A 25-kDa antioxidant enzyme that provides protection against oxidation systems capable of generating reactive oxygen and sulfur species has previously been identified. The nature of the oxidant eliminated by, and the physiological source of reducing equivalents for, this enzyme, however, were not known. The 25-kDa enzyme is now shown to be a peroxidase that reduces $\text{H}_2\text{O}_2$ and alkyl hydroperoxides with the use of hydrogens provided by thioredoxin, thioredoxin reductase, and NADPH. This protein is the first peroxidase to be identified that uses thioredoxin as the immediate hydrogen donor and is thus named thioredoxin peroxidase (TPx). TPx exists as a dimer of identical 25-kDa subunits that contain 2 cysteine residues, Cys$^{47}$ and Cys$^{150}$. Cys$^{47}$-SH appears to be the site of oxidation by peroxides, and the oxidized Cys$^{47}$ probably reacts with Cys$^{150}$-SH of the other subunit to form an intermolecular disulfide. Mutant TPx proteins lacking either Cys$^{47}$ or Cys$^{150}$, therefore, do not exhibit thioredoxin-coupled peroxidase activity. The TPx disulfide is specifically reduced by thioredoxin, but can also be reduced (less effectively) by a small molecular size thiol. The *Saccharomyces cerevisiae* thioredoxin reductase gene was also cloned and sequenced, and the deduced amino sequence was shown to be 51% identical with that of the *Escherichia coli* enzyme.

In the presence of $\text{O}_2$ and an electron donor such as ascorbate or a thiol compound (RSH), iron generates reactive oxygen species that include $\text{O}_2^\cdot$, $\text{H}_2\text{O}_2$, and $\text{HO}^\cdot$ (1, 2). It is generally believed that the following reactions take place:

$$\text{Fe}^{2+} + \text{ascorbate} \rightarrow \text{Fe}^{3+} + \text{semidehydroascorbate radical}$$

**REACTION 1**

$$\text{Fe}^{2+} + \text{RSH} \rightarrow \text{Fe}^{3+} + \text{RS}^\cdot + \text{H}^+$$

**REACTION 2**

$$\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^\cdot$$

**REACTION 3**

$$2\text{O}_2^\cdot + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

**REACTION 4**

$$\text{Fe}^{2+} + \text{HO}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$$

**REACTION 5**

$\text{Fe}^{4+}$ is reduced by the electron donor as shown in reactions 1 and 2. The resulting reduced metal ion then generates $\text{O}_2^\cdot$, $\text{H}_2\text{O}_2$, and $\text{HO}^\cdot$ by the sequential reactions 3, 4, and 5. Hydroxyl radicals (OH$^\cdot$) are powerful oxidants and inflict damage on lipids, proteins, and nucleic acids. Although the semidehydroascorbate radical, the other product of reaction 1, is not particularly reactive and undergoes a disproportionation to ascorbate and dehydroascorbate or a reduction by glutathione (GSH), the thiol radical (RS$^\cdot$) produced in reaction 2 is reactive and can be further converted to the sulfur-containing radicals $\text{RSO}_2^\cdot$ and RSSR$^\cdot$ (3, 4).

These two metal-catalyzed oxidation systems, the ascorbate-oxidation system (Fe$^{3+}$, ascorbate, and dehydroascorbate or a reduction by glutathione (GSH), the thiol radical (RS$^\cdot$) produced in reaction 2 is reactive and can be further converted to the sulfur-containing radicals $\text{RSO}_2^\cdot$ and RSSR$^\cdot$ (3, 4).

We have previously purified a 25-kDa enzyme from yeast (4) and rat brain (5) that prevents damage induced by the thiol oxidation system but not that induced by the ascorbate oxidation system, despite the fact that the degree of oxidative stress is similar for the two systems as judged by the comparable extent of induced inactivation of glutamine synthetase. We postulated that the 25-kDa enzyme eliminates reactive sulfur species such as RS$^\cdot$, RSSR$^\cdot$, or $\text{RSO}_2^\cdot$, providing specificity to the thiol-containing system, and named this protein thi\-specific antioxidant (TSA). However, several lines of evidence described in this report suggest that TSA is not an appropriate name.

