Escherichia coli thiol peroxidase (Tpx, p20, scavengase) is part of an oxidative stress defense system that uses reducing equivalents from thioredoxin (Trx1) and thioredoxin reductase to reduce alkyl hydroperoxides. Tpx contains three Cys residues, Cys\textsuperscript{61}, Cys\textsuperscript{82}, and Cys\textsuperscript{95}, and the latter residue aligns with the N-terminal active site Cys of other peroxidases in the peroxiredoxin family. To identify the catalytically important Cys, we have cloned and purified Tpx and four mutants (C61S, C82S, C95S, and C82S,C95S). In rapid reaction kinetic experiments measuring steady-state turnover, C61S is inactive, C95S retains partial activity, and the C82S mutation only slightly affects reaction rates. Furthermore, a sulfenic acid intermediate at Cys\textsuperscript{61} generated by cumene hydroperoxide (CHP) treatment was detected in UV-visible spectra of 4-nitrobenzo-2-oxa-1,3-diazole-labeled C82S,C95S, confirming the identity of Cys\textsuperscript{61} as the peroxidatic center. In stopped-flow kinetic studies, Tpx and Trx1 form a Michaelis complex during turnover with a catalytic efficiency of 3.0 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}, and the low K\textsubscript{m} (9.0 μM) of Tpx for CHP demonstrates substrate specificity toward alkyl hydroperoxides over H\textsubscript{2}O\textsubscript{2} (K\textsubscript{m} > 1.7 mM). Rapid inactivation of Tpx due to Cys\textsuperscript{61} oxidation is observed during turnover with CHP and a lipid hydroperoxide, 15-hydroperoxyeicosatetraenoic acid, but not H\textsubscript{2}O\textsubscript{2}. Unlike most other 2-Cys peroxiredoxins, which operate by an intersubunit disulfide mechanism, Tpx contains a redox-active intrasubunit disulfide bond yet is homodimeric in solution.

Oxidative stress defenses combat reactive oxygen species (1, 2) such as superoxide (O\textsubscript{2}\textsuperscript{-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and the hydroxyl radical (OH\textsuperscript{•}) generated by the host immune response, environmental factors, and the incomplete reduction of oxygen to water during aerobic respiration, all of which are hazardous to proteins, DNA, and lipids (3, 4). Escherichia coli protects cellular components from oxidative damage by employing a variety of antioxidant defense enzymes, such as hydroperoxidases (catalases) I and II (gene products of katG and katE, respectively) that decompose H\textsubscript{2}O\textsubscript{2} (5), and superoxide dismutases (manganese superoxide dismutase, sodA; iron superoxide dismutase, sodB; copper-zinc superoxide dismutase, sodC) (6) that eliminate O\textsubscript{2}\textsuperscript{-}. Additional defenses in E. coli against alkyl and lipid hydroperoxides are provided by multiple, non-heme peroxidases including 1) the peroxidatic component from the alkyl hydroperoxide reductase system (AhpC)\textsuperscript{1} (7); 2) a weakly active, thioredoxin (Trx)-dependent bacterioferritin-comigratory protein (BCP) (8); and 3) the periplasmic thiol peroxidase (Tpx, p20, scavengase) (9). In addition, a glutathione peroxidase homologue, the gene product of \textit{binE} (10), has been identified in \textit{E. coli}, and preliminary investigations have indicated Trx-dependent peroxidatic activity against organic hydroperoxides (ROOH) and H\textsubscript{2}O\textsubscript{2}.\textsuperscript{2}

AhpC, BCP, and Tpx are all members of the ubiquitous peroxidase (Prx) family within the Trx superfamily of protein folds; however, the three \textit{E. coli} Prx members are highly diverged from one another and are representative of three distinct Prx subfamilies (11–13). Generally, the Prx active site contains a disulfide bond composed of a conserved N-terminal Cys (Cys\textsuperscript{66} from \textit{Salmonella typhimurium} AhpC) and the C-terminal Cys (Cys\textsuperscript{165} from \textit{S. typhimurium} AhpC) from the other subunit of the antiparallel dimer, resulting in two symmetrical active sites per dimer (14). Most Prxs contain two conserved Cys (2-Cys Prxs); however, in some homologues, only the N-terminal Cys is retained (the 1-Cys Prxs) (15). In many instances, the homodimers assemble into toroid-shaped decamers (15), a redox-dependent process in \textit{S. typhimurium} AhpC whereby reduced decamers disassociate into dimers upon oxidation (16). To detoxify peroxides, the reduced N-terminal Cys attacks the peroxide -O–O- bond, with concomitant formation of a Cys sulfenic acid (Cys-SOH) intermediate, which then condenses with the C-terminal Cys to regenerate the stable disulfide bond at the active site (14, 17). For most bacterial Prxs, disulfide reduction is achieved by a specialized electron donor, AhpF (18), whereas many other Prx systems (both bacterial and eukaryotic) receive electrons from a reducing system composed of Trx and Trx reductase (Trxr) (13, 15).

Homologues of \textit{tpx} are distributed throughout most or all eubacterial species, both Gram-negative and Gram-positive, and are found in pathogenic strains such as \textit{Haemophilus influenzae}, \textit{Streptococcus pneumoniae}, and \textit{Helicobacter pylori} (19), but biochemical and genetic analyses have been limited primarily to \textit{E. coli} Tpx. In response to oxidative stress, \textit{E. coli}
up-regulates Tpx expression through an oxygen-responsive promoter element that is repressed by the transcriptional regulators ArcA and Fnr under anaerobic conditions (9, 20, 21). In addition, tpx deletion mutants, while still viable, were more susceptible to oxidative stress and displayed diminished colony sizes and numbers after peroxide exposure (22). In vitro studies have confirmed that Tpx forms a Trx-linked peroxidase system capable of reducing H$_2$O$_2$ and ROOH and protecting against glutathione synthetase inactivation by a mixed function oxidation system (9).

