Ceruloplasmin Has a Distinct Active Site for the Catalyzing Glutathione-Dependent Reduction of Alkyl Hydroperoxide†

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Received February 24, 1999; Revised Manuscript Received June 30, 1999

ABSTRACT: Ceruloplasmin, a blue multi-copper α_2-glycoprotein found in the plasma of all vertebrates, is capable of oxidizing aromatic amines and ferrous iron. Here, we report that human ceruloplasmin exhibits an alkyl hydroperoxide peroxidase activity, which is independent of the oxidase activity. The site-specific modification of the sulfhydryl of cysteine at position 699 in ceruloplasmin completely abolished the antioxidant activity, suggesting that ceruloplasmin is a peroxidase with a cysteinyl thiol as a functional nucleophile. The crystal structure of human ceruloplasmin reveals that the domain containing Cys-699 is apart from the multi-copper complex domains. Taken together, these data suggest that ceruloplasmin has a distinct active site for a glutathione-linked peroxidase activity apart from the copper complex site exerting ferroxidase activity.

Ceruloplasmin (CP),[1] with a molecular mass of 125 kDa, is synthesized in hepatocytes and secreted as a holoprotein with six atoms of copper per molecule. CP is also synthesized locally within human nonhepatic tissues, including the central nervous system (*1*) and lung tissue (*2*). CP is important for normal release of cellular iron (*3*) because of its ferroxidase activity, a catalytic oxidation of Fe^{2+} to Fe^{3+}. An inherited CP-deficient disease, aceruloplasminemia, is known to cause diabetes and neurologic symptoms such as the degeneration of retinal photoreceptors and ganglion cells and the marked neuronal loss in the basal ganglia (*1*, *4*). The presence of inherited aceruloplasminemia with the clinical and pathological features indicates an essential role for CP in human physiology. The human CP concentration in the plasma is increased during pathological processes such as inflammation (*2*) and decreased with aging (*5*). A marked increase in the extent of plasma membrane peroxidation was observed in the patients with aceruloplasminemia (*6*). The expression level of pulmonary CP gene is increased during inflammation and hyperoxia (*2*). This evidence may indicate an antioxidant role of CP via the ferroxidase activity removing ferrous irons, which induce the production of hydroxyl radical from H_2O_2. CP deficiency-related pathology could be caused by an abnormal iron accumulation resulting from the absence of the ferroxidase activity. However, it is also possible that some CP deficiency-related pathology may result from the loss of an antioxidant activity of CP for removing reactive oxygen intermediates such as hydroperoxides. This possibility may be supported by a recent observation that human CP has the ability to remove H_2O_2 (*7*).

In an attempt to clarify this important issue, we searched for a possible antioxidant activity of CP and investigated its antioxidant mechanism against reactive oxygen species. In this study, we report that CP is also capable of catalyzing GSH-dependent reduction of alkyl hydroperoxide rather than H_2O_2, and has a distinct active site for the peroxidase activity apart from the copper complex site exerting ferroxidase activity.

EXPERIMENTAL PROCEDURES

Purification of Ceruloplasmin. Human blood plasma was obtained from freshly drawn heparinized blood. Human plasma proteins were precipitated with 70% ammonium sulfate. The precipitates were used for the purification of ceruloplasmin by a series of subsequent chromatographic steps with DEAE, Sephadex G-75, and phenyl Sepharose CL-4B columns as described previously (*10*). For a final HPLC purification step, a size-permeation column, SynChropack GPC 300 (250 mm × 10 mm), was used. The antioxidant activity of CP was detected by the glutamine synthetase protection assay (*11*) throughout all the purification procedures.

Determination of Antioxidant and Oxidase Activities of Ceruloplasmin. The antioxidant activity of CP was measured in terms of the protecting activity against the inactivation of glutamine synthetase by a MCO system containing 3 μM Fe^{3+} and 10 mM DTT (*10*, *11*). The remaining glutamine synthetase activity was measured at 540 nm. The oxidase activity of CP was measured in terms of its ability to oxidize an aromatic amine, *o*-dianisidine (*12*). The reaction mixture contained 2 mM *o*-dianisidine and 70 mM sodium acetate

† This work was supported by Creative Research Initiative Program of MOST.

