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REMOVALS OF HYDROGEN PEROXIDE AND HYDROXYL RADICAL BY THIOL-SPECIFIC ANTIOXIDANT PROTEIN AS A POSSIBLE ROLE *IN VIVO*

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Summary: Thiol-specific antioxidant protein (Protector Protein; PRP) from *Saccharomyces cerevisiae* was found to remove hydrogen peroxide and hydroxyl radical in the presense of dithiothreitol (DTT). Without DTT as a reducing equivalent, the antioxidant protein did not show the activities for destroying hydrogen peroxide and hydroxyl radical. N-ethylmaleimide (NEM) was observed to prevent the PRP from both removing hydrogen peroxide and protecting the cleavage of DNA. These observations suggest that the sulfhydryl of cysteine in PRP could function as a strong nucleophile to attack and destroy H_2O_2 and ·OH.

To survive, all aerobic organisms are equipped with several antioxidant enzymes which can removed the harmful reactive oxygen species(1). Most cell have efficient mechanism for removing H_2O_2. Two different enzymes reduce H_2O_2 to water: catalase within the peroxisomes and glutathione peroxidase in both the cytosol and the mitochondria. The $\cdot O_2^-$ produced in the cytosol or mitochondria is catabolized to H_2O_2 by superoxide dismutase. A thiol-specific antioxidant protein (Protector Protein, PRP), first found in *Saccharomyces cerevisiae*(2), is the antioxidant protein which specifically protects the inactivation of various enzymes by a nonenzymatic mixed-function oxidation (MFO) system(3-5) containing a sulfhydryl compound as a reducing equivalent (Fe^{3+}, O_2 and DTT: thiol MFO system), but not by the MFO system containing a non-sulfhydryl reducing equivalent such as ascorbate (Fe^{+3}, O_2 and ascorbate: non-thiol MFO system)(2). The yeast PRP synthesis was significantly enhanced by oxidative pressure. Furthermore, PRP was conserved in mammalian tissues(6), indicating its physiological significance.

Despite these advances, very little is known about the reaction mechanism of PRP as an antioxidant enzyme. In this communication, we report the thiol-specific

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removal of both H_2O_2 and ·OH by yeast PRP as a possible reaction mechanism for destroying the harmful reactive oxygen species, implicating the unique physiological function of PRP as a hydroxyl radical scavenger.

Materials and Methods

Assay for Hydrogen Peroxide: The reaction was started by the addition of H_2O_2 to the reaction mixture containing 100 mM Hepes buffer (pH 7.0), 10 mM DTT and PRP at 37 °C. At appropriate reaction time, 0.75 ml of trichloroacetic acid (TCA) solution (12.5 %, v/v) was added to the reaction mixture to stop the reaction, followed by the addition of 0.2 ml of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 ml of 2.5 M KSCN to develop complex showing purple color. The removal of H_2O_2 by PRP was monitored by measurement of absorbance decrease at 450 nm, the absorbance maximum of the complex.

Detection for DNA Cleavage by Nonenzymatic MFO System (DTT, O_2 and Fe^{3+}): Reaction mixture containing 2 μg of PUC 19 plasmid, 3 μM $FeCl_3$, 0.1 mM EDTA and 10 mM DTT was incubated for 4 hr at 37 °C. The resulting reaction mixture was applied to 1 % agarose gel to examine DNA cleavage. The DNA band on agarose gel was stained with EtBr (5 μg/ml).

Other Methods: Protein determination was performed by the method of Bradford(7). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli(8). The protein band was stained with Coomassie Brilliant Blue R-250. A thiol-specific antioxidant property of PRP was determined by monitoring its ability to inhibit the inactivation of *E. coli* glutamine synthetase (GS) by a MFO system containing 10 mM DTT and 3 μM $FeCl_3$ as previously described by Kim et al.(2). The modification of cysteine in PRP with NEM was performed in the presence of 1 mM DTT at 30 °C, pH 8.0 (100 mM Tris-HCl buffer) according to the method described by Lundblad(9). This reaction was followed spectrophotometrically by the decrease in absorbance at 300 nm, the absorbance maximum of NEM.

