PAGE by transition of the fast migrating reduced monomer band (at 21–22 kDa) to the disulfide-linked dimer (at 42–44 kDa). Figure 1 shows that incubation of Prx2 and Prx3 with a slight excess of tyrosine hydroperoxide resulted in rapid dimerization with concomitant loss of the reduced form. With Prx2, the percentage monomer decreased by approx. 50 % within 15 s (Figure 1A, lane 2) and none remained after 5 min (Figure 1B, lane 2). The reaction of Prx3 was even faster, with complete dimerization after a 15 s incubation with the hydroperoxide (Figure 1C, lane 2). A small amount of dimer was invariably present in the Prx starting material. The reactions of hydroperoxide(s) present on tyrosine residues within the tripeptide Gly-Tyr-Gly were somewhat slower. The proportion of monomer decreased over 15 s by approx. 25 % for Prx2 and 60 % for Prx3 (Figures 1A and 1C), with less than 10 % remaining after 5 min (Figure 1B and 1D). These reactions were reversible, as only a single monomer band was observed under reducing conditions (results not shown).

Other amino acids and small peptides with hydroperoxide groups present on the imidazole ring of histidine residues, or the aliphatic side chains of leucine and isoleucine derivatives were examined. For leucine and isoleucine, derivatives with either or both of the N- and C-termini blocked were examined, to test whether this had a major effect on the reaction rate. Both Prx2 and Prx3 reacted very rapidly with the leucine and isoleucine hydroperoxides, regardless of whether they were on the free amino acids or incorporated into peptides, or whether the N- or C-termini were blocked (Table 1). Gels run after a 10–15 s reaction showed complete Prx oxidation, except with leucine hydroperoxide, where a small fraction of the monomer remained at this time. Histidine hydroperoxide reacted more slowly (Table 1), but still gave significant Prx oxidation.

To gain quantitative information on the reactivity of the Prxs, we examined the time course of the reactions and measured rate constants for one of the slower-reacting and one of the faster-reacting hydroperoxides. Histidine hydroperoxide was the only

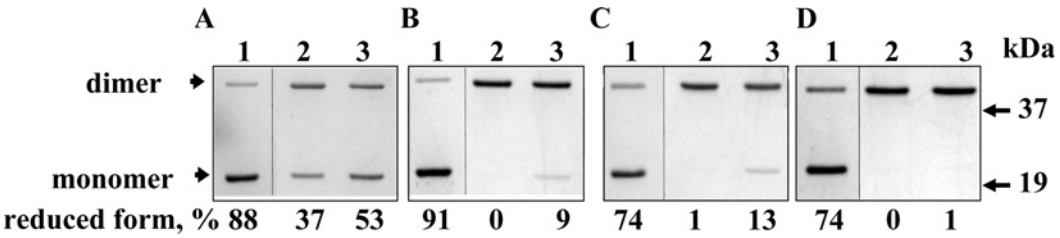

Figure 1 Oxidation of Prx2 and Prx3 by hydroperoxides formed on tyrosine or the tripeptide Gly-Tyr-Gly

(**A** and **B**) Prx2 or (**C** and **D**) Prx3 (7 μM) were incubated alone (lane 1), with 10 μM tyrosine hydroperoxide (lane 2) or Gly-Tyr-Gly hydroperoxide (lane 3) for (**A** and **C**) 15 s or (**B** and **D**) 5 min. Samples were separated by non-reducing SDS/PAGE (15 % gel) and silver stained as described in the Experimental section. In each case, the untreated samples were run on the same gel as the treated samples (intervening lanes have been excised in the preparation of the Figure). Numbers under the lanes refer to the percentage of the protein in the monomeric reduced form, as determined by densitometry.

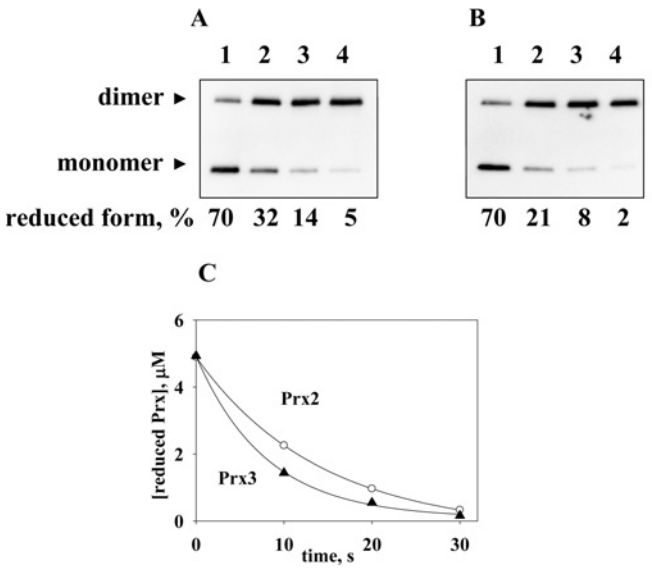

Figure 2 Time course of oxidation of Prx2 or Prx3 by histidine hydroperoxide

(**A**) Prx2 or (**B**) Prx3 (7 μM) were incubated alone (lane 1) or with 20 μM hydroperoxide for 10 s (lane 2), 20 s (lane 3) or 30 s (lane 4). The reactions were stopped and the samples were analysed by non-reducing SDS/PAGE (15 % gels) and Western blotting with the appropriate anti-Prx antibody. Monomer and dimer bands and densitometry analysis is as described for Figure 1. (**C**) Kinetic analysis of densitometry results. The reduced Prx concentrations in (**C**) were estimated from the initial Prx concentration and the percentage monomer in each sample. Curves for Prx2 (○) and Prx3 (▲) represent exponential fits.

species that reacted slowly enough to follow the time course by gel electrophoresis. As shown in Figure 2, with a 4-fold excess of hydroperoxide, dimerization of Prx2 (Figure 2A) and Prx3 (Figure 2B) was essentially complete within 30 s. Plots of the loss of the monomer over time, determined by densitometry (Figures 2C), were fitted to exponential curves and analysed assuming pseudo first-order kinetics. For the determination of the second-order rate constants for the reaction of the peroxidative Prx thiol with the hydroperoxides, it is pertinent that the Prx dimer represents both the single and double disulfide-bonded isoforms of the protein. As such a proportionality constant needs to be incorporated into the rate law. This constant should lie between one (if the reactivity of the dimer isoform with one disulfide bond is much greater than the monomer) and two (if the dimer is much less reactive). We have used the latter assumption and therefore the reported rate constants represent the lower limit for these reactions.

