Reduction of Cysteine Sulfenic Acid by Sulfiredoxin Is Specific to 2-Cys Peroxiredoxins

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Cysteine residues of certain peroxiredoxins (Prxs) undergo reversible oxidation to sulfenic acid (Cys-SO2H) and the reduction reaction is catalyzed by sulfiredoxin (Srx). Specific Cys residues of various other proteins are also oxidized to sulfenic acid, suggesting that formation of Cys-SO2H might be a novel posttranslational modification that contributes to regulation of protein function. To examine the susceptibility of sulfenic forms of proteins to reduction by Srx, we prepared such forms of all six mammalian Prx isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Purified sulfiredoxin reduced the sulfenic forms of the four 2-Cys members (Prx I to Prx IV) of the Prx family in vitro, but it did not affect those of Prx V, Prx VI, or GAPDH. Furthermore, Srx bound specifically to the four 2-Cys Prxs in vitro and in cells. Sulfinic forms of Prx I and Prx II, but not of Prx VI or GAPDH, present in H2O2-treated A549 cells were gradually reduced after removal of H2O2; overexpression of Srx increased the rate of the reduction of Prx I and Prx II but did not induce that of Prx VI or GAPDH. These results suggest that reduction of Cys-SO2H by Srx is specific to 2-Cys Prx isoforms. For proteins such as Prx VI and GAPDH, sulfenic acid formation might be an irreversible process that causes protein damage.

The sulfur atom of cysteine is able to assume several different oxidation states: the −2 state in the sulphydryl group (−SH), the −1 state in disulfide (−S−S−), the 0 state in sulfenic acid (−SOH), the +2 state in sulfonic acid (−SO2H), and the +4 state in sulfonic acid (−SO3H). Reversible oxidation of cysteine to sulfenic or sulfenic acid, both of which are readily reduced by thiols such as glutathione (GSH) and thioredoxin (Trx), is an important type of posttranslational modification that contributes to the regulation of protein function. Sulfenic and sulfonic oxidation states are also found in proteins. However, given that sulfenic and sulfonic acids were not found to be reduced by biological thiols under physiological conditions, the hyperoxidation reactions that give rise to these moieties were thought to be irreversible and to occur only under conditions of extreme oxidative stress or during protein isolation. The cysteine sulfenic acid produced during the catalytic cycle of peroxiredoxins (Prxs) was nevertheless recently found to be reducible in cells (2–6).

Members of the Prx family of peroxidases are present in organisms from all kingdoms (7–9). All Prx enzymes contain a conserved Cys residue in the NH2-terminal portion of the molecule, and most contain an additional conserved Cys in the COOH-terminal region. Prx enzymes exist as homodimers, with the two monomers arranged in a head-to-tail manner, and the dimers are able to aggregate further to form decamers (9). Mammalian cells express six isoforms of Prx (Prx I to VI), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) on the basis of the number and position of Cys residues that participate in catalysis (8). Prx I to Prx IV belong to the 2-Cys Prx subgroup, Prx V to the atypical 2-Cys subgroup, and Prx VI to the 1-Cys subgroup. Prx I to Prx IV thus possess the conserved NH2-terminal Cys (N-Cys) and a COOH-terminal Cys (C-Cys) that are separated by 121 amino acids. During catalysis, peroxides oxidize N-Cys–SH to sulfenic acid, which then reacts with C-Cys–SH of the other subunit to form an intermolecular disulfide. This disulfide is subsequently reduced specifically by Trx. The N-Cys–SH is also the site of oxidation by peroxides in Prx V and Prx VI, neither of which contains a C-Cys. The resulting sulfenic acid of Prx V forms an intramolecular disulfide with a Cys–SH that is separated from N-Cys by 104 amino acids, and the disulfide is reduced by Trx. In the case of Prx VI, the sulfenic acid does not form a disulfide because of the unavailability of another Cys–SH nearby; it can be reduced by nonphysiological thiols such as dithiothreitol (DTT) but not by Trx or GSH.

