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Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes

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ABSTRACT **A cDNA corresponding to a thiol-specific antioxidant enzyme (TSA) was isolated from a rat brain cDNA library with the use of antibodies to bovine TSA. The cDNA clone encoded an open reading frame capable of encoding a 198-residue polypeptide. The rat and yeast TSA proteins show significant sequence homology to the 21-kDa component (AhpC) of *Salmonella typhimurium* alkyl hydroperoxide reductase, and we have found that AhpC exhibits TSA activity. AhpC and TSA define a family of >25 different proteins present in organisms from all kingdoms. The similarity among the family members extends over the entire sequence and ranges between 23% and 98% identity. A majority of the members of the AhpC/TSA family contain two conserved cysteines. At least eight of the genes encoding AhpC/TSA-like polypeptides are found in proximity to genes encoding other oxidoreductase activities, and the expression of several of the homologs has been correlated with pathogenicity. We suggest that the AhpC/TSA family represents a widely distributed class of antioxidant enzymes. We also report that a second family of proteins, defined by the 57-kDa component (AhpF) of alkyl hydroperoxide reductase and by thioredoxin reductase, has expanded to include six additional members.**

Organisms living in aerobic environments require mechanisms that prevent or limit cellular damage caused by reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$) that arise from the incomplete reduction of oxygen during respiration or from exposure to external agents such as light, radiation, redox-cycling drugs, or stimulated host phagocytes (1, 2). Cellular processes also generate reactive sulfur species ($RS^{\cdot}$, $RSSR^{\cdot-}$, $RSOO^{\cdot}$) from thiol compounds (3). The reactive oxygen and sulfur species cause damage to all major classes of biological macromolecules leading to protein oxidation, lipid peroxidation, and DNA base modifications and strand breaks. To counter these destructive processes, cells have evolved protective enzymatic systems, which act to prevent and repair the radical-linked damage (1, 2).

Saccharomyces cerevisiae cells contain a 25-kDa enzyme that protects cellular components against oxidative damage from a system capable of generating reactive sulfur species but not from a system that generates only reactive oxygen species (4). This enzyme has therefore been designated thiol-specific antioxidant (TSA). (TSA was called protector protein in refs. 4 and 5.) Exposure of yeast cultures to oxidative stress caused by 100% O_2 or by addition of Fe^{3+} results in an increase in the synthesis of the 25-kDa protein (5). The yeast TSA gene has been cloned and sequenced, and the encoded protein shows no significant homology to any known catalase, superoxide dismutase, or glutathione peroxidase enzyme (6). Under aerobic conditions, especially under oxidative stress exerted by the presence of peroxides or methyl viologen, the growth rate of a mutant lacking TSA was significantly less than that of wild-type cells. This result suggests that TSA is a physiologically important antioxidant.‖

Salmonella typhimurium and *Escherichia coli* cells contain an alkyl hydroperoxide reductase, which converts alkyl hydroperoxides to their corresponding alcohols (7). When this activity was purified from *S. typhimurium*, it was found to be composed of a 21-kDa component (AhpC) and a 57-kDa component (AhpF) with a bound FAD cofactor. (AhpC and AhpF were previously referred to as C22 and F52, respectively, in ref. 7.) The proposed catalytic mechanism for alkyl hydroperoxide reductase involves substrate peroxide reduction by the AhpC protein with subsequent rereduction of the AhpC by the AhpF coupled to either NADH or NADPH oxidation (7). The locus encoding alkyl hydroperoxide reductase was identified by genetic screens for mutants resistant or hypersensitive to alkyl hydroperoxides, and the corresponding *ahpCF* operon was cloned by complementation of the mutants (8). AhpF was found to show considerable homology to *E. coli* thioredoxin reductase (TR), while no proteins with similarity to AhpC were found (9).

We have now further extended our studies on yeast TSA by purifying TSA from bovine brain and by cloning and sequencing a rat cDNA that encodes TSA. We also show that the AhpC component has TSA activity and that AhpC and TSA define a large family of related proteins present in organisms from all kingdoms. We propose that this family of abundant proteins plays a major role as cellular antioxidant enzymes.

MATERIALS AND METHODS

TSA Activity. TSA and AhpC-dependent inhibition of thiol/Fe^{3+}/O_2-mediated inactivation of glutamine synthetase was measured as described (4).