Results

Removal of H_2O_2 by PRP: Yeast PRP showing the thiol-specific antioxidant properties(2) was found to have a capability to remove H_2O_2 in the presence of DTT, but not in the absence of DTT. Within 10 min the 1 mg/ml of PRP can destruct about 45 % of 10 mM H_2O_2 (Fig. 1-A). The reaction was initiated by adding PRP (1 mg/ml) to the reaction mixture containing 10 mM H_2O_2 and 10 mM DTT at 37 °C, pH 7.0 (100 mM Hepes-HCl buffer). DTT itself did not significantly destroy H_2O_2 (data not shown), but somewhat increased the color reaction (shown on Fig. 1-B and -C). Fig. 1-B shows the H_2O_2-destroying profile as a function of the concentration of PRP. The increasing rate of the destruction of H_2O_2 as a function of PRP concentration indicates that H_2O_2 can be removed by PRP.

Functional Cysteine in PRP: The PRP required DTT to remove H_2O_2 (Fig. 1-A and -B), but catalase eliminated H_2O_2 regardless of the existence of DTT (Fig. 1-C), indicating that DTT having sulfhydryl group did not react with H_2O_2. This thiol-requiring property by PRP was also observed in the thiol-specific protection of PRP

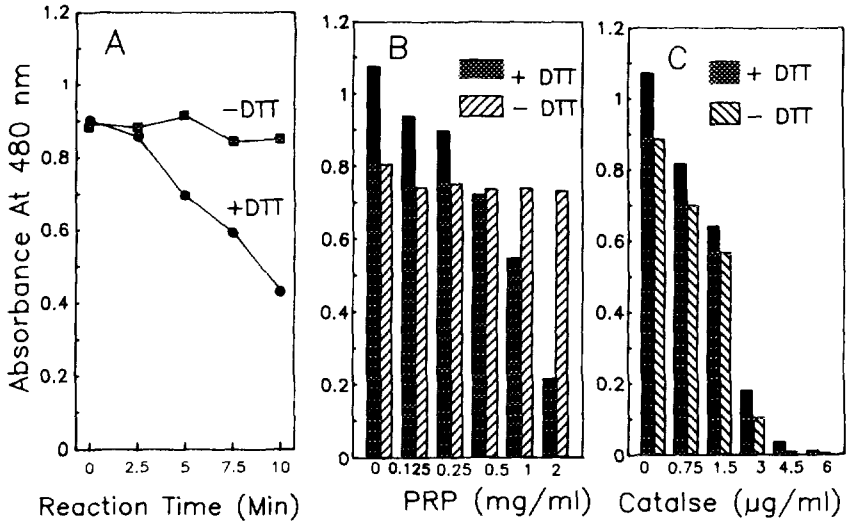

Fig. 1. Removal of H_2O_2 by PRP or catalase . The reactions were started by PRP (B) or catalase (C) to the reaction mixture containing thiol MFO system or non-thiol MFO system. In case of A, 1 mg/ml of PRP was added.

against inactivation of *E. coli* GS(2). To step toward understanding this thiol-dependency, DTT was added to the non-thiol MFO system in the presence of *E. coli* GS. Fig. 2 shows that PRP did not protect the inactivation of *E. coli* GS by the non-thiol MFO system (AFP shown in Fig. 2), but in the presence of DTT and ascorbate, completvely prevent the inactivation (DAFP shown in Fig. 2), suggesting that an inactive form of PRP was converted to the active form by DTT. The thiol-