All the kinetic experiments were carried out with catalase in the reagents to prevent Prx oxidation by traces of H_2O_2. It is theoretically possible that the haem of catalase could promote Prx oxidation by decomposing the hydroperoxides to generate thiol-reactive species [26]. Although this alternative explanation for our findings seems unlikely, based on previous findings that catalase does not accelerate the decomposition of peptide hydroperoxides [15], a further control experiment was carried out. The catalase present in the original histidine hydroperoxide was removed, and the time courses of reaction of Prx2 with this preparation with and without added catalase were compared. As shown in Supplementary Figure S2 http://www.BiochemJ.org/bj/432/bj4320313add.htm, the profiles of Prx2 oxidation were very similar.

The relatively fast oxidation of Prx2 by *N*-acetyl leucine hydroperoxide was followed over 0.6–20 s using a quench-flow method. NEM reacts too slowly with Prx2 to be an efficient quencher under these conditions, so the reaction was stopped by mixing with a large excess of *t*-butyl hydroperoxide to oxidize the remaining Prx2. The loss of *N*-acetyl leucine hydroperoxide was measured by LC/MS. Figure 3 shows the chromatographic profile of the *N*-acetyl leucine hydroperoxide isomers before and after reaction with Prx2. The starting material eluted as three incompletely resolved peaks with the same molecular mass. Previous tandem MS analysis has shown these to be isomers, with the hydroperoxide groups present at different positions [21]. The results suggest that the fast migrating peak corresponds to the α-carbon hydroperoxide and the other peaks represent a mixture of hydroperoxides present on side chain carbon centres [21,27]. Incubation with Prx2 resulted in the rapid progressive loss of the slower-migrating peaks compared with the α-carbon hydroperoxide (Figure 3). The latter remained constant until the slower-migrating peaks completely disappeared, then declined over a longer time period with excess Prx2 (results not shown). Reduction of hydroperoxides by thiols should produce alcohols, with a mass loss of 16 Da, according to:

$$2\mathrm{PrxSH} + \mathrm{ROOH} \rightarrow \mathrm{PrxS-SPrx} + \mathrm{ROH}$$

This was confirmed by performing LC/MS of the reaction mixture at the time when the fast-reacting *N*-acetyl leucine hydroperoxide peaks had disappeared. Selective ion monitoring showed formation of two isomeric species with *m/z* 190, migrating at 7.9 and 8.5 min. Their combined peak area was comparable with that lost from the hydroperoxides.

The results were analysed on the basis of the slower-migrating peaks (representing the side chain isomers) corresponding to approx. 60 % of the 5 μM total hydroperoxides present. The loss in these peaks over time was fitted to a straight line (Figure 3,

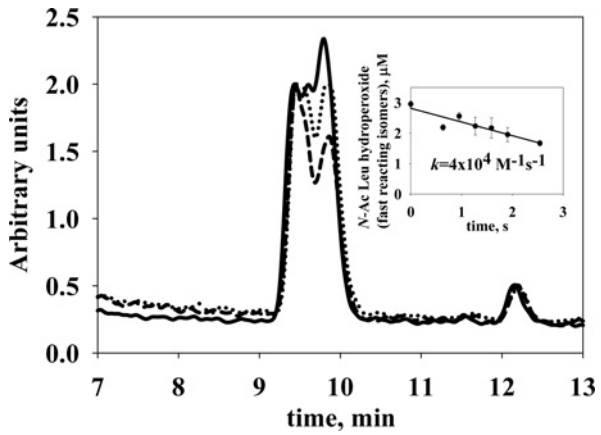

Figure 3 LC/MS chromatogram of *N*-acetyl leucine hydroperoxide loss during reaction with Prx2

N-acetyl leucine hydroperoxide (5 μM) was mixed with 5 mM phosphate (100 mM NaCl) buffer (solid line) or 5 μM Prx2 (80 % reduced) using the quench-flow system for 1.2 s (· · · · · ·) or 2.5 s (- - - -). Unreacted Prx2 was quenched by a 50-fold excess of *t*-butyl hydroperoxide. The inset shows the loss of the slow-migrating peak for a range of mixing times (the error bars show the range for two independent experiments) with the concentration determined on the basis that these peaks represented 60 % of the total hydroperoxide. The second-order rate constant was calculated from the slope of the linear fit using the initial reactant concentrations. To maximize sensitivity, the LC/MS chromatograms were recorded using selective ion monitoring in the range of $m/z = 205$–207 to detect *N*-acetyl leucine hydroperoxide (m/z 206), but not other species in the reaction mixture. Unreacted *N*-acetyl leucine was the dominant peak in the total ion count chromatogram with a retention time of 14.2 min. The peak at 12 min is a contaminant in our system that has an $m/z = 206$, but a fragmentation pattern different from *N*-acetyl leucine hydroperoxide. It was present in all samples and the peak area did not change during the course of the reaction.

inset) from which an initial rate was determined. The second-order rate constant was determined from the rate law given by $d[\text{L-OOH}]/dt = k[\text{L-OOH}][\text{Prx}_{red}]$, where L-OOH represents the side chain *N*-acetyl leucine hydroperoxides and Prx_{red} the reduced Prx monomer. This gave a second-order rate constant of 4×10^4 $\text{M}^{-1} \cdot \text{s}^{-1}$ (Figure 3A, insert). As the assay measures loss of hydroperoxide, this value is only slightly affected by whether the reaction of the second thiol in the dimer containing one disulfide is fast or slow. This rate constant would correspond to a half-life of ~ 3 s under the conditions used in the present study, which is consistent with the gel electrophoresis results.