Yeast and rat genes that encode the 25-kDa protein have been cloned and sequenced (6, 7). The deduced amino acid sequences show no homology to conventional antioxidant enzymes, including superoxide dismutases (yeast Cu,Zn-superoxide dismutase, yeast Mn-superoxide dismutase), catalases (yeast catalase A, yeast catalase T), and peroxidases (yeast cytochrome c peroxidase, mouse glutathione peroxidase, and pig phospholipid hydroperoxide glutathione peroxidase) (7). *Salmonella typhimurium* alkylhydroperoxide reductase has been shown to consist of 22-kDa AhpC and 57-kDa AhpF, an FAD-containing NAD(P)H dehydrogenase (8, 9). The two 25-kDa protein sequences are 40% identical with AhpC. A search of data bases also revealed 26 additional protein sequences that are homologous to the 25-kDa protein and AhpC (6). The homologous proteins, except for AhpC, are not associated with known biochemical functions and may represent a new, widely distributed family of antioxidants. Alignment of the amino acid sequences of the antioxidant family members revealed 2 highly conserved cysteine residues, corresponding to Cys$^{47}$ and Cys$^{150}$.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank$^{10}$/EMBL Data Bank with accession number(s) U10274.

$^1$ The abbreviations used are: TSA, thi\-specific antioxidant; AhpC, a 21-kDa component of alkyl hydroperoxide reductase; AhpF, a 57-kDa component of alkyl hydroperoxide reductase; Trx, thioredoxin; Trx reductase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TPx, thioredoxin peroxidase; kb, kilobase(s).

$^2$ AhpC and AhpF were previously referred to as C22 and F52, respectively, in Ref. 8.
in the yeast 25-kDa protein. The more amino-terminal cysteine is conserved in all family members, whereas the more carboxyl-terminal cysteine is present in most but not all members (6). The oxidized form of the 25-kDa protein exists mainly in a dimeric form linked by two disulfide bonds between Cys47 and Cys170. The 25-kDa protein does not contain any obvious redox cofactor, and the cysteine residues appear to constitute the site of oxidation (10).

The reduced form of AhpC converts alkyl hydroperoxides to the corresponding alcohols with concomitant oxidation of the two sulfhydrys of AhpC to a disulfide bond (8). The regeneration of AhpC sulfhydrys is achieved by AhpF, which transfers reducing equivalents from NADPH to the disulfide of AhpC. The fact that the 25-kDa protein is homologous to AhpC suggests that it may also act on peroxides, and the reduction of the 25-kDa protein disulfide may be achieved by an enzyme with a function similar to that of AhpC. Thus, the 25-kDa protein may possibly function as an antioxidant against both the ascorbate and thiol oxidation systems, and the previously observed specificity for the thiol oxidation system may be attributable to the possibility that thiols, but not cysteine, are able to reduce the 25-kDa protein disulfide.

We now describe the purification of two protein components from yeast that can reduce the 25-kDa protein disulfide at the expense of NADPH and support the antioxidant activity of the 25-kDa protein against the ascorbate oxidation system. The two protein components are shown to be thioredoxin (Trx) and thioredoxin reductase (TR). In the presence of Trx, TR, and NADPH, the 25-kDa protein reduced H$_2$O$_2$ and alkylhydroperoxide. We also describe, for the first time, the cloning and sequencing of the yeast TR gene.

**EXPERIMENTAL PROCEDURES**

**Materials**—A stock solution of FeC1$_2$ was prepared in 0.1 M HCl. Stock solutions of ascorbate and diethionitroreitol (DTT) were prepared in 20 mM Hepes-NaOH (pH 7.0) and treated with Chelex 100 (Bio-Rad). Glutamine synthetase was purified from *Escherichia coli* as described (11). A S. cerevisiae *cerevisiae* strain that is not able to produce the 25-kDa antioxidant has been described (7). Cysteine residues 47 and 170 of the yeast 25-kDa protein were individually replaced by serine, and recombinant proteins (RWT [wild type], RC47S, and RC170S) were expressed in and purified from *E. coli* as described (10).

**Antioxidant Activity Assay—Glutamine synthetase was subjected to inactivation by the ascorbate oxidation system, and the ability of column fractions to protect glutamine synthetase from the oxidative insult was measured in the presence of the 25-kDa protein and NADPH. Glutamine synthetase inactivation was performed in a 50-µl reaction mixture containing 2 µg of glutamine synthetase, 2 µg of 25-kDa protein, 0.4 mM NADPH, 10 mM ascorbate, 12.5 mM FeC1$_2$, 50 mM Hepes-NaOH (pH 7.0), and a portion of column chromatography fractions. After 10 min at 30 °C, the residual glutamine synthetase activity was measured by adding 2 ml of a γ-glutamyltransferase assay mixture as described (4).