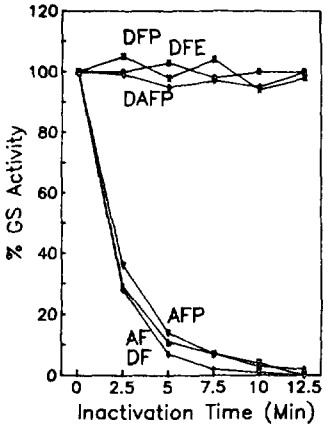

Fig. 2. The effect of DTT on the PRP antioxidant property. The inactivation of *E. coli* GS was initiated by adding 3 μM $FeCl_3$ to the reaction mixture containig MFO components as folllows: D, 10 mM DTT; F, 3 μM $FeCl_3$; E, 1 mM EDTA; A, 10 mM ascorbate; P, 0.5 mg/ml yeast PRP. Details are in Methods.

dependency makes it probable that the cysteines in PRP might be directly involved in the protection mechanism.

To examine the possibility that the cysteine(s) in PRP might act as a catalytic group, PRP was reacted with NEM, Fig. 3-A shows that the H_2O_2-removal activity of PRP decreased as a function of concentration of NEM. In addition, the PRP reacted with NEM (5 mM) did not protect the inactivation of *E. coli* GS by a thiol-MFO system) (Fig. 3-B). Without DTT, NEM did not inactivate the PRP. The loss of the activities by NEM and DTT indicates a catalytic involvement of the reduced cysteine in PRP. It is noteworthy that although 10 mM DTT having sulfhydry group (20 mM sulfhydryl group) did not show significant H_2O_2-removing activity (data not shown), 47 μM of functional cysteine (calculated from 1 mg/ml, base on molecular mass of 21,750 Da for yeast PRP) in PRP removed 45 % of 10 mM H_2O_2 within 10 min reaction time at 37 °C, pH 7.0 (Fig. 1-A). This indicates the strong nucleophilicity of cysteine in PRP.

Removal of Hydroxyl Radical by PRP: Hydroxyl radical has been known to be formed in biological fluid by the reaction of hydrogen peroxide with Fe^{2+} (i.e.,the Fenton reaction). It is believed that the hydroxyl radical is a strong oxidant capable

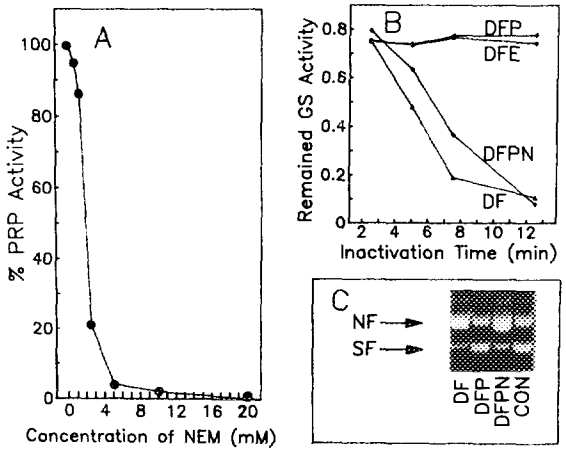

Fig. 3. The effect of NEM on the PRP activities. **(A)** The effect of NEM on H_2O_2-destroying activity of PRP. **(B)** The effect of NEM on the PRP protecting activity against the inactivation of *E. coli* GS by the MFO system containing the components as described in Fig. 2. **(C)** The effect of NEM on the PRP protecting activity against the cleavage of PUC 19 plasmid by the MFO system. After 10 min incubation at 37 °C for the generation of ·OH by the reaction mixture, 0.5 mg/ml of PRP in 10 mM DTT was added to the reaction mixture for an additional 30 min incubation. Two μg of PUC19 plasmid was added to the incubated mixture and further incubated for 4 hr. PN, 0.5 mg /ml of PRP reacted with 5 mM NEM. CON, control DNA incubated without DTT and $FeCl_3$. Other abbreviations are described in Fig. 2. NF, SF; Nicked, Super coilded Form of PUC19 plasmid DNA, respectively. Details are in Methods.

of destroying DNA(10,11). The fact that iron is a potent catalyst of thiol oxidation has been verified by a number of laboratories(11-16). Rowley and Halliwell(14) verified the presence of OH· in a thiol-MFO system. Also Claycamp(11) showed that the oxidation of thiol in neutral buffer can cause significant damage in DNA and its constituents.