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[1] Abbreviations: CP, ceruloplasmin; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; MCO, metal-catalyzed oxidation; TPx, thiol peroxidase; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate.

10.1021/bi990444b CCC: $18.00
Published on Web 08/26/1999

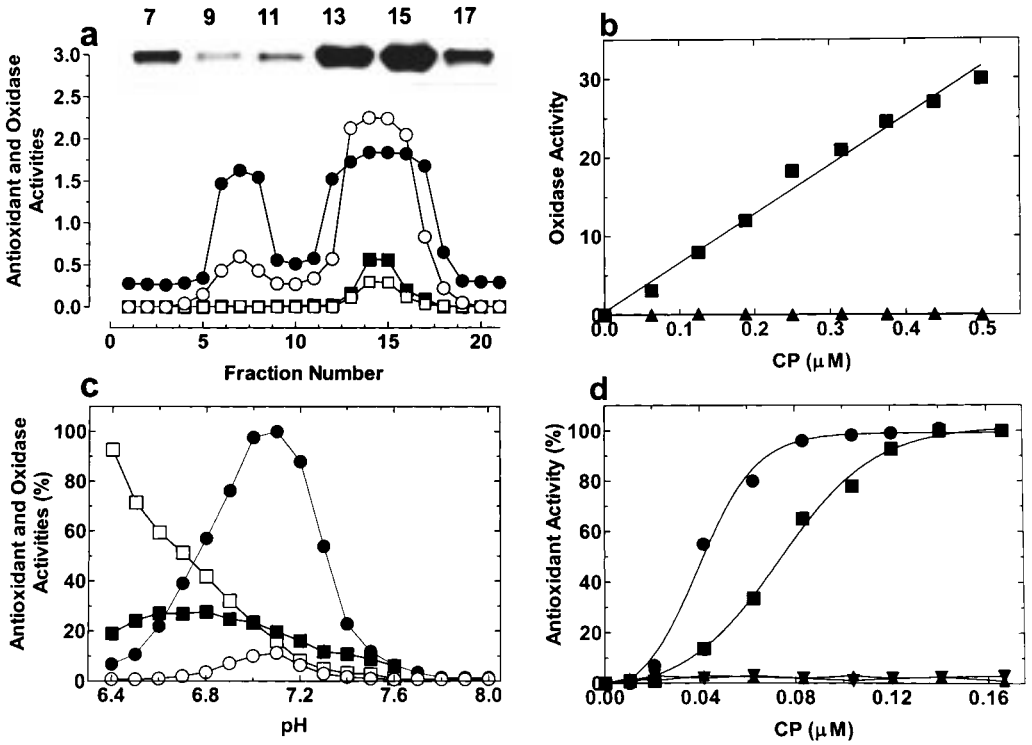

FIGURE 1: Purification and characterization of the antioxidant activity of ceruloplasmin. (a) HPLC results for the final purification of human CP. The proteins were detected at 280 (○) and 610 nm (□). The antioxidant activity (●) was determined by the glutamine synthetase protection assay as described in Experimental Procedures. The oxidase activity (■) was measured in terms of its ability to oxidize an aromatic amine, *o*-dianisidine, described in Experimental Procedures. Each HPLC fraction (8 μL) exhibiting antioxidant activity was separated on an 8% polyacrylamide electrophoresis gel in the absence of SDS. The number in the inset represents the fraction number. (b) Oxidase activities of the late eluting blue-colored protein (CP) (■) and the late eluting colorless protein (apo-CP) (▲) shown in panel a. The oxidase activity is expressed as micromoles of product per minute. (c) pH profiles of the antioxidant and oxidase activities of CP, and the salt effect on the antioxidant and oxidase activity of CP: (●) the antioxidant activity of CP as a function of pH in the presence of 100 mM NaCl and (○) in the absence of NaCl, (□) the oxidase activity of CP as a function of pH in the absence of NaCl and (■) in the presence of 100 mM NaCl. One hundred percent of the antioxidant and oxidase activities represent the maximal activities at optimum pH values of 7.1 and 5.5, respectively. (d) The antioxidant activities of CP (●) and apo-CP (■) which is supported by DTT. The antioxidant activities of CP (▼) and apo-CP (▲) against the inactivation by the MCO system containing ascorbate as a reducing agent instead of DTT. One hundred percent of the antioxidant activity represents the glutamine synthetase activity without the inactivation by the MCO system.