Tpx contains three Cys residues in its primary sequence, Cys$^{61}$, Cys$^{82}$, and Cys$^{95}$. Of these, Cys$^{61}$ aligns with the peroxidatic, N-terminal Cys of other Prxs; whereas Cys$^{95}$ does not align with the conserved C-terminal Cys of other 2-Cys Prxs, it is conserved among all Tpx homologues (19). Previous mutagenesis studies have presented conflicting information about which Cys residues are involved in peroxide attack and have indicated that both Cys$^{61}$ and Cys$^{95}$ are essential for activity, whereas loss of Cys$^{82}$ only slightly attenuates activity (22, 23). In this report, we identify Cys$^{61}$ as the peroxidatic Cys forming a Cys-SOH intermediate and firmly establish its intrasubunit linkage to Cys$^{95}$ in the redox-active disulfide. Although Tpx monomers are not covalently linked, analytical ultracentrifugation studies reported herein demonstrate that the enzyme is a homodimer.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**—SDS, ultrapure glycerine, ultrapure water, EDTA disodium salt, dithiothreitol (DTT), ammonium sulfate, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), Tris base, and other buffer agents were purchased from Research Organics (Cleveland, OH). Bacterial media components were from Difco. Ethanol was obtained from Warner Graham Company (Cockeysville, MD). Isopropyl $\beta$-thiogalactopyranoside and X-gal (5-bromo-4-chloro-3-indolyl-$\beta$-galactopyranoside) were from Inalc (Milan, Italy). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA). Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, Taq DNA polymerase, MgCl$_2$, and dNTPs were from Promega (Madison, WI). Agarose medium, electrophoresis grade, was from Promega (Madison, WI). E. coli medium EEO (electrophoresis grade), was from GE Healthcare. Protein markers (10, 20, 50, and 100 kDa) were from Bio-Rad. DEAE cellulose (DE52; Whatman, Kent, UK) and phenyl-Sepharose (Pharmacia, Uppsala, Sweden) were from Sigma. Ethanol and isopropanol were from Fisher. Acrylamide/bis (40%) solution was purchased from Bio-Rad. NADH was obtained from Roche Molecular Biochemicals. L-1-(Tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from Worthington. Buffer agents were from Worthington. Tpx-consuming strains were grown in LB broth containing 15% (v/v) glycerol. Culture procedures were generally the same as for wild type Tpx with a few exceptions. The pooled C61S mutant Tpx protein from the Phenyl-Sepharose column was loaded onto a 2.5-cm DEAE-cellulose column (DE52; Whatman, Kent, UK) equilibrated with 30 mM potassium phosphate and eluted with a linear gradient from 30 to 80 mM potassium phosphate (1 liter total volume). To completely purify C61S, fractions containing the mutant protein were reapplied to the Phenyl-Sepharose and DEAE-cellulose columns.

**Purification of Recombinant and Mutant Tpx Proteins**—A modification of the previous Tpx purification protocol was used for this study (9). All procedures were carried out in a standard buffer (pH 7.0) consisting of 25 mM potassium phosphate with 1.0 mM EDTA. Briefly, 100 ml of 20-ml cultures of E. coli harboring the appropriate plasmids were added to 1 liter of LB medium containing 0.5 g of ampicillin supplemented with 0.2% glucose in a BioFlo 2000 fermentor (New Brunswick Scientific, Edison, NJ). Isopropyl $\beta$-thiogalactopyranoside (0.4 mM) was added at $A_{600} = 0.9$, and bacteria were harvested by centrifugation 16 h after induction. Pelleted bacteria were disrupted with a Bead Beater (BioSpec Products, Bartlesville, OK), and cell extracts treated with streptolysin-O to precipitate nucleic acids were subjected to 30 and 75% (NH$_4$)$_2$SO$_4$ treatments to precipitate proteins (25). The protein mixture resuspended in standard buffer containing 10% (NH$_4$)$_2$SO$_4$ was applied to a 24 × 2.5-cm Phenyl-Sepharose 6 Fast Flow Column (Amersham Biosciences), washed with 10% (NH$_4$)$_2$SO$_4$ buffer, and eluted with a linear gradient of 0.3 M ammonium H$_2$O$_2$ in 50 ml of buffer. The column was then equilibrated with 0.5 M potassium phosphate (pH 7.0), the protein was loaded onto a Q-Sepharose column (Amersham Biosciences) pre-equilibrated in 5 mM potassium phosphate and eluted with a linear gradient from 5 to 30 mM potassium phosphate (1 liter total volume). Fractions were analyzed for activity using the assay with the appropriate reaction buffer. The purified Tpx and Tpx mutants were stored at $-80^\circ$C.