Separation of Cysteine-Containing Peptides. Purified bovine brain TSA (200 μg) was denatured reductively by treatment with 50 mM Tris·HCl, pH 8.0/6 M guanidine hydrochloride/2 mM dithiothreitol (DTT) and incubated with 10 mM Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid). The resulting 5-thio-2-nitrobenzoic acid-labeled protein was digested with

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Abbreviations: TSA, thiol-specific antioxidant; TR, thioredoxin reductase; DTT, dithiothreitol; ORF, open reading frame.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U06099).

trypsin and three cysteine-containing peptides were purified as described (10). Partial amino acid sequences of the purified peptides were determined by automated Edman degradation.

Antibodies to TSA. We have previously shown that mammalian tissues contain a thiol-specific antioxidant that resembles yeast TSA (5). Bovine brain TSA was purified to homogeneity and used to prepare specific rabbit antibodies (H.Z.C. and S.G.R., unpublished work).

Cloning and Sequencing of TSA cDNA. A rat brain cDNA library in Uni-ZAP XR (Stratagene) was screened with the monospecific antibodies to bovine brain TSA. A positive plaque was isolated, and the pBluescript SK(−) plasmid containing the cDNA insert was excised from the Uni-ZAP XR vector. The *Eco*RI/*Xho* I fragment (0.9 kb) was subcloned into pBluescript SK and sequenced by the *Taq* dye primer cycle sequencing kit (Applied Biosystems) on an automated fluorescent dye DNA sequencer (Applied Biosystems; model 370A).

Sequencing of the AhpC. The 1.1-kb *Eco*RI fragment carrying the 3′ region of the *ahpC* gene and the 5′ region of the *ahpF* gene (8) was cloned into the *Eco*RI site of M13mp18 in both orientations. The oligonucleotides 5′-GACCCGACTGGCGCCCTG-3′, 5′-GTCACGGCCGATACCTTC-3′, 5′-TGGCGTGAAAGACGACGG-3′, and 5′-CGTAAAATTAAAGCAGC-3′ were used as primers in sequencing reactions with the dGTP and dITP nucleotide extension mixtures in the Sequenase kit (United States Biochemical).

S. typhimurium AhpC was purified according to the procedure (7) from the *E. coli ahpΔ5* deletion mutant (TA4315) transformed with the *S. typhimurium* pAQ9 clone of the *ahp* locus (8). Reductive alkylation of AhpC with radiolabeled iodoacetamide, tryptic digestion, purification of radioactive peptides, and amino acid sequence analysis were performed essentially as described (11).

DNA Sequence Analysis. Members of the AhpF/TR and AhpC/TSA families were identified by a recursive search of GenBank and EMBL data bases. The family members were aligned and the clustering trees were generated using Genetics Computer Group's PILEUP [a progressive alignment program (12)] with the default parameters.

RESULTS

Cloning and Sequencing of Rat TSA cDNA. A rat brain Uni-ZAP XR cDNA library was screened with antibodies to bovine brain TSA. An immunologically positive clone was isolated from 1.8×10^5 plaques. To help identify the open reading frame (ORF), we isolated three cysteine-containing peptides from bovine brain TSA and determined partial amino acid sequences: QYTDE (peptide 1, related to peptide 2), LVQAFQYTDEHGNVXPA (peptide 2), LNXEV (peptide 3), where X represents an unidentified amino acid that is likely to be cysteine. The nucleotide sequence of the 0.9-kb cDNA insert revealed an ORF that contains coding sequences for bovine peptides 1, 2, and 3 with one amino acid in each peptide (glutamine and glycine, respectively) substituted by aspartic acid, likely due to species differences. The ORF encodes a polypeptide of 198 amino acid residues with a calculated molecular weight of 21,652. Alignment revealed 65% identity and 76% similarity between rat and yeast TSA. The rat and yeast TSA molecules also showed significant sequence similarity to the AhpC subunit of the *S. typhimurium* alkyl hydroperoxide reductase.