**Identification and Purification of Protein Components That Support the NADPH-dependent Antioxidant Activity of the 25-kDa Protein against the Ascorbate Oxidation System—**Frozen *S. cerevisiae* BJ2926 cells (800 g) were suspended in 2 liters of deionized water and centrifuged at 5000 × g for 10 min. The cell pellet was resuspended with 3 liters of 50 mM Hepes-NaOH (pH 7.0) containing 5 mM phenylmethylsulfonyl fluoride, aprotinin (5 µg/ml), and leupeptin (1 µg/ml). Cells were lysed by 6 passes through a Laboratory Homogenizer (model 15 x; Gaulin, Wilmington, MA) at 9000 p.s.i., and the cell extract was centrifuged at 9000 × g for 30 min. Polyethylene glycol was added to the supernatant to a final concentration of 8%, and the mixture was then stirred for 30 min at 4 °C. The precipitate was collected by centrifugation at 5000 × g for 30 min and resuspended in 600 ml of 20 mM Hepes-NaOH (pH 7.0). Insoluble material was removed by centrifugation at 45,000 × g for 30 min.

The resulting supernatant was applied to an AF-red Toyopearl dye-affinity column (5 × 12 cm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.0). The column was washed with 800 ml of equilibration buffer, and proteins were eluted with a linear NaCl gradient from 0 to 2 x 1 liters of equilibration buffer at a flow rate of 5 ml/min (Fig. 3A, a). Fractions of 16 ml were collected, and 10 µl of each fraction were assayed. Peak fractions (fractions 131 to 138) were pooled, diluted with 20 mM Tris-HCl (pH 7.5), and concentrated in an Amicon concentrator.

One-fourth (5 ml) of the concentrated sample was applied to a Mono Q HR 10/10 column that had been equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted at a flow rate of 2 ml/min with a linear NaCl gradient from 0 to 400 mM for 40 min (Fig. 3A, b). Fractions of 2 ml were collected. Assays of 10 µl of each fraction did not reveal any antioxidant activity. However, when the assay was performed in the presence of 5 µl of the flavoprotein-containing peak centered at 27 min (peak I, Fig. 3A, b), glutamine synthetase-protecting activity was apparent in a peak centered at 17 min. Conversely, when the assay was performed in the presence of 5 µl of the 17-min fraction, a peak of antioxidant was apparent that coincided with the flavoprotein peak I. The Mono Q column yielded another flavoprotein-containing peak (peak II) centered at 19 min. However, with the addition of the peak II flavoprotein to the assay mixture, none of the Mono Q fractions protected glutamine synthetase. These results suggest that the 25-kDa protein-supporting activity consisted of two protein components that eluted in the 17- and 27-min peaks.

The Mono Q chromatographic step was repeated with the three remaining portions of the concentrated sample from the dye-affinity column. The 17-min peak fractions (fractions 16 to 19) and the 27-min peak fractions (fractions 26 and 27) were pooled. Small portions of both peak fractions were subsequently reduced with DTT, and the remaining portions were diluted with 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl (17-min sample) or 20 mM Hepes-NaOH (pH 7.0) (27-min sample) and concentrated.

One-third (3 ml) of the concentrated 27-min peak sample from the Mono Q column was applied at a flow rate of 1 ml/min to a TSK heparin-5PW high performance liquid chromatography column (7.5 × 75 mm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.0). Proteins were eluted at a flow rate of 1 ml/min by a NaCl gradient from 0 to 400 mM for 40 min (Fig. 3B, a). Fractions of 1 ml were collected and assayed for the glutamine synthetase-protecting activity in the presence of 2 µl of the pooled 17-min peak fractions from the Mono Q column. The Mono Q chromatographic step was repeated with the remaining two portions of the concentrated 27-min sample. Peak fractions (fractions 28 to 30) were combined and concentrated to 0.7 ml. One-half (0.35 ml) of the concentrated sample from the TSK heparin-5PW column was applied to a TSK G3000SW column (7.5 × 600 mm) that had been equilibrated with 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. Proteins were eluted at a flow rate of 0.5 ml/min with the same buffer. Fractions of 0.5 ml were collected and assayed in the presence of 2 µl of the pooled 17-min peak fractions from the Mono Q column. Activity was eluted as a single symmetrical peak (Fig. 3B, b). This gel filtration chromatography step was repeated with the remaining concentrated sample from the heparin column. Peak fractions (fractions 21 to 25) were combined, diluted with 20 mM Hepes-NaOH (pH 7.0), concentrated, divided into portions, and stored at −70 °C.