The hydroxyl radical produced in the thiol-MFO system can cause to nick the intact supercoiled form of PUC19 plasmid to the nicked form. The supercoiled form of DNA was mostly converted to the nicked form during the 4 hr-incubation of the DNA in the reaction mixture containing the thiol-MFO system (lane DF in Fig. 3-C). This conversion was completely prevented by the active PRP (lane DFP), but not by the inactive PRP reacted with NEM (lane DFPN). To have an insight into the protection phenomenon, we examined the effects of H_2O_2 on the protection activities of PRP against the cleavage of DNA by thiol-MFO system, If this protection activity might be caused by the capability for destroying H_2O_2 an excess amount of H_2O_2 should retard the protection activity. Fig 4-A indicates that H_2O_2 (10 mM) was significantly destroyed in the reaction mixture containing thiol-MFO system. The rate of H_2O_2 destruction was found to be accelerated by addition of PRP (0.5 mg/ml) to the thiol-MFO system containing H_2O_2 (Fig. 4-A). Fig. 4-B

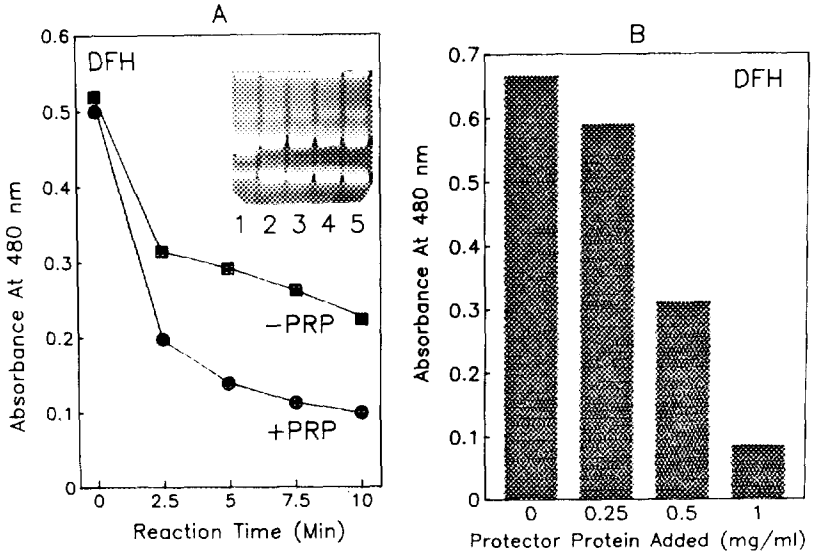

Fig. 4. The effect of PRP on the removal of H_2O_2 by thiol MFO system. (A) Time-coursed H_2O_2-removal by thiol MFO system with 0.5 mg/ml of PRP (+PRP) or without PRP (-PRP). **(Inset in A)** The cleavage of the plasmid by thiol MFO system containing 10 mM H_2O_2. lane 1; without PRP, lanes2-4; with 0.25, 0.5, or 1 mg/ml of PRP, respectively, lane 5; control DNA without thiol MFO components. The reaction were performed as described in Fig. 3-C. **(B)** The H_2O_2 removal as function of PRP concentration for 10 min reaction time. The reaction conditions are the same as those of (A).