Corrected Sequence of AhpC. Although the deduced amino acid sequence for AhpC reported by Tartaglia et al. (9) was consistent with the expected size of this protein, the alignment with TSA sequences suggested that the C-terminal one-third of the protein sequence was probably incorrect. Therefore, we reexamined the DNA sequence of the *ahpC* gene and determined the amino acid sequence of the regions of catalytic importance. Upon resequencing, an additional cytidine nucleotide was identified between nt 358 and 359 and an additional guanine nucleotide was identified between nt 521 and 522 of the coding sequence of *ahpC*. These corrections lead to the prediction of only two cysteine residues in the AhpC protein, at codons 47 and 166, rather than the three previously reported. The amino acid sequence surrounding the predicted cysteine residues was also confirmed by the peptide analysis.

TSA Activity of AhpC. Given the similarities between the *S. typhimurium* AhpC protein and the TSA protein from *S. cerevisiae* and from rat brain, we assayed the oxidative inactivation of glutamine synthetase as a function of AhpC or TSA added (Fig. 1). We found that AhpC was, in fact, highly effective as a protector protein, requiring ≈0.088 mg of AhpC per ml compared to 0.057 mg of TSA per ml for half-maximal protective activity.

AhpC/TSA Protein Family. In a search of the sequence data bases, we have identified >23 additional proteins from a variety of organisms that show similarity to AhpC and TSA. The majority of the sequences are aligned in Fig. 2 and the relationships among them are represented as a dendrogram in Fig. 3. The similarity among the family members extends over the entire sequence and ranges between 23% and 98% identity.

AhpC/TSA family members were identified in 12 microorganisms in addition to *S. typhimurium* and *S. cerevisiae*, including *Amphibacillus xylanus* (D13563), *Bacillus alcalophilus* (14), *Clostridium pasteurianum* (15), *Cryptosporidium parvum* (C. Petersen, personal communication), *Entamoeba histolytica* (16), *E. coli* (D13187), *Helicobacter pylori* (17), *Legionella pneumophila* (S. Rankin and R. Isberg, personal communication), *Methanobacterium thermoautotrophicum* (18), *Mycobacterium avium* (19), *Mycobacterium leprae* (L01095), and *Streptococcus mutans* (D21803). Several mammalian homologs—one bovine, two mouse, and three human—were identified in addition to the rat brain TSA protein. The partial sequence of the bovine homolog corresponds to a bovine ciliary body protein that was identified as a glutathione peroxidase (20). One mouse homolog, encoded by an ORF denoted MSP23, is expressed in mouse peritoneal macrophages (21). A second mouse homolog is encoded by an ORF denoted MER5, a gene that is preferentially expressed in murine erythroleukemia cells and may be linked to cell differentiation (22). One human homolog was identified by the large scale sequencing of cDNA species present in libraries from human brain [clone EST 311 (23)] and from a human liver HepG2 cell line [clones s17a09 and d-0c12 (24)] and as *pag*, a gene overexpressed during proliferation of epithelial cells (25). The partial sequence of a second ho-

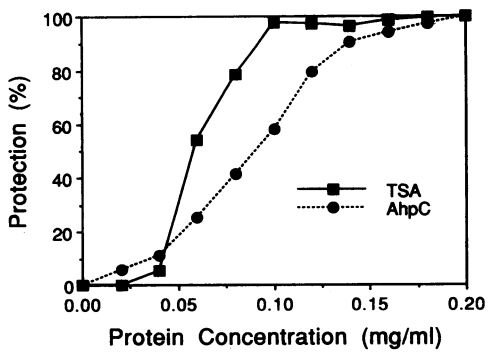

FIG. 1. TSA activity of AhpC. The indicated concentrations of TSA and AhpC were assayed for the ability to inhibit the thiol/Fe^{3+}/O_2-mediated inactivation of glutamine synthetase. A 95% loss of glutamine synthetase activity was observed in the absence of TSA and AhpC.

FIG. 2. Amino acid alignment of AhpC/TSA family. AhpC/TSA family members were aligned by using PILEUP. Sequences that did not align/tree effectively, because the known sequences were only extremely short fragments, were not included. Residues that are present in more than half of the homologs are included in the consensus.

molog (T10952) identified in a cDNA library from pancreatic islets also shows similarity to MER5. Finally, a third human family member is the sixth ORF (ORF6) present in a random sample of cDNA clones (D14662), suggesting that multiple isoforms of the AhpC/TSA family are present in eukaryotes. An mRNA corresponding to an AhpC/TSA-like polypeptide has also been identified in *Bromo secalinas* (27), a wild grass species, and the nematode *Caenorhabditis elegans* (T00682). Two additional proteins with more limited similarity to AhpC/TSA proteins are a bacterioferritin comigratory protein from *E. coli* (28) and a protein encoded by a *Bacterioides fragilis* ORF (29).