Purification of Recombinant and Mutant Tpx Proteins—A modification of a previous Tpx purification protocol was used for this study (9). All procedures were carried out in a standard buffer (pH 7.0) consisting of 25 mM potassium phosphate with 1.0 mM EDTA. Briefly, 100 ml of 20-ml cultures of E. coli harboring the appropriate plasmids were added to 1 liter of LB medium containing 0.5 g of ampicillin supplemented with 0.2% glucose in a BioFlo 2000 fermentor (New Brunswick Scientific, Edison, NJ). Isopropyl $\beta$-thiogalactopyranoside (0.4 mM) was added at $A_{600} = 0.9$, and bacteria were harvested by centrifugation 16 h after induction. Pelleted bacteria were disrupted with a Bead Beater (BioSpec Products, Bartlesville, OK), and cell extracts treated with streptolysin-O to precipitate nucleic acids were subjected to 30 and 75% (NH$_4$)$_2$SO$_4$ treatments to precipitate proteins (25). The protein mixture resuspended in standard buffer containing 10% (NH$_4$)$_2$SO$_4$ was applied to a 24 × 2.5-cm Phenyl-Sepharose 6 Fast Flow Column (Amersham Biosciences), washed with 10% (NH$_4$)$_2$SO$_4$ buffer, and eluted with a linear gradient of 0.3 M ammonium H$_2$O$_2$ in 50 ml of buffer. The column was then equilibrated with 0.5 M potassium phosphate (pH 7.0), the protein was loaded onto a Q-Sepharose column (Amersham Biosciences) pre-equilibrated in 5 mM potassium phosphate and eluted with a linear gradient from 5 to 30 mM potassium phosphate (1 liter total volume). Fractions were analyzed for activity using the assay with the appropriate reaction buffer. The purified Tpx and Tpx mutants were stored at $-80^\circ$C.
Initial attempts at purifying the C95S mutant under the same conditions as wild type resulted in aggregation of the mutant protein, as observed by multiple bands during SDS-PAGE of the subsequent fractions, even after many rounds of purification over the two columns. DTNB titration of the isolated protein even after DTT treatment gave less than one thiol (data not shown), suggesting that Cys11 had become irreversibly oxidized to a sulfenic (Cys-SO₂H) or sulfonic (Cys-SO₃H) acid. The addition of 2 mM DTT to all buffers prior to bacterial disruption and over the course of the purification of C95S gave pure protein after one round of purification on the two columns. C82S was purified in buffers containing 2 mM DTT according to the same protocol as C95S; however, pure C82S,C95S was obtained using a slightly altered protocol. 20% instead of 10% (NH₄)₂SO₄ was used to treat C82S,C95S prior to its application to the Phenyl-Sepharose column equilibrated with 20% (NH₄)₂SO₄, and C82S,C95S was eluted using a gradient of 20% (NH₄)₂SO₄ in 25 mM potassium phosphate with 2 mM DTT to 0% (NH₄)₂SO₄ in deionized water with 2 mM DTT. Elution from the Q Sepharose column was achieved with a gradient of 20–120 mM potassium phosphate in 2 mM DTT. Prior to assay, each mutant was subjected to ultrafiltration and washed with standard buffer to remove DTT and then immediately incubated with equal volumes of TCEP gel.

Other Protein Purifications—Purifications of E. coli TrxR (26) and E. coli Trxl (27) were carried out as described previously (25, 30). To further quantify Tpx proteins by denaturation and over the course of the purification of C95S gave pure protein after one round of purification on the two columns. C82S was purified in buffers containing 2 mM DTT according to the same protocol as C95S; however, pure C82S,C95S was obtained using a slightly altered protocol. 20% instead of 10% (NH₄)₂SO₄ was used to treat C82S,C95S prior to its application to the Phenyl-Sepharose column equilibrated with 20% (NH₄)₂SO₄, and C82S,C95S was eluted using a gradient of 20% (NH₄)₂SO₄ in 25 mM potassium phosphate with 2 mM DTT to 0% (NH₄)₂SO₄ in deionized water with 2 mM DTT. Elution from the Q Sepharose column was achieved with a gradient of 20–120 mM potassium phosphate in 2 mM DTT. Prior to assay, each mutant was subjected to ultrafiltration and washed with standard buffer to remove DTT and then immediately incubated with equal volumes of TCEP gel.

Fluorescein-5-Maleimide Labeling of Proteins—Wild type, C61S, C82S, C95S, and C61S,C95S Tpx (100 μg each), prereduced with DTT that was removed by ultrafiltration, were reacted with fluorescein-5-maleimide (10 equivalents) under denaturing (4 μl urea) and reducing (100 eq of TCEP) conditions for 16 h at 4 °C in standard buffer. Protein samples (5 μg) were separated on 18%, 20-cm-long SDS-polyacrylamide gels. Densitometry to assess the protein contents of fluorescein-labeled bands was conducted using the Quantity One quantitation software from Bio-Rad, and image files were generated with a Chemimagler 5500 digital imaging system from Alpha Innotech Corp. (San Leandro, CA) and a near UV light source and UV filter. For quantitation, six bands was conducted using the Quantity One quantitation software from Alpha Innotech Corp. (San Leandro, CA) unless otherwise noted. Microbiuret assays for proteins to determine extinction coefficients, disulfide assays with 2-nitro-5-thiosulfobenzoate, and thiol quantification with DTNB were conducted as described previously (25, 30). To further quantify Tpx proteins by absorbance, the following experimentally determined extinction coefficients at 280 nm were used: Tpx and C82S,C95S, 10,200 M⁻¹ cm⁻¹; C95S, 3500 ± 200 M⁻¹ cm⁻¹; C82S, 4200 ± 600 M⁻¹ cm⁻¹; C61S, 5300 ± 700 M⁻¹ cm⁻¹. Other extinction coefficients used were as follows: E. coli TrxR, 11,300 M⁻¹ cm⁻¹ (454 nm) (31); E. coli Trxl, 13,700 M⁻¹ cm⁻¹ (280 nm) (32); S. typhimurium AhpC, 24,300 M⁻¹ cm⁻¹ (280 nm) (25); S. typhimurium AhpF, 13,100 M⁻¹ cm⁻¹ (450 nm) (29); E. coli glutaredoxin 1, 12,400 M⁻¹ cm⁻¹ (280 nm) (33); NADPH, 6200 M⁻¹ cm⁻¹ (540 nm); NADH, 6220 M⁻¹ cm⁻¹ (340 nm); 2-nitro-5-thiosulfonate (TNB⁻), 14,150 M⁻¹ cm⁻¹ (412 nm) (34).

