Removal of Hydrogen Peroxide by Thiol-specific Antioxidant Enzyme (TSA) Is Involved with Its Antioxidant Properties

TSA POSSESES THIOL PEROXIDASE ACTIVITY*

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The thiol-specific antioxidant protein (TSA) protects glutamine synthetase from inactivation by a metal-catalyzed oxidation (MCO) system comprised of dithiothreitol (DTT)/Fe³⁺/O₂ but not by the ascorbate/Fe³⁺/O₂ MCO system. The removal of sulfur-centered radicals or H₂O₂ has been proposed as the protective mechanism of TSA. Like catalase, TSA prevents the initiation of the rapid O₂ uptake phase during MCO of DTT but causes only partial inhibition when added after the reaction is well into the propagation phase. Stoichiometric studies showed that the antioxidant property of TSA is, at least in part, due to its ability to catalyze the destruction of H₂O₂ by the overall reaction 2 RSH + H₂O₂ → RSSR + H₂O. Results of kinetic studies demonstrate that the removal of H₂O₂ by TSA correlates with its ability to protect glutamine synthetase from inactivation. In the presence of thioredoxin, TSA is more active, whereas C170S (an active mutant of TSA in which cysteine 170 was replaced by a serine) and open reading frame 6 (a human antioxidant protein homologous to TSA with only one conserved cysteine residue) are only slightly affected. The thiol specificity of the protective activity of TSA derives from the fact that the oxidized form of TSA can be converted back to its sulfhydryl form by treatment with thiols but not by ascorbate.

The interaction of reactive oxygen species with biomolecules has been implicated in the etiology or manifestation of several pathological processes (1). To guard against this oxygen toxicity, organisms have developed a battery of antioxidant defenses (2, 3). The preventive antioxidant systems include enzymes that decompose peroxides and superoxide anion and compounds that sequester metal ions. These types of antioxidants reduce or eliminate the generation of free radicals. Chain-breaking antioxidants, such as ascorbate and α-tocopherol, scavenge transient free radicals and inhibit the attack of these reactive species on biological targets.

TSA1 is an antioxidant protein originally isolated from Saccharomyces cerevisiae (4) and later found to be widely distributed in mammalian tissues (5). It specifically protects enzymes from damage by thiol-dependent MCO systems (4). The most studied antioxidant property of TSA is the protection of glutamine synthetase from oxidative inactivation (4–7). TSA is not able to prevent glutamine synthetase and other enzymes from oxidative inactivation if a nonsulfhydryl reducing agent replaces a thiol compound in the reaction mixture. No catalase, glutathione peroxidase, or superoxide dismutase activities were associated with TSA (4). TSA does not have prosthetic groups, such as heme or flavin, or tightly bound metal ions. Based on these features, it was proposed that this 25-kDa protein could remove sulfur radical species (4).

Recently, it was shown by means of EPR spectroscopy that TSA can prevent the accumulation of thyl radical-spin trap adducts formed in reaction mixtures containing DTT (or glutathione)/Fe³⁺/O₂ and in the peroxidase-catalyzed oxidation of DTT (or glutathione) (8); however, whether TSA prevents the formation of thyl radicals or scavenges them once they are formed remains to be determined.

Lim et al. (9) showed that high concentrations of TSA (1 mg/ml) can accelerate the decomposition of H₂O₂ (10 μM) by DTT/Fe³⁺/O₂ MCO system. However, because the rate of H₂O₂ elimination did not correlate with protection of DNA cleavage by TSA, the authors suggested that the removal of other reactive species would be involved with the antioxidant properties of TSA. Chae et al. (10) and Kwon et al. (11) have shown that in the presence of thioredoxin, thioredoxin reductase, and NADPH lower concentrations of TSA are required to decompose H₂O₂ and protect glutamine synthetase from inactivation by DTT MCO systems.

