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Reversible Oxidation of the Active Site Cysteine of Peroxiredoxins to Cysteine Sulfinic Acid

IMMUNOBLOT DETECTION WITH ANTIBODIES SPECIFIC FOR THE HYPEROXIDIZED CYSTEINE-CONTAINING SEQUENCE*

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We previously suggested that oxidation of the active site cysteine of peroxiredoxin (Prx) I or Prx II to cysteine sulfinic acid in H_2O_2-treated cells is reversible (Woo, H. A., Chae, H. Z., Hwang, S. C., Yang, K.-S., Kang, S. W., Kim, K., and Rhee, S. G. (2003) *Science* 300, 653–656). In contrast, it was recently proposed that sulfinylation of Prx II, but not that of Prx I or Prx III, is reversible (Chevallet, M., Wagner, E., Luche, S., van Dorssealaer, A., Leize-Wagner, E., and Rabilloud, T. (2003) *J. Biol. Chem.* 278, 37146–37153). The detection of sulfinylated proteins in both of these previous studies relied on complex proteomics analysis. We now describe a simple immunoblot assay for the detection of sulfinylated Prx enzymes that is based on antibodies produced in response to a sulfonylated peptide modeled on the conserved active site sequence. These antibodies recognized both sulfinic and sulfonic forms of Prx equally well and allowed the detection of sulfinylated Prx enzymes in H_2O_2-treated cells with high sensitivity and specificity. With the use of these antibodies, we demonstrated that not only the cytosolic enzymes Prx I and Prx II but also the mitochondrial enzyme Prx III undergo reversible sulfinylation. The generation of antibodies specific for sulfonylated peptides should provide insight into protein function similar to that achieved with antibodies to peptides containing phosphoserine or phosphothreonine.

Peroxiredoxins (Prxs)[1] are a family of peroxidases that reduce hydrogen peroxide (H_2O_2) and alkyl hydroperoxides to water and alcohol, respectively, with the use of reducing equivalents provided by thiol-containing proteins such as thioredoxin (1–3). All Prx enzymes exist as homodimers, and Prx I to Prx IV, which are members of the 2-Cys Prx subgroup, each contain two conserved Cys residues corresponding to Cys^{51} and Cys^{172} of mammalian Prx I (4). Prx I and Prx II exist in the cytosol; Prx III, which is synthesized with a mitochondrial targeting sequence, is imported into and matures within mitochondria (1, 5); and Prx IV is a secreted protein (1, 6, 7). The NH_2-terminal conserved cysteine (Cys^{51} of Prx I) of Prx I to IV, which exists as the thiolate anion even at neutral pH as a result of its ionic interaction with the positively charged residue Lys^{127} (8, 9), is selectively oxidized by H_2O_2 to Cys-SOH. The unstable Cys^{51}–SOH reacts with Cys^{172}–SH of the other subunit of the homodimer to form an intermolecular disulfide, which is subsequently reduced by thioredoxin (Trx) (10). Because Cys^{51} and Cys^{172} are situated far apart, with their sulfur atoms separated by ~13 Å (9), formation of the disulfide is a slow process and the sulfenic intermediate is occasionally hyperoxidized to sulfinic acid (Cys–SO_2H), resulting in inactivation of peroxidase activity (10–12).

Proteins that contain hyperoxidized cysteine residues (Cys-SO_2H or Cys-SO_3H) are detected as the more acidic satellite spots of the spots corresponding to the reduced form of the protein on two-dimensional polyacrylamide gels (11, 12). On examination of the redox state of Prx in several mammalian cell lines that had been metabolically labeled with ^{35}S, we observed that, on two-dimensional gels, the ^{35}S-labeled acidic spots corresponding to sulfinylated Prx I and Prx II increased in intensity during exposure of cells to H_2O_2 and then underwent a shift back to the spots corresponding to the respective reduced forms after removal of H_2O_2 in the presence of the protein synthesis inhibitor cycloheximide (13). This observation led us to propose that the sulfinylation reaction is reversible in cells (13).

Given that an acidic shift on two-dimensional gels is also caused by protein phosphorylation, as is the case with Prx (14), mass spectral analysis of the acidic forms of proteins has been necessary to ascertain the presence of hyperoxidized cysteine residues. To develop an alternative approach to the complex procedure involving isotopic labeling of cells, two-dimensional electrophoresis, and mass spectrometry for the detection of proteins containing hyperoxidized cysteine residues, we prepared rabbit antibodies to a sulfonylated peptide based on the active site sequence common to mammalian Prx I to IV. With the use of immunoblot analysis with these antibodies, we reinvestigated Prx oxidation and now not only confirm the reversibility of sulfinylation of cytosolic Prx isoforms but also demonstrate reversibility of the sulfinylation of mitochondrial Prx.