One-fourth of the concentrated 17-min peak sample from the Mono Q column was applied to a TSK G3000SW column (7.5 × 600 mm) that had been equilibrated with 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. Proteins were eluted at a flow rate of 0.5 ml/min with the same buffer. Fractions of 0.5 ml were collected and assayed in the presence of 2 µl of the pooled 27-min peak fractions from the Mono Q column. Activity was eluted as a single peak coincided with the major protein peak (Fig. 3C, a). This chromatographic step was repeated with the remaining three peaks of the concentrated 17-min peak sample from the Mono Q column, and the peak fractions were pooled and concentrated.

One-half of the concentrated sample from the TSK G3000SW gel filtration column was applied to a Vydac C$_8$ column (4.6 × 250 mm) that had been equilibrated with 10 mM sodium phosphate (pH 7.0). Proteins were eluted with a linear gradient of 0 to 30% acetonitrile in the same buffer for 10 min, followed by a second linear acetonitrile gradient of 30 to 70% for 40 min, at a flow rate of 1 ml/min. Fractions of 1 ml were collected, and, after removal of acetonitrile by evaporation, the two peaks of glutamine synthetase-protecting activity (fraction 24 and fractions 27 to 29) (Fig. 3C, b) were pooled separately and stored at −70 °C.

**RESULTS**

The 25-kDa Protein Is Reduced by DTT but Not by Ascorbate—We compared the abilities of ascorbate and DTT to reduce the oxidized form of the 25-kDa protein by taking advantage of the fact that the oxidized form exists as a dimer and the
Reduced form as a monomer under denaturing conditions. The dimer was converted to monomer by 10 mM DTT but not by 10 mM ascorbate (Fig. 1). This result supports the possibility that the 25-kDa protein could not prevent the damage induced by the ascorbate oxidation system because its sulfhydryl groups could not be regenerated.

Identification of a 25-kDa Protein-reducing Activity in Yeast Extract—We undertook a search for an enzyme that would support the antioxidant activity of the 25-kDa protein against the ascorbate oxidation system. Protection of glutamine synthetase against the ascorbate oxidation system was measured in the presence of various combinations of the 25-kDa protein, yeast extract, and NADPH or NADH. The combination of the 25-kDa protein, yeast extract, and NADPH provided the greatest protection (Fig. 2A). NADH was ineffective. The combination of crude extract and NADPH afforded a similar extent of protection as the combination of the 25-kDa protein, crude extract, and NADPH (Fig. 2B), probably because the yeast extract contained a sufficient amount of the 25-kDa protein (the 25-kDa protein is an abundant protein, constituting 0.3 to 0.7% of total soluble protein in yeast (5)). This conclusion was supported by the observation that an extract from a yeast mutant that cannot produce the 25-kDa protein did not protect glutamine synthetase in the presence of NADPH, whereas the mutant extract supplemented with the 25-kDa protein provided protection (Fig. 2B).

The 25-kDa Protein-reducing Activity Comprises Two Components—Purification of the putative 25-kDa protein-reducing enzyme was attempted from yeast extract. Column fractions were monitored for their ability to protect glutamine synthetase against the ascorbate oxidation system in the presence of the 25-kDa protein and NADPH (Fig. 3). Chromatography on a Toyopearl dye-affinity column yielded a single peak of protection activity (Fig. 3A, a). After subsequent chromatography on a Mono Q column, however, activity was not detectable in any fraction. The observation that the pool of all eluted proteins exhibited protection activity suggested that the 25-kDa protein-reducing activity is likely attributable to more than one component. Given that NADPH-dependent redox enzymes often contain FAD as a prosthetic group, we identified, by absorbance at 450 nm, peaks I and II (centered at 27 and 19 min, respectively) as potential flavoprotein-containing fractions from the Mono Q column (Fig. 3A, b). We then re-evaluated the protection activity of each of the Mono Q fractions after supplementation with either peak I or peak II. A protection activity peak centered at 17 min was detected when fractions were supplemented with flavoprotein peak I. Supplementation of each of the Mono Q fractions with the 17-min fraction yielded an activity peak centered at 27 min. These results suggested that the 25-kDa protein-regenerating activity was attributable to two components, one of which eluted at 17 min and the other, a flavoprotein, at 27 min from the Mono Q column.

Purification and Identification of the Flavoprotein Component—The flavoprotein that eluted in the 27-min peak from the Mono Q column was purified to homogeneity by two sequential chromatographic steps on a TSK heparin-5PW column and a TSK G3000SW gel filtration column (Fig. 3B). The molecular mass of the active protein was estimated as 66 kDa from the gel filtration chromatography (data not shown). However, the purified protein yielded a single band with an apparent molecular mass of 34 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) (not shown), suggesting it exists as a dimer of two identical subunits under nondenaturing conditions. The ultraviolet-visible absorbance spectrum of the purified protein has revealed peaks at 273, 379, and 457 nm and a shoulder at 480 nm (not shown), which are characteristics of flavoproteins.

