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Dimerization of thiol-specific antioxidant and the essential role of cysteine 47

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ABSTRACT **Thiol-specific antioxidant (TSA) from yeast contains cysteine residues at amino acid positions 47 and 170 but is not associated with obvious redox cofactors. These two cysteines are highly conserved in a family of proteins that exhibit sequence identity of 23–98% with TSA. The roles of Cys-47 and Cys-170 in yeast TSA were investigated by replacing them individually with serine and expressing the mutant TSA proteins (RC47S and RC170S, respectively), as well as wild-type TSA (RWT), in *Escherichia coli*. Wild-type TSA purified from yeast (YWT) and RWT were both shown to exist predominantly as dimers, whereas RC47S and RC170S existed mainly as monomers under a denaturing condition. This observation suggests that the dimerization of YWT and RWT requires disulfide linkage of Cys-47 and Cys-170. The presence of the Cys-47–Cys-170 linkage in YWT was directly shown by isolation of dimeric tryptic peptides, one monomer of which contained Cys-47 and the other contained Cys-170. A small percentage of YWT, RWT, RC47S, and RC170S molecules formed dimers linked by Cys-47–Cys-47 or Cys-170–Cys-170 disulfide bonds. The antioxidant activity of the various TSA proteins was evaluated from their ability to protect glutamine synthetase against the dithiothreitol/Fe^{3+}/O_2 oxidation system. YWT, RWT, and RC170S were equally protective, whereas RC47S was completely ineffective. Thus, Cys-47, but not Cys-170, constitutes the site of oxidation by putative substrate.**

A 25-kDa antioxidant enzyme, which exhibits neither the enzymic activity of nor similarity in amino acid sequence to conventional antioxidants, such as superoxide dismutase, catalase, and peroxidase, has been purified from *Saccharomyces cerevisiae* (1) and bovine brain (2). The purified enzyme protects cellular components against oxidation systems in which a thiol functions as a reducing equivalent [for example, dithiothreitol (DTT)/Fe^{3+}/O_2]. The enzyme was thus named thiol-specific antioxidant (TSA).

TSA genes have been cloned from yeast (3) and rat brain (2) and sequenced. The two deduced amino acid sequences are 65% identical to each other and 40% identical to AhpC, which, together with the flavin adenine dinucleotide-containing protein, AhpF, has been proposed to constitute *Salmonella typhimurium* alkyl hydroperoxide reductase (4, 5). Several lines of evidence suggest an antioxidant role for TSA and Ahp (AhpC and AhpF) proteins *in vivo* (4). A search of data bases revealed 23 additional protein sequences that are homologous to TSA and AhpC (2). These proteins appear to represent a newly discovered widely distributed family of antioxidant enzymes (named the AhpC/TSA family). Alignment of the AhpC/TSA family members revealed two highly conserved cysteine residues, which correspond to Cys-47 and Cys-170 in yeast TSA. The N-terminal cysteine is conserved in all family members and the C-terminal cysteine is conserved in all except six members.

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We have now investigated the role of these conserved cysteines by replacing them with serine in yeast TSA. Both Cys-47 and Cys-170 were shown to be necessary for the maintenance of the dimeric structure of oxidized TSA, and Cys-47, but not Cys-170, is essential for antioxidant activity measured *in vitro*.

MATERIALS AND METHODS

PCR-Mediated, Site-Directed Mutagenesis. The 1.0-kb *Eco*RI fragment that contains the complete yeast TSA coding sequence was ligated into cloning vector pBluescript SK(+) (Stratagene). The resulting plasmid pBS-TSA, served as a PCR template for generation of cassettes for wild-type and mutant TSA proteins. The forward primer 5′-TSA (5′-CGAGAATTCACAATGGTCGCTCAA-3′) contains an *Eco*RI site (underlined) and nt −3 to 12 of the yeast TSA sequence, which include the initiation codon (overlined). The reverse primer 3′-TSA (3′-GTTTATTCTGCGAACGGGCCCAT-5′) contains a *Sma* I site (underlined) and the sequence complementary to nt 585–599 of TSA, which includes the stop codon (overlined). 5′-C47S (5′-CACTTTCGTCAGTCCAACCGAA-3′) and 3′-C47S (3′-GTGAAAGCAGTCAGGTTGGCTT-5′), the sequences of which are complementary, correspond to nt 132–153 of the TSA sequence with a 1-bp mismatch (underlined) that converts the codon for Cys-47 to a serine codon. 5′-C170S (5′-CTGTCTTGCCAAGTAACTGGACTCCAG-3′) and 3′-C170S (3′-GACAGAACGGTTCATTGACCTGAGGTC-5′) are complementary and correspond to nt 500–526 of TSA, with a 1-bp mismatch (underlined) that converts the codon for Cys-170 to a serine codon.