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[1] The abbreviations used are: Prx, peroxiredoxin; Trx, thioredoxin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum.

These results are thus discrepant with the recent observation by Chevallet *et al.* (15) that the sulfinylation of Prx II, but not that of Prx I or Prx III, is reversible.

EXPERIMENTAL PROCEDURES

Preparation of a Sulfonylated Prx Peptide—A peptide (DFTFVCPTEI), which corresponds to the active site of mammalian Prx I to IV, was oxidized by dissolving 5 mg of the peptide in 50 μl of performic acid (freshly prepared by mixing formic acid and H_2O_2, 9:1 (v/v)) and incubating the mixture for 1 h at 25 °C. The peptide (1 mg) was then dried for 15 min under vacuum without heating, and the resulting residue was dissolved in 500 μl of water. A portion (10 μg) of the oxidized peptide was then analyzed by high-performance liquid chromatography on a Vydac C_{18} column that had been equilibrated with 0.1% trifluoroacetic acid in water; elution was performed over 60 min with a linear gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid. The major peak (>95%) eluted at 34.0 min and was subjected to analysis with a Voyager-STR matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer to confirm the sulfonic oxidation state of the peptide.

Antibody Production—The sulfonylated Prx peptide (2 mg) was coupled to 10 mg of keyhole limpet hemocyanin (Pierce) by incubation overnight at room temperature in the presence of 7 mM glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0). The peptide-hemocyanin conjugate was mixed with incomplete Freund's adjuvant for the initial injection and with complete Freund's adjuvant for booster injections. After the initial injection with 1 mg of peptide, rabbits were subjected to two booster injections, each of 500 μg of peptide, administered (at multiple subcutaneous sites) at 4-week intervals. Antisera (20–60 ml) were collected 1 week after the second booster injection, and the immunoglobulin G fraction was precipitated with 50% (w/v) ammonium sulfate. Antibodies that recognized the nonoxidized Prx peptide were removed by treating the immunoglobulin G fraction with the thiol peptide coupled to Affi-Gel-15 affinity resin (Bio-Rad).

Preparation of Oxidized Prx I—Recombinant human Prx I (5 μg) was incubated in a 200-μl reaction mixture containing 200 μM NADPH, 2.5 μM recombinant human Trx, 46 nM rat Trx reductase, 1 mM H_2O_2, and 50 mM Hepes-NaOH (pH 7.0). The oxidation reaction was initiated by the addition of H_2O_2 and continued for 30 min at 30 °C. The sulfinic oxidation state of Cys^{51} of Prx I was confirmed by MALDI-TOF mass spectrometry (12).

Cell Culture—HeLa (human cervical cancer) cells were maintained in Dulbecco's minimum essential medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin. Raw264.7 (mouse macrophage) cells were maintained in DMEM supplemented with 10% FBS. A549 (human lung epithelial type II) cells were maintained in Ham's F-12 nutrient mixture medium (Invitrogen) supplemented with 10% FBS. Cells were treated with and allowed to recover from H_2O_2 and cell lysates were prepared as described previously (12, 13).

Immunoblot Analysis—Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 14% gel or by two-dimensional gel electrophoresis as described previously (12, 13). The separated proteins were transferred eletrophoretically to a nitrocellulose membrane, which was then incubated with the antibodies to the sulfonylated Prx peptide. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce). The membrane was also probed with rabbit antibodies to Prx I, to Prx II, or to Prx III, the specificity of which has been described previously (16, 17).

RESULTS AND DISCUSSION

The NH_2-terminal conserved cysteine (Cys^{51} of Prx I) of 2-Cys Prx enzymes, which include mammalian Prx I to IV, is located within a signature motif, DFTFVCPTEI (1, 18). To explore the possibility of immunological detection of Prx proteins containing a hyperoxidized cysteine residue, we prepared rabbit antibodies to the sulfonylated signature peptide. To assess the specificity of the antibodies, we combined oxidized (sulfinylated) and nonoxidized forms of Prx I in various ratios and then subjected equal amounts of these mixtures to immunoblot analysis with the antibodies (Fig. 1*A*). The intensity of the Prx I band detected by the antibodies increased as the mole fraction of oxidized Prx I increased, suggesting that the antibodies are specific for oxidized Prx. The specificity of the antibody preparation was also apparent from immunoblot analysis of lysates derived from HeLa cells, A549 cells, or Raw264.7 cells that had been treated or not with H_2O_2 (Fig. 1*B*). For all three cell types, the antibodies to the sulfonylated Prx peptide detected Prx isoforms (one band for Prx I and Prx II, which contain the same number of amino acid residues, and another for Prx III) only in the H_2O_2-treated cells, not in the nontreated cells.