The sequence of the amino-terminal 15 residues and the partial sequences of five tryptic peptides of the purified flavoprotein were determined: VXNKVXITGSGPAAH (amino terminal), VDLSSKPF (peptide 1), MHLPGFEETXWQK (peptide 2), VGSK (peptide 3), KNXETD (peptide 4), and QAIXAGX (peptide 5), where X represents an unidentified amino acid. A search of the GenBank database revealed that the amino-terminal sequence was homologous to the amino-terminal sequence of E. coli TR. Of 13 residues identified, 8 were identical and 3 were conservative substitutions. In addition, E. coli TR contains regions that showed homology to peptides 1, 2, and 5 and is known to be a dimer of 35-kDa subunits, each of which contains one tightly bound FAD molecule. TR purified from bakers’ yeast also consists of two 38-kDa subunits (12, 13). Furthermore, TR activity associated with the purified flavoprotein was directly demonstrated with the use of an assay that involves the conversion of 5,5’-dithiobis(2-nitrobenzoic acid) by reduced Trx to a colored product as described (12).

Purification and Identification of the Second Component of the 25-kDa Protein-reducing Activity—The second component...
required for the 25-kDa protein-reducing activity was purified to homogeneity from the 17-min peak fractions of the Mono Q column by successive chromatographic steps on a TSK G3000SW gel filtration column and a Vydac C<sub>18</sub> reversed-phase column (Fig. 3C). The reversed-phase chromatography yielded two protection activity peaks, 1 and 2, each of which contained a single protein of 12.6 kDa and 12.4 kDa, respectively, as estimated by SDS-PAGE (Fig. 4).

Sequences of the amino-terminal 12 residues were determined to be VTQFKTASEFDS (12.4-kDa protein) and VTQLK-SASEYDSDS (12.6-kDa protein). These sequences are identical with those of yeast Trx I (which contains 102 amino acids) and Trx II (103 amino acids), respectively (14–16).

**Cloning and Sequencing of Yeast Thioredoxin Reductase Gene**—Although our results indicated that the flavoprotein component of the 25-kDa protein-reducing activity was likely TR, the complete amino acid sequence of yeast TR was not known and many other FAD-containing disulfide oxidoreductases share similar sequences. We therefore prepared rabbit antibodies to purified yeast TR and used them to screen an S. cerevisiae genomic DNA library in an attempt to clone and sequence the yeast TR gene. An immunologically positive clone with a 2.6-kb insert was isolated. The insert contained three internal Acc<sub>1</sub> sites, cleavage at which generated four fragments of 0.5, 1.1, 0.2, and 0.8 kb. Sequencing revealed that the 1.1-kb and 0.2-kb fragments together yielded an open reading frame that contained sequences encoding the amino-terminal 15 residues and the five tryptic peptides of the purified flavoprotein (Fig. 5). The open reading frame encodes a polypeptide of 319 amino acids with a calculated molecular mass of 35,908 Da. The amino acid sequences of E. coli and S. cerevisiae TR molecules were compared by a dot matrix plot (not shown). The resulting diagonal line without large gaps indicated that most regions of the two sequences are well conserved. Alignment revealed 51% identity and 69% similarity between E. coli and S. cerevisiae TR. In addition, yeast TR, like the E. coli enzyme (17), has a CXXC motif for the redox-active cysteines and consensus sequences for the binding of FAD and NADPH.

These results confirmed that the flavoprotein we purified from yeast extract is TR. The calculated isoelectric point of yeast TR is 5.36 and the extinction coefficient at 278 nm is 23,380 M<sup>-1</sup> cm<sup>-1</sup>, which is equivalent to 0.69 absorbance unit mg<sup>-1</sup> ml<sup>-1</sup>. Previously, a partial reading frame capable of encoding the carboxy-terminal 59 amino acids of TR was detected adjacent to the S. cerevisiae TRP4 gene, which encodes the tryptophan biosynthetic enzyme anthranilate phosphorolysase (18).

