Reversing the Inactivation of Peroxiredoxins Caused by Cysteine Sulfinic Acid Formation

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and/or $K_{1_{DD}}$. Evidence that the two rate constants are similar for sensitive and robust 2-Cys Prxs is that the enzymes have highly conserved active sites (implying $k_{2(DS)}$ is similar) and also have conserved positions of $C_a$ and $C_p$ (implying $k_{2(S)}$ changes little). On the basis of the structural evidence, we suggest that stabilizing the fully folded conformation (i.e., optimizing $K_{1_{DD}}$) is the most important evolutionary variable in this case. This model predicts that any changes that disrupt the packing of the C-terminus of a sensitive enzyme will increase $K_{1_{DD}}$, and decrease the rate of inactivation. Biochemical support for the model comes from a mutagenesis study on the role of the C-terminal extension of the yeast 2-Cys Prx, TpxY (32). That work showed that the C-terminus could be highly altered with little effect on enzyme activity but that almost any change caused the normally sensitive enzyme to become robust to inactivation by over-oxidation (see fig. S2).

Thus, the sensitivity of 2-Cys Prxs to inactivation by over-oxidation is not a limitation of the 2-Cys Prx catalytic mechanism, but it is a feature that has been selected for during the evolution of eukaryotes. Recent in vivo observations are consistent with the following model of 2-Cys Prx function in eukaryotes. In a resting cell, 2-Cys Prxs are present in large amounts (38) and keep ambient levels of peroxides very low to ensure that no signaling is triggered. In contrast, a transient intracellular peroxide burst (1) would produce peroxide at levels sufficient to rapidly inactivate 2-Cys Prxs, as has been observed in vivo during peroxide signaling in cells treated with tumor necrosis factor (25). Then peroxide could act as a messenger by interacting with other proteins, like the recently characterized yeast oxidant receptor peroxidase 1 (39) and peroxide-sensitive phosphatases (2). At this stage, the activities of the less abundant antioxidant enzymes, glutathione peroxidase and catalase, would be crucial to "mop up" excess peroxide and mitigate against its toxic effects. When too much 2-Cys Prx is present, the peroxide burst would not be sufficient to inactivate all of it, and signaling would be blocked as is seen in cells that overexpress 2-Cys Prxs and block peroxide activation of NF-kB (11, 12). This provides a rationale for the correlation in some cancer cells between defec-

Some proteinaceous cysteine residues are sensitive to oxidation by H$_2$O$_2$ because their environment promotes ionization of the thiol (Cys–SH) group, even at a neutral pH, to the thiolate anion (Cys–S$^-$), which is more readily oxidized to sulfenic acid (Cys–SOH) than

References and notes
is Cys–SH (1, 2). The sulfenic acid group is usually unstable and either reacts with any accessible thiol to form a disulfide or undergoes further oxidation to sulfenic acid (Cys–SOH); the disulfide group is stable and resistant to further oxidation (3, 4).

Members of the peroxiredoxin (Prx) family of proteins contain such an H2O2-sensitive Cys residue (5–7). These peroxidases, which exist as homodimers, catalyze the reduction of H2O2 by using reducing equivalents that are provided by thioredoxin (Trx) (8, 9). An H2O2-sensitive Cys, corresponding to Cys51 in mammalian Prx I and Prx II, is conserved in all Prx enzymes. The conserved Cys51–SH is selectively oxidized by H2O2 to Cys–SOH, which then reacts with Cys172–SH of the other subunit to form an intermolecular disulfide. The disulfide is then specifically reduced by Trx (6, 8). However, the sulfur atoms of Cys51 and Cys172 are relatively far apart (−13 Å) and the formation of an intermolecular disulfide between these residues is a slow process (10, 11). Thus, the Cys51–SOH intermediate is occasionally oxidized to Cys–SO2H before it is able to form a disulfide (8, 12–14). Given that sulfenic acid is not susceptible to reduction by thioredoxin, Prx enzymes with a Cys–SO2H group are catalytically inactive.

Hydrogen peroxide, which is generated as a result of respiration as well as in response to a variety of extracellular stimuli, including cytokines and peptide growth factors, serves as an intracellular messenger at low concentrations but induces cell death at higher concentrations (1, 15). By removing H2O2, Prx enzymes modulate various receptor signaling pathways and protect cells from oxidatively induced death (16–18). The proportion of Prx enzymes in the sulfenic state (19), which is small in cells cultured under basal conditions, increases markedly in cells exposed to H2O2, or tumor necrosis factor–α (12–14). Hydrogen peroxide also oxidizes exposed methionine residues to methionine sulfoxide, and mammalian cells contain two types of methionine sulfoxide reductase that reverse the oxidation reaction using reducing equivalents from Trx (20). Proteins that have been modified oxidatively at other amino acid residues are usually not repaired, however, and are removed by proteolysis (21).

