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Binding of peroxiredoxin 6 to substrate determines differential phospholipid hydroperoxide peroxidase and phospholipase A_2 activities

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ABSTRACT

Peroxiredoxin 6 (Prdx6) differs from other mammalian peroxiredoxins both in its ability to reduce phospholipid hydroperoxides at neutral pH and in having phospholipase A_2 (PLA_2) activity that is maximal at acidic pH. We previously showed an active site C47 for peroxidase activity and a catalytic triad S32-H26-D140 necessary for binding of phospholipid and PLA_2 activity. This study evaluated binding of reduced and oxidized phospholipid hydroperoxide to Prdx6 at cytosolic pH. Incubation of recombinant Prdx6 with 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine hydroperoxide (PLPCOOH) resulted in peroxidase activity, cys47 oxidation as detected with Prdx6-$SO_{2(3)}$ antibody, and a marked shift in the Prdx6 melting temperature by circular dichroism analysis indicating that PLPCOOH is a specific substrate for Prdx6. Preferential Prdx6 binding to oxidized liposomes was detected by changes in DNS-PE or bis-Pyr fluorescence and by ultrafiltration. Site-specific mutation of S32 or H26 in Prdx6 abolished binding while D140 mutation had no effect. Treatment of A549 cells with peroxides led to lipid peroxidation and translocation of Prdx6 from the cytosol to the cell membrane. Thus, the pH specificity for the two enzymatic activities of Prdx6 can be explained by the differential binding kinetics of the protein; Prdx6 binds to reduced phospholipid at acidic pH but at cytosolic pH binds only phospholipid that is oxidized compatible with a role for Prdx6 in the repair of peroxidized cell membranes.

Introduction

Peroxiredoxins are a widely distributed superfamily of peroxidases that use thiols to reduce enzymatically H_2O_2 and other hydroperoxide substrates [1]. These enzymes have been classified as 2-cys, 1-cys or atypical based on the number of conserved cysteines and consequent mechanism of catalysis [2–6]. Peroxiredoxin 6 is the only mammalian 1-cys peroxiredoxin and unlike the other peroxiredoxins, utilizes glutathione (GSH) [7–9] or ascorbate [10] rather than thioredoxin as the physiological reductant. In addition to H_2O_2 and short chain aliphatic hydroperoxides, Prdx6 can reduce phospholipid hydroperoxides such as phosphatidylcholine hydroperoxide (PCOOH)[1] with a relatively high rate constant similar to that for H_2O_2 reduction ($\sim 10^6\ M^{-1}\ s^{-1}$) [7,11]. This property is thought to be of major importance in the ability of Prdx6 to protect cells against oxidant stress. Reduction of the enzyme by GSH requires an intermediary step of protein glutathionylation mediated by π glutathione *S*-transferase (πGST) [8,12]. Based on the crystal structure of the protein, the catalytic Cys is situated at the base of a pocket that restricts accessibility of the resolving thiol for interaction with oxidized intermediate and restoration of the reduced state of the protein [13]. Thus, glutathionylation likely induces a conformational change that is conducive to GSH access.

Prdx6 alone among the peroxiredoxins possesses phospholipase A_2 (PLA_2) activity in addition to its peroxidatic function [5,9,11]. The PLA_2 activity of Prdx6 has been demonstrated to have an important role in the metabolism of lung surfactant phospholipids [14]. The PLA_2 activity is maximal at pH 4 while the peroxidase activity is maximal in the pH 7–8 range [9]. Compatible with the proposed physiological role of these two activities, the presence of Prdx6 has been demonstrated in both the neutral pH (cytosol) and acidic pH (lysosomes; lamellar bodies) compartments of rat lungs [15,16]. Our previous studies to evaluate PLA_2 activity at pH 4 showed binding of Prdx6 to the phospholipid substrate as an important step leading to hydrolysis of the *sn*-2 phospholipid bond [17]. These studies provided evidence that the phospholipid head group bound at the protein surface, in the vicinity of the PLA_2 catalytic triad, while the *sn*-2 acyl chain is inserted into the protein core allowing alignment of the –OOH group to the catalytic Cys. Thus, specific positioning between the substrate and protein could explain the dual enzymatic function of Prdx6. The present study was designed to evaluate binding of Prdx6 to substrate at