Prx oxidation by protein hydroperoxides

Hydroperoxides generated on BSA either by photolysis or by γ-irradiation were able to oxidize Prx2 (Figure 4A) and Prx3 (Figure 4B). Although some dimerization was seen upon the addition of unirradiated albumin to each Prx, the magnitude of the change was substantially greater with the hydroperoxide-containing samples. Reaction rates were assessed by following dimer formation for Prx2 (Figure 5A) and Prx3 (Figure 5B) treated with γ-irradiated albumin. Both followed a similar time course, with most of the reaction completing within 5 min. Plots of the densitometry results (Figure 5C) indicate a fast initial decrease in the monomer band followed by a slower decay that could be fitted to an exponential curve. Non-irradiated BSA also gave an initial fall in the reduced form of the Prxs (Figure 4), but no further oxidation was observed. Assuming that the second phase represents reactions with hydroperoxides on the protein, pseudo first-order rate constants were calculated from the exponential fits of the second phase of monomer loss At a hydroperoxide concentration of 20 μM, these gave values of 0.006 s^{-1} and 0.014 s^{-1} for Prx2 and Prx3 respectively and (using a proportionality constant of 2) correspond to second-order rate constants in the 10^2 $\text{M}^{-1} \cdot \text{s}^{-1}$ range (Table 2).

Table 2 Second-order rate constants ($\text{M}^{-1} \cdot \text{s}^{-1}$) for the reactions of Prxs with various hydroperoxides

ND, not determined.

Hydroperoxide	Prx2	Prx3
Lysozyme hydroperoxide	40	90
BSA hydroperoxide	160	360
Histidine hydroperoxide	2×10^3	3×10^3
N-acetyl leucine hydroperoxide	4×10^4	ND

Lysozyme hydroperoxides, formed by photolysis, also caused time-dependent oxidation of Prx2 (Figure 6A) and Prx3 (Figure 6B). The reactions were slower than with BSA hydroperoxides formed by γ-irradiation and did not show an initial rapid phase of dimerization. Exponential fitting of the densitometry data (Figure 6C) gave second-order rate constants that were 4-fold lower than those for BSA (Table 2).

Oxidation of erythrocyte Prx2 by amino acid and protein hydroperoxides

To test whether Prxs are competitive targets for peptide hydroperoxides in a physiological setting, intact erythrocytes were exposed to *N*-acetyl leucine methyl ester hydroperoxide. Being small and neutral, we reasoned that this molecule should be able to enter the cells. Erythrocytes contain high concentrations of Prx2 and are well endowed with GSH/GPx, the system normally considered as detoxifying hydroperoxides [28]. Reactions were carried out in PBS without added glucose. This restricts the supply

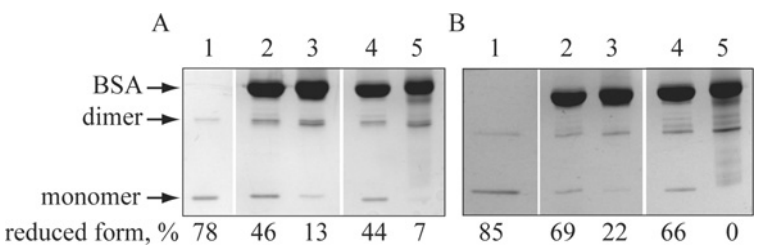

Figure 4 Oxidation of Prx2 and Prx3 by hydroperoxides formed on BSA

(**A**) Prx2 and (**B**) Prx3 (7 μM) was incubated for 30 min alone (lane 1) or with 20 μM BSA hydroperoxides formed either by photo-oxidation in the presence of Rose Bengal (lane 3) or γ-irradiation (lane 5). Non-illuminated BSA with Rose Bengal (lane 2) or untreated BSA (lane 4) were used as controls. Samples were separated by non-reducing SDS/PAGE (12 % gels) and silver stained. Monomer and dimer bands plus a higher molecular mass band, corresponding to BSA, are marked. Other separation conditions and analysis methods are as described in Figure 1.

