Immunoblot Detection of Proteins That Contain Cysteine Sulfinic or Sulfonic Acids with Antibodies Specific for the Hyperoxidized Cysteine-Containing Sequence

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Summary

The cysteine residues in the active sites of certain proteins, including peroxiredoxin (Prx), Parkinson’s disease protein DJ-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are more readily oxidized to sulfenic acid (Cys-SOH) than are other cysteines because of their environment. The unstable sulfenic acid undergoes further hyperoxidation to sulfinic (Cys-SO2H) and sulfonic (Cys-SO3H) acids. Hyperoxidation to sulfinic acid was recently implicated as a reversible posttranslational modification responsible for regulation of protein function. Studies of such a role have been hampered, however, by the technically demanding nature of assays for hyperoxidized proteins. Here we describe the production and characterization of antibodies to hyperoxidized peptides modeled on the active sites of Prx, DJ-1, and GAPDH. The utility of these antibodies in the study of protein function should prove similar to that of antibodies to peptides containing phosphoserine or phosphothreonine.

Introduction

Reactive oxygen species (ROS) are produced by all aerobic organisms as a by-product of aerobic metabolism. In addition, ultraviolet and γ irradiation of cells results in the production of ROS. A substantial increase in the intracellular concentration of ROS is generally associated with deleterious effects, including cell death by apoptosis or necrosis, in pathological conditions such as inflammation and ischemia-reperfusion. The generation of ROS also appears to be required for many normal cellular functions, including transduction of cell surface receptor signaling.

Thiols of cysteine residues in proteins are the most common targets of ROS in cells. The cysteine residues in the active sites of certain proteins are sensitive to oxidation by ROS 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. The sulfenic acid is usually unstable and either reacts with any accessible thiol to form a disulfide or undergoes further oxidation to sulfonic acid (Cys-SO₂H): the disulfide group is stable and resistant to further oxidation. The hyperoxidation to sulfonic acid 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 sulfenic acid; in contrast, cysteine sulfonic acid (Cys-SO₃H) was not detected. Hyperoxidation to sulfinic acid has been observed with a number of proteins including various isoforms of peroxiredoxin (Prx), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the Parkinson’s disease protein DJ-1.

Prxs protect cells from oxidative stress by removing hydroperoxides produced as a result of normal cellular metabolism. Mammalian cells express six isoforms of Prx (Prx I to Prx VI), which can be classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) on the basis of the number and position of cysteine residues that participate in catalysis. Prx I to Prx IV, which belong to the 2-Cys subgroup, possess two conserved cysteine residues. Prx V and Prx VI, which belong to the atypical 2-Cys and 1-Cys subgroups, respectively, contain only one conserved cysteine residue.

In the catalytic cycle of 2-Cys Prx enzymes, the NH₂-terminal conserved Cys-SH is first converted to cysteine sulfenic acid (Cys-SOH) by a peroxide. The unstable sulfenic intermediate then reacts with the COOH-terminal conserved Cys-SH of the other subunit in the homodimer to form a disulfide, which is subsequently reduced by a thiol-containing reductant, such as thioredoxin, to complete the catalytic cycle. As a result of the slow rate of its conversion to a disulfide, the sulfenic intermediate is occasionally oxidized further to cysteine sulfonic acid (Cys-SO₃H), leading to inactivation of peroxidase activity. Examination of the fate of inactivated sulfinic 2-Cys Prxs led to the unexpected finding that sulfinic acid formation is a reversible process. The enzyme responsible for the reduction of the hyperoxidized
Prxs was identified in yeast (6) and mammals (7) and was named sulfiredoxin (Srx). Reduction by Srx is specific to 2-Cys Prxs; the sulfenic forms of Prx V and Prx VI are thus not reduced by Srx (8). Moreover, Srx acts on neither the sulfenic form of GAPDH nor DJ-1 (8). This specificity is due to the fact that Srx physically associates with the 2-Cys Prxs but not with other sulfenic proteins.

In addition to peroxidase activity, 2-Cys Prxs function as molecular chaperones. While the hyperoxidation of the active site cysteine results in inactivation of peroxidase activity, the chaperone function is enhanced by the hyperoxidation (9). DJ-1 also has chaperone activity, and only the sulfenic form of DJ-1 has been shown to have significant antiaggregation properties against α-synuclein (10). Hyperoxidation induces the translocation of DJ-1 from the cytosol to the outer mitochondrial membrane and protects against mitochondrial damage (2). Accordingly, hyperoxidation of critical cysteine residue to sulfenic acid has recently drawn wide attention as a novel posttranslational modification responsible for regulation of protein function. Studies of such a role have been hampered, however, by the technically demanding nature of assays for sulfanylated proteins. 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 (2D) polyacrylamide gels. However, given that such an acidic shift is also caused by protein phosphorylation, as is the case with Prx I (11) and GAPDH (12), mass spectral analysis of the acidic forms of proteins is required to ascertain the presence of hyperoxidized Cys residues. Even with mass spectral analysis, quantitation of hyperoxidized proteins has been possible only by the application of complex procedures.