We investigated the fate of Prx I that contained the Cys51–SO2H group. Raw 264.7 mouse macrophage cells were metabolically

![Fig. 1. Reversible oxidation of Prx I. 35S-labeled Raw 264.7 cells were exposed for 10 min to 100 μM H2O2 (A) or 500 μM H2O2 (B) in HBSS, washed, and incubated for various times in DMEM, supplemented with 10% FBS and with cysteine and methionine, each at a concentration of 200 μg/mL. Cellular proteins were then analyzed by 2D gel electrophoresis followed by either autoradiography or immunoblot analysis with antibodies to Prx I. The regions of the autoradiograms and immunoblots corresponding to molecular sizes (vertical) of 22 to 28 kD, and isoelectric points (horizontal) of 7.6 to 8.2 are shown. The positions of oxidized (Ox) and reduced (Re) Prx I are indicated. The times shown on the left side of the gels represent total time elapsed, beginning with the 10-min period of H2O2 treatment. The percentage of the reduced form was determined from the autoradiograms or immunoblots and is shown plotted against total time elapsed, as indicated. Quantitative data are means ± SEM of values from three independent experiments. (C) Electrospray ionization mass spectra of reduced Prx I (upper panels) and oxidized Prx I (lower panels), both of which were purified from H2O2-treated Hela S3 cells (30) (fig. S2). The spectra of multiply charged ions with mass/charge ratio (m/z) values and corresponding total charges (left panels) and deconvoluted spectra derived from these component ions with calculated masses (right panels) are shown.](www.sciencemag.org)
labeled with tracer amounts of a mixture of [35S]cysteine and [35S]methionine in a medium lacking nonradioactive sulfur-containing amino acids. They were then washed with Hank’s balanced salt solution (HBSS), exposed for 10 min to 100 or 500 μM H2O2 in HBSS, and then incubated in culture medium supplemented with unlabeled cysteine and methionine, each at a concentration of 200 μg/ml (22). At various time points, cellular proteins were analyzed by two-dimensional (2D) gel electrophoresis and autoradiography, and the Prx I spots on the 2D gels were identified by immunoblot analysis (23) (Fig. 1, A and B). Oxidized Prx enzymes are detected as more acidic satellite spots of the spots corresponding to the reduced enzymes on 2D gels (12–14).

Both the autoradiogram and the immunoblot showed that Prx I exists almost exclusively in the reduced form in cells not treated with H2O2, as has previously been demonstrated (12–14). In contrast, the proportion of the reduced enzyme decreased to ~25% of the total Prx I in cells treated with 100 μM H2O2 for 10 min (Fig. 1A). After the removal of H2O2, the amount of reduced enzyme increased rapidly and that of the oxidized enzyme decreased concomitantly. However, the regeneration of the reduced enzyme reached a plateau at ~70 to 80% recovery in cells treated with 100 μM H2O2, suggesting that a proportion of the oxidized Prx I molecules is refractory to reduction—probably as a result of damage caused by further oxidation of Cys51–SO3H to cysteic acid or by the oxidation of other amino acid residues. Almost 100% of the Prx I was oxidized in cells treated with 500 μM H2O2 for 10 min (Fig. 1B). The recovery of the reduced enzyme was slower in these cells than in those treated with 100 μM H2O2, possibly reflecting a diminished reducing power of cells exposed to the higher concentration of oxidant.

The exposure of HeLa cells to H2O2 also elicited an acidic shift of Prx I similar to that observed in Raw 264.7 cells (14). To identify the modification responsible for the acidic shift, reduced and oxidized Prx I were purified from H2O2-treated HeLa cells (Fig. S2), and the molecular masses of the two forms were determined by electrospray ionization mass spectrometry (Fig. 1C). The difference of 32 mass units between the reduced and oxidized enzymes suggests the presence of two additional oxygen atoms in the oxidized species. Furthermore, in-gel tryptic digestion of Prx I in the normal and acidic spots and mass spectral analysis of the resulting peptides showed that the acidic shift was due specifically to the formation of the sulfur oxyacid exclusively at Cys51, although Prx I contains three additional Cys residues (Fig. S3).