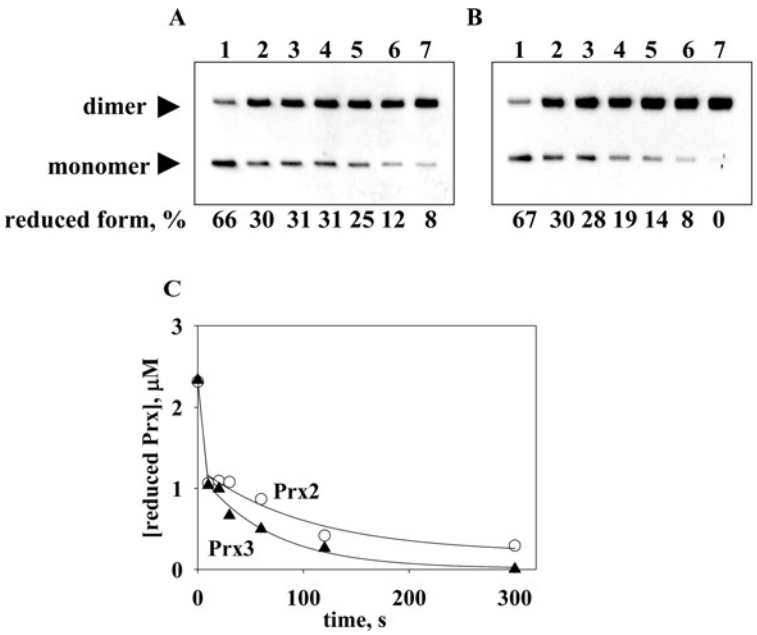

Figure 5 Time course of oxidation of Prx2 and Prx3 by BSA hydroperoxide

(**A**) Prx2 or (**B**) Prx3 (3.5 μM) was incubated alone for 5 min (lane 1) or with 20 μM BSA hydroperoxide generated by γ-irradiation for 10 s, 20 s, 30 s, 1 min, 2 min or 5 min (lanes 2–7 respectively). The reactions were stopped and the samples were analysed by non-reducing SDS/PAGE (15 % gels) and Western blotting with the (**A**) anti-Prx2 antibody or (**B**) anti-Prx3 antibody. (**C**) Kinetic analysis of densitometry results. The reduced Prx concentrations were estimated from the initial Prx concentration and the percentage monomer in each sample. Curves for Prx2 (○) and Prx3 (▲) represent exponential fits.

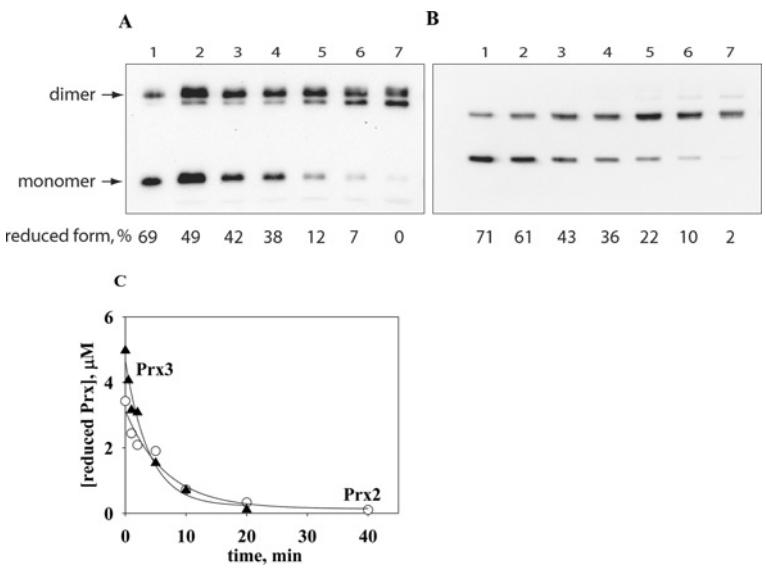

Figure 6 Time course of oxidation of Prx2 or Prx3 by hydroperoxides formed on lysozyme

(**A**) Prx2 or (**B**) Prx3 (7 μM) was incubated alone (lane 1) or with 30 μM lysozyme hydroperoxide generated by photolysis for 30 s, 1 min, 2 min, 5 min, 10 min or 20 min (lanes 2–7 respectively). The reactions were stopped and the samples were analysed by non-reducing SDS/PAGE (10 % gels) and Western blotting with the (**A**) anti-Prx2 antibody or (**B**) anti-Prx3 antibody. (**C**) Kinetic analysis of densitometry results. The reduced Prx concentrations were estimated from the initial Prx concentration and the percentage monomer in each sample. Curves for Prx2 (○) and Prx3 (▲) represent exponential fits.

of NADPH for recycling Prx2 or GSH, such that Prx oxidation results in dimer accumulation and oxidized GSH accumulates as GSSG [29]. As shown in Figure 7(A), Prx2 was initially present as the reduced monomer in the erythrocytes and was unaffected by a 10 min exposure of the cells to the non-irradiated amino acid. Incubation with the hydroperoxide caused concentration-dependent oxidation of Prx2. More than 80 % was oxidized to the dimer with 11 μM hydroperoxide and higher concentrations gave complete dimerization. In contrast, GSH levels were unaffected by up to 55 μM hydroperoxide and only partial loss was seen at the highest concentration used (Figure 7B).

The reaction of BSA hydroperoxide with erythrocyte Prx2 and GSH was also examined. In this case a concentrated lysate was used to overcome the membrane permeability barrier,

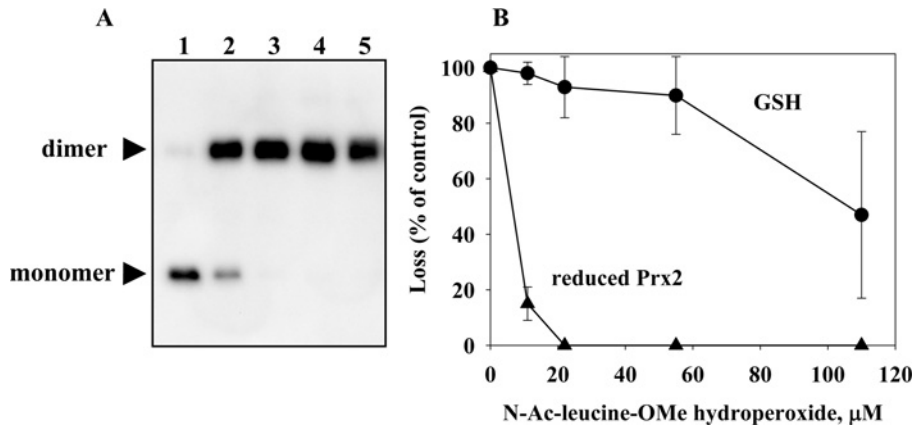

Figure 7 Prx2 and GSH oxidation in erythrocytes incubated with *N*-acetyl leucine methyl ester hydroperoxide

Isolated human erythrocytes (10^8 cells/ml in PBS) were mixed with an equal volume of γ-irradiated or non-irradiated *N*-acetyl leucine methyl ester as described in the Experimental section. (**A**) Western blot of Prx2 in erythrocytes. Lane 1, non-irradiated *N*-acetyl leucine methyl ester control at a concentration equivalent to the irradiated amino acid containing 110 μM hydroperoxide; lanes 2–5, *N*-acetyl leucine methyl ester hydroperoxide (11, 22, 55 and 110 μM in peroxide equivalents respectively). Monomer and dimer bands are marked. (**B**) Dependence of Prx2 and GSH oxidation in erythrocytes on *N*-acetyl leucine methyl ester hydroperoxide concentration (the error bars show the range for two independent experiments). For Prx2 the ranges at concentrations above 11 μM were zero.