An expression cassette for wild-type TSA was generated by PCR with pBS-TSA as a template and 5′-TSA and 3′-TSA as primers. The PCR products were digested with *Eco*RI and *Sma* I and inserted into pBluescript SK(+) according to the manufacturer's instructions (FMC). The resulting plasmid was designated pBS-TSA-WT, and the entire sequence of the expression cassette (*Eco*RI/*Sma* I fragment) was confirmed by nucleotide sequencing.

To generate the recombinant protein (RC47S) in which Cys-47 is replaced by serine, we synthesized two PCR products that overlap in the sequence containing the same mutation with two separate PCRs, one with primers 5′-TSA and 3′-C47S and the other with primers 5′-C47S and 3′-TSA. The two primary PCR products were then mixed, denatured, and allowed to reanneal, producing two possible heteroduplex products. The heteroduplexes with recessed 3′ ends were extended by *Pfu* DNA polymerase to produce a frag-

Abbreviations: DTT, dithiothreitol; TSA, thiol-specific antioxidant; TNB, 5-thio-2-nitrobenzoic acid; RWT, recombinant wild-type protein; YWT, yeast wild-type protein.

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ment that was the sum of the two overlapping products, and the extended fragment was then amplified with the use of primers 5'-TSA and 3'-TSA. The secondary PCR products were digested with *Eco*RI and *Sma* I, separated on a low-melting point agarose gel, and introduced into pBluescript SK(+). The cassette (*Eco*RI/*Sma* I fragment) that encoded the recombinant protein (RC170S), in which Cys-170 is replaced by serine, was produced in the same manner as the cassette for RC47S, except that primers 5'-C170S and 3'-C170S were used instead of 5'-C47S and 3'-C47S, respectively. The entire sequences of the cassettes for RC47S and RC170S were confirmed by nucleotide sequencing. The cassettes that encoded the recombinant wild-type (RWT), RC47S, and RC170S proteins were transferred to the *Escherichia coli* expression vector pKK223-3 with the use of the *Eco*RI and *Sma* I sites to generate pKK-WT, pKK-C47S, and pKK-C170S, respectively.

Assay of TSA. The oxidative inactivation of glutamine synthetase by DTT/Fe^{3+}/O_2 can be prevented by TSA. TSA was thus assayed by monitoring its ability to inhibit the DTT/Fe^{3+}/O_2-mediated inactivation as described (1).

Overexpression of TSA. *E. coli* strain DH5αF'IQ carrying the appropriate expression plasmid was cultured (25 ml) overnight, transferred to 500 ml of fresh LB medium supplemented with ampicillin (100 μg/ml), and cultured further for 10 hr with vigorous shaking. The 500-ml culture was transferred to 10 liters of LB medium in a Microferm Fermentor (New Brunswick Scientific), and, when the absorbance at 600 nm of the culture reached 0.6–0.8, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 4 hr of induction, the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −70°C until use.

Sequencing of Tryptic Peptides: 5-Thio-2-nitrobenzoic Acid (TNB)-Conjugated Tryptic Peptides from Reduced TSA. Purified TSA (50 μg) was denatured reductively by treatment with 50 mM Tris·HCl, pH 8.0/6 M guanidine hydrochloride/2 mM DTT. The sulfhydryl groups were labeled with TNB by adding Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), to a final concentration of 10 mM. The TNB-conjugated TSA was precipitated with trichloroacetic acid [final concentration, 10% (wt/vol)], and the pellet was washed with acetone, suspended in 50 mM Tris·HCl (pH 8.0), and digested with trypsin overnight at 30°C. The resulting peptides were applied to a Vydac C_{18} column (4.6 × 250 mm), and they were eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid over 60 min. Five peptides (a–e) containing cysteine residues were readily identified by monitoring elution at 210 and 328 nm (see Fig. 6*A*).

Tryptic Peptides from Nonreduced TSA. Purified TSA (200 μg) in 50 mM Tris·HCl (pH 8.0) was digested with trypsin overnight at 30°C and the resulting peptides were analyzed on a Vydac C_{18} column as described above (see Fig. 6*B*).