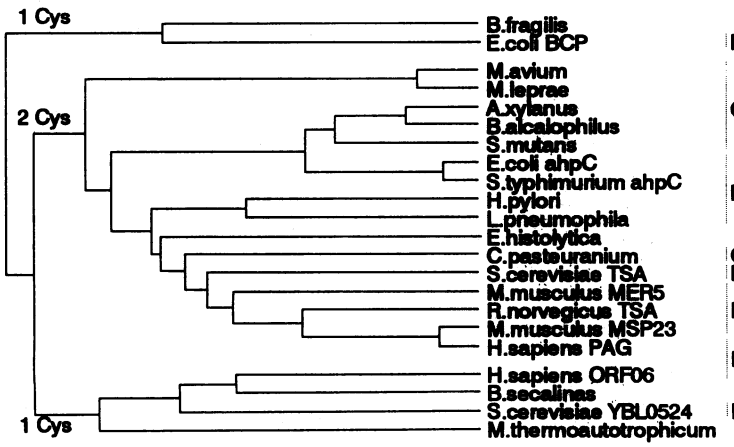

FIG. 3. Clustering tree for the AhpC/TSA family. The tree was generated by PILEUP and then manually transformed into a topologically equivalent structure in order to emphasize deviations and consistencies with accepted phylogenies of the species involved. The bacterial relationships and nomenclature were taken from Woese (13).

AhpF/TR Protein Family. The AhpC component of the alkyl hydroperoxide reductase is reduced by the AhpF component. Previous studies showed that AhpF and TR formed a distinct family of disulfide-containing oxidoreductase proteins distinct from other flavoprotein disulfide oxidoreductases such as glutathione reductase, dihydrolipoamide reductase, and mercuric reductase (9, 30).

We identified eight additional members of the AhpF/TR family by data base searches (Fig. 4). Tree analysis of the sequences shows a relatively clear bifurcation between the AhpF-like and TR-like subfamilies. Five sequences belong to the AhpF subfamily including *S. typhimurium* AhpF, a partial sequence for *E. coli* AhpF (D13187), NADH dehydrogenase in an alkalophilic *Bacillus* strain (now designated *B. alcalophilus*) (14), NADH oxidase from *A. xylanus* (D13563), and a partial reading frame from *Pseudomonas cepacia* (31). All of the AhpF-like sequences from *S. typhimurium*, *E. coli*, *B. alcalophilus*, and *A. xylanus* sequences possess additional amino acids at the N terminus, which are absent from all members of the TR subfamily for which the N terminus is known (sequence comparison not shown). This region, which accounts for nearly 40% of the full-length polypeptide in AhpF and the NADH dehydrogenase, has been implicated in membrane association in the NADH dehydrogenase protein. The members of the second AhpF/TR subfamily, which more closely resemble TR, include TR sequences from *E. coli* and *Streptomyces clavuligerus* (30, 32), a partial *Oryza sativa* (rice) cDNA (D15855), a partial reading frame adjacent to the *S. cerevisiae* TRP4, and an ORF upstream of the *C. pasteurianum* rubredoxin gene (15).

DISCUSSION

Here we report the isolation of cDNA corresponding to rat TSA and the corrected sequence of *S. typhimurium* AhpC, a component of alkyl hydroperoxide reductase. The newly discovered AhpC amino acid sequence was found to be ≈40% identical to those of TSA proteins from *S. cerevisiae* and rat brain. In addition, we found that the purified AhpC protein protects glutamine synthetase against damage by DTT/Fe^{3+}/O_2, but not by ascorbate/Fe^{3+}/O_2, suggesting that AhpC protein carries TSA activity. Attempts to identify any peroxidase activity inherent in the TSA protein have been hampered by the lack of a known reductase protein as a counterpart for the NAD(P)H-dependent AhpF of alkyl hydroperoxide reductase. AhpF participates in the reductive half-reaction of the peroxide reductase activity of AhpC (see below). While DTT can serve as a chemical reductant of TSA, direct interaction between DTT and the ethyl hydroperoxide substrate has precluded its use in assays for alkyl hydroperoxide reductase activity (data not shown). We also report that a large family of proteins (designated AhpC/TSA family) is homologous to AhpC and TSA and that the family of proteins defined by AhpF and TR has expanded to include six additional members.