TSA shares regions of sequence similarity with a number of proteins from bacteria to mammals (5). Among these proteins, only alkyl hydroperoxide reductase from Salmonella typhimurium was associated with a cellular function (5). Alkyl hydroperoxide reductase, like other peroxide-removing enzymes, is under control of a positive regulatory gene, oxyR (12), which suggests an antioxidant function for the TSA family of proteins. Indeed, high concentrations of either mercaptoethanol, iron, or O₂ induce the synthesis of TSA (13). Moreover, a S. cerevisiae mutant, in which the TSA gene was disrupted, is more sensitive to oxidative stress induced by peroxides than the wild-type yeast (6). In spite of its apparent importance to cells, the antioxidant mechanism of TSA remained unknown. The removal of sulfur radicals (4, 6, 8) or of H₂O₂ (10, 11, 14) has been implicated in the antioxidant process. As shown here the antioxidant property of TSA is due at least in part to its ability to remove H₂O₂ and thereby prevent the formation of reactive species during the iron-catalyzed oxidation of thiol compounds. Furthermore, by direct measurement of the reaction...
EXPERIMENTAL PROCEDURES

Materials—Catalase (bovine liver) and glucose oxidase (Aspergillus niger) were obtained from Calbiochem; alcohol oxidase (Pichia pastoris) was from United States Biochemical Corp., horseradish peroxidase (bovine erythrocytes) and oxidized and reduced forms of DTT and DH LA were from Sigma; Escherichia coli glutamine synthetase was purified as described before (15), and its activity was assayed by the glutamyntransferase method at pH 7.57 (4). Yeast TSA has two highly conserved cysteine residues (Cys-47 and Cys-170) (5). Two mutant proteins, C47S and C170S, where the respective cysteine residues were replaced by serine, were prepared as described previously (7). Yeast TSA, wild-type TSA, and the mutant proteins expressed in P. pastoris (20 nM) were obtained from Calbiochem; alcohol oxidase (P. pastoris) was kindly provided by Dr. Kanghwa Kim. O2 uptake—O2 dissolved in aqueous solution was measured polarographically at 37 °C in 50 mM Hepes-NaOH (pH 7.4), using a Clark electrode placed in a water-jacketed cell assembly from YSI Inc. (model 5300). The calibration of the O2 monitor was made as described before (36). Under our experimental conditions, the saturating concentration of O2 is 200 μM. O2 uptake was initiated by the addition of the reducing agent (ascorbate, DH LA, or DTT), unless specifically noted. Stock solutions of DTT (0.5 mM) were made in 0.5 mM HCl. The profile of DTT oxidation is comprised of a lag phase of several minutes, followed by a rapid O2 uptake step when the maximal rate is reached (Fig. 1). The maximum rate was determined from the slope of the steepest, nearly linear segment of the O2 uptake curve. Extrapolation of the linear segment to the x axis identifies the time which the lag phase is ended. The distance between the zero time and the x axis intercept represents the lag phase length.

H2O2 Concentration—H2O2 concentration was determined by two methods. (a) Spectrophotometric determination of the yellowish Fe3+/Fe2+ complex produced when Fe3+ was oxidized by H2O2 in the presence of sodium thiocyanate at acid pH (17). Reactions were stopped by HCl addition (final concentration 0.2 M). Reaction mixtures with TSA or other proteins were centrifuged at 12,000 × g, and the supernatants were analyzed. (b) Fluorescent measurement of the product homovanillic acid by H2O2 (18). Because thiols are substrates for horseradish peroxidase (8, 19), sulfhydryl groups were alkylated with N-ethylmaleimide, prior to the determination of H2O2 concentration. All fluorimetric studies were done in a Photon Technology International model LS-100 spectrophotometer, with slit width at 5 nm. Calibration curves were generated for each day, with known amounts of H2O2 (e240 = 43.6 m−1 cm−1, Ref. 17).

Test for Protein Inactivation—To determine if catalase or TSA was inactivated during incubation with DTT/Fe3+/O2 or with H2O2, after various times of incubation, arsenite (50 mM) was added to the reaction mixtures to prevent oxidation of the unreacted DTT (20). The mixtures were dialyzed against several batches of 20 mM Hepes (pH 7.4), and the protein solutions were concentrated with a Centricon-10 device (Amicon Co.). The concentrations of TSA and catalase were determined by absorbance measurements at 280 (4) and 406 nm (21), respectively. Catalase activity was determined by its ability to catalyze the decomposition of H2O2, using the thiocyanate-Fe3+ method. TSA activity was measured by its ability to inhibit O2 consumption associated with DTT oxidation catalyzed by EDTA-Fe3+. The activities of catalase and TSA were compared with fresh solutions of these proteins.