Wild type, C61S, C82S, and C82S,C95S Tpx (100 μg each), prereduced with DTT and then incubated with NBD chloride were carried out as described previously for H. pylori Trxl (35), with a few exceptions. Each mutant (171 μg each) was loaded into the remaining sectors as a reference. To prepare proteins for di-
solvent removal, exhaustive tryptic digestion of Tpx, C61S, or C95S in either oxidized or reduced forms was carried out as described (30). Tryptic maps were generated by injecting samples into a Rainin Dynamax HPLC system equipped with an Aquapore RP-300 C8 column (4.6 × 100 mm) and were eluted using a 90-min gradient consisting of 5–60% Solvent B (Solvent A was 0.1% trifluoroacetic acid in deionized ultrapure H2O; Solvent B was 70% acetonitrile with 0.08% trifluoroacetic acid in H2O). Peaks were detected at 215 and 254 nm on a Dynamax UV-DII dual wavelength detector. To isolate the disulfide-containing fragments, peaks at 68 (P1) and 70 min (P2) from oxidized Tpx were isolated and then further purified on a shallower gradient. Acetonitrile and trifluoroacetic acid were then removed by vacuum centrifugation, and solutions were brought to a final volume of 100 μl with deionized H2O prior to analysis by ESI-MS.

RESULTS

Characterization of Purified Tpx and Mutants—Homogeneous, recombinant wild type Tpx and mutant proteins were isolated using a modification of the previous purification protocol (9), and all mutants were purified in the presence of 2 mM DTT to prevent overoxidation of free Cys residues (14). Mutant enzymes were difficult to prepare under oxidizing conditions due to irreversible protein dimerization, and C95S purified without DTT displayed significantly less activity than C95S purified under reducing conditions. DTNB titrations of denatured, prereduced wild type Tpx and mutants confirmed the expected thiol content for each (Table I). Additionally, ESI-MS of Tpx indicated a mass of 17,700 atomic mass units, 135 atomic mass units less than the mass of 17,835 atomic mass units predicted by the tpx open reading frame, indicating the loss of the initiating Met from E. coli Tpx. Each single mutant exhibited a mass of 17,686 atomic mass units, corresponding to a 16 atomic mass units loss due to the Cys to Ser mutation, whereas the 17,672-atomic mass units mass for C82S,C95S confirmed the double Cys to Ser mutation. The Cys to Ser mutations are conservative, and spectral and circular dichroism scans conducted in the far ultraviolet region revealed no gross structural perturbations among the mutants compared with the wild type protein (data not shown).

As mutant proteins were isolated from E. coli expressing low amounts of wild type Tpx, labeling experiments were conducted using fluorescein-5-maleimide to shift protein molecular weights according to the number of cysteine residues (an increase in 427 g/mol per fluorescein-labeled cysteine residue) and to enhance quantitation by densitometry. Using long gels and 18% acrylamide, singly, doubly, and triply labeled proteins were nicely separated (data not shown). Including data from kinetic studies described below, which give an upper limit of 0.4% for the degree of contamination of the C61S mutant by wild type Tpx (if all activity is due to the presence of wild type), densitometry of the gel samples taking into account this information indicates a wild type Tpx contamination level of less than 5%. Verification of the single (C82S,C95S), double (C61S, C82S, and C95S mutants), and triple (wild type Tpx) labeling of these proteins by fluorescein was obtained by mass spectrometry. Furthermore, purification protocols for C61S and C82S,C95S mutants were significantly different from that for the wild type enzyme, making contamination by the wild type enzyme less likely in these cases. Oligomeric State of Tpx—Earlier studies using a gel filtration column standardized with molecular weight markers suggested that oxidized Tpx was a monomer of 16.8 katomic mass units (9). In addition, the 2-atomic mass units difference for Tpx ESI-MS masses obtained under reducing (17,698.4 ± 0.62 atomic mass units) and nonreducing (17,696.7 ± 0.23 atomic mass units) conditions and the nonreducing SDS-PAGE analysis (see below) exclude the possibility of a covalent dimer for oxidized Tpx. Nonetheless, most other 2-Cys Prx proteins examined thus far, except for human PrxV (38), exist as covalent dimers when oxidized and noncovalent dimers or higher order oligomers when reduced (15). The oligomeric state of Tpx in solution was, therefore, examined by analytical ultracentrifugation sedimentation equilibrium experiments. Data analyses of three separate data sets each (40–340 μM) gave weight-average molecular weights for Tpx of 29,500 and 32,000 for the oxidized and reduced enzyme, respectively, indicating that two Tpx monomers self-associate in solution independent of redox state. Tpx dimerization is not concentration-dependent above 40 μM, because similar molecular weights are obtained at different speeds and all three concentrations. Detection of dimeric molecular weights for both C61S and C95S under the same experimental conditions indicates that these mutations do not perturb self-association.