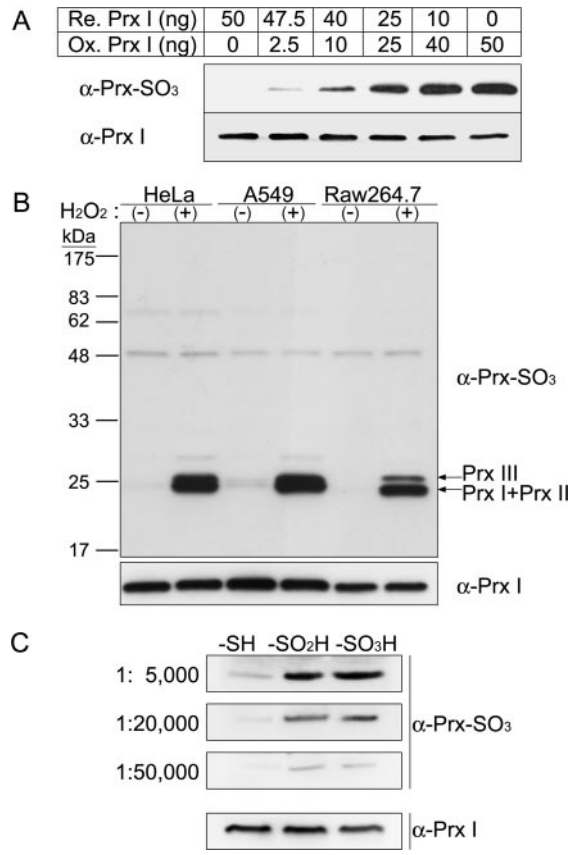

FIG. 1. **Characterization of rabbit antibodies to a sulfonylated Prx peptide.** *A*, nonoxidized Prx I (*Re. Prx I*) and Prx I hyperoxidized on Cys^{51} (*Ox. Prx I*) were mixed in the indicated ratios to yield samples each containing a total of 50 ng of Prx I. The samples were subjected to immunoblot analysis with the antibodies to the sulfonylated Prx peptide (*α-Prx-SO$_3$*) and with antibodies to Prx I (*α-Prx I*). *B*, HeLa, A549, or Raw264.7 cells were incubated for 30 min in the absence or presence of 1 mM H_2O_2, after which cell lysates (25 μg of protein) were subjected to immunoblot analysis with α-Prx-SO_3 and α-Prx I. Prx I and Prx II, which contain the same number of amino acid residues, comigrate. *C*, sulfonylated Prx I was produced by incubation of 5 μg of the oxidized (sulfinylated) protein (prepared as described under "Experimental Procedures") in 2% SDS sample buffer for 30 min at 30 °C with 1 mM H_2O_2. The sulfonic oxidation state of the protein was confirmed by mass spectrometry. Ten nanograms each of nonoxidized Prx I (*-SH*), sulfinylated Prx I (*-SO$_2$H*), and sulfonylated Prx I (*-SO$_3$H*) were then subjected to immunoblot analysis with the indicated dilutions of α-Prx-SO_3 and with α-Prx I under anaerobic conditions. All solutions used for the analysis were purged with nitrogen gas to remove solubilized oxygen, and sodium thioglycolate (final concentration, 0.1 mM) was added to the electrophoresis buffer to prevent oxidation during SDS-PAGE.

The active site Cys-SH of Prx is oxidized by H_2O_2 to sulfinic acid both *in vitro* and *in vivo* (10–13). The sulfinylated enzyme is resistant to further oxidation, but it undergoes a slow autoxidation to the sulfonylated form when the enzyme is denatured (12). Oxidized Prx that has been subjected to SDS-PAGE thus contains both sulfinylated and sulfonylated forms of the protein. Given that a sulfonylated peptide was used to generate the rabbit antibodies, we investigated the possibility that the antibodies recognize sulfonylated Prx but not the sulfinylated form of the enzyme. We prepared sulfonylated Prx I by further