**Comparison of the 25-kDa Protein-reducing Activities of DTT and the Thioredoxin System**—Incubation with the Trx system (Trx, TR, and NADPH) caused immediate reduction of the dimeric form of the 25-kDa protein (data not shown) similar to...
The conversion induced by DTT (Fig. 1). The 25-kDa protein-supporting functions of DTT and the Trx system were compared by measuring their abilities to prevent glutamine synthetase inactivation in the presence of various concentrations of the 25-kDa protein (Fig. 6). Glutamine synthetase was inactivated by the ascorbate-oxidation system for the evaluation of the Trx system and by the DTT-oxidation system for the evaluation of DTT. The concentration of Fe²⁺ was adjusted such that 90% of glutamine synthetase was inactivated after incubation for 10 min in the absence of the 25-kDa protein. The concentration of the 25-kDa protein required for 50% protection of glutamine synthetase in the presence of the Trx system was ~0.3 μM, compared to ~2.3 μM in the presence of DTT. We have previously shown that the 25-kDa protein is less effective as an antioxidant with glutathione or lipoic acid than with DTT as a provider of reducing equivalents (4). These results suggest that the Trx system, not a thiol such as glutathione, is likely to reduce oxidized 25-kDa protein in cells.

The extent of glutamine synthetase protection increased in a saturable, dose-dependent manner with 25-kDa protein (Fig. 6), Trx (not shown), and TR (not shown) concentration. Trx I was slightly more efficient as a hydrogen donor than Trx II.

### Peroxidase Activity of the 25-kDa Protein

We examined the 25-kDa protein for peroxidase activity toward H₂O₂ by directly following the decrease in H₂O₂ in the presence of the Trx system (Fig. 7). The rate of the H₂O₂ removal was fast initially and then decreased gradually. Peroxidase activity toward the 25-kDa protein for peroxidase activity toward 25-kDa protein was measured by directly monitoring the decrease in H₂O₂ in the presence of the Trx system.

### Deduced Amino Acid Residues

The regions corresponding to determined partial amino acid sequences of yeast Tr are underlined; amino acids in parentheses represent residues that cannot be determined unambiguously. The redox-sensitive motif (Cys-Ala-Val-Cys) is highlighted with a shaded rectangle. The two consensus sequences for the binding of FAD are marked with triangles (▲), and the consensus sequence for the binding of NADPH with squares (■).
concentration increased. For equivalent concentrations of peroxide, the decrease in rate was more rapid with t-butyl hydroperoxide than with H$_2$O$_2$; the reaction essentially stopped several minutes after the addition of millimolar concentrations of t-butyl hydroperoxide (Fig. 8B). Cumene hydroperoxide also elicited NADPH oxidation, and the oxidation rate decreased rapidly with time as for t-butyl hydroperoxide (data not shown). The decrease in rate was not attributable to exhaustion of substrate or to product inhibition by NADP$^+$; NADP$^+$ competes poorly with NADPH for TR (the $K_m$ for NADPH is 1.2 $\mu$M and the $K_m$ for NADP$^+$ is 15 $\mu$M (19)). The addition of NADP$^+$ at a concentration similar to that of NADPH did not have a marked effect on the NADPH oxidation rate (data not shown). The decrease in rate was a first-order process, as judged from analysis (not shown) of the time course shown in Fig. 8A, and appeared attributable to inactivation of 25-kDa protein by peroxides.

**FIG. 8. NADPH oxidation coupled by TR, Trx, and the 25-kDa protein to the reduction of H$_2$O$_2$ (A), t-butyl hydroperoxide (B), and putative H$_2$O$_2$ generated by Fe$^{3+}$ and ascorbate (C).** NADPH oxidation was monitored as $A_{260}$ in a 500-ml reaction mixture containing 25 mM Hepes-NaOH (pH 7.0), 0.15 $\mu$M TR, 0.45 $\mu$M Trx II, 2.1 $\mu$M 25-kDa protein, 0.25 mM NADPH, and peroxide or the peroxide-generating system. The background NADPH oxidation in the absence of peroxide (or Fe$^{3+}$) was subtracted. Dotted lines are for 1 mM H$_2$O$_2$ (A), 1 $\mu$M t-butyl hydroperoxide (B), or 20 $\mu$M FeCl$_3$, and 10 mM ascorbate (C); solid lines are for 5 mM H$_2$O$_2$ (A), 5 mM t-butyl hydroperoxide (B), and 40 $\mu$M FeCl$_3$ and 10 mM ascorbate (C).