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[1] *Abbreviations used:* Prdx6, peroxiredoxin 6; PCOOH, phosphatidylcholine hydroperoxide; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PLPCOOH, hydroperoxide of PLPC; *t*-BOOH, *tert*-butyl hydroperoxide; PLA_2, phospholipase A_2; MJ33, 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol; GSH, glutathione; GST, glutathione *S*-transferase; CD, circular dichroism; TBARS, thiobarbituric acid reactive substances.

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cytosolic pH compatible with its peroxidase function. These results can explain the role of cytosolic Prdx6 in protection against oxidant stress.

Materials and methods

Materials

1,2-Bis palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), egg yolk phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS), and cholesterol (chol) were purchased from Avanti-Polar Lipids (Birmingham, AL). *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DNS-PE), and 1,2-bis(1-pyrenedecanoyl1)-*sn*-glycero-3-phosphocholine (bis-Pyr-PC) were purchased from Molecular Probes (Eugene, OR). MJ33 (1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol), mercaptosuccinic acid, H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH), catalase and all other chemicals were purchased from Sigma (St. Louis, MO). The polyclonal antibody to Prdx has been described previously [18]. Antibody to oxidized Prdx6 (sulfinic/sulfonic Cys) was obtained from Lab Frontier (Seoul, Korea). PLPC hydroperoxide (PLPCOOH) was prepared by treatment of PLPC with 15-lipoxygenase obtained from Cayman Chemical (Ann Arbor, MI) and was purified using standard procedures [7,18]. Anti-caveolin-1 monoclonal antibody was from BD Bioscience (San Jose, CA). A459 cells were obtained by the American Type Culture Collection (ATCC, Manassas, VA).

Expression of recombinant Prdx6

Rat wild type and mutant Prdx6 proteins (S32A, H26A, D140A and C47S) were expressed in pETBlue-1 Novagen, Madison, WI) as described previously [9,17]. S32L, S32T and S32 V were prepared using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), following protocols recommended by the supplier. Mutagenic oligonucleotide primer pairs were purchased from Operon Biotechnologies (currently Eurofins Mwg/Operon, Huntsville, AL). The sequences are shown in Supplementary Data. S32, H26 and D140 are the putative amino acids constituting the PLA_2 catalytic triad and C47 is the catalytic residue for peroxiredoxin activity as described earlier [17]. Rat Prdx6 contains a single Cys residue and lacks the additional non-catalytic Cys present in the human and mouse proteins [17,19].

Proteins were purified by column chromatography which gave a homogeneous product as determined by SDS–PAGE and Western blot (not shown). Size-exclusion FPLC (YMC-10 column, 500 × 8 mm ID, 120 Å pore size from Waters, Milford, MA using 1.0 ml/min isocratic elution with 0.15 M NaCl in 0.1 M phosphate buffer, pH 7.0 [8] demonstrated a single peak for the S32A mutant at 25 kDa while two major peaks were seen for the other proteins compatible with the 25 kDa monomeric and 50 kDa homodimeric forms [8,17]. Treatment with NBD-Cl followed by spectrophotometric detection of the NBD-Prdx6 adduct at 347 nm, using an extinction coefficient of 13,400 $M^{-1}\,cm^{-1}$ [20], indicated that ~70% of cys in recombinant wild type protein was oxidized to the sulfenic acid state (data not shown). Prdx6 was phosphorylated in vitro by incubation with extracellular regulated kinase 2 (Erk 2) as described previously [21].