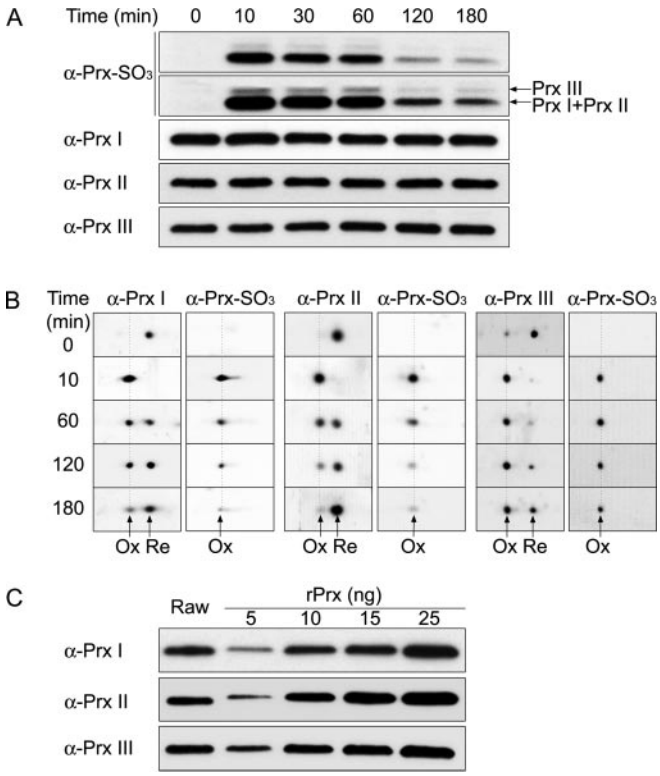

FIG. 2. **Reversible sulfinylation of Prx I, Prx II, and Prx III in Raw264.7 cells monitored by immunoblot analysis with the sulfinylation-sulfonylation-specific antibodies.** *A* and *B*, Raw264.7 cells were exposed to 200 μM H_2O_2 for 10 min, washed with Hanks' balanced salt solution, and incubated for various times in DMEM supplemented with 10% FBS and cycloheximide (10 μg ml^{-1}). Cell lysates (25 and 150 μg of protein, respectively) were analyzed by SDS-PAGE (*A*) or two-dimensional PAGE (*B*) followed by immunoblot analysis with the sulfinylation-sulfonylation-specific antibodies (*α-Prx-SO$_3$*) or with antibodies to Prx I (*α-Prx I*), to Prx II (*α–Prx II*), or to Prx III (*α-Prx III*), as indicated. In all panels, the times shown represent the total time elapsed, including the 10-min period of H_2O_2 treatment. The blot with α-Prx-SO$_3$ shown in *A* was exposed to x-ray film for 30 s (*upper panel*) or 60 s (*lower panel*). The region of the two-dimensional gels shown in *B* corresponds to molecular sizes (*vertical*) of 22–28 kDa and to isoelectric points (*horizontal*) of 7.6–8.2 for Prx I, 4.9–5.6 for Prx II, and 5.9–6.4 for Prx III. The positions of oxidized (*Ox*) and reduced (*Re*) Prx enzymes are indicated. *C*, lysates (5 μg for Prx I and Prx II, 10 μg for Prx III) prepared from untreated Raw264.7 cells and the indicated amounts of recombinant Prx I, Prx II, or Prx III were subjected to immunoblot analysis with the corresponding antibodies.

oxidizing the sulfinylated enzyme with H_2O_2 under denaturing conditions and then subjected the two forms to immunoblot analysis in an anaerobic chamber with reagents rendered free of oxygen. The resulting blots revealed no substantial difference in intensity between the sulfinylated and sulfonylated Prx I bands, even when the antibodies were diluted until the blot intensities became faint (Fig. 1*C*). Thus, although a sulfonylated peptide was used as the immunogen, the resulting antibodies recognize both the sulfinylated and sulfonylated forms of Prx with similar avidity, rendering it unnecessary to take special precautions to oxidize Prx completely to the sulfonic state before immunoblot analysis.