Conserved Cysteines Among AhpC/TSA Protein Family. One cysteine residue is conserved in all family members and a majority of the proteins also have a second conserved cysteine residue. The presence or absence of the second cysteine is correlated with the conservation of sequences in the neighborhood of the first cysteine. In the 2-Cys AhpC/TSA family members, the sequence surrounding the first cysteine is FvCP, whereas in the 1-Cys branch (see below) the sequence is PvCT. For some 1-Cys proteins, such as the *E. coli* bacterioferritin comigratory protein, the region surrounding the second cysteine seems to be missing altogether. These data suggest that the invariant N-terminal cysteine is more likely to be critical in catalysis [see accompanying paper (33)].

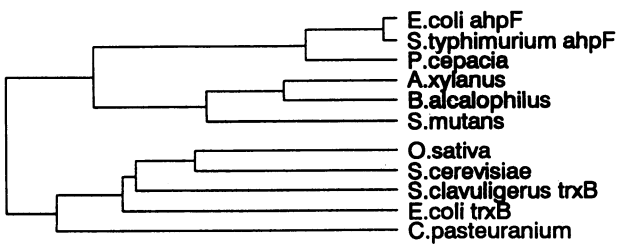

FIG. 4. Clustering tree for AhpF/TR family.

Evolutionary Relationships Among AhpC/TSA Protein Family. Inspection of the AhpC/TSA protein family alignment and the derived phylogeny indicates several striking features of the family (Fig. 3). Most notably, the three deepest branches all contain members from multiple kingdoms instead of branching along species lines. Furthermore, Gram-positive and purple (Gram-negative) bacterial sequences are not segregated into specific nodes. An example is the 2-Cys branch containing AhpC from *E. coli* and *S. typhimurium* (purple bacteria) along with the *B. alcalophilus* and *A. xylanus* (Gram-positive) sequences. All four of these sequences contain a highly conserved TL(ak)PSLD(li)VGKI C-terminal motif, which is not evident in any other sequence in the protein data bases. Additional incongruities can be found among the eukaryotic sequences. For example, two of the human sequences are in distinct branches with many nodes separating the sequences. At least four distinct AhpC/TSA homologs (represented by rat TSA, mouse MSP23/human PAG, mouse MER5, and human 1-Cys ORF6) are present in mammals. The presence of multiple forms is consistent with the multiple bands detected in the zoo blot presented by Prospéri *et al.* (25).

Organization of AhpC/TSA and AhpF/TR Genes. Interestingly, at least eight of the genes encoding AhpC/TSA homologs are found in close proximity to genes encoding proteins with other oxidation–reduction activities. In *S. typhimurium* and *E. coli*, the *ahpC* gene is directly upstream of *ahpF* (9). In *B. alcalophilus* and *A. xylanus*, the ORFs encoding the AhpC/TSA homologs are just upstream of the genes encoding the NADH dehydrogenase or NADH oxidase proteins, respectively, which belong to the AhpF/TR family. *C. pasteurianum* also encodes both an AhpC/TSA-like protein and an AhpF/TR-like protein but the AhpF/TR-like ORFA is upstream of a putative ORFB followed by the AhpC/TSA-like ORFC (15). In *M. thermoautotrophicum*, the ORF with AhpC/TSA similarity is upstream of the gene encoding a SOD activity (18), and in *L. pneumophila*, the AhpC/TSA homolog is downstream of a gene encoding a glutaredoxin-like protein (S. Rankin and R. Isberg, personal communication).

Antigenic Properties of the AhpC Proteins. It is striking that the AhpC/TSA has been identified in nine pathogenic organisms and was initially characterized as a species-specific antigen in four of these organisms. Three gastrointestinal pathogens—*E. histolytica* (16, 34), *C. parvum*, and *H. pylori* (17)—were found to have the AhpC/TSA homolog. *S. typhimurium*, *E. coli*, and *B. fragilis* also inhabit the gastrointestinal tract and can be pathogenic. *M. avium* causes tuberculosis-like pulmonary disease in immunocompromised patients and *M. leprae* is also a major human pathogen (19). An AhpC/TSA-like gene was also identified in a selection for *L. pneumophila* genes that are expressed at higher levels when the bacteria are growing within macrophages (S. Rankin and R. Isberg, personal communication). The AhpC/TSA homologs may not be the direct cause of pathogenesis but may be essential for the organisms to defend against oxidants generated by macrophages and neutrophils. The relative abundance of some of these AhpC/TSA homologs may be at least partially responsible for their identification as antigens.