Immunoblot Analysis. TSA proteins on nitrocellulose filters were incubated with antibodies to yeast TSA. Immunoreactive proteins were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories).

RESULTS

Physical Characterization of Mutant TSA Proteins. Cysteine residues 47 and 170 of yeast TSA were individually replaced by serine, and recombinant TSA proteins (RWT, RC47S, and RC170S) were expressed in *E. coli* cells containing the appropriate expression cassette in the vector pKK223-3, under the control of the isopropyl β-D-thiogalactopyranoside-inducible *Taq* promoter.

Immunoblot analysis of crude extracts of *E. coli* showed that RWT, RC47S, and RC170S proteins were expressed at similar levels, which were ≈20 times that of native TSA in yeast (YWT) (Fig. 1). The mobility of RC47S on reducing SDS/polyacrylamide gels was slightly slower than those of YWT, RWT, and RC170S. RWT, RC47S, and RC170S were purified from *E. coli* extracts (Fig. 2), and YWT was purified from *S. cerevisiae* extract (1). The purified proteins were analyzed on SDS/polyacrylamide gels with and without 2-mercaptoethanol and visualized by either Coomassie blue staining (Fig. 3) or immunoblot analysis (Fig. 4). In the reducing gels, all four of the purified proteins were detected at molecular sizes corresponding to the monomeric form (Figs. 3*A* and 4*A*). However, in the nonreducing gels, the mobility patterns were complex (Figs. 3*B* and 4*B*), as shown schematically in Fig. 4*C*. The major band (D1) of YWT and RWT corresponded to the molecular size of a dimer, whereas the major band (M1) of RC47S and RC170S corresponded to the molecular size of a monomer. In addition, YWT and RWT each yielded two minor bands (D2 and D3) in the dimeric region, whereas RC47S and RC170S each yielded at least one additional monomeric band (M2) just above M1 and one band each (D4 for RC47S and D5 for RC170S) in the dimer region. The minor bands were more evident in the immunoblot (Fig. 4*B*), which was deliberately incubated for an extended period with the substrate for the alkaline phosphatase-conjugated secondary antibody.

These results are consistent with the notion that both YWT and RWT exist predominantly as dimers (D1) formed through disulfide linkage of Cys-47 and Cys-170. With either of the two cysteines replaced by serine, RC47S and RC170S cannot form such a dimer and exist mainly as monomers (M1) under a denaturing condition.

Reductive Dissociation of TSA Dimers. Progressive reduction of YWT was monitored by incubation for 5 min at room temperature with various concentrations of DTT (Fig. 5). In the absence of DTT, the major band, D1, and the two minor bands, D2 and D3, were visible. As the DTT concentration increased, D1, D2, and D3 decreased gradually, with a concomitant increase in monomers M1, M2, and M3. In addition, a new band, D6, appeared first at 0.25 mM DTT, increased gradually up to 2 mM DTT, and decreased thereafter. The intensity of D6 at 2 mM DTT was greater than the sum of those of D2 and D3 in the absence of DTT, suggesting

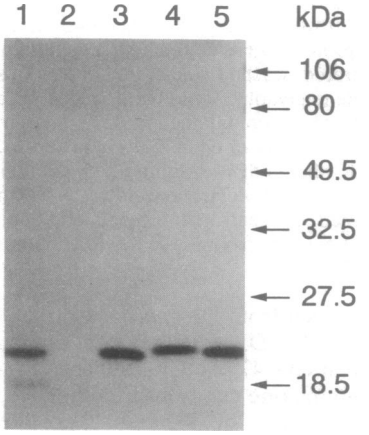

FIG. 1. Expression of wild-type and mutant TSA proteins in *E. coli*. Crude protein extracts of *S. cerevisiae* (10 μg) and *E. coli* (1 μg) were separated by SDS/PAGE on a 12% gel in the presence of 2-mercaptoethanol and subjected to immunoblot analysis with antibodies to yeast TSA. Lanes 1, *S. cerevisiae*; 2, *E. coli* transformed with expression vector (pKK223-3) alone; 3, *E. coli* transformed with pKK-WT, which encodes RWT; 4, *E. coli* transformed with pKK-C47S, which encodes RC47S; 5, *E. coli* transformed with pKK-C170S, which encodes RC170S. Molecular size markers are indicated.

