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INHIBITION OF METAL-CATALYZED OXIDATION SYSTEMS BY A YEAST PROTECTOR PROTEIN IN THE PRESENCE OF THIOREDOXIN

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SUMMARY: A protector protein from *Saccharomyces cerevisiae* specifically prevents the inactivation of enzymes caused by a thiol/Fe^{3+}/O_2 metal-catalyzed oxidation system but not by an ascorbate/Fe^{3+}/O_2 system. Ascorbate/Fe^{3+}/O_2-mediated damage of enzymes could be prevented by the protector protein only in the presence of reduced thiol. We demonstrate that two proteins from yeast, thioredoxin plus another protein having properties similar to that expected to thioredoxin reductase, when presented with NADPH and the yeast protector protein prevented inactivation of *E. coli* glutamine synthetase by the ascorbate/Fe^{3+}/O_2 system. This system also removes hydrogen peroxide effectively. We also demonstrate evidence suggesting that the NADPH-dependent thioredoxin system reactivates protector protein by reversible disulfide-dithiols exchange.

Metal-catalyzed oxidation (MCO) systems comprised of transition metals, O_2, and electron donors such as sulfhydryl compounds and ascorbate, catalyze the inactivation of many enzymes, the peroxidation of lipid, and damage to DNA (1-6). It has been shown that the MCO systems generate reactive oxygen species such as hydrogen peroxide, superoxide anion, hydroxyl radical, as well as reactive sulfur species (7, 8). In order to minimize the deleterious effects of such reactive species, all aerobic organisms are equipped with several antioxidant enzymes, including superoxide dismutases, catalase, and peroxidases (9). We have shown that a novel 25 kDa protector protein from *S.*

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cerevisiae specifically prevents the inactivation of enzymes (10) and strand breaks in DNA (6) caused by a thiol/Fe^{3+}/O_2 MCO system but not by an ascorbate/Fe^{3+}/O_2 MCO system. The fact that the yeast protector protein can be induced in response to an increase in oxidative pressure indicates its physiological significance (11). However, neither the role of this protein *in vivo* nor the mechanism of action of the protein *in vitro* is elucidated clearly.

Previous studies have revealed that ascorbate/Fe^{3+}/O_2 MCO system-mediated inactivation of enzymes could be protected by the protector protein only in the presence of reduced thiol (12). Therefore, it has been suggested that thiol could play a dual role as a reducing equivalent for MCO system and an activator of protector protein via the reduction of disulfide to dithiols. It has been reported that a thiol group of cysteine in protector protein is involved in catalysis. Chemical modification with thiol-specific reagents such as N-ethylmaleimide inhibits the antioxidant activity of protector protein (12). Enzymes that depend on the thiol group of a cysteine residue for catalytic activity are prone to oxidation of such a group during purification and little activity can be measured unless low-molecular-mass thiol compounds are added. It can be assumed that a cysteine residue of protector protein may be easily oxidized due to its strong nucleophilicity. The oxidized cysteine residue can be recovered by reaction with excess thiol *in vitro* (12). However, it is not clear whether there is a mechanism that allows recovery of protector protein *in vivo*. Accordingly, we have examined the possibility that cellular redox-active proteins, such as thioredoxin, may be able to reactivate protector protein. The NADPH-dependent thioredoxin system (NTR) has been known as a general disulfide reductase and catalyzes reduction of exposed S-S bridges in a variety of proteins (13, 14).

In this communication, we have shown that two cytosolic protein components from *S. cerevisiae*, thioredoxin and presumably thioredoxin reductase, in the presence of NADPH can replace nonphysiological thiols such as dithiothreitol (DTT) in the protection of *E. coli* glutamine synthetase against ascorbate/Fe^{3+}/O_2 MCO system. We also found that protector protein with NTR has the ability to remove hydrogen peroxide.

MATERIALS AND METHODS

Yeast protector protein from *S. cerevisiae* BJ926 cultures was purified to homogeneity on SDS-polyacrylamide gel by the method of Kim *et al.* (10). Two protein components, thioredoxin and presumably thioredoxin reductase, were also purified to homogeneity. Other chemicals, DTT, NADPH, NADH, 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), hydrogen peroxide, and ascorbate were purchased from Sigma.