Liposome preparation

Unilamellar liposomes were prepared from a mixture of DPPC: egg PC: chol: PG (50:25:15:10, mol/mol) by extrusion under pressure [17]. We have used this composition for liposomes as a mimic for the lung surfactant. For fluorescently labeled liposomes, 2 mol% of egg PC was equivalently replaced with DNS-PE or bis-Pyr-PC. To prepare an oxidized liposomal phospholipid substrate, PLPC was substituted for egg PC; these liposomes were peroxidized by exposure to an $^{\bullet}OH$-generating system (10 μM Cu^{2+} in the presence of 0.20 mM ascorbate) [22] for 45 min at room temperature followed by dialysis for 2 h against PBS (pH 7.4) using a 10 kDa molecular mass cut-off Slide-A-Lyzer® dialysis cassette (Pierce, Rockford, IL). Liposomal lipids were extracted [23] and analyzed by HPLC using PLPC and PLPCOOH as standards [18]; ~70% of PLPC in the liposomes was oxidized by the Cu^{2+}/ascorbate treatment. The reduced phospholipid (PLPC) was detected by absorbance at 205 nm and the oxidized phospholipid (PLPCOOH) at 235 nm [18]. Analysis by dynamic light scattering (DLS 90 Plus, Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY) showed a homogeneous population of liposomes with a diameter of ~100–120 nm for the standard vesicles and minimal effect of oxidation or Prdx6 addition as shown previously for study at pH 4 [17]. Assuming a surface area per phospholipid of ~63 $Å^2$ [17] and a liposome radius of ~500 Å, each vesicle theoretically contains ~100,000 phospholipid molecules.

Enzymatic activity

Peroxidase activity was measured by the initial slope of the change in NADPH fluorescence in the presence of GSH and GSH reductase with H_2O_2, PLPCOOH, or oxidized liposomes as substrate in 40 mM PBS, pH 7.4, 5 mM EDTA, 1 mM NaN_3, and πGST equimolar to Prdx6; for assay with PLPCOOH, 0.1% Triton X-100 was added [7]. Protein concentration was measured using the Bradford Protein Assay (BioRad, Hercules, CA). To analyze products of the peroxidase reaction, the lipids were extracted from the reaction mixture containing oxidized liposomes at 2 and 5 min [23], dried under N_2, dissolved in propanol-2, and analyzed by reverse phase HPLC as described previously [18]. PLA_2 activity was measured from the appearance of ^{3}H-palmitate during a 1-h incubation with the standard liposomes containing DPPC labeled with 9, 10-^{3}H-palmitate in the *sn*-2 position. Incubation was in Tris-EGTA buffer, pH 7.4 in the absence of Ca^{2+}. At the end of incubation with either reduced or oxidized liposomes, lipids were extracted, separated by TLC, and analyzed by scintillation counting as described previously [24].

Protein fluorescence

Protein fluorescence was measured with a PTI spectrofluorometer (Photon Technology International, Inc., Lawrenceville, NJ) equipped with a single photon counting system for fluorescence intensity detection, dual fluorescence and absorbance channels, and a temperature controlled sample holder using excitation and emission slits of 1 and 2 nm, respectively. For tryptophanyl fluorescence, 0.5 μM protein in 40 mM PBS, pH 7.4, in a 5 × 5 mm quartz cuvette at 22 °C was assayed with excitation at 295 nm to minimize input of phenylalanine and tyrosine residues; final spectra were corrected by subtracting the appropriate controls [17].

Fluorescence detection of Prdx6 binding to liposomes

Prdx6 binding was studied with fluorescent liposomes labeled with N-DNS-PE or bis-Pyr-PC [17]. The buffers used for these studies were equilibrated with 100% N_2 before fluorescence measurements (45 min at 4 °C) to minimize the possibility of O_2-induced quenching. Studies were performed at 22 ± 0.5 °C with constant stirring. Kinetics were determined using the time-based ratiometric mode of the fluorometer with recording of one measurement per second for ~1000 s before and ~3000 s after addition of protein to liposomes (100 μM total phospholipid in 40 mM PBS, pH 7.4). Fluorescence was monitored at 415/505 nm for N-DNS-PE or 382/470 nm for bis-Pyr-PC. The binding analysis is based on shielding by bound Prdx6 of the DNS chromophore on the liposome surface from the surrounding buffer resulting in a short wavelength shift of DNS fluorescence (from 505 to 415 nm) or, for bis-Pyr-PC,