Most 2-Cys Prxs are linked by an intersubunit disulfide bond at the active site in their oxidized state (25, 39). However, previous SDS-polyacrylamide gel studies of wild type Tpx revealed only a 19-kDa band under reducing and nonreducing conditions, suggesting the formation of an intrasubunit, rather than intersubunit, disulfide bond upon oxidation (9, 19). In the present study, a second species (~36 kDa) was observed on SDS-PAGE conducted under nonreducing conditions only when a thiol-specific blocking agent, methyl methanethiosulfonate, was excluded from sample preparations prior to denaturation (data not shown), indicating that any covalent dimerization of Tpx is artifactual. Furthermore, during SDS-PAGE analysis under nonreducing conditions, only a very small downward shift in protein migration is observed upon oxidation (from an apparent molecular mass of 18.5 to 18.2 kDa, data not shown), consistent with intersubunit disulfide bond formation as analyzed further using tryptic mapping techniques.

Identification of the Intrasubunit Disulfide Linkage—To assess the redox state of Cys residues and identify the location of the putative intrasubunit disulfide linkage, a series of biochemical assays were conducted on oxidized and reduced wild type Tpx. Reduced Tpx contained no disulfide bonds (as quantified by 2-nitro-5-thiosulfobenzoate assays) and 1.90 ± 0.03 or 2.60 ± 0.09 free thiols/monomer under native or denaturing conditions, respectively (Table I), indicating the presence of one buried, slow reacting Cys thiol. After the addition of 1 eq of
peroxide to prereduced Tpx, the two accessible thiol groups were lost (Table I), and one disulfide bond (0.90 ± 0.01) per monomer was gained.

To identify the disulfide-forming Cys residues, tryptic maps of oxidized and reduced, pyridylethylated Tpx were generated by HPLC and compared at 215 and 254 nm (Fig. 1). Instead of two strong peaks at 254 nm in the reduced, pyridylethylated chromatograms, four strong peaks were observed around 54, 56, 58, and 74 min, which allowed for the identification of Cys-containing peptides. After comparing the oxidized and reduced tryptic maps at 215 nm, two new peaks representing the disulfide-containing peptides were detected in the oxidized map at 68 P1 and 70 min P2, representing the disulfide-containing peaks (Fig. 1). P1 and P2 were reisolated on a shallower gradient and then reduced and pyridylethylated and reanalyzed by HPLC. P1 and P2 each gave two new peaks corresponding to two of the 254 nm absorbing peaks observed in the original chromatogram of the reduced, pyridylethylated protein (data not shown). Masses of P1 (3830 atomic mass units) and P2 (3702 atomic mass units) as determined by ESI-MS correspond to Cys61, and Cys85-containing peptides and verify that Cys61 and Cys85 compose the active site intrasubunit disulfide bond (Fig. 1). The presence of adjacent tryptic digest sites upstream of Cys61 resulted in two disulfide-containing peaks due to the partial digestion after Arg47.

Kinetic Characterization of Tpx and Inactivation during Catalysis—The Trx1-linked peroxidase activity of Tpx and its differential activity with ROOH and H2O2 were reported earlier (9, 19). In this study, the true kcat and kcat/Km values of Tpx for Trx1, CHP, and H2O2 were determined from Hanes-Woolf plots of the initial rate data (Table II). All lines in the Hanes-Woolf primary plots intersect the origin, indicating a bisubstrate, ping-pong (substituted) reaction mechanism for Tpx, which can be depicted as a sequence of consecutive reactions,

\[
\text{Tpx}_{\text{red}} + \text{Trx}_{\text{red}} \leftrightarrow \text{Tpx}_{\text{red}} + \text{Tpx}_{\text{red}} \\
\text{Tpx}_{\text{red}} + \text{ROOH} \rightarrow \text{Tpx}_{\text{ox}} + \text{ROH} + \text{H}_2\text{O}
\]

Reactions 1 and 2

where ROH is the corresponding alcohol. These redox reactions are analogous to those observed for other peroxidases from H. pylori and Crithidia fasciculata (35, 40) that are recycled by Trx or a Trx homologue, but unlike those systems that display an infinite \(K_m\) for Trx, Tpx interacts with Trx1 in a saturable manner with a \(K_m\) of 22.5–25.5 \(\mu\)M (Table II).

Tpx steady-state reactions followed for longer than 5 s exhibited rapidly declining NADPH oxidation rates over time for CHP, but not \(H_2O_2\), that were dependent on the concentration of peroxide. This observed inactivation of Tpx required a threshold level of CHP, whereby at high levels of CHP (>150 \(\mu\)M), but not at low CHP levels (<100 \(\mu\)M), activity diminished rapidly and nonlinearly and resulted in incomplete consumption of peroxide (Fig. 2). Activity could not be regained with the addition of new substrates; however, supplementation of reactions with fresh Tpx restored NADPH oxidation, which again diminished rapidly due to inactivation of the newly added Tpx (data not shown). Inactivated reaction mixtures (i.e. 200 \(\mu\)M CHP and levels of NADPH, TrxR, Trx1, and Tpx matching those used for the steady-state assays) that had been incubated with DTT and subsequently washed could not regenerate activity in the presence of fresh NADPH and CHP. These data suggest that inactivation is due to an irreversible overoxidation process that is most likely occurring at the active site Cys-SOH, a transient species that can be readily and irreversibly overoxidized by excess peroxide to Cys-SO2H or Cys-SO3H (14, 41). Prereduced Tpx incubated overnight in excess CHP retained full activity, indicating that inactivation requires Tpx turnover. ESI-MS verification of active site Cys overoxidation was obtained by injecting freshly inactivated reaction mixtures and immediately determining Tpx mass after turnover. After subtracting the contribution of TrxR and Trx1 ions from the reaction mixture spectra, the mass of Tpx was found to be 17,732 atomic mass units, an increase in 32 atomic mass units, which is consistent with the addition of two oxygen atoms. Together, these data strongly suggest that during turnover, Tpx’s active site Cys is converted to Cys-SO2H or Cys-SO3H (14, 41).
creasing amounts of 15-HPETE followed by the addition of a
centrations, but at higher [15-HPETE] (10 μM) activity with higher concentrations of 15-HPETE (10
min in one syringe and then were mixed with varying amounts of CHP and 400 μM (open triangles), 200 μM (closed squares), and 400 μM (open diamonds) in another syringe (final concentrations after mixing; see “Experimental Procedures”). Reaction progress was monitored at 340 nm on a stopped flow spectrophotometer at 25 °C, and rates were extrapolated from the linear portion of the curve (0–1.5 s) using linear regression analysis. At 100 μM CHP, Tpx was not inactivated and gave linear absorbance changes for the duration of the reaction and full peroxide consumption (open triangles). At higher CHP concentrations, Tpx activity diminished rapidly and nonlinearly without complete consumption of CHP or NADPH (closed squares and open diamonds). Stopped-flow data were collected every 50 ms, but only data from every 1.5 s are represented by the symbols.