A thiol-specific antioxidant activity of protector protein was determined by monitoring its ability to inhibit the inactivation of *E. coli* glutamine synthetase by a MCO system as previously described by Kim *et al.* (10). Fifty μl of reaction mixture containing 5 μg of glutamine synthetase, 10 mM DTT or ascorbate, 3 μM $FeCl_3$, 50 mM HEPES, pH 7.0, and an antioxidant system was incubated at 30° C. The remaining activity of glutamine synthetase was measured by addition of 10 μl of the inactivation reaction mixture to 2 ml of γ-glutamyltransferase assay mixture as described previously (15).

To determine the removal of hydrogen peroxide, the reaction was started by the addition of 0.5 mM H_2O_2 to 0.1 ml of reaction mixture containing 1 mM NADPH, 25 μg/ml thioredoxin, 25 μg/ml thioredoxin reductase, protector protein, and 40 mM HEPES, pH 7.2, and then incubation at 37° C. At appropriate reaction time, 160 μl of TCA solution (12.5%, w/v) was added to the 20 μl of reaction mixture to stop the reaction, followed by the addition of 40 μl of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 20 μl of 2.5 N KSCN to develop the complex, producing a purple color. The removal of H_2O_2 by protector protein in the presence of NTR was monitored by measurement of the decrease in absorbance at 480 nm, the absorbance maximum of the complex.

Conversion of dimer form to monomer form of protector protein in the denaturing condition was followed by 15% SDS-PAGE performed by the method of Laemmli (16). Reversion of the protector protein disulfide to dithiols by nonphysiological thiol was carried out by treating with a sample buffer containing 0.1 M 2-mercaptoethanol. Reaction with NTR, containing 2 mM NADPH, 16 μg/ml each of thioredoxin and thioredoxin reductase, 200 μg/ml of protector protein was performed at 30° C for 20 min. These samples were treated with a sample buffer lacking 2-mercaptoethanol.

RESULTS

Two protein components were purified from the yeast crude extract. SDS-PAGE indicates that two proteins have molecular weights of 12.7 kDa and 35 kDa. The spectrum of the purified 35 kDa component has maxima at 270, 380, and 448 nm and a shoulder at 474 nm. The visible spectrum is characteristic of a flavoprotein. The NH_2-terminal sequence for the first 15-amino acid residues were determined by sequential Edman degradation. The NH_2-terminal 15-amino acid sequence of the 12.7 kDa component was identical to the previously reported yeast thioredoxin (17). Although the sequence of yeast thioredoxin reductase is not known, the 15 NH_2-terminal amino acid sequence of the 35 kDa component is partially homologous to the *E. coli* thioredoxin reductase (18). Comparison of the NH_2-terminal sequence of 35 kDa protein and *E. coli* thioredoxin reductase is as follows (X represents an unidentified amino acid; Conserved amino acids are indicated by an asterisk):

35 kDa protein	V★H★N★I★V★T★I★I★X★S★X★P★A★A★H
E. coli Thioredoxin reductase	GTTKHSKLLILGSGPAGY

These two proteins were identified as thioredoxin and presumably thioredoxin reductase based on molecular weight, absorption spectrum, and NH_2-terminal sequence. The activity of the NTR system was confirmed by the assay with DTNB (19). Omission of either component resulted in the loss of ability to reduce DTNB.

Figure 1 shows time-dependent inactivation of glutamine synthetase by the DTT/Fe^{3+}/O_2 MCO system and its prevention in the presence of NTR and protector protein. Incubation for 5 min under standard inactivation conditions (3 μM Fe^{3+}, 10 mM DTT, 30° C) caused complete inactivation of glutamine synthetase whether NTR (1 mM NADPH, 20 μg/ml each of thioredoxin and thioredoxin reductase) is present or not, whereas only 55% inactivation of glutamine synthetase was observed in the presence of 20