Oxidation of Thiol—Oxidation of the DTT sulfhydryl groups was determined using Ellman’s reagent (5,5′-dithiobis(2-nitrobenzoic acid) (22). Concentration of oxidized DTT was determined during reversed-phase HPLC analysis (Vydac C-18 column, 25 cm, number 218TP54) by its absorbance at 210 nm. The flow was 1 ml/min, and a linear gradient of 0–14% acetonitrile in aqueous 0.05% TFA (0–14 min) was used. Under these conditions, the retention times of reduced and oxidized DTT are 8 and 10 min, respectively. Calibrations curves for the oxidized form of DTT were generated using commercially available standards (Sigma). Besides the co-elution between the sample and authentic standards, the identity of the oxidized form of DTT was confirmed by its characteristic UV spectrum (ε260 = 273 M−1 cm−1) (23), which was obtained by a high performance liquid chromatography-diode array system (Hewlett-Packard 1090).

Determination of Sulfhydryl Groups in TSA—TSA (1.8 mg/ml) was treated with 50 mM Hepes-NaOH (pH 7.4), Hepes + 10 mM DTT, Hepes + 100 mM DH LA, or Hepes + 10 mM ascorbate for 5 min at room temperature (final volume = 100 μl). The buffer and aqueous solutions were sparged with argon to remove O2 during a 15-min period. Two milliliters of 50 mM Hepes (pH 7.4) were added, and the protein was concentrated with a Centrifuge-10 (Amicon Co.) microconcentrator, washed four times with deaerated buffer, and again concentrated. Protein concentration was determined by its absorbance at 280 nm (4), and the sulfhydryl groups’ quantity was measured with Ellman’s reagent (22).

RESULTS

TSA and Catalase Have Similar Effects on the Iron-catalyzed Oxidation of Thiol Compounds—The Fe3+/catalyzed oxidation of DTT is a complicated biphasic process (Fig. 1) characterized by a lag phase of several minutes; during this time the slow uptake of O2 correlates with the accumulation of H2O2 which

2 S. W. Kang, I. Baine, and S. G. Rhee, unpublished results.

3 H. Z. Chae, H. J. Kim, K. Kim, S. W. Kang, and S. G. Rhee, unpublished results.
initiates propagation of a radical chain of reactions, leading ultimately to a very rapid uptake of O$_2$ and disappearance of sulfhydryl groups. In the presence of catalase the accumulation of H$_2$O$_2$ is prevented, and the rapid phase of O$_2$ uptake does not take place (Table I and Fig. 1). The possibility that TSA, like catalase, owes its protective action to the removal of H$_2$O$_2$ is supported by the data in Table I and Fig. 1, showing that TSA and catalase exert similar effects on the pattern of O$_2$ uptake by different thiol-dependent MCO systems. Thus, the effects of TSA and catalase on the lag time and maximal rates of O$_2$ uptake are the same, whatever the iron complex or thiol compound tested (Table I). Like catalase, TSA inhibits completely the rapid O$_2$ uptake phase when added prior to starting the reaction but causes only partial inhibition when added after the reaction is well into the fast O$_2$ uptake phase (Fig. 1, A and B). These data suggest that TSA, like catalase, exerts its effect by the removal of H$_2$O$_2$. The fact that catalase and TSA causes only partial inhibition of O$_2$ uptake when added after the reaction is well into propagation phase (Fig. 1, A and B) is not due to partial inactivation of these antioxidant enzymes. When tested by the method described under “Experimental Procedures,” Test for Protein Inactivation, TSA and catalase were fully active following its reisolation from the reaction mixtures. Similar effects are provoked by these antioxidant enzymes again, when concentrations of TSA and catalase were reduced to levels of 40 and 20%, respectively, of those for the experiment described in Fig. 1, A and B. In this case, the lag phase was substantially increased, but the maximal rate of O$_2$ uptake was almost unaltered (Fig. 1C). We suggest that at the lower concentrations used in the experiment described in Fig. 1C, TSA and catalase were unable to remove all of the H$_2$O$_2$ generated, but they did reduce the steady-state level of H$_2$O$_2$ and consequently caused a delay in the initiation of events leading to propagation of the free radical chain involved in the rapid oxidation of DTT.

The maximum rate of O$_2$ uptake in the EDTA-Fe$^{3+}$-catalyzed reaction is almost the same whether DTT, DHLA, or ascorbate is used as the electron donor. However, TSA does not inhibit the ascorbate-dependent O$_2$ uptake, even when it was added at 12 times the concentration required to prevent the dithiol-dependent reaction (Table I). This differential effect of TSA on O$_2$ uptake by the dithiol- and ascorbate-catalyzed oxidations systems is similar to its effect on the glutamine synthetase inactivation by these systems. TSA prevents the inactivation of glutamine synthetase by the DTT system but has little or no effect on glutamine synthetase inactivation by the ascorbate system (4).