Fig. 2. Steady-state kinetic analysis of Tpx with various concentrations of CHP. All three protein components (TrxR (1.5 μM), Trx1 (10 μM), and Tpx (1 μM)) were incubated in 150 μM NADPH for 5 min in one syringe and then were mixed with varying amounts of CHP (0 μM (closed circles), 100 μM (open triangles), 200 μM (closed squares), and 400 μM (open diamonds)) in another syringe (final concentrations after mixing; see “Experimental Procedures”). Reaction progress was monitored at 340 nm on a stopped flow spectrophotometer at 25 °C, and rates were extrapolated from the linear portion of the curve (0–2 s) using linear regression analysis. At 100 μM CHP, Tpx was not inactivated and gave linear absorbance changes for the duration of the reaction and full peroxide consumption (open triangles). At higher CHP concentrations, Tpx activity diminished rapidly and nonlinearly without complete consumption of CHP or NADPH (closed squares and open diamonds). Stopped-flow data were collected every 50 ms, but only data from every 1.5 s are represented by the symbols.

oxide than for t-butyldihydroperoxide (30.7 versus 66.6 μM). Examination of Tpx reactivity with a physiological alkyl hydroperoxide, a relatively insoluble fatty acid hydroperoxide, 15-HPETE, resulted in a complex pattern of [15-HPETE]-de-

TABLE II

| Peroxide | $k_{cat}$ s$^{-1}$ | $K_m$ μM | Peroxide | $k_{cat}$/K_m (Trx1) | $k_{cat}$/K_m (peroxide) |
|----------|-----------------|--------|----------|---------------------|-------------------------|
| H$_2$O$_2$ | 76.0 ± 8.1 | 25.5 ± 2.9 | 15-HPETE | 1730 ± 360 | 3.0 × 10^6 |
| CHP | 70.1 ± 7.1 | 22.5 ± 4.7 | 15-HPETE | 9.1 ± 1.8 | 3.1 × 10^6 |

whether or not other E. coli enzymatic disulfide/dithiol reductants could serve as reductase systems. Because Trx2 (trxC) is up-regulated in response to oxidative stress (43, 44) and exhibits similar disulfide reductase capabilities as Trx1 (44, 45), we postulated that Trx2 may be able to substitute for Trx1. However, replacement of Trx2 in the steady-state Tpx assay did not result in NADPH oxidation (data not shown). Previous work also indicated that Tpx was active in the presence of high levels of glutathione (10–20 mM) (9); however, replacing the TrxR/Trx1 reductase system with glutathione reductase and glutathione (up to 40 mM) did not support Tpx turnover in our stopped-flow assays (data not shown). Additionally, neither E. coli glutaredoxin 1 nor E. coli AhpF were capable of reducing Tpx, indicating that Tpx peroxidase activity specifically requires Trx1.

Cys$^{61}$ Is the Peroxidatic Center in Tpx—To address which of the three Cys residues in Tpx are involved in the direct reductase, Cys to Ser mutants were analyzed under steady-state assay conditions using a stopped-flow spectrophotometer. Unlike in previous studies, where both C61S and C95S were inactive (22, 23), we detected activity with C95S (∼20% of wild type activity with 10 μM Trx1) but not with C61S in our assays (Fig. 5), indicating that Cys$^{61}$ is the essential peroxidatic Cys of the disulfide pair (the activity of C61S is about 0.4% that of wild type Tpx and may be entirely due to a very small amount of wild type contamination in the C61S protein). C95S is also more sensitive to inactivation than wild type Tpx, as illustrated by less robust reaction rates that are more quickly diminished even in the presence of low concentrations of peroxide (data not shown). The loss of Cys$^{82}$ does not have a large effect on activity (−72% activity compared with wild type Tpx); interestingly, removal of both Cys$^{61}$ and Cys$^{95}$ in the double mutant, C82S,C95S, dramatically decreases activity below that of C95S to about 10% of wild type activity (Fig. 5), suggesting a modest, stabilizing effect of Cys$^{82}$ in the absence of Cys$^{95}$. When the single Cys Tpx mutants were assayed with varying amounts of Trx1 (0–80 μM) at one concentration of CHP (50 μM), we found that C82S retained the same low $K_{cat,app}$ for Trx1 as wild type Tpx (14.8 versus 12.3 μM), whereas C95S had a much higher $K_{cat,app}$ for Trx1 (50.7 μM). Our labeling and densitometry data verifying less than 5% contamination of the mutants by wild type Tpx, taken together with the value of 20% for the rate of C95S compared with wild type Tpx (at 10 μM Trx1) and the unique kinetic properties of this mutant (higher $K_{cat,app}$ for Trx1 and more rapid inactivation during turnover), clearly indicate that C95S does retain activity, although removal of part of the Tpx redox-active disulfide center adversely affects its normal catalytic activity with Trx1 and exacerbates its inactivation by peroxides.