Studies with 2-Cys Prxs, mainly Prx I, have indicated that the N-Cys–SOH intermediate is occasionally further oxidized to sulfenic acid before it is able to form a disulfide with C-Cys–SH, resulting in inactivation of peroxidase activity (10, 11). This oxidation to sulfenic acid was recently found to be a reversible step (2–4), with the back reaction being catalyzed by each of two ATP-dependent reductases designated sulfiredoxin (Srx) and sestrin (5, 6). The N-Cys–SH of Prx V or Prx VI also undergoes hyperoxidation to sulfonic acid (4). Furthermore, oxidation of Cys to sulfonic acid does not appear to be restricted to Prx enzymes. Critical Cys residues of many other proteins, including α1-antitrypsin (12), carbonic anhydrase III (13), glyco-
eraldehyde-3-phosphate dehydrogenase (GAPDH) (14), protein-tyrosine phosphatase 1B (15), and metalloproteinases (16), are also oxidized to sulfenic acid. Indeed, 1.4% of the Cys residues of soluble proteins in rat liver were detected as sulfenic acid, whereas sulfonic acid was not detected (17). The cycle of thiol to sulfenic acid might thus represent a redox switch by which the function of a wide variety of proteins is regulated in response to a change in intracellular redox status. The operation of such a thiol-sulfenic switch would appear to require the existence of sulfenic reductases of broad specificity. We have examined the ability of Srx to catalyze the reduction of various sulfenic proteins, and we now show that Srx appears to act specifically in reduction of the sulfenic acid groups of 2-Cys Prx proteins.

EXPERIMENTAL PROCEDURES

Materials—Preparation of recombinant human Prx I, III, IV, V, and VI (18–21), recombinant rat Srx (22), and recombinant human Trx1 (18) was described previously. Rabbit antisera specific for rat Srx (22), for sulfonlated 2-Cys Prxs (3), for Prx isoform (18–21) or for human Trx1 (23), have also been described. Mouse monoclonal antibodies to β-actin, to the hemagglutinin epitope (HA), to GAPDH, or to glutathione S-transferase (GST) were obtained from Abcam, Santa Cruz Biotechnology, Chemicon International, and Santa Cruz Biotechnology, respectively. Rabbit muscle GAPDH was obtained from Sigma. Prx III was purified from outdated human erythrocytes as described (24).

Preparation of Sulfenic Proteins—Human 2-Cys Prx I (Prx I to IV) were oxidized in the presence of H2O2 (1 mM), human Trx1, and DTT as described previously for Prx I (2, 3). A similar procedure was followed for the preparation of sulfenic forms of Prx V with the exception that the concentration of H2O2 was increased to 3 mM for Prx V. Recombinant human Prx VI (250 μg) or rabbit muscle GAPDH (250 μg) was incubated for 10 min at 30 °C in a 250-μl reaction mixture containing 4 mM H2O2 and 50 mM Tris-HCl (pH 7.5). The sulfenic state of the oxidized proteins was verified by electrospray ionization-mass spectrometry (ESI-MS) as described (2, 3).

Generation of Antibodies Specific for Sulfenic Proteins—Three peptides, APTPGCSKTH, DFTPVCTTEL, and KIISNASCTTN, which correspond to the oxidation-sensitive sites of mammalian Prx V and Prx VI, and mammalian GAPDH, respectively, were oxidized and used to generate rabbit antibodies specific for the sulfonlated proteins as described previously (3).

Construction of Expression Vectors—Complementary DNA encoding human Srx was cloned into the BamHI and NotI sites of pEBG-SrfI (kindly provided by Y. Liu, NIA, National Institutes of Health) or the XbaI and BamHI sites of pCGN (kindly provided by W. Herr, Cold Spring Harbor Laboratory) for the expression of a GST-Srx fusion protein or HA-tagged Srx, respectively. Cells were transfected with these vectors with the use of FuGENE 6 (Roche Applied Science).

RESULTS

Srx Reduces the Cys Sulfenic Acid of 2-Cys Prx Enzymes but Not That of Atypical 2-Cys Prx, 1-Cys Prx, or GAPDH in Vitro—Sulfenic forms of the six human Prx isoforms (Prx I to VI) were prepared, and their sulfenic oxidation state was verified by ESI-MS (data not shown). They were then incubated with Srx in the presence of MgCl2 and ATP, and the resulting assay mixtures were subjected to two-dimensional PAGE followed by immunoblot analysis (Fig. 1). Sulfenic Prx are detected at a more acidic position than are the corresponding reduced proteins because of the presence of the negatively charged sulfenic group. Although the rates of reduction differed, Srx gradually regenerated the reduced forms of the four 2-Cys Prxs. Srx failed to reduce the sulfenic forms of Prx V and Prx VI, however. Similar results were obtained when the regeneration of the reduced enzymes was monitored on the basis of recovery of Trx-dependent peroxidase activity (data not shown). To confirm the different reactivities of Prxs with Srx, we incubated a mixture of the sulfenic forms of Prx I, Prx III, and Prx VI with Srx. The two-dimensional analysis of the reaction mixture confirmed that Prx I and Prx III, but not Prx VI, were substrates of Srx (data not shown).