### Table I

| Reducing agent | Addition | Maximum rate (μM O$_2$/min) | Lag phase (min) |
|---------------|----------|----------------------------|----------------|
| DTT (5 mM)    | No       | 6.1                        | 16.0           |
| DTT (5 mM)    | + TSA    | 0.9                        | _b_            |
| DTT (5 mM)    | + Catalase| 0.8                        | _b_            |
| DTT (5 mM)    | + EDTA   | 30.0                       | 9.5            |
| DTT (5 mM)    | + EDTA + TSA | 0.3                   | _b_            |
| DTT (5 mM)    | + EDTA + catalase | 0.2                     | _b_            |
| DHLA (5 mM)   | + EDTA   | 34.1                       | 7.2            |
| DHLA (5 mM)   | + EDTA + TSA | 0.3                      | _b_            |
| DHLA (5 mM)   | + EDTA + catalase | 0.2                    | _b_            |
| Ascorbate (10 mM) | + EDTA | 30.6                       | 2.4            |
| Ascorbate (10 mM) | + EDTA + TSA | 28.8                     | 2.3            |
| Ascorbate (10 mM) | + EDTA + catalase | 31.6                     | 2.6            |
| Ascorbate (10 mM) | + EDTA + catalase | 8.8                      | 2.1            |

a The same as other reaction mixtures, except that TSA concentration is 300 μM.
b In these cases, no fast oxygen uptake phase was detected. Therefore, no lag phase could be measured.

### Table II

| Reaction mixtures | Additions | Maximum rate (μM O$_2$/min) | Lag phase (min) |
|-------------------|-----------|-----------------------------|----------------|
| No                |           | 30                          | 9.3            |
| Trx$^+$ I (6.4)   |           | 28.1                        | 11.8           |
| C47S (1.0)        |           | 30.6                        | 12.3           |
| C170S (1.0)       |           | 0.1                         | _b_            |
| C170S (0.6)       |           | 14.2                        | 72.9           |
| C170S (1.0) + Trx I (6.4) |         | 0.1                         | _b_            |
| C170S (0.6) + Trx I (6.4) |     | 6.3                         | 325.2          |
| TSA (0.8)         |           | 0.1                         | _b_            |
| TSA (0.6)         |           | 25.4                        | 38.1           |
| TSA (0.24) + Trx I (6.4) |     | 0.1                         | _b_            |
| TSA (0.18) + Trx I (6.4) |     | 16.1                        | 26.2           |
| Rat Trx (6.4)     |           | 28.3                        | 9.3            |
| ORF6 (0.25)       |           | 0.1                         | _b_            |
| ORF6 (0.16)       |           | 22.5                        | 83.9           |
| ORF6 (0.16) + rat Trx (6.4) |     | 0.1                         | _b_            |
| ORF6 (0.08) + rat Trx (6.4) |     | 23.1                        | 129.5          |

a Trx, thioredoxin.
b In these cases, no fast oxygen uptake phase was detected. Therefore, no lag phase could be measured.

L. E. S. Netto and E. R. Stadtman, unpublished results.
80 \( \mu M \) of \( H_2O_2 \) is produced (Fig. 2). Thus, 1 mol of \( O_2 \) is consumed for each mole of \( H_2O_2 \) formed. When added separately to such incubation mixtures, neither DTT (0.25 \( mM \)) nor TSA (6 \( \mu M \)) had a significant effect on the rates of \( O_2 \) uptake or of \( H_2O_2 \) production. The simultaneous addition of DTT and TSA to the incubation mixture had little if any effect on the rate of \( O_2 \) uptake by the glucose oxidase system, but the amount of \( H_2O_2 \) that accumulated during the 20-min incubation period was reduced from a value of 80 \( \mu M \) (in the absence of DTT + TSA) to a value of 20 \( \mu M \) in the presence of both TSA and DTT (Fig. 2). It is noteworthy that under these experimental conditions, TSA at a concentration of 6 \( \mu M \) caused the decomposition of 60 \( \mu M \) of \( H_2O_2 \); thus, TSA acts catalytically.

The \( H_2O_2 \)-removing activity of TSA is not due to catalase contamination because (a) no \( O_2 \) is produced by TSA after \( H_2O_2 \) addition into the \( O_2 \) monitor; (b) the addition of azide, a catalase inhibitor, does not eliminate the inhibitory effect of TSA on the \( O_2 \) uptake by DTT, or its ability to protect glutamine synthetase from inactivation, and (c) catalase, but not TSA, prevents \( O_2 \) uptake by ascorbate (Table I).