(pH 5.5). After incubation for 10 min at 37 °C, the oxidase reaction was stopped by the addition of 2 volumes of 9 M H_2SO_4. The peroxidase activity of CP was determined by directly measuring the amount of remaining *tert*-butyl hydroperoxide by using ferrithiocyanate as described previously (*13*). The peroxidase activity was also indirectly measured in terms of GSSG. The concentration of GSSG was determined by following the decrease in the amount of NADPH at 340 nm in the presence of 1 unit of glutathione reductase and 0.15 mM NADPH.

HPLC Separation of Tryptic Peptides. Ceruloplasmin was reductively denatured by a 6 M guanidine hydrochloride solution containing 1 mM DTT and 100 mM Tris-HCl (pH 8.0). The cysteinyl thiol was labeled with TNB by an excess of DTNB for 1 h at 30 °C. The TNB-linked protein was washed with 7 volumes of acetone. The resulting precipitate was suspended in 100 mM Tris-HCl buffer (pH 8.0), and following the digestion with trypsin for 3 h at 30 °C. The additional digestion with fresh trypsin was carried out overnight. The tryptic peptides were separated by a Vydac C_8 column with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid over the course of 30 min at a flow rate of 1 mL/min. The peptides were detected using Shimadzu SPD-M10A_{vp} and RF-10A_{XL} detectors.

RESULTS

Purification and Characterization of the Antioxidant Activity of Human Ceruloplasmin. We homogeneously purified two types of antioxidant proteins to electrophoretic homogeneity by employing HPLC size exclusion chromatography as a final purification step (Figure 1a). The major peaks were found to be homogeneous on polyacrylamide gel electrophoresis in the presence of SDS. All active fractions from HPLC were also separated on an 8% polyacrylamide gel in the absence of SDS. The intensity of the protein band on the gel was well correlated with the strength of antioxidant activity (inset of Figure 1a). The early eluting colorless protein exhibited the same mobility on native polyacrylamide gel electrophoresis as the late eluting blue-colored protein exhibiting absorbance at 610 nm. The early and late eluting proteins were identified as apo-CP and holo-CP, respectively, by the comparison of the amino acid sequences of their tryptic peptides (data not shown). As expected, the early eluting protein, apo-CP, did not exhibit oxidase activity (Figure 1b). The apo-CP, however, still exhibited antioxidant activity as did CP, although the antioxidant activity of apo-CP was decreased (Figure 1d). These results suggest that apo-CP and CP have antioxidant activity. The decrease in the antioxidant activity could be explained in terms of the