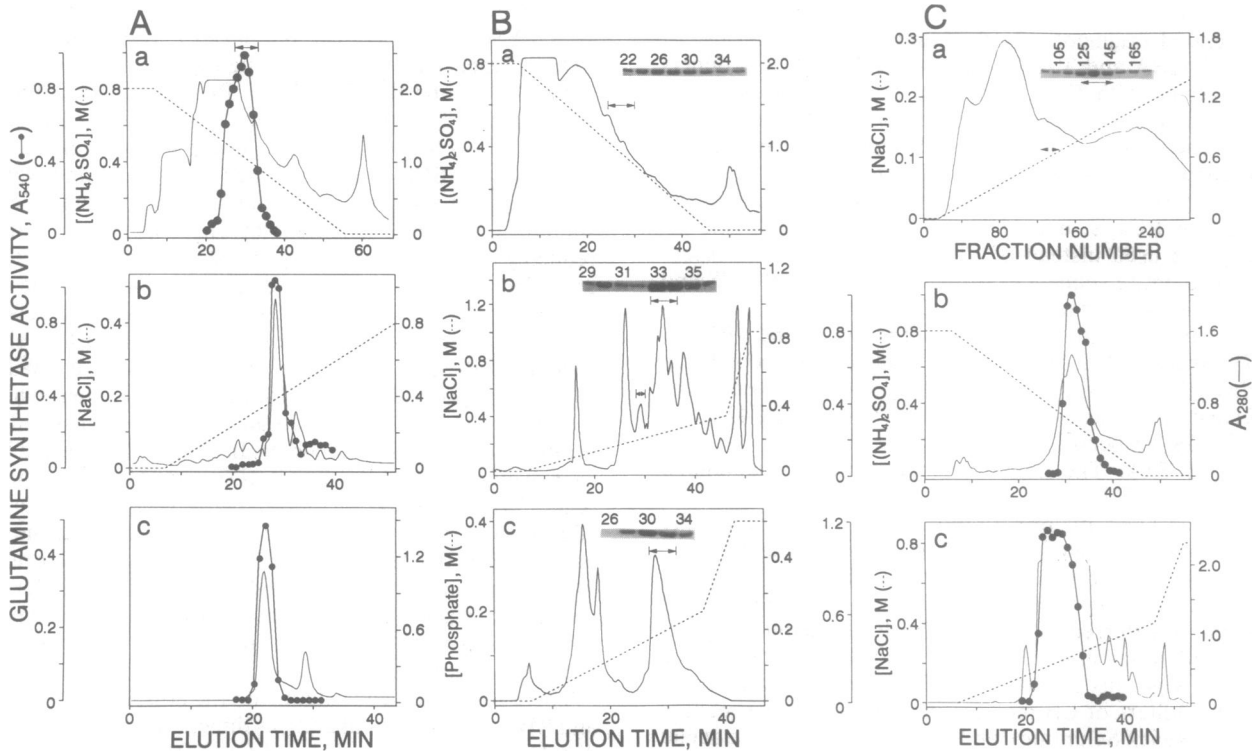

FIG. 2. Because of their different chromatographic behaviors, RWT, RC47S, and RC170S TSA proteins were purified from the extract of the appropriate *E. coli* strain by different combinations of column chromatographic steps. During purification, RWT was assayed by its ability to inhibit the inactivation of glutamine synthetase. The RC47S, which lacks antioxidant activity, was detected by immunoblot analysis with antibodies to yeast TSA as described (6). RC170S was assayed either by immunoblot analysis (during the first chromatographic step) or by the protection assay (during the second and third chromatographic steps). (*A*) Purification of RWT. A 30–60% ammonium sulfate fraction of *E. coli* extract was applied to an HPLC preparative TSK phenyl-5PW column (21.5 × 150 mm) that had been equilibrated with 20 mM Hepes (pH 7.2) and 0.8 M ammonium sulfate. Proteins were eluted by a decreasing ammonium sulfate gradient from 0.8 to 0 M for 50 min (*a*). The leading fractions of the broad activity peak (fractions 24–27) contained catalase activity as measured by the capacity to remove H_2O_2 (7). Thus, the trailing fractions of the activity peak (fractions 30–34) were combined, concentrated, and washed. The washed sample was applied to a TSK DEAE 5-PW HPLC column (21.5 × 150 mm) that had been equilibrated with 20 mM Tris·HCl (pH 7.6) and proteins were eluted by a linear NaCl gradient from 0 to 0.5 M for 50 min (*b*). The trailing fractions of the activity peak still contained catalase activity. Thus, only two peak fractions (fractions 27 and 28) were collected, concentrated, and applied to a TSK-G3000SW HPLC gel filtration column that had been equilibrated with a solution containing 100 mM sodium phosphate (pH 7.0) and 100 mM NaCl. Proteins were eluted with the same buffer (*c*). Peak fractions (fractions 19–21) were pooled, concentrated, aliquoted, and stored at −70°C. (*B*) Purification of RC47S. Fractionation by ammonium sulfate and separation on a TSK phenyl-5PW column (15 × 210 mm) were achieved as described for RWT. Peak fractions (fractions 22–34), detected by immunoblot analysis (*a*) were pooled, concentrated, washed, and applied to a Mono Q HR 10/10 column (Pharmacia). Consecutive applications of NaCl gradients from 0 to 0.4 M for 40 min and from 0.4 to 1.0 M for 5 min resulted in two TSA peaks (*b*). Peak fractions (fractions 30, 31, and 33–35) were combined, concentrated, and washed. The washed sample was applied to an HPLC hydroxyapatite HCA column (4 × 75 mm), and proteins were eluted with a sodium phosphate gradient from 0 to 200 mM for 30 min (*c*). Peak fractions (fractions 28–33) were pooled, concentrated, washed, and stored in portions at −70°C. (*C*) Purification of