We here describe the production of antibodies to sulfonlated peptides modeled on the active sites of Prx isoforms, DJ-1, and GAPDH. Immunoblot analysis with the respective antibodies detected hyperoxidized Prxs, DJ-1, or GAPDH in H₂O₂-treated cells with high sensitivity and specificity. The utility of these antibodies in the study of protein function should prove similar to that of antibodies to peptides containing phosphoserine or phosphothreonine.

Materials and Methods

Preparation of recombinant human Prx I, V, and VI was previously described (13–15). Rabbit antisera specific for each Prx isoform were obtained from Young In Frontier (Seoul, Korea). Rabbit muscle GAPDH proteins and mouse monclonal antibodies to GAPDH were obtained from Sigma (St. Louis, MO) and Millipore (Billerica, MA), respectively. Aprotinin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were obtained from ICN Biomedicals (Costa Mesa, CA). Mouse monoclonal antibodies to DJ-1 were kindly provided by Dr. Benoit Giasson (University of Pennsylvania School of Medicine).

Preparation of sulfonlated peptides

Five peptides, DFTFVCPTEL, AFTPBCSKTH, DFTPVCTTEL, KI5NASCTTN, and GLIJAICAGPTA, which correspond to the active site of 2-Cys Prxs (Prx I to IV), Prx V, Prx VI, GAPDH, and DJ-1, respectively, were oxidized by dissolving 5 mg of each in 50 μl of performic acid (freshly prepared by mixing formic acid and hydrogen peroxide, 9:1 (v/v), and incubating the mixture for 1 h at 25°C. The peptides were then dried for 15 min under vacuum without heating, and the resulting residue was dissolved in 500 μl of water to give a concentration of 2 mg/ml. Portions (10 μg) of the oxidized peptides were 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. Major peaks (>95% for each peptide) were collected and subjected to analysis with a Voyager-STR matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) instrument to confirm their sulfonic oxidation state.

Antibody production

Sulfonlated peptides (2 mg) were individually coupled to 10 mg of keyhole limpet hemocyanin (Pierce Biotechnology, Rockford, IL) 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 conjugates were 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 corresponding nonoxidized peptide were removed by treating the immunoglobulin G fraction with the thiol peptide coupled to Affigel-15 affinity gel (Bio-Rad Laboratories, Hercules, CA).

Preparation of oxidized proteins

Recombinant human Prx I (250 μg) was incubated in a 250 μl reaction mixture containing 20 μg recombinant human Trx1, 1 mM EDTA, 10 mM DTT, 1 mM H₂O₂, and 50 mM Tris-HCl (pH 7.5). The oxidation reaction was started by the addition of H₂O₂ and continued for 30 min at 30°C. Addition of 5 μl of 50 mM H₂O₂ to the reaction mixture and incubation for 30 min were repeated three times. A similar procedure was followed for the preparation of the sulfenic form of Prx V with the exception that the concentration of H₂O₂ was increased to 5 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 residual DTT and H₂O₂ were removed by ultrafiltration. The sulfenic state of the oxidized proteins was verified by MALDI-TOF mass spectrometry.

Cell culture, treatment with H₂O₂, and preparation of cell extracts

HeLa (human cervical cancer) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) 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 (5 x 10⁶ cells/100 mm plate) were washed with Hank’s balanced salt solution (HBSS) (Invitrogen) before the addition of H₂O₂. After the cells were incubated with 100 μM H₂O₂ for 1 h, the medium was aspirated, and the cells were washed 2-3 times with HBSS before being harvested and analyzed.
solution (HBSS) twice and exposed to the indicated amount of H$_2$O$_2$ in 10 ml of prewarmed HBSS for 10 min. Then cells were rinsed twice with cold HBSS and lysed in 1 ml of lysis buffer (20 mM HEPES, pH7.4, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor [5 g/ml leupeptin, 5 g/ml aprotinin, and 1 mM AEBSF]).

**Two-dimensional (2D) gel electrophoresis**

To remove nonprotein impurities such as salts and nucleic acids, trichloroacetic acid was added to the cell lysates (250 mg of protein) to a final concentration of 10%, and the proteins were allowed to precipitate on ice for 30 min. The precipitated proteins were washed twice with ice-cold acetone and then resuspended in 250 ml of Rehydration buffer (GE Healthcare, Uppsala, Sweden) containing 3 ml of DeStreak Solution (GE Healthcare). After removal of insoluble material, each sample was applied to 13 cm Immobiline DryStrips (pH 3 to 10, nonlinear; GE Healthcare). Isoelectric focusing was performed with an IPGPhor unit (GE Healthcare), after which the focused proteins were subjected to reduction and alkylation on the strips as recommended by the manufacturer. The second-dimension SDS-PAGE was conducted on 12% gels, then subjected to immunoblot analysis.