shows that the accelerated rate increrased as function of concentration of PRP, confirming the capability of PRP for H_2O_2 destruction. However, an excess of H_2O_2 did not retard the protection against the DNA cleavage even at the unsaturated concentration of PRP, 0.25 mg/ml (inset in Fig. 4-A, and -B). These results indicate that the protection activity againt DNA cleavage by the thiol-MFO system may be owing to the removal of activated oxygen species, presumablely, OH·, not to the removal of H_2O_2. This interpretation can be strongly supported by the observations that even an excess amount (5 mg/ml) of catalase destroying H_2O_2 more faster than PRP (shown in Fig. 1-C) did not protect the cleavage of DNA , but 0. 5 mg/ml of PRP completely prevented the cleavage of DNA (Fig. 5-A). To determine the protection potency of PRP against the DNA cleavage by the thiol-MFO system, the concentration of PRP was varied from 500 μg to 16 μg / ml. From the result shown on Fig. 5-B, it was known that the concentration of protein required to protect perfectly the inactivation was between 62.5 μg and 31.5 μg per ml of reaction mixture (lane 8 and 9 of Fig. 5-B). The pattern of protection as a function of PRP concentration shows an all-or-nothing pattern, indicating that PRP eliminates the reactive hydroxyl radical.

Discussions

The hydroxyl radical is one of the most harmful species in oxygen toxicity, but little is known about an antioxidant enzyme removing ·OH directly, although several

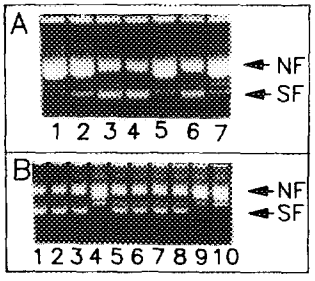

Fig. 5. The cleavage of PUC19 plasmid by the various thiol MFO systems.
(A) Effect of catalase on the cleavage of DNA by various MFO system.
(lane 1): DTT + Fe^{3+} + H_2O_2. (lane 2): DTT + Fe^{3+}. (lane 3): DTT + Fe^{3+} + H_2O_2 + PRP (0.5 mg/ml). (lane 4): DTT + Fe^{3+} + PRP. (lane 5): DTT + Fe^{3+} + H_2O_2 + catalase (5 mg/ml). (lane 6): control DNA. (lane 7): DTT + Fe^{3+} + catalase.
(B) The protection potency of PRP against the cleavage of DNA by thiol MFO system. (lane 1): Control DNA. (lane 2): Fe^{3+} only. (lane 3): DTT only. (lane 4): DTT + Fe^{3+}. (lanes 5-10): DTT + Fe^{3+} + 500, 250, 125, 62.5, 31.3, 15.6 μg/ml of PRP, respectively. NF; Nicked Form of Plasmid. SF; Super coiled Form. The reaction procedure was carried out as described in Fig. 3.

antioxidants such as glutathione and ascorbate have been known to be chemical protectors against ·OH *in vivo*. However, these chemical antioxidants can manifest paradoxical pro-oxidant actions wherein they promote rather than inhibit oxidizing damage. Most often this occurs through acceleration rather than inhibition of the Fenton-like production of ·OH by a suitable metal catalyst that causes reduction of H_2O_2 from dismutation of $\cdot O_2^-$ or from some oxidases(17).

Recently, we purified a new type of antioxidant protein (Protector Protein: PRP) requiring thiol for its antioxidant activities. The PRP did not show any activities of known antioxidant enzymes such as catalases and peroxidases including glutathione peroxidase(2). The PRP shows both capabilities for destroying H_2O_2 and ·OH, and its activities were completely inhibited by NEM in the presence of DTT, suggesting that the cysteine in PRP involves in catalytic group acting as a strong nucleophile enough to destroy H_2O_2 and ·OH significantly. Analogies with the cysteine in PRP showing a strong nucleophilicity were reported by other laboratories. The ovothiol, a mercaptoimidazole, is more effective than egg catalase in destroying H_2O_2(18). They suggested that the ovothiol remarkably abundant (ca, 5 mM) in the eggs of marine invertebrates, functions as an antioxidant. The ovothiol preventing oxidative damage at fertilization is more effective than egg catalase in destroying H_2O_2. The capability of ovothiol was proven to be due to the strong nucleophilicity(19). The thiol groups of ovothiol and glutathione are chemically distinct. The mercaptoimidazole in solution at physiological pH is both more nucleophile and more reactive as an one-electron donor. Wayner et al. (20) reported that a human plasma proteins having sulfhydryl group show the peroxy radical-trapping antioxidant activity which is completely inhibited by the reaction with NEM.