It is unlikely that the increase in reduced Prx I was due to de novo synthesis because newly synthesized protein should not be detectable by autoradiography. The cells were labeled with only trace amounts of [35S]amino acids and were subsequently washed and supplemented with a large excess of unlabeled sulfur-containing amino acids. In addition, analysis of the samples used to generate Fig. 1A by 1D gel electrophoresis and immunoblotting revealed that the total amount of Prx I did not change during exposure of the cells to H2O2 and subsequent incubation for up to a total of 4 hours (24). Thus, the increase in the amount of reduced Prx I after removal of H2O2 reflected conversion of the oxidized enzyme rather than de novo synthesis.

To confirm that de novo protein synthesis did not contribute to our results, we labeled Raw 264.7 cells with [35S]methionine and [35S]cysteine, exposed them to 500 μM H2O2 for 10 min, and then monitored the conversion of oxidized Prx I to the reduced form in the presence of the protein synthesis inhibitor cycloheximide. Incubation of the cells with cycloheximide for 2 or 3 hours resulted in the reappearance of the reduced enzyme in amounts corresponding to ~25 and 40%, respectively, of the total Prx I (Fig. 2). The rate of recovery was similar to that observed in the absence of the protein synthesis inhibitor (Fig. 1B). It was not possible to monitor the recovery of reduced Prx I for periods of more than 3 hours, because the oxidatively stressed cells appeared unhealthy and began to die in the presence of cycloheximide.

Prokaryotes are able to convert various organic sulfonic acids to the corresponding aldehyde and sulfone by the action of alkanesulfonate monooxygenase (25). Although an equivalent enzyme has not been described in eu- karyotes, we considered the possibility that “desulfurization” caused the reversal of the acidic shift of Prx. We therefore subjected [35S]-
by oxidation of methionine include repair of promatrix metalloproteinases (28) and nitrile hydrate (29).”

References and Notes
19. The Cys-SO2H group is generally unstable and is readily oxidized by H2O2 or slowly autooxidized to cystic acid (Cys-SO2H) (3). However, the Cys-SO2H of Prx I remains unchanged so long as the enzyme is not denatured, probably because it is buried inside the active-site pocket and stabilized by a salt bridge with Arg127, the same basic residue that contributes to stabilization of the thiolate anion of Cys51 (17). The oxidation state of Cys51 of Prx I was determined to be sulfonic when analyzed by 2D gel electrophoresis in the second dimension (3).
22. For labeling of Raw 264.7 cells with 35S-containing amino acids, the cells (5 × 106) well in six-well plates) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), washed with labeling medium (DMEM free of cysteine and methionine; Life Technologies), and then incubated for 4 hours at 37°C in 1 ml of labeling medium supplemented with 5 mM dithiothreitol (Life Technologies) and a mixture of (0.2 M/mL) of [35S]methionine and [35S]cysteine (TRANSLABEL, ICN). The cells were then washed twice with HBSS, exposed for 10 minutes to the indicated concentrations of H2O2 in 2 ml of prewarmed HBSS, washed again with HBSS, and incubated for the indicated times at 37°C with DMEM supplemented with 10% FBS and with cysteine and methionine, each at a concentration of 200 μM/ml, which is equivalent to between 3 and 7 times the concentration of these amino acids that are normally present in culture medium and several thousand times that of the 35S-labeled amino acids. The cells were finally rinsed twice with ice-cold HBSS and scraped into 1 ml of 10% trichloroacetic acid.
23. The precipitated 35S-labeled proteins were washed twice with ice-cold acetone and then resuspended in 0.5 ml of Rehydration Buffer (Amersham Biosciences) containing 20 mM diethanol. After removal of insoluble material, one-half of each sample (250 μl) was diluted with 200 μl of Rehydration Buffer containing 20 mM diethanol and applied to 24-cm immobilized pH gradient (IPG) strips (pH 3 to 10, nonlinear; Amersham Biosciences). Isoelectric focusing was performed with an IPGPhor unit (Amersham Biosciences), after which the focused proteins were subjected to reduction and alkylation on the IPG strips as recommended by the manufacturer. Electrophoresis in the second dimension was performed on 12% polyacrylamide gels with the use of an Ettan DALT II System (Amersham Biosciences). The 35S-labeled proteins in the 2D gels were visualized and quantified with the use of a FLA-3000 image analyzer (Fujiﬁlm). For immunoblot analysis of Prx enzymes, proteins were transferred electrohoresitically from 2D gels to a nitrocellulose membrane and probed with rabbit antibodies speciﬁc for Prx I or II. Immune complexes were detected with alkaline phosphatase–conjugated secondary antibodies (KPL). CDP-Star (Tropix) chemiluminescence reagent, and a Kodak Image Station 440.
30. Materials and methods are available as supporting material on Science Online.
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Materials and Methods
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Figs. S1 to S3
References