From the data of several experiments, summarized in Table III, it is evident that the TSA-catalyzed consumption of 1 mol of \( H_2O_2 \) is associated with the disappearance of 2 eq of sulfhydryl groups and the formation of one disulfide bond according to Equation 1. This stoichiometry is the same whether the \( H_2O_2 \) is generated by the glucose-glucose oxidase system, or by a methanol-alcohol oxidase system, or is added directly to the reaction mixture. Studies with the mutant proteins show a good correlation between their peroxidase activity and their antioxidant property. The active mutant enzyme in which cysteine 170 is replaced by serine is just as active in removing \( H_2O_2 \) as is the wild-type enzyme (Table III). The inactive mutant protein, C47S, is unable to remove \( H_2O_2 \) and to protect glutamine synthetase from oxidative inactivation (Table III, legend). It follows from the stoichiometry of the TSA-catalyzed reaction that TSA possesses thiol peroxidase activity (see Reaction 1).

Thioredoxin Effect—Thioredoxin stimulates the antioxidant activity of TSA but only slightly affects C170S (Table II). The minimum concentration of C170S required to inhibit the initiation of the fast \( O_2 \) uptake step is the same in the absence and in the presence of thioredoxin (1 \( \mu M \)). In contrast, the minimum concentration of TSA needed to prevent the initiation of the rapid phase of \( O_2 \) uptake is three to four times higher in the absence (0.8 \( \mu M \)) than in the presence of thioredoxin (0.24 \( \mu M \)). These results suggest that the C-terminal cysteine of TSA is involved with the stimulatory effect of thioredoxin. The large differences of the maximum rates of \( O_2 \) uptake observed in a

![Graph](image)

**FIG. 2.** \( H_2O_2 \) removal by TSA. \( H_2O_2 \) was generated enzymatically by the glucose (5 \( mM \))/glucose oxidase (0.1 unit/ml) system in 50 \( mM \) Hepes (pH 7.4) at 37 \( ^\circ C \). The reactions were started by the addition of glucose and were stopped by the addition of HCl (0.5 \( M \) final concentration). Additions to the glucose/glucose oxidase system were none (●), DTT (0.25 \( mM \)) (■), TSA (0.15 \( mg/ml \)) (*), or DTT (0.25 \( mM \)) + TSA (0.15 \( mg/ml \)) (○). Inset, \( O_2 \) uptake was monitored during the production of \( H_2O_2 \) by the glucose/glucose oxidase system. In the reaction mixtures containing DTT, a very small amount of \( O_2 \) was consumed before glucose addition.

| Reaction mixture | Time | \( H_2O_2 \) produced (\( \mu M \)) | \( H_2O_2 \) removed (\( \mu M \)) | \(-SH\) consumed (\( \mu M \)) | \(-SS-\)produced (\( \mu M \)) | D | D |
|------------------|------|-------------------------------|---------------------------------|---------------------|-------------------|----|----|
| G.O. (0.23 units/ml) | 10   | 57.8                          | 27.0                            | 45.0                | ND*               | 1.7 | ND |
| TSA (0.5 mg/ml)    | 20   | 104.1                         | 41.4                            | 75.0                | ND                | 1.8 | ND |
| DTT (0.25 \( mM \)) |      |                               |                                 |                     |                   |     |     |
| A.O. (0.2 units/ml)| 10   | 56.3                          | 56                              | 115.0               | ND                | 2.1 | ND |
| C170S (0.4 mg/ml)  | 20   | 80.6                          | 79.7                            | 186.0               | ND                | 2.3 | ND |
| DTT (0.5 \( mM \)) |      |                               |                                 |                     |                   |     |     |
| G.O. (0.5 units/ml)| 10   | 149.0                         | 111.0                           | 245                 | ND                | 2.2 | 1.1|
| C170S (0.3 mg/ml)  | 20   | 275.0                         | 176.1                           | 349.0               | 142               | 2.0 | 0.8|
| DTT (0.5 \( mM \))|      |                               |                                 |                     |                   |     |     |
| \( H_2O_2 \) (60 \( \mu M \)) | 10   | 60.0a                         | 38.8                            | 78.8                | ND                | 2.0 | ND |
| TSA (0.4 mg/ml)    | 20   | 60.0a                         | 56                              | 114.0               | ND                | 2.0 | ND |
| DTT (0.25 \( mM \))|      |                               |                                 |                     |                   |     |     |
| \( H_2O_2 \) (60 \( \mu M \)) | 20   | 60.0a                         | 58.7                            | 109.6               | 66.5              | 1.9 | 1.1|
| TSA (0.08 mg/ml)   |      |                               |                                 |                     |                   |     |     |
| Trx I (0.08 mg/ml) |      |                               |                                 |                     |                   |     |     |
| DTT (0.25 \( mM \)) |      |                               |                                 |                     |                   |     |     |
| \( H_2O_2 \) (60 \( \mu M \)) | 10   | 60.0a                         | 59.2                            | 117.1               | ND                | 2.0 | ND |
| C170S (0.4 mg/ml)  |      |                               |                                 |                     |                   |     |     |
| DHLA (0.25 \( mM \))|      |                               |                                 |                     |                   |     |     |