PRP shows a distinct protecting property against the cleavage DNA by thiol-MFO system regardless of the presence of H_2O_2. However, the *E. coli* catalase destroying H_2O_2 more fast than PRP did not prevent cleaving DNA by thiol-MFO system in the same condition as that of PRP reaction. This result clearly indicates that in addition to the H_2O_2-destroying activity, PRP has the activity destroying a activated oxygen species from H_2O_2 in the presence of thiol-MFO system, wherein the production of ·OH should occur. The concentration of PRP required to protect perfectly the cleavage of DNA is between 31.5 μg and 62.5 μg/ml. This potency is ten-times high as the potency for protecting the site-specific inactivation of protein (i.e., 500 μg/ml). Based on the results described above, we suggest that the activity for scavenging ·OH rather than the activity for destroying H_2O_2 has physiological importance. The *in vivo* function of PRP remains to be explored further, but the PRP warrants further investigation as a new type of antioxidant enzyme.

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References

1. Fridovich, I. & Freeman,B. (1986) *Annu. Rev. Physiol.* **48**, 693-702
2. Kim,K.H., Kim,I.H., Lee,K.Y., Rhee,S.G. & Stadtman,E.R. (1988) *J. Biol. Chem.* **263**, 4704-4711
3. Fucci,L., Oliver,C.N., Coon,M.J. & Stadtman,E.R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1521-1535
4. Stadtman,E.R. (1988) *Trends Biochem. Sci.* **11**, 11-12
5. Kim,K., Rhee,S.G. & Stadtman,E.R. (1985) *J. Biol. Chem.* **260**, 13394-13397
6. Kim,I.H., Kim,K. & Rhee,S.G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6018-6022
7. Bradford,M.M. (1976) *Anal. Biochem.* **72**, 248-254
8. Laemmli,U.K. (1970) *Nature (London)* **227**, 680-685
9. Lundblad, R.L.. & Noyes, C.M. (1984) *Chemical Reagents for Protein Modification* **1**, p.55-93, CRC Press,Inc.
10. Chevio,M. (1988) *Free Radical Biol. & Med.* **260**, 757-764
11. Claycamp, H.G. (1987) *Biochem. Biophys. Res. Commun.* **144**, 432-437
12. Tien,M., Buchner,J.R. & Aust,S.D. (1982) *Biochem. Biophys. Res. Commun.* **107**, 279-285
13. Lamfrom,H. & Nielsen, S.O. (1957) *J. Am. chem. Soc.* **79**, 1966-1970
14. Rowley, D.A. & Halliwell, B. (1982) *FEBS Lett.* **138**, 33-36
15. Halliwell,B. & Gutteridge,J.M.C. (1985) *Free Radical in Biology and Medicine* p. 119, Clarendon Press, Oxford
16. Misra, H.P. (1974) *J. Biol. Chem.* **249**, 2151-2155
17. Borg,D.C. & Schaich,K.M.(1989) *CRC Hanbook of Free Radicals & Antioxidants in Biomedicine* **1**, 63-80, CRC Press Inc.
18. Turner,E., Hager,L.J. & Shapiro, B.M. (1988) *Science* **242**, 939-941
19. Holler,T.D. & Hopkins, P.B. (1988) *J. Am. Chem. Soc.* **110**, 4837-4838
20. Wayner,D.D.M., Burton,G.W., Ingold,K.U., Barclay,L.R.C. & Locke, S.J. (1987) *Biochim. Biophys. Acta* **924**, 408-419