We also prepared sulfenic forms of rabbit muscle GAPDH. GAPDH contains four Cys residues, among which the catalytic site Cys127-SH, which is activated by the proton-extracting residue His176, is sensitive to oxidation. Incorporation of two oxygen atoms into Tsa1 and GAPDH during oxidation was detected by ESI-MS. Specific oxidation of Cys149 of GAPDH was revealed by collision-induced dissociation-tandem mass spectrometry of tryptic peptides (data not shown). We have previously developed a procedure to monitor the reduction of sulfenic 2-Cys Prx enzymes by immunoblot analysis (after one-dimensional PAGE) with antibodies (anti-2-Cys Prx-SO2−) that specifically recognize both sulfenic and sulfonic forms of these proteins. We also produced anti-Prx V-SO2−, anti-Prx VI-SO2−, and anti-GAPDH-SO2− antibodies. The specificity of these antibodies was apparent from the observation that they detected oxidized (sulfenic) forms but not nonoxidized forms of the corresponding target proteins in immunoblot analysis (Fig. 2). The reduction of the sulfenic forms of Prx I, Prx V, Prx VI, and GAPDH was examined by immunoblot analysis with the corresponding antibodies specific for the hyperoxidized state (Fig. 2). Consistent with the results obtained by two-dimensional analysis, the immunoblot intensity of the Prx I band detected by anti-2-Cys Prx-SO2− gradually decreased with time of incubation with Srx, whereas that of the band detected with antibodies to Prx I remained unchanged (Fig. 2). In contrast, the band intensities of oxidized Prx V, Prx VI, and GAPDH revealed by the corresponding antibodies to the hyperoxidized state remained unchanged during incubation with Srx. These data indicated that the reduction reaction catalyzed by mammalian Srx is specific to mammalian 2-Cys Prx enzymes.

Substrate Specificity of Sulfiredoxin

FIG. 1. Reduction of sulfenic Prx enzymes by Srx in vitro. Sulfenic human Prxs (5 μM) were incubated at 30 °C in a 50-μl reaction mixture containing 1 μM rat Srx, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM creatine phosphate, creatine phosphokinase (25 milliunits/ml), 1 mM MgCl2, 1 mM ATP, and 5 mM GSH. A portion (15 μl) of the mixture was removed after incubation for 0, 1, or 2 h (Ox, 1 h, 2 h) and was subjected to 2D PAGE followed by immunoblot analysis with antibodies specific for the corresponding Prx isozyme. Similar amounts of reduced Prxs (Re) were also subjected to the same analysis. The positions of sulfenic (Ox) and reduced (Re) Prxs are indicated.

Srx Binds to 2-Cys Prx Enzymes but Not to Atypical 2-Cys or 1-Cys Prxs—We expressed a GST fusion protein of human Srx (GST-Srx) in HeLa cells and precipitated the fusion protein from the lysates of H2O2-treated cells with GSH-agarose beads. Bead-bound proteins were fractionated by SDS-PAGE, and major bands corresponding to proteins that coprecipitated with GST-Srx were excised from the gel, subjected to in-gel digestion with trypsin, and identified by matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) mass spectrometry (data not shown). All major bands, with the exception of that corresponding to a 50-kDa protein, were identified as Prx IV, GST-Mu, Prx III, Prx I, and Prx II (Fig. 3A). The 50-kDa band was found to be associated with GSH-agrose beads even when GST-Srx was not expressed in HeLa cells (data not shown). HeLa cells express all six mammalian Prx enzymes; the abundance of Prx I, Prx II, and Prx VI is in the range of 3–7 μg/mg of soluble protein and that of Prx III and Prx V is in the range of 0.3–0.5 μg/mg (8). However, only 2-Cys Prxs, not Prx V nor Prx VI, were found associated with GST-Srx.

Human Srx contains a single Cys residue at position 99 that is essential for reductase activity and has been proposed to
form a thiosulfinate with the sulfenic moiety of Prx during the catalytic cycle (5). To test whether the Srx-Prx interaction indeed involves Cys99, we constructed a mutant (C99S) of human Srx in which Cys99 is replaced by Ser. HeLa cells transfected with expression vectors for GST-Srx, GST-Srx(C99S), or GST alone were treated with H2O2, after which GST or the GST fusion proteins were precipitated from cell lysates. Immunoblot analysis of the precipitates with antibodies specific for the sulfenic forms of 2-Cys Prxs, Prx V, or Prx VI, or GAPDH, respectively. The blots were reprobed with antibodies to Prx I, Prx V, Prx VI, and GAPDH, respectively. Reduced forms (Re) of the proteins were similarly analyzed.