**Immunoblot analysis**

Cell lysates were fractionated by SDS-PAGE on a 14% gel or by 2D gel electrophoresis as described above. The separated proteins were transferred electrophoretically to a nitrocellulose membrane, which was then incubated with the antibodies to the sulfonlated peptides. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (GE Healthcare). The membranes were then stripped and re-probed with antibodies to the corresponding whole proteins.

**Results**

To explore the possibility of immunological detection of hyperoxidized Cys-containing proteins, we prepared rabbit antibodies to five sulfonlated peptides based on the active site sequences of 2-Cys Prx (DFTFVCPTEI), Prx V (AFTPGC SKTH), Prx VI (DFTPVCTTEL), GAPDH (KIISNASCTTN), and DJ-1(GLIAAICAGPTA). Because the active-site sequence (DFTFVCPTEI) is the same for 2-Cys Prxs (Prx I to IV) and because the sizes of Prx I and Prx II are identical, the sulfonic forms of Prx I and Prx II cannot be differentiated by immunoblot of a one-dimensional gel. Sulfonic Prx III and Prx IV, however, can be distinguished because their sizes are different from that of Prx I/II and from each other. The Prx peptide sequence is common to four mammalian isoforms of the protein (Prx I to IV).

To assess the specificity of the antibodies generated in response to the sulfonlated Prx peptide, 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. 4–1A). 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 preparations was also apparent from immunoblot analysis with antibodies to the sulfonylated Prx VI peptide (α-Prx VI-SO3) and with antibodies to Prx VI (α-Prx VI). HeLa or A549 cells were incubated for 30 min in the absence or presence of 1 mM H2O2, after which cell lysates (25 μg of protein) were subjected to immunoblot analysis with α-Prx VI-SO3 and α-Prx VI.

FIG. 4–3. Characterization of antibodies to sulfonylated Prx VI peptide. A, Nonoxidized (Re) and Prx VI hyperoxidized on Cys-47 (Ox) were mixed in the indicated ratios to yield samples each containing a total of 100 ng of Prx VI. The samples were subjected to immunoblot analysis with antibodies to the sulfonylated Prx VI peptide (α-Prx VI-SO3) and with antibodies to Prx VI (α-Prx VI). B, HeLa or A549 cells were incubated for 30 min in the absence or presence of 1 mM H2O2, after which cell lysates (25 μg of protein) were subjected to immunoblot analysis with α-Prx VI-SO3 and α-Prx VI.

Similar experiments with a mixture of oxidized and nonoxidized GAPDH revealed the specificity of the antibodies prepared with the sulfonylated GAPDH peptide for oxidized GAPDH (Fig. 4–2A). The antibodies to the sulfonylated GAPDH peptides also detected GAPDH only in the H2O2-treated cells—not in the nontreated cells (Fig. 4–2B).

The antibodies prepared with the sulfonylated Prx VI peptide showed similar specificity for oxidized Prx VI both in recombinant proteins and cell lysates (Fig. 4–3A and B). Proteins that contain hyperoxidized cysteine residues (Cys-SO2H or Cys-SO3H) are detected at a more acidic position than are the corresponding reduced proteins on 2D gels because of the presence of the negatively charged sulfinic group. By incubation with H2O2 in cells, Prx I, II, III, and VI were partially hyperoxidized and were separated as acidic, hyperoxidized spots and basic, normal spots by 2D gels. The antibodies specific to the hyperoxidized 2-Cys Prx or Prx VI recognized the acidic spots but not the normal spots (Fig. 4–4).

The anti–Prx V-SO3 antibodies could recognize the hyperoxidized recombinant Prx V proteins specifically; however, the hyperoxidized Prx V band in cell lysate was barely detectable (Fig. 4–5A). Unlike typical 2-Cys Prxs, Prx V formed intramolecular disulfide bonds (15) and was highly resistant to the sulfinylation by H2O2 (unpublished data). DJ-1 is a ubiquitously expressed protein of the DJ-1=ThiJ=PfpI superfamily. DJ-1 functions as an atypical peroxiredoxinlike peroxidase. Furthermore, oxidative conditions induce the formation of sulfenic acid of Cys-106 of DJ-1 (16). The antibodies to the sulfonylated Cys-106 DJ-1 peptides recognized the oxidized DJ-1 from the H2O2-treated cells (Fig. 4–5).

FIG. 4–5. Characterization of antibodies to sulfonylated Prx V and DJ-1 peptides. A, Nonoxidized (Re) and hyperoxidized on Cys-48 (Ox) Prx V recombinant proteins and 50 μg of cell extracts from HeLa cells, incubated for 30 min in the absence or presence of 1 mM H2O2, were subjected to immunoblot analysis with α-Prx V-SO3 and α-Prx V. B, HeLa cells were incubated for 30 min in the absence or presence of 1 mM H2O2, after which cell lysates (25 μg of protein) were subjected to immunoblot analysis with α-DJ-1-SO3 and α-DJ-1.

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References

1. Hamann M, Zhang T, Hendrich S, and Thomas JA. Quantitation of protein sulfenic and sulfonylic acid, irreversibly

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