RC170S. Proteins precipitated from the *E. coli* homogenate by 8% polyethylene glycol (8 kDa) were loaded onto a DEAE-Sephacel anion-exchange column (5 × 60 cm) that had been equilibrated with 50 mM Tris·HCl (pH 7.6) containing 1 mM DTT. After washing with 1 liter of equilibration buffer, proteins were eluted with a linear NaCl gradient from 0 to 0.5 M in 6 liters of equilibration buffer (*a*). Peak fractions (fractions 70–80) were pooled, brought to 0.8 M ammonium sulfate, and applied to a TSK phenyl-5PW column (21.5 × 150 mm) that had been equilibrated with 20 mM Hepes (pH 7.2) containing 0.8 M ammonium sulfate and 1 mM DTT. Proteins were eluted by a decreasing ammonium sulfate gradient from 0.8 to 0 M for 40 min (*b*). Peak fractions (fractions 20–23) were pooled, concentrated, washed, and applied to a Mono Q HR 10/10 column. Proteins were eluted with linear NaCl gradients from 0 to 0.4 M for 40 min and from 0.4 to 1.0 M for 5 min (*c*). Peak fractions (fractions 24–27) were combined, concentrated, washed, and stored in aliquots at −70°C.

that D6 was derived from D1 and not from D2 and D3. It is likely, therefore, that D1 is a dimer that contains 2 disulfide linkages between Cys-47 and Cys-170 and is reduced first to D6, which contains one such disulfide linkage, before complete reduction to M1 or M2. M1 and M2 are likely monomers with different conformations. It is not clear whether the faint band M3 corresponds to another monomeric form or to a fragment derived from a nicked dimer. The appearance of M3 was variable from experiment to experiment.

Treatment of RC47S and RC170S with 10 mM DTT resulted in elimination of D4 and D5 (data not shown), suggesting that D4 and D5 are also dimers linked by a disulfide bond.

Identification of the Cys-47–Cys-170 Linkage as the Major Disulfide Bond in Wild-Type TSA. Reduced YWT (treated with DTT) and oxidized YWT (untreated) were digested with trypsin and the resulting peptides were separated on an HPLC C_{18} column (Fig. 6 *A* and *B*). Sulfhydryl groups of reduced YWT were labeled with 5,5′-dithiobis-(2-nitrobenzoic acid) before the digestion, thus making it possible to identify the cysteine-containing (TNB-labeled) peptides a–e (Fig. 6*A*). Peak f was shown to represent TNB-conjugated DTT. Comparison of Fig. 6 *A* and *B* indicated that the five TNB-conjugated peptides (a–e) were derived only from reduced TSA, whereas peptides I and II were present only in the tryptic digest of nonreduced TSA. This result suggests that peptides I and II were candidates for peptides containing disulfide linkages.

After reduction by 2 mM DTT, peptide I yielded four new peptides (I-1, I-2, I-3, and I-4) (Fig. 6*C*) and peptide II yielded five new peptides (II-1, II-2, II-3, II-4, and II-5) (Fig. 6*D*).