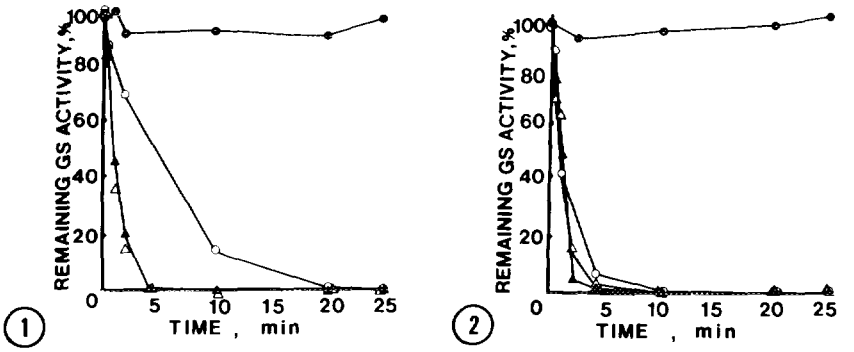

Fig. 1. Protection of glutamine synthetase against DTT/Fe^{3+}/O_2 MCO system. The inactivation mixture contained 10 μg of glutamine synthetase, 10 mM DTT, 3 μM $FeCl_3$, and 50 mM HEPES, pH 7.0, in a total volume of 100 μl. At various times aliquots (10 μl) were removed and assayed for glutamine synthetase activity. Also added were none (△), 20 μg protector protein (○), NTR (1 mM NADPH, 20 μg/ml thioredoxin, 20 μg/ml thioredoxin reductase) (▲), NTR and protector protein (●).

Fig. 2. Protection of glutamine synthetase against ascorbate/Fe^{3+}/O_2 MCO system. The inactivation conditions were as in Fig. 1, except that DTT was replaced by ascorbate. Also added were none (△), 20 μg/ml protector protein (○), NTR (1 mM NADPH, 20 μg/ml thioredoxin, 20 μg/ml thioredoxin reductase) (▲), NTR and protector protein (●).

μg/ml of protector protein without NTR. Although the activity of glutamine synthetase was completely abolished with a 20 min incubation even in the presence of protector protein, the combination of NTR and protector protein almost completely protected enzyme activity. Omission of one component from NTR, either NADPH, thioredoxin or thioredoxin reductase, did not support restoration of protector protein as an antioxidant enzyme. The ascorbate/Fe^{3+}/O_2 MCO system completely inactivates glutamine synthetase regardless of the existence of protector protein or NTR in the reaction mixture. However, protector protein was able to prevent the inactivation of enzyme in the presence of NTR comprised of 1 mM NADPH, 20 μg/ml each of thioredoxin and thioredoxion reductase as shown in Fig. 2. When NTR was added to protector protein, much less protector protein was required to provide the same degree of protection. Figure 3 shows the plot of per cent protection as a function of protein concentration. NTR and protector protein provided complete protection when added in sufficient amount. The concentration required for 50% (IC_{50}) protection against the DTT/Fe^{3+}/O_2 MCO system-induced inactivation in the absence of NTR was 19 μg/ml. The IC_{50} for both DTT/Fe^{3+}/O_2 and ascorbate/Fe^{3+}/O_2 MCO systems in the presence of NTR was 1.8 μg/ml. This suggests that NTR has a higher efficiency than that of DTT in restoring the antioxidant activity of protector protein. In comparison to DTT, the physiological low-mass-thiol, glutathione, did not show comparable activity even with higher concentrations. A protector protein concentration of 260 μg/ml was required for an IC_{50} against ascorbate/Fe^{3+}/O_2 MCO system in the presence of 10 mM GSH. The relative activity of thioredoxin in restoring the protecting activity of protector protein was actually several

