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 H2O2-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 sulfenylation of Prx II, but not that of Prx I or Prx III, is irreversible (Chevatte, M., Wagner, E., Luche, S., van Dorsselaer, A., Leize-Wagner, E., and Rabilloud, T. (2003) J. Biol. Chem. 278, 37146–37153). The detection of sulfonlated proteins in both of these previous studies relied on complex proteomics analysis. We now describe a simple immunoblot assay for the detection of sulfonlated Prx enzymes that is based on antibodies produced in response to a sulfonlated peptide modeled on the conserved active site sequence. These antibodies recognized both sulfonic and sulfinic forms of Prx equally well and allowed the detection of sulfonlated Prx enzymes in H2O2-treated cells with high sensitivity and specificity. With the use of these antibodies, we demonstrated that not only the cystolic enzymes Prx I and Prx II but also the mitochondrial enzyme Prx III undergo reversible sulfonation. The generation of antibodies specific for sulfonlated peptides should provide insight into protein function similar to that achieved with antibodies to peptides containing phosphoserine or phosphothreonine.

Peroxiredoxins (Prxs)3 are a family of peroxidases that reduce hydrogen peroxide (H2O2) and alkyl hydroperoxides to water and alcohol, respectively, with the use of the reducing equivalents provided by thiol-containing proteins such as thioredoxin (1–3). All Prx enzymes exist as homodimers, and Prx I and Prx IV, which are members of the 2-Cys Prx subgroup, each contain two conserved Cys residues corresponding to Cys45 and Cys172 of mammalian Prx I (4). Prx I and Prx II exist in the cysteol; 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 NH2-terminal conserved cysteine (Cys51 of Prx I) to 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 Lys127 (8, 9), is selectively oxidized by H2O2 to Cys-SOH. The unstable Cys51–SOH reacts with Cys172–SH of the other subunit of the homodimer to form an intermolecular disulfide, which is subsequently reduced by thioredoxin (Trx) (10). Because Cys51 and Cys172 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 sulfonic acid (Cys–SO2H), resulting in inactivation of peroxidase activity (10–12).

Proteins that contain hyperoxidized cysteine residues (Cys–SO2H or Cys–SO3H) 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 35S, we observed that, on two-dimensional gels, the 35S-labeled acidic spots corresponding to sulfonlated Prx I and Prx II increased in intensity during exposure of cells to H2O2 and then underwent a shift back to the spots corresponding to the respective reduced forms after removal of H2O2 in the presence of the protein synthesis inhibitor cycloheximide (13). This observation led us to propose that the sulfonation 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 sulfonlated peptide based on the active site sequence common to mammalian Prx I to IV. With the use of immunoblot analysis with these antibodies, we reinvigated Prx oxidation and now not only confirm the reversibility of sulfonation of cystolic Prx isoforms but also demonstrate reversibility of the sulfonation of mitochondrial Prx.
These results are thus discrepant with the recent observation by Chevallet et al. (15) that the sulfination of Prx II, but not that of Prx I or Prx III, is reversible.

**EXPERIMENTAL PROCEDURES**

Preparation of a Sulfonlated Prx Peptide—A peptide (DFT-FVCPTPEI), 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 H2O2, 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 C18 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 MALDI-TOF mass spectrometry to confirm the sulfonic oxidation state of the peptide.

Antibody Production—The sulfonlated 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% (v/v) ammonium sulfate. Antibodies that recognized the nonoxidized Prx peptide were removed by treating the immunoglobulin G fraction with the thiold peptide coupled to Affi-Gel-15 affinity resin (Bio-Rad).

Preparation of Oxidized Prx I—Recombinant human Prx I (1.5 μg) was incubated in a 200-μl reaction mixture containing 200 μM NADPH, 2.5 μM recombinant human Trx, 46 μM rat Trx reductase, 1 mM H2O2, and 50 mM Hepes-NaOH (pH 7.0). The oxidation reaction was initiated by the addition of H2O2 and continued for 30 min at 30 °C. The sulfonic oxidation state of Cys51 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 H2O2 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 electrophoretically to a nitrocellulose membrane, which was then incubated with the antibodies to the sulfonlated Prx peptide. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce). The membrane was also probed with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce). The membrane was also probed with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce). The membrane was also probed with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce).

RESULTS AND DISCUSSION

The NH2-terminal conserved cysteine (Cys51 of Prx I) of 2-Cys Prx enzymes, which include mammalian Prx I to IV, is located within a signature motif, DFT-FVCPTPEI (1, 18). To explore the possibility of immunological detection of Prx proteins containing a hyperoxidized cysteine residue, we prepared rabbit antibodies to the sulfonlated signature peptide. To assess the specificity of the antibodies, we combined oxidized (sulfonlated) and nonoxidized forms of Prx I in various ratios and then subjected equal amounts of these mixtures to immunoblot analysis with the antibodies (Fig. 1A). The intensity of the Prx I band detected by the antibodies increased as the molar 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, which contain both sulfinated and sulfonlated forms of the protein. Given that a sulfonlated peptide was used to generate the rabbit antibodies, we investigated the possibility that the antibodies recognize sulfonlated Prx but not the sulfinated form of the enzyme. We prepared sulfonlated Prx I by further...
oxidizing the sulfinylated enzyme with H$_2$O$_2$ 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. 1C). 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$_2$O$_2$ for 10 min and then incubated for various times in H$_2$O$_2$-free medium in the presence of cycloheximide, after which cell lysates were subjected to immunoblot analysis (Fig. 2A). As observed in Fig. 1B, 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$_2$O$_2$. The intensity of the band corresponding to oxidized Prx I and Prx II gradually decreased with time after removal of H$_2$O$_2$, 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/III 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. 2C). 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$_2$O$_2$ from the culture medium, the sulfinylated forms of both Prx I and Prx II must have been reduced in response to H$_2$O$_2$ withdrawal.

The lysates derived from Raw264.7 cells after treatment with and removal of H$_2$O$_2$ 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$_2$O$_2$, and this shift was followed by the gradual reversion of the immunoreactive spots to the normal position after H$_2$O$_2$ removal (Fig. 2B). 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 sulfenic 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. 3A) and HeLa (Fig. 3B) cells that had been exposed to 100 μM H$_2$O$_2$ 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$_2$O$_2$. Given that the abundance of Prx I is much greater than that of Prx III in A549 cells (Fig. 3C), 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 sulfenic acid. Chevallet et al. (15) thus suggested that reversion of the acidic spot corresponding to sulfinylated Prx I to the normal spot...
corresponding to the reduced enzyme may not be the result of bona fide reduction of sulfenic acid to thiol. This observation indicates that mechanisms other than sulfenic 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 sulfenic 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 sulfenic 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 sulfonic 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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