*ND, not determined.

*\( H_2O_2 \) was added exogenously.
very narrow range of protein concentrations is due to the fact that the rate during the lag phase (about 0.1 μM/min) is much slower than during the maximal rates (6–30 μM/min) (Table II). This stimulatory effect is also observed when the ability of TSA to express thiol peroxidase is measured in the presence and absence of thioredoxin. In the presence of thioredoxin I, lower concentrations of TSA (0.08 mg/ml) are needed to remove H$_2$O$_2$ at about the same rate that TSA (0.4 mg/ml) decomposes H$_2$O$_2$ in the absence of thioredoxin (Table III, lines 4 and 5).

ORF6 is a human protein homologous to TSA, that like C170S, has only one conserved cysteine, corresponding to the N-terminal cysteine of TSA (5). From the results described in Table II, it appears that ORF6, in analogy to C170S, can use DTT but not thioredoxin as an electron donor, because the minimum concentration of ORF6 required to prevent the initiation of the rapid phase of O$_2$ uptake is only slightly higher in the absence (0.25 μM) than in the presence (0.16 μM) of thioredoxin. As C170S, ORF6 can remove H$_2$O$_2$ in the presence of small molecular weight thiols such as DTT or DHLA, but not in the presence of the thioredoxin system.3 Contrary to TSA, ORF6 does not protect glutamine synthetase from inactivation by the ascorbate/Fe$^{3+}$/O$_2$ MCO system in the presence of the thioredoxin system.3 Therefore, it appears that the conserved C-terminal cysteine of TSA-like proteins is involved in the ability of thioredoxin to stimulate the antioxidant activity of these enzymes.

**TSA Possesses Thiol Peroxidase Activity**

![Image](http://www.jbc.org/)

**Fig. 3.** Correlation between glutamine synthetase protection (A and C) and H$_2$O$_2$ removal (B and D) by TSA. DTT (5 mM) and Fe$^{3+}$ (5 μM) were incubated at 37°C in Hepes buffer (50 mM, pH 7.4), in the presence (A and B) and absence (C and D) of TSA (0.2 mg/ml). The glutamine synthetase concentration used in these experiments was 0.1 mg/ml. H$_2$O$_2$ was added at 0 μM (*), 10 μM (○) in the absence of TSA; • in the presence of TSA; and 70 μM (○) in the absence of TSA; ♦ in the presence of TSA. H$_2$O$_2$ concentrations were measured by the horseradish peroxidase-homovanillic acid method described under “Experimental Procedures.” The inset is the semi-log graph of D.

TSA Removal of H$_2$O$_2$ Generated by the DTT-dependent MCO Correlates with the Protection of Glutamine Synthetase—Using a sensitive fluorimetric method (horseradish peroxidase-homovanillic method), we were able to detect steady-state concentrations of H$_2$O$_2$ generated during the unchelated Fe$^{3+}$-catalyzed oxidation of DTT (Fig. 3B). Glutamine synthetase is almost fully inactivated during the initial 10-min period (Fig. 3A, *–*), when a steady-state level of H$_2$O$_2$ formation of about 3 μM is achieved (Fig. 3B). In the presence of TSA, no H$_2$O$_2$ could be detected (Fig. 3D, *–* and glutamine synthetase is fully protected from oxidative inactivation (Fig. 3C, *–*). Therefore, it seems likely that the ability of TSA to protect glutamine synthetase from inactivation by the DTT-dependent MCO system is due to the removal of H$_2$O$_2$. In fact, the H$_2$O$_2$ removing enzymes, catalase and GSH peroxidase, also have the ability to protect glutamine synthetase from inactivation by the MCO comprised of DTT/Fe$^{3+}$/O$_2$ (4).