Identification of the Sulfenic Acid Form of Cys$^{61}$—NBD chloride labeling and x-ray crystallography have directly demonstrated the presence of a R-SOH intermediate on the peroxi-
datic Cys of several Prx homologues (17, 46). During catalysis, the labile Cys-SOH is quickly attacked by the other half-cysteine of 2-Cys Prxs to reform the redox active disulfide; therefore, to stabilize and trap Cys-SOH, the C-terminal Cys of the active site disulfide pair must be removed. Because C95S and C82S, but not C61S, display a reduced thiol titer upon oxida-
Kinetic Mechanism of E. coli Tpx

Fig. 3. Scheme of TrxR/Trx1 reduction and electron transfer pathways for Tpx during catalysis and inactivation. During Tpx turnover, a small proportion of Tpx becomes overoxidized and is removed from the reaction cycle (path A). The remaining Tpx reforms the redox-active disulfide to continue the catalytic cycle (path B). Eventually, after multiple turnovers in the presence of ≥150 μM CHP, the enzyme primarily converts to the R-SO₂H species, few disulfide-containing species remain, and activity declines, leading to irreversible inactivation. This scheme depicts the overall flow of electrons but not necessarily the precise redox forms of TrxR involved in turnover.

Fig. 4. Steady-state assay of Tpx as a function of 15-HPETE concentration. Reaction mixtures in one syringe containing NADPH (150 μM), Trx1 (10 μM), TrxR (1.5 μM), and Tpx (1 μM) were mixed with varying amounts of 15-HPETE (0–90 μM) in another syringe on the stopped-flow spectrophotometer at 25 °C (final concentrations after mixing). Each rate is the average of three experiments and was obtained by linear regression analysis of the linear portion of the reaction progression (0–1 s) prior to inactivation. Standard peroxidase buffer was used in all cases as described under “Experimental Procedures.”

Fig. 5. Steady-state kinetic analysis of wild type and mutant Tpx proteins. Tpx reaction mixtures in one syringe containing NADPH (150 μM), Trx1 (10 μM), TrxR (1.5 μM), and varying amounts (0–1 μM) of Tpx (closed squares), C82S (open circles), C95S (closed triangles), C82S,C95S (open triangles), or C61S (closed circles) were mixed with CHP (50 μM) on the stopped-flow spectrophotometer in standard peroxidase buffer (final concentrations after mixing; see “Experimental Procedures”). Each rate is the average of three experiments conducted at 25 °C and was obtained by linear regression analysis of the linear portion of the reaction (0–1 s) prior to inactivation.

Fig. 6. Spectrophotometric analysis of NBD-labeled Tpx mutants. Prereduced C82S,C95S treated with 1 eq of cumene hydroperoxide (solid line) or no peroxide (dashed line) was modified with NBD chloride (10× for 5 min. To remove excess reagent, treated samples were washed with 5 ml of buffer by ultrafiltration, and then labeled proteins were analyzed from 200 to 600 nm.

DISCUSSION

Although Tpx was excluded initially from the Prx family (9) due to its low sequence identity (17% compared with E. coli AhpC), the high z score (>70) for the alignment of Tpx with 2-Cys mammalian Prx homologues in fold and function assignment system (FFAS) analysis (47) confirms homology of Tpx with the Prxs. Not surprisingly then, Tpx shares many features of the Prx catalytic mechanism, including a reliance on the conserved N-terminal Cys for peroxide reduction (15). Of Tpx’s three Cys residues (Cys⁶¹, Cys⁸², and Cys⁹⁵), Cys⁶¹ was shown in our mutagenesis studies to be essential for attack of the peroxide’s -O–O- bond (Fig. 4) and to form a Cys-SOH intermediate upon oxidation (Fig. 6). Our assay data, collected using stopped-flow spectrophotometry, captured initial reaction rates before overoxidation of Cys⁶¹, and it is likely that prior inability

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to detect mutant C95S activity (22, 23) was due to the rapid overoxidation of Cys63 during purification in the absence of DTT and/or during the manual mixing procedures used in assay methods. Tpx does not form the hallmark intersubunit disulfide linkage of typical 2-Cys Prxs, and instead Cys63 links with Cys50 in an unusual intramolecular disulfide bond, a reaction intermediate common to atypical 2-Cys Prxs such as human PrxV (15). Whereas Cys50 does not align with the C-terminal Cys of other 2-Cys Prxs, the participation of Cys63 as a disulfide partner signals that Cys63 is functionally equivalent to the "resolving" Cys of other 2-Cys Prxs (15).