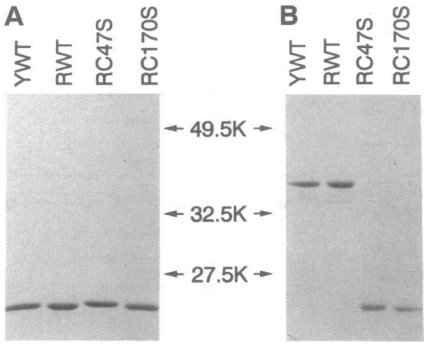

FIG. 3. SDS/PAGE analysis of purified wild-type TSA and mutant TSA proteins. Purified proteins (5 μg in 50 μl) were mixed with an equal volume of reducing buffer (0.125 M Tris·HCl, pH 6.8/4% SDS/20% glycerol/10% 2-mercaptoethanol) (*A*) or nonreducing buffer (the reducing buffer minus 2-mercaptoethanol) (*B*), and then heated at 95°C for 5 min. The samples were separated by SDS/PAGE on a 12% gel and visualized with Coomassie blue. Positions of molecular size standards are indicated in kDa.

Peptides I-1, I-2, I-3, and I-4 appeared to be identical to peptides II-1, II-2, II-3, and II-4, respectively, because of their identical retention times. In addition, II-1 (I-1) and II-2 (I-2) were presumed related to TNB-modified peptides a and b, because, under the chromatography conditions used, TNB-modified peptides eluted 1–3 min later than the corresponding unmodified peptides. For the same reason, peptides II-3 (I-3), II-4 (I-4), and II-5 were presumed related to TNB-modified peptides c, d, and e, respectively. The sequence of the N-terminal five residues was determined for peptides I-1, II-1, II-2, II-3, II-4, and II-5. As expected, the sequences of I-1 and II-1 were identical. Alignment with the TSA sequence (Fig. 7) revealed that peptides II-1 (I-1) and II-2 both contained Cys-170, whereas peptides II-3, II-4, and II-5 all contained Cys-47. This assignment was further supported by the observation that the Cys-47-containing peptides contain a significantly greater number of hydrophobic residues and should therefore elute later than the Cys-170-containing peptides. The ultraviolet spectrum of peptides I-1 and I-2 showed characteristics of tryptophan-containing peptides, whereas peptides I-3 and I-4 showed characteristics of peptides containing tyrosine and phenylalanine but not tryptophan (data not shown).

From these results, we conclude that peaks I and II (Fig. 6*B*) both represent a mixture of peptides that are linked by a disulfide bond between one of the two (or three) Cys-47-containing peptides and one of the two Cys-170-containing peptides.

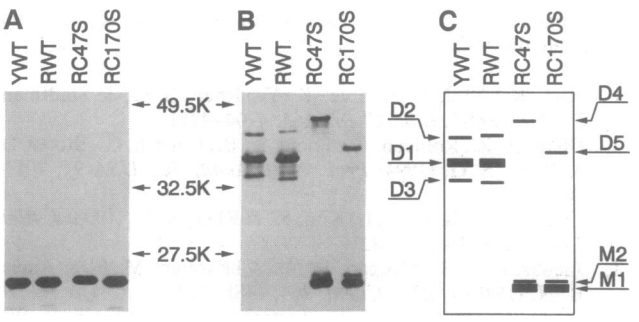

FIG. 4. Immunoblot analysis of purified wild-type and mutant TSA proteins. Purified proteins (0.5 μg) were treated with reducing (*A*) or nonreducing (*B*) buffer and subjected to SDS/PAGE as indicated in the legend to Fig. 3. The proteins were then transferred to nitrocellulose filters and subjected to immunoblot analysis with antibodies to TSA. (*C*) Schematic representation of blot in *B*. Positions of molecular size standards (kDa) are indicated.

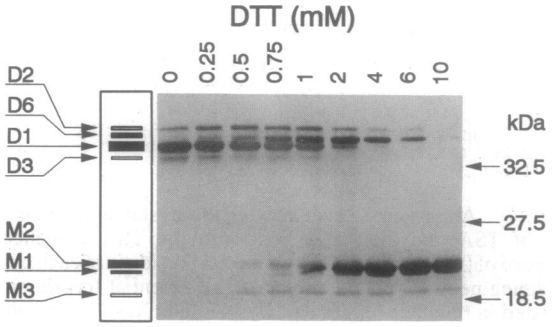

FIG. 5. Reduction of purified TSA by DTT. Purified YWT (0.5 μg) was incubated with various concentrations of DTT for 5 min at room temperature and then subjected to nonreducing SDS/PAGE. Separated proteins were subjected to immunoblot analysis with antibodies to TSA. Various dimeric and monomeric forms of TSA are shown schematically on the left. Positions of molecular size standards are indicated.