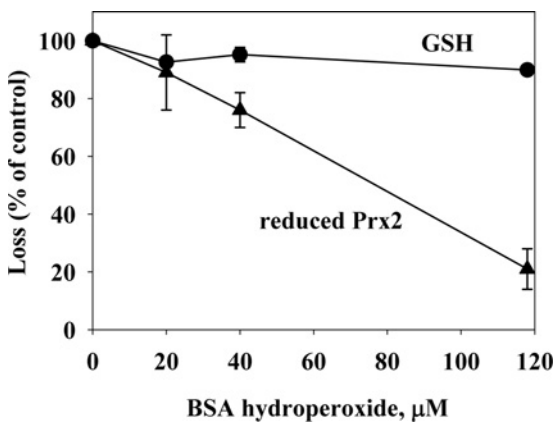

Figure 8 Prx2 and GSH oxidation in erythrocyte lysate incubated with BSA hydroperoxide

Erythrocyte lysates were treated with BSA hydroperoxide for 15 min as described in the Experimental section. The results are for two experiments for GSH measurements and four for Prx2. Reduced Prx2 in untreated lysates was 67 ± 6 % of the total Prx2. Non-irradiated BSA at a concentration corresponding to the highest BSA hydroperoxide added to lysate resulted in no change in reduced Prx2.

with minimal disruption of the intracellular environment. The protein hydroperoxide caused concentration-dependent oxidation of Prx2, whereas GSH loss was negligible (Figure 8).

DISCUSSION

Our present results indicate that protein and amino acid hydroperoxides react rapidly with the typical two-cysteine Prxs, Prx2 and Prx3. Although the difference between the two Prxs was not large, Prx3 consistently showed higher reactivity. For the amino acids and peptides studied, second-order rate constants varied from 4×10^4 $M^{-1} \cdot s^{-1}$ for the reaction with *N*-acetyl leucine hydroperoxide to 2×10^3 $M^{-1} \cdot s^{-1}$ for histidine hydroperoxide. The rate with *N*-acetyl leucine hydroperoxide appears representative of the other leucine or isoleucine analogues studied, and slightly faster than for tyrosine and Gly-Tyr-Gly hydroperoxides. The results for *N*-acetyl leucine suggest that the hydroperoxide on the α-carbon reacts more slowly than those on the side chain. This may be due to the more hindered tertiary hydroperoxide on the α-carbon being less accessible to the active site than the primary species on the side chain methyl groups.

Although the rate constants for the amino acids are not as high as for H_2O_2, they are approaching values measured for small alkyl hydroperoxides [20]. The protein hydroperoxides reacted more slowly, but nevertheless gave rate constants of $\sim 100\ M^{-1} \cdot s^{-1}$ or greater (Table 2) and oxidized each Prx within a few minutes. Protein hydroperoxide groups will be present on different residues, depending on how they were generated. Photolysis of Rose Bengal produces singlet oxygen, which preferentially targets tryptophan, tyrosine and histidine, whereas γ-irradiation favours aliphatic side chains and α-carbons of the peptide backbone [2,30–34]. These hydroperoxides would be expected to vary in reactivity, and the measured rates will be a composite depending on the residues modified and their accessibility. The observed reactivity of the protein hydroperoxides suggests that their access to the Prx active site is more restricted compared with hydroperoxides formed on free amino acids. However, the lower reactivity of lysozyme than the larger BSA suggests that size of the protein itself is not a major determinant.

The peroxidative cysteine residue of Prxs is much more reactive with H_2O_2 than can be accounted for by its low pK_a. However, high reaction rates are not observed with typical thiol reagents. For example, iodoacetamide and acrolein react very poorly with Prx2 and Prx3 [17,18,35,36], and rate constants for amino acid chloramines and Prx2 are $< 10\ M^{-1} \cdot s^{-1}$ compared with $\sim 10^3\ M^{-1} \cdot s^{-1}$ for low-pK_a protein thiols [29,37]. The amino acid hydroperoxides, and even the protein hydroperoxides, are more reactive than these reagents. Thus the Prx active sites appear well designed to react with R-OOH groups while protecting the reactive cysteine from other thiol reactants.

Previous studies of peptide and protein hydroperoxides have shown that they are reduced slowly by GSH and other small molecule thiols [15]. Small peptide hydroperoxides are also able to inactivate various thiol-dependent enzymes. For some, this is slow; e.g. protein tyrosine phosphatases and SERCA underwent partial inactivation on incubation with >100 μM hydroperoxide

for 0.5–1 h [9]. GAPDH (k of $\sim 50\ M^{-1} \cdot s^{-1}$ estimated from data in [38]) and caspase 3 [8] were inactivated more rapidly, although tryptophan hydroperoxide (not investigated in the present study) had the greatest effect and tyrosine hydroperoxide caused only $\sim 20\%$ inactivation over 2 min. Protein hydroperoxides did inactivate protein tyrosine phosphatases and lysosomal cathepsins [10,39], but over a longer time frame than observed in the present study with the Prxs. Although kinetic data are not available for all of these reactions, it appears that amino acid and peptide hydroperoxides are at least 1–2 orders of magnitude more reactive with Prxs than other thiol proteins; Prxs are the only thiol proteins shown to react with protein hydroperoxides efficiently.