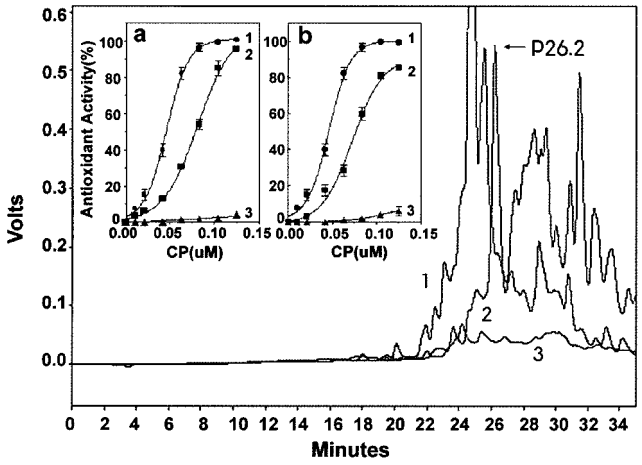

FIGURE 2: Chemical modification of the antioxidant activity of CP (inset) and HPLC separation of the tryptic peptides of CP. For chemical modification, curve 2 in inset a represents the inhibition of the antioxidant activity of CP by 10 mM iodoacetamide without preincubation of CP with DTT and curve 3 the complete inhibition of the activity by 10 mM iodoacetamide after preincubation of CP with 2 mM DTT. Curve 2 in inset b represents the inhibition of the antioxidant activity of CP by 5 mM *N*-(1-pyrene)maleimide without preincubation of CP with DTT and curve 3 the complete inhibition of the activity by 5 mM *N*-(1-pyrene)maleimide after preincubation of CP with 1 mM DTT. Curves 1 shown in insets a and b represent the control antioxidant activity of CP without the reaction with a modification agent. All modification reactions, including the control experiment, were carried out in 100 mM Hepes/NaOH (pH 7.0) and 100 mM NaCl at 30 °C for 4 h. Profile of tryptic peptides. Profiles 2 and 3 depict data the tryptic digest of the *N*-(1-pyrene)maleimide-modified CP after preincubation with DTT and that without preincubation with DTT, respectively. Profile 1 shows the *N*-(1-pyrene)maleimide-labeled tryptic peptides of CP under reductive denaturation conditions after the modification of CP by iodoacetamide in the presence of DTT. The *N*-(1-pyrene)-maleimide-labeled tryptic peptides were detected using a fluorescence detector at 376 nm.

previously reported conformational change of CP caused by removing copper ion from CP (*8*, *9*). The antioxidant activity of CP was measured in terms of the remaining glutamine synthetase activity in the presence of the MCO system comprised of Fe^{3+}, O_2, and DTT (*10*). The MCO system involves the Fe^{2+}-dependent production of hydroxyl radical from H_2O_2, which is capable of oxidizing glutamine synthetase. Therefore, the ferroxidase activity of CP could contribute to the antioxidant activity in our assay system by converting Fe^{2+} to Fe^{3+}. CP did not exhibit the antioxidant activity in the MCO system containing ascorbate as a reducing equivalent instead of DTT (Figure 1d). CP exhibited the same antioxidant activity in the MCO system whose transition metal constituent was replaced by Cu^{2+} (data not shown). Human erythrocyte GSH peroxidase and "TPx" exhibited such a thiol-dependent antioxidant activity against the inactivation of glutamine synthetase by the MCO system (data not shown). These results suggest that the antioxidant activity of CP required a thiol group as a reducing agent, and the antioxidant activity does not result from the ferroxidase activity of CP, but from its ability to remove reactive oxygen intermediates such as alkyl hydroperoxides and hydrogen peroxide.

To differentiate the antioxidant activity from the oxidase activity of CP, each pH- and salt-dependent activity was studied. As shown in Figure 1c, the optimum pH for the antioxidant activity was about 7.1 in the presence of 100 mM NaCl. In the absence of NaCl, the antioxidant activity significantly decreased to 10% of that in the presence of NaCl at the optimum pH. However, in contrast to the simple stimulatory effect of NaCl on the antioxidant activity of CP, the effect of NaCl on the oxidase activity was very complicated as previously reported (*12*). The antioxidant activity profile as a function of NaCl concentration showed that about 10 mM NaCl was required for maximal activity (data not shown). To identify which ion has the ability to activate the antioxidant activity, we examined the effects of various salts consisting of monovalent cation and halide ion. Except for fluoride ion, all salts containing halide anions increased the activity regardless of their counterpart cations (data not shown). The extent of the activation of the antioxidant activity by various halide ions revealed that chloride ion was the most effective ion, exhibiting the optimum size effect of chloride ion. The activation of the antioxidant activity and the inactivation of the oxidase activity of CP by chloride ion are likely to be caused by a chloride ion-induced conformational change. A recent report showed that incubation of human CP with a physiological