Antioxidant Activity of Mutants. The abilities of YWT, RWT, RC47S, and RC170S to protect glutamine synthetase against oxidative inactivation by DTT/Fe^{3+}/O_2 were evaluated (Fig. 8). YWT, RWT, and RC170S showed similar

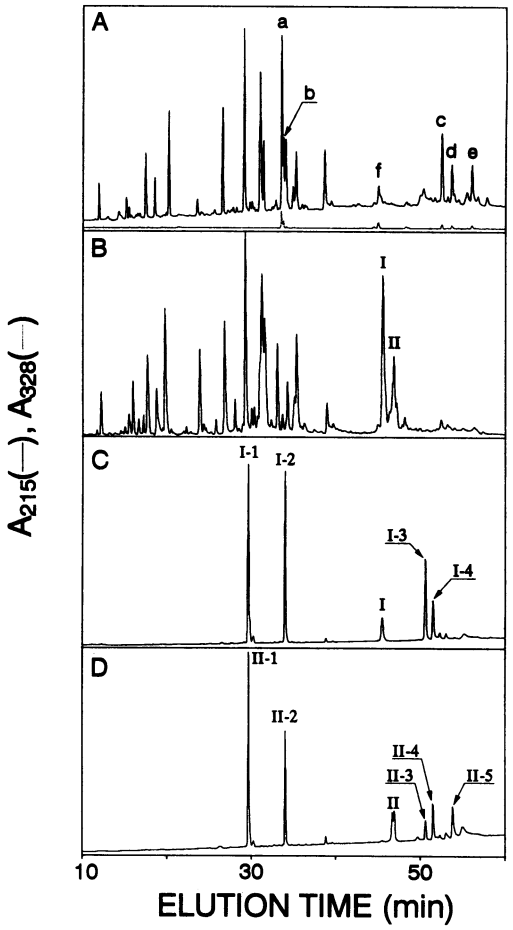

FIG. 6. Isolation of disulfide-linked tryptic peptides of TSA. (*A*) Tryptic peptides of reduced, TNB-labeled YWT were separated on a C_{18} column, and elution was monitored at 210 nm (upper trace) and 328 nm (lower trace). TNB-conjugated compounds are indicated by peaks a–f. (*B*) Elution of tryptic peptides of nonreduced YWT from the C_{18} column monitored by absorbance at 210 nm. Peaks I and II likely represent disulfide-linked peptides. (*C*) Elution (210 nm) of peptides derived from peptide I after reduction with DTT. (*D*) Elution (210 nm) of peptides derived from peptide II after reduction with DTT.

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                                                    47
TSA         KKTAVVDGVFDEVSLDKYKGKYVVLAFIPLAFTFVCPTEIIAFSEAAKKF
II-5          TAVVD.............................C............
II-4(I-4)                       GKYVV..........C............
II-3(I-3)                         YVVLA.........C............

                                  170
TSA         RLVEAFQWTDKNGTVLPCNWTPGAATIKN
II-2(I-2)    LVEAF..........C..........
II-1(I-1)               NGTVL.C..........
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FIG. 7. Amino acid sequences of the cysteine-containing peptides of TSA and their alignment with the TSA sequence. The sequence of the five N-terminal amino acid residues of each cysteine-containing peptide was determined. The potential trypsin sites are indicated in boldface letters and Cys-47 and Cys-170 are indicated.

antioxidant activities, whereas RC47S failed to provide protection. RWT and RC170S showed additive effects. RC47S did not have any effect on the protection by RWT, suggesting that the inactivity of RC47S was not attributable to the presence of an inhibitor in the RC47S preparation (data not shown).