The *H. pylori* and *E. histolytica* proteins are quite abundant, and in *S. cerevisiae* TSA constitutes 0.7% of the total soluble protein (5).

Oxidants Eliminated by AhpC/TSA. With the exception of AhpC, none of the AhpC/TSA members is associated with known biochemical reactions. AhpC together with AhpF can convert a variety of alkyl hydroperoxides (such as the physiologically relevant thymine hydroperoxide and linoleic acid hydroperoxide, as well as nonphysiological cumene hydroperoxide) (7). The activity may act on oxidized DNA or nucleic acid bases since increased expression of the alkyl hydroperoxide reductase activity can suppress elevated spontaneous mutagenesis in *E. coli* and *S. typhimurium* strains defective for the *oxyR*-regulated defense response to hydrogen peroxide (35, 36). Additionally or alternatively, alkyl hydroperoxide reductase may act on oxidatively damaged membrane or lipids. Whether the true function of TSA is the removal of reactive sulfur species is not known. We have proposed that reactive sulfur species ($RS^{\bullet}$, $RSSR^{\bullet -}$, $RSOO^{\bullet -}$, $RSOO^{\bullet}$, or $RSO^{\bullet}$) are substrates of TSA for several reasons. *(i)* The antioxidant activity of TSA is observed only when a thiol compound is added to the metal-catalyzed oxidation system as electron donor; when thiol is replaced with another electron donor [ascorbate, NAD(P)H/NAD(P)H oxidase, or hypoxanthine/xanthine oxidase], no antioxidant activity is observed (4). *(ii)* TSA does not exhibit detectable catalase, superoxide dismutase, or glutathione peroxidase activity (4). *(iii)* The deduced amino acid sequence of yeast TSA shows no homology to any of the reactive oxygen species-specific antioxidant enzymes, such as superoxide dismutases, catalases, and peroxidases. *(iv)* Recently, the capacity of TSA to remove reactive sulfur species was also directly demonstrated by electron paramagnetic resonance spectroscopy (37).

An alternative possibility is that TSA, like AhpC, eliminates a peroxide and that the oxidized TSA generated can be converted back to the reduced form *in vitro* only by a thiol and not by other electron donors (for example, ascorbate). DTT can be converted to sulfur-containing radicals in the presence of a peroxide and the removal of peroxide by TSA would result in the decrease of sulfur-containing radicals as observed by electron paramagnetic resonance. A recent report suggests that TSA at high concentrations (for example, 1 mg/ml) can remove H_2O_2 (38). Further tests to detect any peroxidase activity await identification of a protein factor equivalent to AhpF that also may exist in eukaryotic cells.

We propose that proteins in the large AhpC/TSA family act as general reductants within the cell. The proteins may perform general "reducing" functions, possibly analogous to the general "folding or chaperoning" functions carried out by the highly conserved heat shock proteins. In support of this hypothesis, some of the homologs were identified on the basis of their redox activities and at least eight of the AhpC/TSA proteins are encoded near other genes encoding proteins that have oxidation–reduction activities. The proteins may protect against the damage caused by reactive oxygen and sulfur species generated intracellularly during respiration or during interaction with intracellular iron. However, since *M. thermoautotrophicum* and *C. pasteurianum* are anaerobes, the proteins may also protect against oxidants generated externally.

The continued characterization of the individual family members as well as comparative studies of the AhpC/TSA-like family should help to elucidate the functions of these highly conserved proteins.

Note. During revision of this manuscript, three additional members of the AhpC/TSA family were identified at the cDNA level. Two encode natural killer enhancing factor (NKEF) A and B (accession nos. L19184 for NKEF-A and L19185 for NKEF-B) (26). The third (accession no. L14286) was obtained from a human brain cDNA library.

Note Added in Proof. We recently found that TSA can reduce peroxides in the presence of proper reducing equivalents. We thus propose to name the AhpC/TSA homologs the peroxidoxin family.

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