We next used the sulfinylation-sulfonylation-specific antibodies to monitor the formation and disappearance of sulfinylated Prx enzymes. Raw264.7 cells were exposed to 200 μM H_2O_2 for 10 min and then incubated for various times in H_2O_2-free medium in the presence of cycloheximide, after which cell lysates were subjected to immunoblot analysis (Fig. 2A). As observed in Fig. 1*B*, the sulfinylation-sulfonylation-specific antibodies yielded a pronounced band for both Prx I and Prx II and a lower intensity band for Prx III only in cells exposed to H_2O_2. The intensity of the band corresponding to oxidized Prx I and Prx II gradually decreased with time after removal of H_2O_2, whereas the intensities of the bands detected with antibodies to Prx I, to Prx II, or to Prx III remained unchanged during the time course. Exposure of the immunoblot obtained with the sulfinylation-sulfonylation-specific antibodies for a longer period of time also revealed a gradual decrease in the intensity of the oxidized Prx III band, although the rate of this decrease was lower than that apparent with the oxidized Prx I/II band. Given that new protein synthesis was blocked by cycloheximide, these results are indicative of conversion of the oxidized enzymes rather than of their turnover (degradation of oxidized enzymes and *de novo* synthesis). We also subjected Raw264.7 cell lysate, together with purified Prx I, Prx II, and Prx III, to immunoblot analysis with antibodies to each Prx isoform (Fig. 2*C*). By comparing the resulting immunoblot intensities, we estimated that the amounts of Prx I, Prx II, and Prx III in Raw264.7 cells are ~3, ~2, and ~0.7 μg/mg of soluble protein, respectively. Given that the amounts of Prx I and Prx II are similar in these cells and that the immunoblot intensity of the Prx I/II band obtained with the sulfinylation-sulfonylation-specific antibodies decreased to near zero after removal of H_2O_2 from the culture medium, the sulfinylated forms of both Prx I and Prx II must have been reduced in response to H_2O_2 withdrawal.

The lysates derived from Raw264.7 cells after treatment with and removal of H_2O_2 were also subjected to two-dimensional electrophoresis before immunoblot analysis. Antibodies to each isoform revealed a near complete acidic shift of Prx I, Prx II, and Prx III on exposure of the cells to H_2O_2, and this shift was followed by the gradual reversion of the immunoreactive spots to the normal position after H_2O_2 removal (Fig. 2*B*). The sulfinylation-sulfonylation-specific antibodies recognized the acidic spots but not the original or recovered normal spots for the three Prx enzymes. These observations thus support the notion that reduction of the sulfinic moiety is the major mechanism underlying the reversion of acidic Prx enzymes to the normal form (13).

Finally, with the use of immunoblot analysis with the sulfinylation-sulfonylation-specific antibodies, we monitored the sulfinylation of Prx isoforms in A549 (Fig. 3*A*) and HeLa (Fig. 3*B*) cells that had been exposed to 100 μM H_2O_2 for 10 min and then allowed to recover in the presence of cycloheximide. For A549 cells, the intensity of the band corresponding to sulfinylated Prx I and Prx II gradually decreased to near zero after removal of H_2O_2. Given that the abundance of Prx I is much greater than that of Prx II in A549 cells (Fig. 3*C*), these results show that the sulfinylation of Prx I is reversible in these cells, as was that of Prx II. Although the rates were slower for HeLa cells than for Raw264.7 or A549 cells, the reduction of the three oxidized Prx isoforms was also apparent in HeLa cells.

While this work was in progress, Chevallet *et al.* (15) reported that, among the four isoforms of Prx (Prx I, II, III, and VI) they studied, sulfinylation of only Prx II was reversible during recovery of cells from oxidative stress. This study (15) was performed by mass spectrometric analysis of spots on two-dimensional gels derived from HeLa cells that had been pulse-labeled with deuterated lysine. The researchers concluded that the regeneration of reduced Prx III and Prx VI was achieved by *de novo* synthesis, not through reduction of the sulfinylated enzymes. The oxidized Prx I molecules in the acidic spot returned to the normal spot as observed with Prx II. Unexpectedly, however, mass spectrometry revealed that the returned Prx I molecules still harbored cysteine sulfinic acid. Chevallet *et al.* (15) thus suggested that reversion of the acidic spot corresponding to sulfinylated Prx I to the normal spot