DISCUSSION

The plausible dimeric arrangements of various TSA molecules are shown in Fig. 9. Wild-type TSA (YWT and RWT) exists mainly in the dimeric form D1, in which the constituent molecules are linked by two identical disulfide bonds between Cys-47 and Cys-170. The dimeric form D6 is generated as an intermediate during the reduction of D1 to the monomer; D6 likely contains only one disulfide linkage between Cys-47 and Cys-170. In two minor dimeric forms, D2 and D3, of YWT and RWT, the constituent molecules likely are linked by disulfide linkages between corresponding cysteine residues: in D2, either a Cys-47–Cys-47 or a Cys-170–Cys-170 linkage; in D3, both Cys-47–Cys-47 and Cys-170–Cys-170 linkages, resulting in a more compact arrangement and a faster mobility on SDS/PAGE than D2. RC47S and RC170S, each missing one cysteine, cannot form dimers with a Cys-47–Cys-170 linkage and exist mainly as monomers. D4 and D5 represent minor dimeric forms of RC47S and RC170S, respectively. These various dimeric forms are not artifacts of purification procedures. Immunoblot analyses of yeast extract (YWT) and *E. coli* extracts (RWT, RC47S, and RC170S) showed D1 as the major form of the wild-type enzymes and monomers as the major forms of the mutant enzymes. Dimers D2, D3, D4, and D5 were also readily detected.

Cys-47, but not Cys-170, is essential for TSA activity. This observation suggests that the antioxidation reaction does not involve the formation of either intramolecular or intermolecular disulfide linkages between Cys-47 and Cys-170. A mechanism involving an intramolecular disulfide linkage has been proposed for the alkyl hydroperoxide reductase reaction

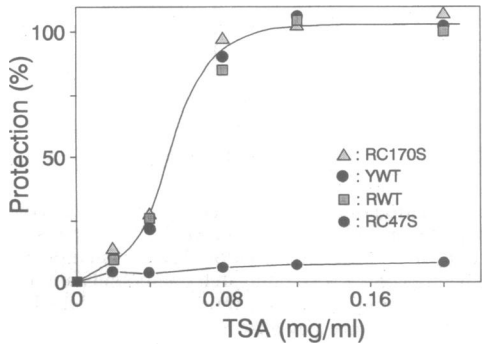

FIG. 8. Protective effect of wild-type and mutant TSA proteins against oxidative inactivation of glutamine synthetase by DTT/Fe^{3+}/O_2.

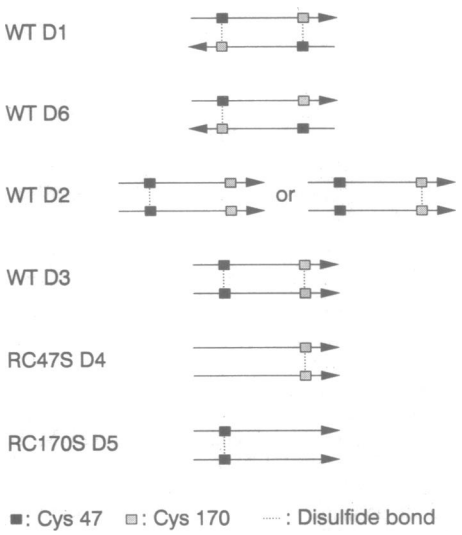

FIG. 9. Schematic representation of various TSA dimeric arrangements. Arrow indicates C terminus.

catalyzed by AhpC and AhpF (4). In this mechanism, reduction of peroxide to alcohol is accompanied by oxidation of two sulfhydryl groups of AhpC to a disulfide. The AhpC contains only two cysteines that correspond to Cys-47 and Cys-170 of TSA. The sulfhydryl groups of AhpC are regenerated by AhpF, which transfers reducing equivalents from NAD(P)H to the disulfide of AhpC. Sequence homology between TSA and AhpC and the relative locations of the two conserved cysteines, together with the results of the present study, suggest that intramolecular disulfide formation is probably not an intermediate step in AhpC action. An intramolecular disulfide is generated during the reaction cycle of many redox-active proteins, including oxidoreductases, thioredoxin, and glutaredoxin (8). In these proteins, the redox-active cysteines are separated by only two or four amino acids. However, the conserved cysteine residues in the AhpC/TSA family proteins are separated by a much larger number of amino acids. Furthermore, whereas the conserved N-terminal cysteine (Cys-47 in TSA) is present in all 25 AhpC/TSA family proteins, the conservation of the C-terminal cysteine (Cys-170 in TSA) is not absolute.

Given that TSA does not contain redox cofactors, such as heme, flavin, or metal ions, Cys-47 is likely the site of oxidation by substrate. The exact chemical nature of the oxidized Cys-47 and the role of Cys-170 in TSA/AhpC family proteins await further studies.

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