Investigations of Prx interactions with their reducing systems have revealed two different kinetic patterns where 1) the formation of enzyme-substrate complexes is an observable phenomenon (25, 26, 48, 49) or 2) reduction of the Prx is a bimolecular process, whereby infinite values for $K_m$ and $V_{max}$ characterize activity (13, 35). The specific reduction of Tpx by Trx1 occurs with a $K_m$ of 25 $\mu$M and a catalytic efficiency of $3 \times 10^6$ M$^{-1}$ s$^{-1}$ (Table II). This rate is about 10-fold faster than rates of $10^5$ M$^{-1}$ s$^{-1}$ achieved generally by other Prx systems (13) and much faster than the weak peroxidase activity displayed by BCP ($10^4$ M$^{-1}$ s$^{-1}$), calculated from apparent maximal velocity ($V_{max}$) and $K_m$ values obtained under non-steady-state conditions for linoleic acid hydroperoxide (8)). On the basis of catalytic efficiency, Tpx is the most potent reductant of alkyl hydroperoxides in E. coli when compared with the other two Prx family members, AhpC and BCP. Whereas some Prxs interact with Trx in a nonsaturable manner, it is unknown if this type of activity arises as a result of differential affinity for Trx binding or because the $K_m$ for the reducing substrate is higher than the concentrations tested.

Earlier reports of the greater catalytic efficiency of Tpx with t-butyl hydroperoxide over H$_2$O$_2$ (9) were confirmed in our own kinetic studies with CHP and H$_2$O$_2$ as substrates (giving true $K_m$ values of 9.1 and 1750 $\mu$M, respectively) (Table II). As a result, Tpx does not seem to exhibit the requirements for a minimal peroxide binding site as observed for the Trx-dependent Prx from H. pylori (35). In light of this, the nature of the Tpx peroxidatic binding site is likely to be complex, and due to its high affinity for CHP, hydrophobic interactions may be the predominating forces that influence substrate interaction with the enzyme.

Previously, it was shown that the primary role of AhpC was to keep concentrations of H$_2$O$_2$ in exponentially growing E. coli quite low ($10^{-8}$ to $10^{-6}$ M) (7), and the role of AhpC in organic hydroperoxide detoxification was questioned because bacteria do not synthesize the polyunsaturated fatty acids required for lipid peroxidation. However, the ability of Tpx and other Prxs, including yeast type II thioredoxin peroxidase (50), to preferentially decompose organic hydroperoxides over H$_2$O$_2$ suggests that Tpx’s central role in vivo involves the reduction of complex ROOH, whereas AhpC mainly reduces H$_2$O$_2$. Several lines of evidence also highlight the importance of Prxs in bacterial ROOH resistance, including the isolation of mutants with increased resistance to organic solvents linked directly to a mutation in E. coli AhpC (51). Other bacterial peroxidases, such as Ohr from Xanthomonas campestris, are specifically up-regulated by organic hydroperoxides (52, 53), implying that defense against these peroxides is requisite for bacterial survival. It is also quite possible that during pathogenesis, bacteria take up polyunsaturated fatty acids from the host (54), creating the potential for bacterial lipid hydroperoxide formation. Whereas the exact nature and concentration of organic hydroperoxides that Prxs are exposed to in vivo is unknown, the existence of Prxs with selectivity for these peroxides suggests the possibility of more complex peroxides as Prx substrates.

Inactivation has been observed for many different Prx homologues during activity assays (14, 55–58), and, when investigated, irreversible overoxidation at the active site Cys is responsible for the loss of activity (57), with the terminal species being sulfenic acid in some cases (15). If Tpx inactivation requires a threshold level of peroxide that is substantially higher than in vitro concentrations, then the observed in vitro overoxidation for Tpx is due to the use of very high substrate concentrations in steady-state assays. This seems probable, considering that the endogenous in vivo concentrations of H$_2$O$_2$ are reportedly quite low in the absence of exogenous sources (59). Exposure to peroxide at endogenous concentrations of 2 $\mu$M or more is growth-inhibitory (7), and in these cases, peroxidase activity may no longer prevent oxidative damage to cellular components. Whereas the in vivo concentrations of other ROOH in bacteria are not known, it is likely that they would not exceed H$_2$O$_2$ levels. Therefore, subjecting Tpx during assays to exceptionally high peroxide levels may artificially promote overoxidation, a future consideration for all Prx steady-state analyses done without the benefits of stopped-flow reaction techniques.

To explain the existence of multiple Prxs, the differential cellular localization of Tpx and AhpC has been cited. Tpx has been characterized as a periplasmic protein (9, 60), despite the lack of a signal sequence for periplasmic transport, whereas AhpC has been localized to the cytoplasm (60). E. coli proteomics studies by Link et al. (60) showed that during the growth phase in minimal media and without induction by H$_2$O$_2$, Tpx (1.6 $\mu$M) is 3.5-fold less abundant than AhpC and 2.5-fold more abundant than BCP. The relatively high expression of all three Prxs indicates a sustained requirement for peroxide detoxification during growth, and in these cases, a periplasmic peroxidase metabolizing peroxides prior to cytosolic entry would be quite beneficial. Although Trx1 is relatively abundant (0.3% of the cellular protein) (61), its cytoplasmic location (62) would restrict access of Tpx to its reductant and decrease the catalytic efficiency of the Tpx system. The designation of Tpx as a periplasmic protein should then be treated with caution until more careful localization studies are completed.

Detailed kinetic analyses of Tpx reaction rates have allowed us to clarify the roles of the three Cys in E. coli Tpx herein and demonstrate the essentiality of Cys63. The shared reliance on Cys-SH formation and disulfide bond formation points to mechanistic similarities between Tpx and other members of the Prx family. Further kinetic and mechanistic studies on Prx family members may begin to delineate the functional roles of multiple Prxs in the same organism, the presence of which most likely signifies considerable selection pressure from oxidative stress and the need to combat reactive oxygen species.

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