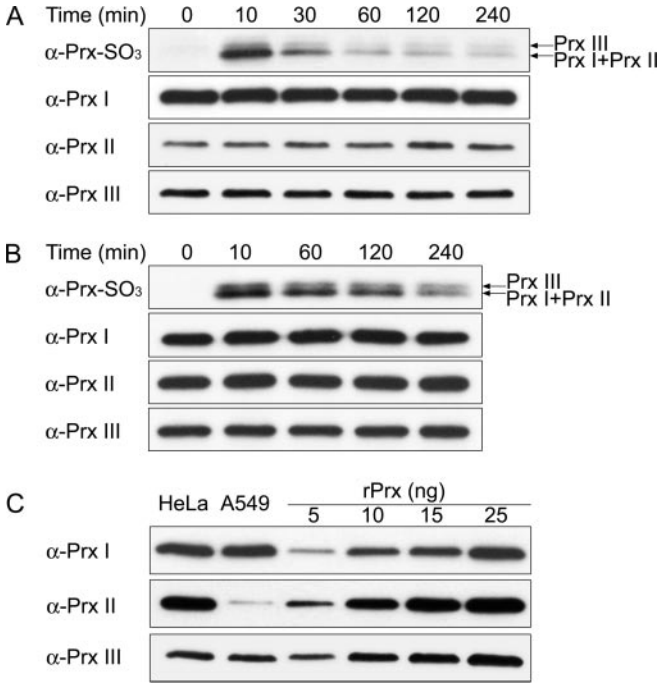

FIG. 3. **Reversible sulfinylation of Prx I, Prx II, or Prx III in A549 and HeLa cells monitored by immunoblot analysis with the sulfinylation-sulfonylation-specific antibodies.** *A* and *B*, A549 cells (*A*) and HeLa cells (*B*) were exposed to 100 μM H_2O_2 for 10 min and then allowed to recover from oxidative stress as in Fig. 2A, after which cell lysates (25 μg of protein) were subjected to immunoblot analysis with the indicated antibodies. In all panels, the times shown represent the total time elapsed, including the 10-min period of H_2O_2 treatment. *C*, lysates (5 μg for Prx I and Prx II, 10 μg for Prx III) of untreated HeLa or A549 cells and the indicated amounts of recombinant Prx I, Prx II, or Prx III were subjected to immunoblot analysis with the corresponding antibodies.

corresponding to the reduced enzyme may not be the result of *bona fide* reduction of sulfinic acid to thiol. This observation indicates that mechanisms other than sulfinic acid reduction (such as dephosphorylation, decarboxylation, deacylation, and arginylation) can result in the shift of Prx I from the acidic spot to the normal spot. Our immunoblot analysis with the sulfinylation-sulfonylation-specific antibodies, however, failed to detect sulfinylated or sulfonylated Prx I in the normal spot of Prx I molecules returned from the acidic spot, suggesting that reduction of sulfinic acid is the major mechanism underlying the acidic-to-normal shift. Although we cannot explain this discrepancy, there are two caveats to the interpretation of the study of Chevallet *et al.* (15). First, MALDI-TOF mass spectrometry is a sensitive, but not a quantitative, technique. Although it can detect small amounts of sulfinylated enzyme, it cannot determine the percentage of molecules in the normal spot that contain sulfinic acid. Second, the rate of reversion of the oxidized spot to the normal spot was slow in HeLa cells, with the consequence that the mass spectral analysis of the normal spot was performed with <10% of total Prx I molecules (Fig. 7 in Ref. 15).

Given that the amino acid sequence of Prx I is 80% identical to that of Prx II and that both proteins are located in the cytosol, it is not surprising that both enzymes manifest the same redox chemistry in response to oxidative stress. Prx III also shares 60% sequence identity with Prx II but is located in mitochondria. Our data indicate that sulfinylated Prx III is also reduced during the recovery of cells from oxidative stress, albeit at a rate slower than that apparent for Prx I or Prx II, suggesting that a sulfinyl-reducing system is also present in mitochondria. We were not able to examine the other three mammalian isoforms of Prx (Prx IV to VI) in the present study, because Prx IV (a secreted protein) is not expressed in the three cell types analyzed and the sulfinylation-sulfonylation-specific antibodies do not recognize oxidized Prx V or Prx VI.

In conclusion, our study is the first to describe the preparation and characterization of antibodies that specifically recognize sulfinylated or sulfonylated proteins. Our immunoblot method offers distinct advantages relative to existing techniques for the detection of such proteins that require isotopic labeling of cells, two-dimensional electrophoresis, and mass spectrometry (13, 15). The reversible sulfinylation of Prx has been proposed to constitute a mechanism for the regulation of H_2O_2 signaling (3, 19). Sulfinylation does not appear to be a rare event, given that 1–2% of the cysteine residues of soluble proteins from rat liver were detected as cysteine sulfinic acid; in contrast, cysteine sulfonic acid was not detected (20). Moreover, given that Prx I was recently shown to function as a tumor suppressor in aging mice (21), reactivation of sulfinylated Prx I by reduction might be important for the prevention of carcinogenesis. The immunoblot assay described here is likely to facilitate characterization of this reduction mechanism and of the biological function of sulfinylation.

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