**In addition, the combination of TR and the 26-kDa protein failed to protect glutamine synthetase from the ascorbate oxidation system when the Trx system served as hydrogen donor (Fig. 11A). Direct assay of peroxidase activity also revealed the inactivity of RC170S and RC47S (Fig. 11B), suggesting the indispensability of both cysteines for peroxidase activity.**

**DISCUSSION**

In our attempt to identify the physiological hydrogen donor that supports the catalytic activity of the 25-kDa yeast antioxidant protein, we purified two protein components that together mediate the flow of electrons from NADPH to the oxidized form of the 25-kDa protein. One component was identified as Trx or Trx II, and the other as TR. The Trx system (Trx, TR, NADPH) was a more potent hydrogen donor for the 25-kDa protein than DTT on the basis of ability to support the antioxidant activity of the 25-kDa protein (Fig. 6). In addition, the combination of Trx and TR was the major electron carrier detectable from yeast extract when NADPH and NADH were used as the ultimate electron donor (Figs. 2A and 3A). These results indicate that our previous assumption (4, 5, 7) was incorrect and that the physiological hydrogen donor for the catalytic function of the 25-kDa protein is not a thiol like glutathione but the Trx system.
Our current data suggest that, in the presence of the Trx system, the 25-kDa protein reduces peroxides with Trx as the immediate hydrogen donor and protects glutamine synthetase against the ascorbate oxidation system by eliminating H$_2$O$_2$ (Figs. 7 and 8). Thus, we propose to rename the 25-kDa protein thioredoxin peroxidase (TPx). It is now clear that the apparent thiol specificity observed previously for TPx, which gave rise to the name TSA, is attributable to the fact that the TPx disulfide, rather than the thiol moiety, is the immediate hydrogen donor and thus the active center of the enzyme. TPx exists predominantly as a dimer linked by two identical disulfide bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center. The sequence of events that are likely to occur during the formation of peroxides associated with the NADPH oxidation system is summarized in Fig. 12. TR consists of two identical subunits linked by noncovalent bonds. Each subunit has one tightly bound FAD molecule and a redox-sensitive disulfide in its active center.
The TPx mutant lacking Cys$^{17}$, RC47S, is inactive regardless of whether the reducing equivalents are provided by DTT or by the Trx system, whereas RC170S is active in the presence of DTT and inactive in the presence of the Trx system (Fig. 11). These results suggest the model shown in Fig. 13A. In this scheme, Cys$^{17}$-SH is the primary site of substrate peroxide reduction and is directly oxidized by ROOH to yield ROH and cysteine sulfenic acid (Cys$^{17}$-SOH). The Cys$^{17}$-SOH then reacts with Cys$^{170}$-SH of the other subunit to produce H$_2$O and an intermolecular disulfide. Cysteine sulfenic acid was previously proposed as a stable intermediate of the redox-sensitive half-cystine in oxidized NADH peroxidase, its stability being mainly attributable to the absence of nearby protein thiols (21). The sulfenic acid is readily oxidized to sulfonic acid (Cys$^{17}$-SO$_2$H) by peroxides, a reaction that has been suggested to be responsible for the irreversible inactivation of NADH peroxidase by H$_2$O$_2$ (21). The Cys$^{17}$-SOH of TPx may also be further oxidized if the reaction of Cys$^{17}$-SOH and Cys$^{170}$-SH requires significant distortion of the protein backbone and is thus sufficiently slow to allow the encounter of Cys$^{17}$-SOH with peroxide molecules; this scenario may be especially relevant in the presence of high concentrations of peroxides. Such inactivation of TPx by substrates is likely responsible for our previous failure to detect peroxidase activity of TPx (4, 20). A slight reduction of H$_2$O$_2$ by a high concentration of TPx (1 mg/ml) and DTT has, however, been observed (22). Although there is no tangible evidence for the sulfenic acid intermediate, the reaction scheme shown in Fig. 12A is the simplest mechanism that is compatible with the observations that both Cys$^{17}$ and Cys$^{170}$ are essential for TPx activity, the oxidized TPx is a dimer containing disulfides between Cys$^{17}$ and Cys$^{170}$, and TRP is highly susceptible to inactivation by peroxides. It is also possible that the reaction mechanism involves an intermediate with sulfonated to a nearby nitrogen analogous to the reaction mechanism proposed for glutathione peroxidase (see below).

In the model shown in Fig. 13A, it is possible that a small diffusible thiol molecule could replace Cys$^{170}$-SH in the formation of a disulfide with Cys$^{17}$-SH. Such a scenario is shown in Fig. 13B and would explain why the TPx mutant RC170S protects glutamine synthetase against the thiol oxidation system (10). An analogous mechanism has been proposed for seleno-protein glutathione peroxidase, which contains a redox-active half-selenocysteine and no other sulfur or second selenium in proximity (23); on reaction with a peroxide molecule, the Cys-SeH is oxidized to an intermediate that was proposed to be selenic (Cys-SeOH) or selenenium bonded to nitrogen (Cys-Se-N) (23, 24) and which is subsequently reduced by 2 molecules of GSH, first to Cys-Se-S-G and then to Cys-SeH and GSSG.