In the present study, Prx2 and Prx3 were oxidized by the hydroperoxides to disulfide-bonded dimers. This process is reversible, with the Trx/TR system (cytoplasmic Trx1/TR1 for Prx2 and mitochondrial Trx2/TR2 for Prx2) recycling the proteins physiologically [16]. Recycling of Prx dimers formed in cells treated with H_2O_2 or chloramines has been observed [29,40] and the same should apply for dimers formed as a result of oxidation by amino acid and protein hydroperoxides. The efficiency of recycling depends on the cellular levels of Trx and TR. It is very slow in erythrocytes because they contain very little TR [40], hence the observed dimer accumulation, but is more rapid in other cell types [16]. In the sense that the reduced Prxs are not consumed during this cycle, this process constitutes a catalytic mechanism for breaking down amino acid and protein hydroperoxides. However, when recycling is slow, it is perhaps more realistic to consider the Prxs as scavengers of the hydroperoxides that can be recycled. Thiol-mediated reduction of hydroperoxides produces the corresponding alcohol derivatives. We showed this with *N*-acetyl leucine hydroperoxide, where consumption of the parent compound was accompanied by the formation of a product of 16 Da, and alcohol formation is also expected for the protein hydroperoxides. Therefore the reaction with Prxs cannot regenerate the parent protein. The hydroperoxides are, however, reactive species that are capable of generating secondary radicals and causing further cell damage, so Prxs can be regarded as providing antioxidant defence against these species.

A possible alternative for providing such defence are GPxs. We have tested GPx1 previously [15], although without carrying out quantitative kinetic analysis. In contrast with the Prxs, GPx1 was unable to break down BSA or lysozyme hydroperoxide. GPx1 in the presence of GSH did catalyse the consumption of hydroperoxides on tyrosine and tryptophan residues, with these reactions both occurring on a similar time scale. Thus both systems could potentially react with small peptide hydroperoxides in a physiological setting but only the Prxs should be active against protein forms. To test this proposal directly in intact cells, we compared the oxidation of Prx2 and GSH in erythrocytes exposed to a cell-permeable amino acid hydroperoxide, under conditions where recycling of Prx2 dimer and GSSG was limited. Our finding that Prx2 became fully oxidized at low hydroperoxide concentrations that caused no GSH loss indicates that Prx2 was the preferred target. GSH loss was observed at higher concentrations, indicating that GPx could come into the play after Prx2 becomes oxidized. With BSA hydroperoxide added to the cytoplasm of lysed erythrocytes, Prx2 but not GSH became oxidized. This is consistent with GPx not reacting with protein hydroperoxides and provides strong support for Prxs being favoured targets for these oxidants. Results from a related study where a number of cellular thiol enzyme activities were measured suggest that these enzymes are even less favoured hydroperoxide targets than the GSH system [41].

Hydroperoxides are major products of oxidative stress. Some are likely to form on amino acids and peptides (as well as lipids) but, based on higher cellular abundance, hydroperoxides on proteins are likely to be much more prevalent. Prxs are ubiquitously expressed and present in cells at high concentrations [40,42,43], so should be well placed to provide antioxidant defence against these species. The focus of the present study has been on Prx2 and Prx3, but other members of the Prx family have high peroxide reactivity that might also extend to amino acid or protein derivatives. It can be estimated from the rate constants for Prx2 and Prx3 that they should remove peptide hydroperoxides with half lives of 1–10 s and protein hydroperoxides within a few minutes. Prxs should therefore prevent accumulation when these reactive species are produced intracellularly, and protect against secondary damage to other targets.

In summary, the first Prxs were recognized as proteins that reduce alkyl hydroperoxides. Many members of the family have subsequently been shown to be efficient at removing these species as well as H_2O_2. The results presented in this paper extend the range of substrates for Prxs to include hydroperoxides formed on free amino acids, peptides and proteins. Their well designed active site for reducing -OOH groups should enable Prxs to protect against peroxides formed on a diverse array of molecules.

AUTHOR CONTRIBUTION

Alexander Peskin carried out the kinetic analyses, contributed to the experimental design and prepared the manuscript; Andrew Cox and Péter Nagy carried out the kinetic analyses, contributed to the experimental design and reviewed the manuscript; Philip Morgan and Michael Davies prepared hydroperoxides and advised on their use; Mark Hampton contributed to the experimental design and reviewed the manuscript; Christine Winterbourn contributed to the experimental design and prepared the manuscript.

FUNDING

This work was supported by the Marsden Fund, the Health Research Council of New Zealand, and the Australian Research Council, through the ARC Centres of Excellence [grant number CE0561607] and Discovery Projects [grant number DP0988311] schemes.

REFERENCES

1 Davies, M. J. (2005) The oxidative environment and protein damage. Biochim. Biophys. Acta **1703**, 93–109
2 Gebicki, S. and Gebicki, J. M. (1993) Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. Biochem. J. **289**, 743–749
3 Simpson, J. A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, J. M. and Dean, R. T. (1992) Long-lived reactive species on free-radical-damaged proteins. Biochem. J. **282**, 621–624
4 Gieseg, S., Duggan, S. and Gebicki, J. M. (2000) Peroxidation of proteins before lipids in U937 cells exposed to peroxyl radicals. Biochem. J. **350**, 215–218
5 Fu, S., Davies, M. J., Stocker, R. and Dean, R. T. (1998) Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem. J. **333**, 519–525
6 Fu, S., Dean, R., Southan, M. and Truscott, R. (1998) The hydroxyl radical in lens nuclear cataractogenesis. J. Biol. Chem. **273**, 28603–28609
7 Nagy, P., Kettle, A. J. and Winterbourn, C. C. (2009) Superoxide-mediated formation of tyrosine hydroperoxides and methionine sulfoxide in peptides through radical addition and intramolecular oxygen transfer. J. Biol. Chem. **284**, 14723–14733
8 Hampton, M. B., Morgan, P. E. and Davies, M. J. (2002) Inactivation of cellular caspases by peptide-derived tryptophan and tyrosine peroxides. FEBS Lett. **527**, 289–292
9 Dremina, E. S., Sharov, V. S., Davies, M. J. and Schoneich, C. (2007) Oxidation and inactivation of SERCA by selective reaction of cysteine residues with amino acid peroxides. Chem. Res. Toxicol. **20**, 1462–1469
10 Headlam, H. A., Gracanin, M., Rodgers, K. J. and Davies, M. J. (2006) Inhibition of cathepsins and related proteases by amino acid, peptide, and protein hydroperoxides. Free Radical Biol. Med. **40**, 1539–1548
11 Davies, M. J., Fu, S. and Dean, R. T. (1995) Protein hydroperoxides can give rise to reactive free radicals. Biochem. J. **305**, 643–649
12 Luxford, C., Morin, B., Dean, R. T. and Davies, M. J. (1999) Histone H1- and other protein- and amino acid-hydroperoxides can give rise to free radicals which oxidize DNA. Biochem. J. **344**, 125–134
13 Gebicki, S. and Gebicki, J. M. (1999) Crosslinking of DNA and proteins induced by protein hydroperoxides. Biochem. J. **338**, 629–636