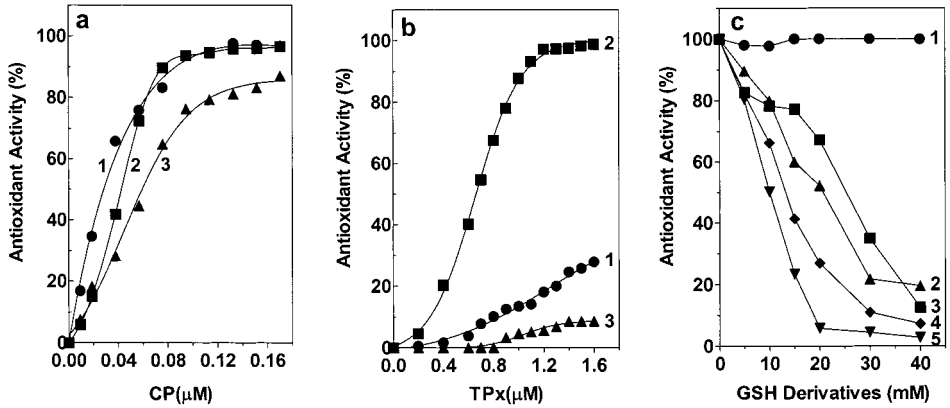

FIGURE 3: Glutathione-linked antioxidant activity of ceruloplasmin. (a) The antioxidant activity of ceruloplasmin (CP) supported by GSH (curve 1), DTT (curve 2), and *N*-acetylcysteine (curve 3). (b) The antioxidant activity of a 25 kDa thiol peroxidase (TPx) from human erythrocytes supported by GSH (curve 1), DTT (curve 2), and *N*-acetylcysteine (curve 3). The concentrations of GSH, DTT, and *N*-acetylcysteine were 20, 10, and 20 mM, respectively. (c) The inhibition of the antioxidant activity of CP by GSH derivatives. Curve 1 depicts the control antioxidant activity without the derivative. Curves 2−5 depict the inhibitory effects on antioxidant activities of CP by *S*-lactoylGSH, *S*-methylGSH, *S*-(*p*-nitrobenzyl)GSH, and *S*-(chlorophenacyl)GSH, respectively, in the presence of 20 mM GSH.