A role of the thiol peroxidase activity of TSA in protecting glutamine synthetase from inactivation by the DTT MCO system is supported further by the results of studies in which H$_2$O$_2$ was added to reaction mixtures at concentrations 10, 40, and 72 μM, in both the presence and absence of TSA. In the absence of TSA, H$_2$O$_2$ at all levels is decomposed at moderate rates, presumably due to reactions initiated by the Fe$^{3+}$-catalyzed conversion of H$_2$O$_2$ to hydroxyl and/or ferryl radicals, which are likely responsible for glutamine synthetase inactivation (1). In the absence of TSA, at all levels of H$_2$O$_2$ tested, glutamine synthetase was almost completely inactivated long before all of the H$_2$O$_2$ was decomposed (Fig. 3, A and B). In contrast, when TSA is present, the rate of H$_2$O$_2$ decomposition is accelerated due to the thiol peroxidase activity; therefore, the time required to decompose all of the H$_2$O$_2$, and consequently the time that glutamine synthetase is exposed to H$_2$O$_2$ (and therefore exposed to hydroxyl and/or ferryl radicals) varies.
TSA Possesses Thiol Peroxidase Activity

Fig. 4. Correlation between glutamine synthetase protection (A) and H$_2$O$_2$ removal (B) by TSA, at low Fe$^{3+}$ concentration. DTT (5 mM), Fe$^{3+}$ (0.5 mM), and H$_2$O$_2$ were incubated at 37 °C in Hepes buffer (50 mM (pH 7.4)), in the absence (●) and in the presence of TSA at 1.6 (*), 3.2 (■), and 6.4 μM (○). The glutamine synthetase (0.1 mg/ml) was used in A. H$_2$O$_2$ concentrations were measured by the horseradish peroxidase-homovanillic acid method described under "Experimental Procedures." In the inset of B, the reciprocal of the H$_2$O$_2$ half-life is plotted against TSA concentrations.

depending upon the initial concentrations of H$_2$O$_2$. Accordingly, when the initial H$_2$O$_2$ concentrations were 0, 10, 40, and 72 μM, the fractional activity of glutamine synthetase declined rapidly to stable values equal to 98, 80, 25, and 8%, at 0.1, 0.5, 2, and 10 min, respectively. In all cases, the onset of glutamine synthetase protection from inactivation (Fig. 3C) coincided with the disappearance of H$_2$O$_2$ (Fig. 3D). It is evident from these results that the protective action of TSA correlates with its ability to remove all of the H$_2$O$_2$. The role of the peroxidase activity of TSA in its protective ability is supported further by the data in Fig. 4, where the concentration of Fe$^{3+}$ was only one-tenth the level (i.e. 0.5 μM) used in the above studies. Under these conditions, in the absence of TSA, the inactivation of glutamine synthetase (Fig. 4A, ●–●) and the decomposition of H$_2$O$_2$ (Fig. 4B, ●–●) are slower than with higher concentrations of Fe$^{3+}$ (Fig. 3). Therefore, when added at 6.4, 3.2, or 1.6 μM, TSA was able to eliminate H$_2$O$_2$ (60 μM) (Fig. 4B) before more than 10, 30, or 50% of the total glutamine synthetase, respectively, was inactivated (Fig. 4A). Once again, the onset of glutamine synthetase protection from inactivation coincided with the disappearance of H$_2$O$_2$. The correlation between the thiol peroxidase activity and the protective property of TSA is evident from Fig. 5, where it is shown that the time required for TSA to prevent glutamine synthetase from inactivation is directly proportional to the half-life of H$_2$O$_2$ at three concentrations of the antioxidant enzyme.

The rate of H$_2$O$_2$ decomposition (expressed as the reciprocal of the half-life of H$_2$O$_2$) is proportional to the concentration of TSA (Fig. 4B, inset). It is noteworthy that for the data in Fig. 4, the concentration of H$_2$O$_2$ (60 μM) initially present was 37, 19, and 9 times greater than the respective concentrations of TSA, 1.6, 3.2, and 6.4 μM, used. It is therefore obvious that under all conditions tested TSA was acting catalytically in the removal of H$_2$O$_2$.