14 Luxford, C., Dean, R. T. and Davies, M. J. (2002) Induction of DNA damage by oxidised amino acids and proteins. Biogerontology **3**, 95–102
15 Morgan, P. E., Dean, R. T. and Davies, M. J. (2004) Protective mechanisms against peptide and protein peroxides generated by singlet oxygen. Free Radical Biol. Med. **36**, 484–496
16 Rhee, S. G., Chae, H. Z. and Kim, K. (2005) Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radical Biol. Med. **38**, 1543–1552
17 Peskin, A. V., Low, F. M., Paton, L. N., Maghzal, G. J., Hampton, M. B. and Winterbourn, C. C. (2007) The high reactivity of peroxiredoxin 2 with H_2O_2 is not reflected in its reaction with other oxidants and thiol reagents. J. Biol. Chem. **282**, 11885–11892
18 Cox, A. G., Peskin, A. V., Paton, L. N., Winterbourn, C. and Hampton, M. B. (2009) Redox potential and peroxide reactivity of human peroxiredoxin 3. Biochemistry **48**, 6495–6501
19 Jeong, W., Cha, M. K. and Kim, I. H. (2000) Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/Alkyl hydroperoxide peroxidase C (AhpC) family. J. Biol. Chem. **275**, 2924–2930
20 Parsonage, D., Karplus, P. A. and Poole, L. B. (2008) Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. Proc. Natl. Acad. Sci. U.S.A. **105**, 8209–8214
21 Morgan, P. E., Pattison, D. I., Hawkins, C. L. and Davies, M. J. (2008) Separation, detection, and quantification of hydroperoxides formed at side-chain and backbone sites on amino acids, peptides, and proteins. Free Radical Biol. Med. **45**, 1279–1289
22 Gay, C., Collins, J. and Gebicki, J. M. (1999) Hydroperoxide assay with the ferric–xylenol orange complex. Anal. Biochem. **273**, 149–155
23 Lim, Y. S., Cha, M. K., Yun, C. H., Kim, H. K., Kim, K. and Kim, I. H. (1994) Purification and characterization of thiol-specific antioxidant protein from human red blood cell: a new type of antioxidant protein. Biochem. Biophys. Res. Commun. **199**, 199–206
24 Peskin, A. V. and Winterbourn, C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. Free Radical Biol. Med. **30**, 572–579
25 Cotgreave, I. A. and Moldeus, P. (1986) Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems. J. Biochem. Biophys. Methods **13**, 231–249
26 Romero, F. J., Ordonez, I., Arduini, A. and Cadenas, E. (1992) The reactivity of thiols and disulfides with different redox states of myoglobin: redox and addition reactions and formation of thiyl radical intermediates. J. Biol. Chem. **267**, 1680–1688
27 Fu, S. L. and Dean, R. T. (1997) Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins. Biochem. J. **324**, 41–48
28 Low, F. M., Hampton, M. B. and Winterbourn, C. C. (2008) Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. Antioxid. Redox Signaling **10**, 1621–1630
29 Stacey, M. M., Peskin, A. V., Vissers, M. C. and Winterbourn, C. C. (2009) Chloramines and hypochlorous acid oxidize erythrocyte peroxiredoxin 2. Free Radical Biol. Med. **47**, 1468–1476
30 Agon, V. V., Bubb, W. A., Wright, A., Hawkins, C. L. and Davies, M. J. (2006) Sensitizer-mediated photooxidation of histidine residues: evidence for the formation of reactive side-chain peroxides. Free Radical Biol. Med. **40**, 698–710
31 Davies, M. J. (2004) Reactive species formed on proteins exposed to singlet oxygen. Photochem. Photobiol. Sci. **3**, 17–25
32 Wright, A., Bubb, W. A., Hawkins, C. L. and Davies, M. J. (2002) Singlet oxygen-mediated protein oxidation: evidence for the formation of reactive side chain peroxides on tyrosine residues. Photochem. Photobiol. **76**, 35–46
33 Gracanin, M., Hawkins, C. L., Pattison, D. I. and Davies, M. J. (2009) Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products. Free Radical Biol. Med. **47**, 92–102
34 Davies, M. J. (1996) Protein and peptide alkoxyl radicals can give rise to C-terminal decarboxylation and backbone cleavage. Arch. Biochem. Biophys. **336**, 163–172
35 Nelson, K. J., Parsonage, D., Hall, A., Karplus, P. A. and Poole, L. B. (2008) Cysteine pK_a values for the bacterial peroxiredoxin AhpC. Biochemistry **47**, 12860–12868
36 Myers, C. R. and Myers, J. M. (2009) The effects of acrolein on peroxiredoxins, thioredoxins, and thioredoxin reductase in human bronchial epithelial cells. Toxicology **257**, 95–104
37 Peskin, A. V. and Winterbourn, C. C. (2006) Taurine chloramine is more selective than hypochlorous acid at targeting critical cysteines and inactivating creatine kinase and glyceraldehyde-3-phosphate dehydrogenase. Free Radical Biol. Med. **40**, 45–53
38 Morgan, P. E., Dean, R. T. and Davies, M. J. (2002) Inhibition of glyceraldehyde-3-phosphate dehydrogenase by peptide and protein peroxides generated by singlet oxygen attack. Eur. J. Biochem. **269**, 1916–1925
39 Gracanin, M. and Davies, M. J. (2007) Inhibition of protein tyrosine phosphatases by amino acid, peptide, and protein hydroperoxides: potential modulation of cell signaling by protein oxidation products. Free Radical Biol. Med. **42**, 1543–1551
40 Low, F. M., Hampton, M. B., Peskin, A. V. and Winterbourn, C. C. (2007) Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. Blood **109**, 2611–2617
41 Rahmanto, A. S., Morgan, P. E., Hawkins, C. L. and Davies, M. J. (2010) Cellular effects of peptide and protein hydroperoxides. Free Radical Biol. Med. **48**, 1071–1078
42 Wood, Z. A., Schroder, E., Robin, H. J. and Poole, L. B. (2003) Structure, mechanism and regulation of peroxiredoxins. Trends. Biochem. Sci. **28**, 32–40
43 Chae, H. Z., Kang, S. W. and Rhee, S. G. (1999) Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. Methods Enzymol. **300**, 219–226