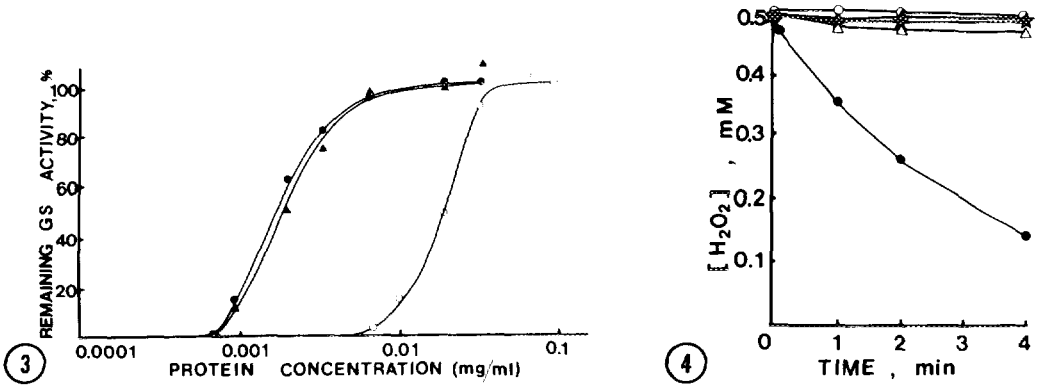

Fig. 3. Protection of glutamine synthetase by protector protein against MCO systems in the presence of NTR. Variable amounts of protector protein were added into the inactivation mixture (50 μl) containing 0.5 μg glutamine synthetase, 3 μM $FeCl_3$, and 50 mM HEPES, pH 7.0. Also added were 10 mM DTT (○), 10 mM DTT and NTR (1 mM NADPH, 20 μg/ml thioredoxin, 20 μg/ml thioredoxin reductase) (●), 10 mM ascorbate and NTR (▲).

Fig. 4. Removal of H_2O_2 by protector protein in the presence of NTR. The incubation mixture contained 0.5 mM H_2O_2 in 40 mM HEPES, pH 7.2, in a total volume of 100 μl. Also added were none (○), 1 mM NADPH (▲), NTR (1 mM NADPH, 25 μg/ml thioredoxin, 25 μg/ml thioredoxin reductase) (△), NTR with 1 mM NADH instead of NADPH (✱), NTR and 45 μg/ml protector protein (●).

orders of magnitude greater than DTT, since 2.8 μM thioredoxin had at least 10-fold higher activity than 10 mM DTT. These observations support the possibility that a cellular redox control system such as NTR has a crucial role in restoring the activity of protector protein.

Yeast protector protein showed a capability to remove H_2O_2 in the presence of NTR. Within 4 min the 45 μg/ml of protector protein was able to remove about 70% of 0.5 mM H_2O_2 as shown in Fig. 4. Removal of H_2O_2 was only observed when the complete NTR was included. Neither NADPH nor NTR significantly destroyed H_2O_2 in the absence of protector protein and protector protein had the capability to remove H_2O_2 only in the presence of NTR. NADH cannot replace NADPH as a reducing equivalent. These results indicate that NTR alone cannot react with H_2O_2. Thus thioredoxin reductase is apparently able to transfer reducing equivalents from NADPH to protector protein via thioredoxin and reduced protector protein restores the ability to remove H_2O_2.

To demonstrate the presence of different forms of protector protein due to reductive activation by either an artificial reducing agent such as 2-mercaptoethanol or NTR, SDS-PAGE was used to analyze the products of such processes. In the presence of 2-mercaptoethanol (0.1 M) in the sample buffer, dimer formed by disulfide linkage of the protector protein was converted to the monomer form as shown in lane 1 of Fig. 5. The reduction of protector protein disulfide by the NTR was determined using SDS-PAGE without reducing agent (such as 2-mercaptoethanol and DTT) in the sample buffer.

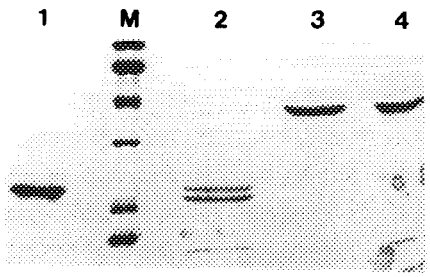

Fig. 5. SDS-PAGE of protector protein in the presence of reducing agents. The 200 μg/ml protector protein was treated by 0.1 M 2-mercaptoethanol (lane 1), complete NTR (2 mM NADPH, 20 μg/ml thioredoxin, 20 μg/ml thioredoxin reductase) (lane 2), omission of NADPH from complete NTR (lane 3), or omission of thioredoxin and thioredoxin reductase from complete NTR (lane 4), and then analyzed by SDS-PAGE (15%). M indicates molecular marker (Top to bottom: phosphorylase b = 94 kDa, bovine serum albumin = 67 kDa, ovalbumin = 45 kDa, carbonic anhydrase = 31 kDa, soybean trypsin inhibitor = 21.5 kDa, lysozyme = 14.4 kDa).