DISCUSSION

The ability of TSA to protect glutamine synthetase from inactivation by the thiol-MCO system is largely, if not entirely, attributable to its thiol peroxidase activity, as described by Reaction 1. This view is supported by the following observations. (a) The effect of TSA on O$_2$ uptake is qualitatively similar to the effect of catalase (Table I and Fig. 1). (b) When no H$_2$O$_2$ is added, the inactivation of glutamine synthetase in the absence of TSA (Fig. 3A, ●–●) is correlated with the production of H$_2$O$_2$ by the DTT-dependent MCO system (Fig. 3B, *–*). (c) In the presence of TSA no H$_2$O$_2$ accumulates from the DTT-dependent MCO system (Fig. 3D, *–*), and glutamine synthetase is fully protected from inactivation (Fig. 3C, *–*). (d) Under all experimental conditions studied (various TSA, H$_2$O$_2$, and Fe$^{3+}$ concentrations), the TSA protection of glutamine synthetase from inactivation correlated with removal of H$_2$O$_2$ (Figs. 3–5) (see Reaction 1).

Our data indicate that H$_2$O$_2$ is consumed by at least two pathways, (i) the DTT-dependent MCO system with the production of very reactive species, such as hydroxyl radical and ferryl ion (1), or (ii) the thiol peroxidase activity of TSA, with the production of water and disulfides. Therefore, by decreasing the concentration of Fe$^{3+}$ (Fig. 4), the rate of pathway i is decreased but not the rate of pathway ii. Under the conditions of Fig. 4, TSA at low concentrations (1.6, 3.2, and 6.4 μM) is able to accelerate H$_2$O$_2$ (60 μM) decomposition, suggesting that TSA protects glutamine synthetase through the catalytic removal of H$_2$O$_2$. Indeed, the fact that TSA (6 μM) removes 60 μM of H$_2$O$_2$ generated by the glucose/glucose oxidase system (Fig. 2) in the absence of transition metals clearly shows that TSA acts catalytically.

The ability of TSA to protect glutamine synthetase from inactivation by the DTT-MCO system but not by the ascorbate-MCO system is finally explained by the demonstration that, as isolated, TSA is present in a oxidized state. The thiol specificity is due to the fact that DTT and DHLA are able to convert the oxidized form of TSA back to its sulfhydryl derivative, whereas ascorbate is unable to do so (see “Results,” Thiol-dependent
Conversion of TSA to Its Sulphydryl Form.

In parallel studies, it was shown by Chae et al. (10) and Kwon et al. (11) that the reduction of TSA can be achieved also by thioredoxin in the presence of NADPH and thioredoxin reductase. Significantly, when coupled to the thioredoxin-thioredoxin reductase system, TSA is able to protect glutamine synthetase from inactivation by the ascorbate-DTT system, suggesting that the thioredoxin-thioredoxin reductase system is the biologically active electron donor for TSA activation. Curiously, the ability of the thioredoxin system to support the antioxidant activity of TSA is completely lost when either one of the cysteine residues, Cys-47 or Cys-170, is replaced by a serine residue by means of site-specific mutagenesis (10) (Table III). In contrast, substitution of Cys-47 by serine renders TSA inactive in the protection of glutamine synthetase by the DTT-MCO system, whereas replacement of Cys-170 with a serine residue has no effect on protective activity (7), nor on the O_2 uptake by the DTT-dependent MCO system (Table II), nor on the DTT peroxidase activity of TSA (Table I). It is well known that thioredoxin possesses disulfide reductase activity. For example, thioredoxin catalyzes the reduction of disulfide bonds in insulin by DTT and DHLA (24). In the present study, we show that the ability of TSA to inhibit the metal-catalyzed oxidation of DTT is substantially increased by thioredoxin, whereas the antioxidant activities of C170S and ORF6 are only slightly affected by thioredoxin (see “Results,” Thioredoxin Effect). It thus appears that the C-terminal cysteine residues corresponding to cysteine 170 of TSA are involved in the binding and/or activity of thioredoxin. In the absence of thioredoxin, the minimum concentration of ORF6 (0.25 mM) needed to prevent the initiation of the fast O_2 uptake phase is significantly lower than the concentrations (0.8–1 mM) required by TSA and C170S (Table II), suggesting that ORF6 might have a higher affinity for DTT and/or H_2O_2.

The rate of peroxide disappearance is higher when the initial concentration of H_2O_2 is 40 μM than when the initial concentration is 70 μM (Fig. 3D, inset). It appears that TSA is susceptible to substrate inhibition at high concentrations of H_2O_2.

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Removal of Hydrogen Peroxide by Thiol-specific Antioxidant Enzyme (TSA) Is Involved with Its Antioxidant Properties: TSA POSSESSES THIOL PEROXIDASE ACTIVITY

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