Received 28 July 2010/2 September 2010; accepted 14 September 2010
Published as BJ Immediate Publication 14 September 2010, doi:10.1042/BJ20101156

Biochem. J. (2010) **432**, 313–321 (Printed in Great Britain) doi:10.1042/BJ20101156

online data

SUPPLEMENTARY ONLINE DATA

Removal of amino acid, peptide and protein hydroperoxides by reaction with peroxiredoxins 2 and 3

Alexander V. PESKIN*[1], Andrew G. COX*, Péter NAGY*, Philip E. MORGAN†, Mark B. HAMPTON*‡, Michael J. DAVIES† and Christine C. WINTERBOURN*‡

*Free Radical Research Group, Department of Pathology, University of Otago, Christchurch, New Zealand, †Free Radical Group, The Heart Research Institute, Sydney, Australia, and ‡National Research Centre for Growth and Development, The Liggins Institute, University of Auckland, Grafton, Auckland, New Zealand

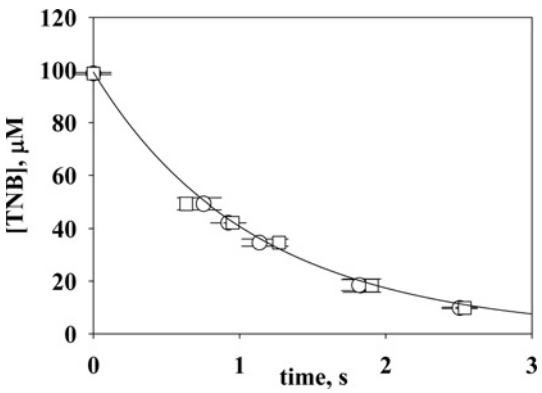

Figure S1 Calibration of tubes to estimate the reaction times in quench-flow experiments

The experimental results of the TNB (5-thio-2-nitrobenzoic acid) oxidation by taurine chloramine were used to calculate the delay times of the quench-flow apparatus with known of TNB and chloramine concentrations and the second-order rate constant of the reaction (○). Each data point corresponds to the tube of a certain length. The reaction time was also calculated by using the speed of the stepper motor and the volume of the tubes (□). The line represents the simulated kinetic trace for $k = 970\ M^{-1} \cdot s^{-1}$ [1]. Error bars are the S.D. ($n = 2$).

[1] To whom correspondence should be addressed (email alexander.peskin@otago.ac.nz).

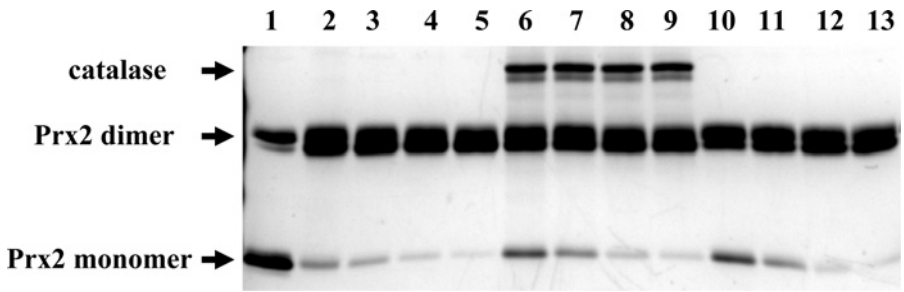

Figure S2 Non-reducing SDS/PAGE showing that catalase does not interfere with Prx2 oxidation by amino acid hydroperoxide

Prx2 (5 μM) was incubated alone (lane 1) or with 20 μM His hydroperoxide for 10 s (lanes 2, 6 and 10), 20 s (lanes 3, 7 and 11), 30 s (lanes 4, 8 and 12) or 40 s (lanes 5, 9 and 13). Catalase (50 μg/ml) was originally present in histidine hydroperoxide solution to remove H_2O_2 produced during preparation. This was removed by passing through an Amicon filter. Prx2 was incubated with histidine hydroperoxide in the absence of catalase (lanes 2–5), with catalase (50 μg/ml) (lanes 6–9) or with the original histidine hydroperoxide containing a final catalase concentration on dilution of 1 μg/ml (lanes 10–13).

REFERENCE

1 Peskin, A. V. and Winterbourn, C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. Free Radical Biol. Med. **30**, 572–579

Received 28 July 2010/2 September 2010; accepted 14 September 2010
Published as BJ Immediate Publication 14 September 2010, doi:10.1042/BJ20101156