We also examined the interaction between Srx and Prx with the yeast two-hybrid system. Yeast cells transformed with a bait plasmid for human Srx and prey plasmids for Prx I, Prx II, Prx III, or Prx IV grew poorly on selective medium (Fig. 3C). These results thus provided further support for the notion that Srx interacts only with 2-Cys Prxs.

Overexpression of Srx Promotes the Reduction of Sulfenic Forms of 2-Cys Prxs but Not That of Atypical 2-Cys Prx, 1-Cys Prx, or GAPDH—To study the specificity of Srx in cells, we transfected A549 human lung epithelial cells with an expression vector for HA-tagged human Srx or the corresponding empty vector (Fig. 4). We have previously shown that attachment of GST to or deletion of 16 amino acid residues from the NH2 terminus of human and rat Srx did not affect its reductase activity measured in vitro (22). The transfected cells were exposed to 1 mM H2O2 for 10 min to induce protein sulfinylation and were then incubated for various times in the absence of H2O2 but in the presence of cycloheximide. Cell lysates were then subjected to immunoblot analysis with antibodies to 2-Cys Prxs, atypical 2-Cys Prx, 1-Cys Prx, and GAPDH—anti-2-Cys Prx-SO2, anti-Prx VI-SO2, and anti-GAPDH-SO2 (Fig. 4). The band recognized by anti-2-Cys Prx-SO2 was broad or partially separated because the molecular sizes of Prx I, Prx II, and Prx III are similar. A gradual reduction of sulfenic 2-Cys Prxs was apparent in the control cells, and this rate was greatly increased in the cells expressing HA-Srx. Treatment of cells with H2O2 induced the sulfinylation of Prx VI and GAPDH; however, reduction of the sulfinic forms of Prx VI and GAPDH was not apparent either in control cells or in cells expressing HA-Srx. Prx IV and Prx V were not analyzed in these experiments because of their low concentrations in A549 cells. These results thus indicated that reduction by Srx is specific for 2-Cys Prx enzymes in cells.

**DISCUSSION**

Our results indicate that Srx specifically binds and reduces the sulfinic forms of 2-Cys Prx isoforms but not those of other proteins exemplified by atypical 2-Cys Prx, 1-Cys Prx, and GAPDH. The binding requires neither the sulfinic oxidation state of the substrate protein nor the active site Cys of Srx. Srx was first identified and characterized in yeast as a sulfinyl reductase.
equipped with H2O2-removing enzymes, which include cata-
lytic reactive hydroxyl radical. All aerobic cells are therefore
self is not especially reactive, it can be converted to the highly
reactive hydrogen peroxide. This function of H2O2
features acquired during evolution to accommodate the intra-
cellular messenger function of H2O2. This function of H2O2
removes the low levels of H2O2 produced as a result of normal
cellular metabolism and oxidative insults. Protection of the
generated H2O2 molecules from destruction by Prx, which are present in high concentrations in the cytosol to
remove the low levels of H2O2 produced as a result of normal
cellular metabolism and oxidative insults. Protection of the
signaling function of H2O2 is probably transiently provided by
the built-in mechanism of inactivation of the two cytosolic
2-Cys Prx enzymes, Prx I and Prx II, mediated by H2O2.

Whereas Srx is restricted to the cytosol (22), Prx III, Prx IV,
and Prx V are localized predominantly to other compartments
(8). Prx III is a mitochondrial protein synthesized with a mito-
chondrial targeting sequence, Prx IV is posttranslationally pro-
cessed in the endoplasmic reticulum and secreted into the extracellular space, and Prx V is present in peroxisomes
and mitochondria. Although Srx is able to reduce sulfenic Prx III
in vitro, it remains unclear whether the reduction of Prx III in
cells is actually mediated by Srx. It is possible that a sestrin
isoform is responsible for this reduction. The substrate specif-
city and subcellular distribution of sestrins are not known.

Prx VI is also a cysteic protein (8), but the sulfenic form of
Prx VI is neither a substrate of Srx nor reduced in cells. Sulfenic
GAPDH was also not reduced either by Srx in vitro or in cells.
On the basis of these observations, we propose that, in contrast
to the reversible phosphorylation of proteins, not all sulfenic
proteins are susceptible to reduction in cells. At present, only
the 2-Cys Prx isoforms have been shown to cycle between thiol
and sulfenic acid states. For certain proteins such as Prx VI and
GAPDH, sulfenic acid formation might be an irreversible
process that causes protein damage.

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