The schemes shown in Fig. 13 require maintenance of the dimeric arrangement of TPx throughout the catalytic cycle, even in the absence of the Cys$^{17}$-Cys$^{170}$ disulfide linkage. Indeed, wild-type TPx, RC47S, and RC170S all exist as dimers or higher oligomers under reducing, nondenaturing conditions, as judged from the PAGE performed in the presence of 2-mercaptoethanol (not shown). In the presence of SDS and 2-mercaptoethanol, however, all three TPx molecules migrate as monomers (10). However, we cannot exclude the possibility that the dimeric TPx

Fig. 12. The sequence of events that couple peroxide reduction to NADPH oxidation.

Fig. 13. Mechanism of peroxide reduction by wild-type TPx in the presence of Trx (A) or by the TPx mutant RC170S in the presence of a thiol (B). Closed circle indicates amino terminus.

is an artifact of purification and that TPx operates as a monomer during catalysis, forming an intramolecular disulfide.

TPx and AhpC are similar in size, exhibit 40% identity in amino acid sequence, contain 2 conserved cysteine residues that align perfectly, and reduce peroxides ultimately at the expense of NAD(P)H. For all their similarities, TPx and AhpC differ in several characteristics: AhpC is rapidly inactivated by H$_2$O$_2$ (8), whereas TPx is more sensitive to alkyl hydroperoxides (Fig. 8); and regeneration of reduced AhpC is achieved by a single protein (AhpF), whereas the reduction of the TPx disulfide requires both Trx and TR. AhpF has been identified in prokaryotes but not in eukaryotes. Yeast TPx cannot be reduced by S. typhimurium AhpF. AhpF (from S. typhimurium, 521 amino acids) is significantly larger than TR (from yeast, 318 amino acids), but the amino acid sequence alignment reveals that the carboxyl-terminal 311 residues of AhpF are 33% identical with TR. The two homologous regions contain the consensus binding sites for FAD and NAD(P)H as well as the redox-sensitive CXXC sequence, all of which align perfectly between the two protein sequences (25, 26). AhpF contains two CXXC motifs: one (Cys$^{129}$ and Cys$^{132}$) in the amino-terminal region that does not have a corresponding region in TR, and the other (Cys$^{347}$ and Cys$^{349}$) in the carboxyl-terminal region that aligns with the only CXXC (Cys$^{142}$ and Cys$^{145}$) motif of TR.

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3 H. Z. Chae and S. G. Rhee, unpublished observations.
These observations suggest that the amino-terminal 210 residues of AhpF may serve as a hydrogen carrier from Cys$^{45}$-SH and Cys$^{46}$-SH to AhpC, analogous to Trx, which carries hydrogens from Cys$^{142}$-SH and Cys$^{143}$-SH of TR to TPx.

We have identified 26 proteins that exhibit homology to TPx and AhpC (6). These proteins are present in organisms from all kingdoms and have not been associated with known biochemical reactions (6). The similarities among these proteins extend over the entire sequence, and 2 cysteines, which correspond to Cys$^{47}$ and Cys$^{179}$ in yeast TPx, are highly conserved. The amino-terminal cysteine is conserved in all family members, and the carboxyl-terminal cysteine in all except six proteins. It is, therefore, reasonable to speculate that these additional 26 proteins are also peroxidases, with the conserved amino-terminal cysteine being the primary redox catalytic site. We propose to name this family of peroxidases the peroxiredoxin family. The diversity in the amino acid sequences of the family members probably reflects several different mechanisms involved in the regeneration of reduced peroxiredoxin. The members that contain the two conserved cysteines may be reduced by a mechanism that involves either a single protein, like AhpF, or two proteins, like Trx and TR. On the other hand, the members that contain only 1 conserved cysteine might require the participation of a small thiol like GSH, as in the case of glutathione peroxidase and RC170S.

The peroxiredoxin family thus likely represents a widely distributed class of enzymes that directly reduce H$_2$O$_2$ and various alkyl hydroperoxides with hydrogens derived from NAD(P)H via various routes. TPx, previously called TSA, is a member of the peroxiredoxin family whose immediate hydrogen donor is Trx. To our knowledge, TPx is the first peroxidase to be identified that uses Trx as hydrogen donor. Because TPx is ubiquitous and abundant in mammalian tissues, it, together with glutathione peroxidase, would provide a major pathway of H$_2$O$_2$ elimination. The discovery of TPx, therefore, adds a previously unidentified antioxidant function to the thioredoxin system.

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