Complete NTR (2 mM NADPH, 20 μg/ml each of thioredoxin and thioredoxin reductase) also led to cleavage of the disulfide bridge in protector protein (lane 2). Omission of NADPH or thioredoxin and thioredoxin reductase did not cause conversion of a dimer to monomer as shown in lanes 3 and 4 of Fig. 5.

DISCUSSION

Although not much is known about the mechanism of protector protein, a plausible mechanism has been suggested by the fact that this protein can protect *E. coli* glutamine synthetase from inactivation caused by the ascorbate/Fe^{3+}/O_2 MCO system only in the presence of DTT (12). The fact that thiol itself can cause inactivation in the presence of Fe^{3+} and that protector protein can protect this type of damage only in the presence of thiol suggests that the inactive form of protector protein can be converted to the active form by thiols. Higher concentrations of DTT (1-20 mM) can act as a direct hydrogen donor for the enzyme. However, the present study provides the first direct evidence that this mechanism may be feasible under physiological conditions, and the antioxidant activity of protector protein is restored by the cellular reducing catalyst NTR through a redox control mechanism.

Studies with purified protein revealed that protector protein contains neither a heme or a flavin prosthetic group nor tightly bound metal ions, but it does contain two cysteine residues (20). Although the three dimensional structure of this protein is not known, it can be assumed that these two cysteine residues reside in the vicinity of the active site and may be involved in modulation of the activity by a dithiol-disulfide exchange reaction.

Thioredoxin was initially known as a hydrogen donor for ribonucleotide reductase, the essential enzyme providing deoxyribonucleotides for DNA replication (13). Recent research on thioredoxin in many organisms has revealed a variety of different functions

(21, 22). Thioredoxin is now considered as a powerful general reductant of protein disulfides. Thioredoxin is maintained in the reduced form by the flavoprotein thioredoxin reductase, which transfers reducing equivalents to the protein from NADPH (13).

Our observations indicate that NTR restores the scavenging effect for reactive oxygen species of oxidized protector protein by facilitating proton transfer to the disulfide on protector protein through its catalytic action. This system is similar to alkyl hydroperoxide reductase from *Salmonella typhimurium* in which coupling of NAD(P)H oxidation to peroxide reduction is mediated by electron transfer between a redox active thiol on the F52a protein and the disulfide on C22 (23). It has been shown that the F52a protein is highly homologous to the *E. coli* thioredoxin reductase (24). It is likely that the key function of protector protein coupled with NTR is the removal of H_2O_2 because both ascorbate/Fe^{3+}/O_2 and DTT/Fe^{3+}/O_2 systems may produce H_2O_2. However, the possibility that the protector protein has a role to remove other oxygen radicals (12) or sulfur radicals (10) cannot be ruled out. Since NTR can reduce the dose requirement for the protector protein about 10 times compared to DTT even with a thousand times less concentration on a molar basis, protector protein coupled with NTR may be implicated as an important antioxidant enzyme system *in vivo*.

Our findings not only demonstrate the presence of a novel modality in the activation of protector protein, but also provide strong evidence that cysteine residues on protector protein may be directly involved in its protective effect on reactive oxygen species as previously implicated. However, it remains to be examined whether NTR plays a part in a redox exchange of protector protein *in vivo* and whether reversion of redox status is effective in the protection of cells from reactive oxygen species. Recently, it has been reported that the amount of thioredoxin is greatly increased in some cell lines by treatment with a low-dose of hydrogen peroxide (22). Although there is no evidence supporting this hypothesis, it could be proposed that the transient oxidative stress elicited by hydrogen peroxide might induce the production of thioredoxin which would then activate protector protein that had been oxidized in the process of defense against reactive oxygen species.

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