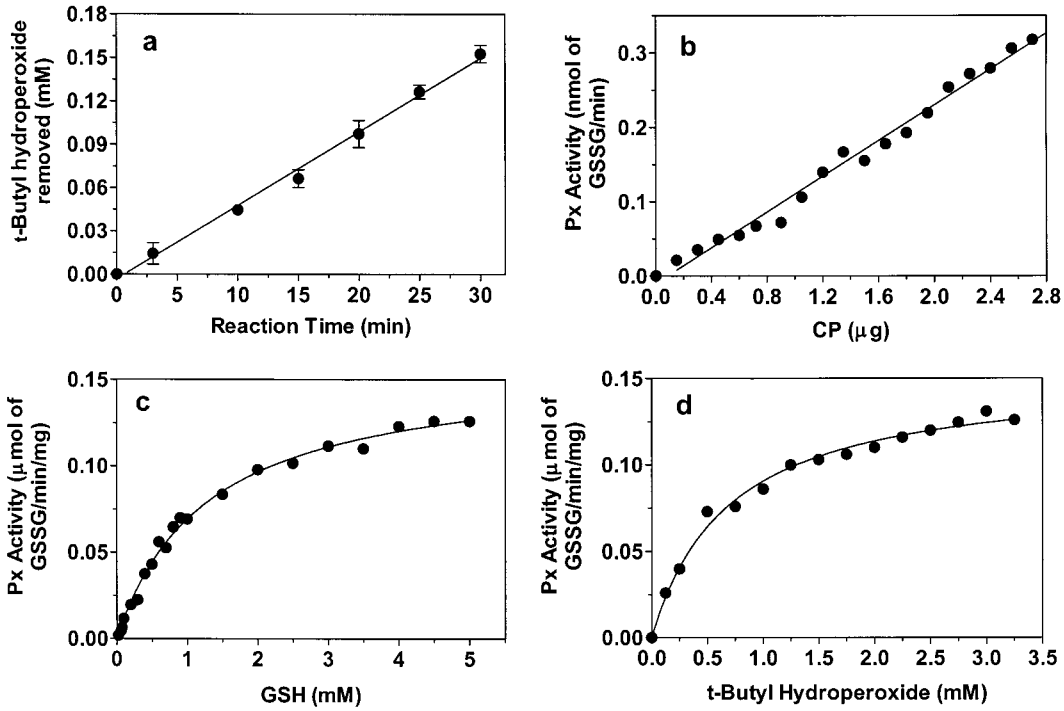

FIGURE 4: GSH-linked alkyl hydroperoxide peroxidase activity of ceruloplasmin. (a) Removal of *tert*-butyl hydroperoxide by CP supported by GSH. The peroxidase reaction was carried out in a reaction mixture containing 100 mM NaCl, 3 mM *tert*-butyl hydroperoxide, 5 mM GSH, 0.48 μM (60 μg/mL) CP, and 100 mM Hepes/NaOH (pH 7.0) at 30 °C. The amount of *tert*-butyl hydroperoxide remaining was directly determined. Each data point is the mean (±standard deviation) of five independent experiments. (b) The peroxidase activity of CP as a function of CP concentration supported by glutathione. The peroxidase activity was indirectly measured in terms of the amount of oxidized GSH (GSSG). The reaction mixture consisted of 3 mM *tert*-butyl hydroperoxide, 5 mM GSH, 100 mM NaCl, and 100 mM Hepes/NaOH (pH 7.0) at 30 °C. The peroxidase activity is expressed as nanomoles of GSSG per minute. (c) The peroxidase activity of CP as a function of GSH concentration. The activity was measured in a reaction mixture consisting of 3 mM *tert*-butyl hydroperoxide, varying amounts of GSH, 100 mM NaCl, 0.48 μM CP, and 100 mM Hepes/NaOH (pH 7.0) at 30 °C. The peroxidase activity was expressed as micromoles of GSSG per minute per milligram of CP. (d) The peroxidase activity of CP as a function of *tert*-butyl hydroperoxide concentration. The activity was measured in a reaction mixture consisting of varying amounts of *tert*-butyl hydroperoxide, 5 mM GSH, 100 mM NaCl, 0.48 μM CP, and 100 mM Hepes/NaOH (pH 7.0) at 30 °C.

concentration of chloride at neutral pH induced a dramatic conformational change (*14*).

The different pH profiles and chloride effects of the antioxidant and oxidase activities suggest independent catalytic activities exist between them.

Identification of a Functional Cysteinyl Thiol Group within Ceruloplasmin. We previously reported a new type of peroxidase with a molecular mass of 25 kDa in human brain (*15*) and red blood cell (*16*). The protein, named TPx, exerted the same thiol-dependent antioxidant activity against the inactivation of glutamine synthetase by the MCO system as CP. In addition, its functional group was identified as a highly conserved cysteine residue. Recently, the functional cysteine residue was shown to exist in a catalytic domain by X-ray crystallographic study (*15*, *17*). To examine the possibility that a cysteine within CP may act as a functional group on the antioxidant activity as TPx, CP was reacted with a cysteine residue-specific modification agent. As shown in the inset of Figure 2, iodoacetamide and *N*-(1-pyrene)-maleimide completely inhibited the antioxidant activity of CP under the reaction condition in which thiols can be generated by selectively reducing cystine disulfides with DTT. These results suggest the existence of a redox-active cysteine, which acts as a functional nucleophile. If it is assumed that CP shares the similar catalytic cycle of TPx (*13*, *18*), the functional cysteine could be oxidized to form a disulfide bond with its counterpart cysteine during the antioxidative catalysis, which involves the transfer of hydrogen by a redox active sulfhydryl group. To identify the functional cysteine of CP, we analyzed the tryptic peptides from the inactivated CP by iodoacetamide and fluorescent *N*-(1-pyrene)maleimide (Figure 2). As shown in peptide elution profile 2 of Figure 2, a single peptide peak emitting fluorescence at 376 nm was eluted at 26.2 min when the tryptic peptides from the CP reductively modified with *N*-(1-pyrene)maleimide were chromatographed. However, any significant fluorescent peak was not eluted from the tryptic peptides from the CP incubated with *N*-(1-pyrene)-

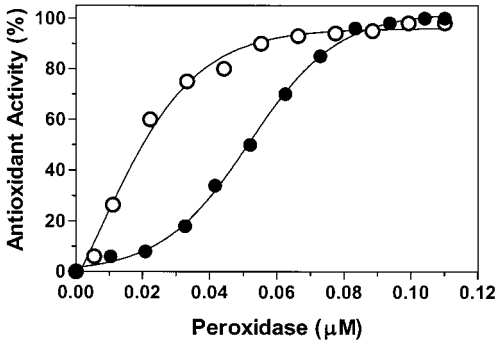

FIGURE 5: Comparison of CP antioxidant activity with those of other thiol peroxidases. The antioxidant activities of CP (●) and bovine erythrocyte GSH peroxidase (○) were determined with the glutamine synthetase protection assay as described in Experimental Procedures.

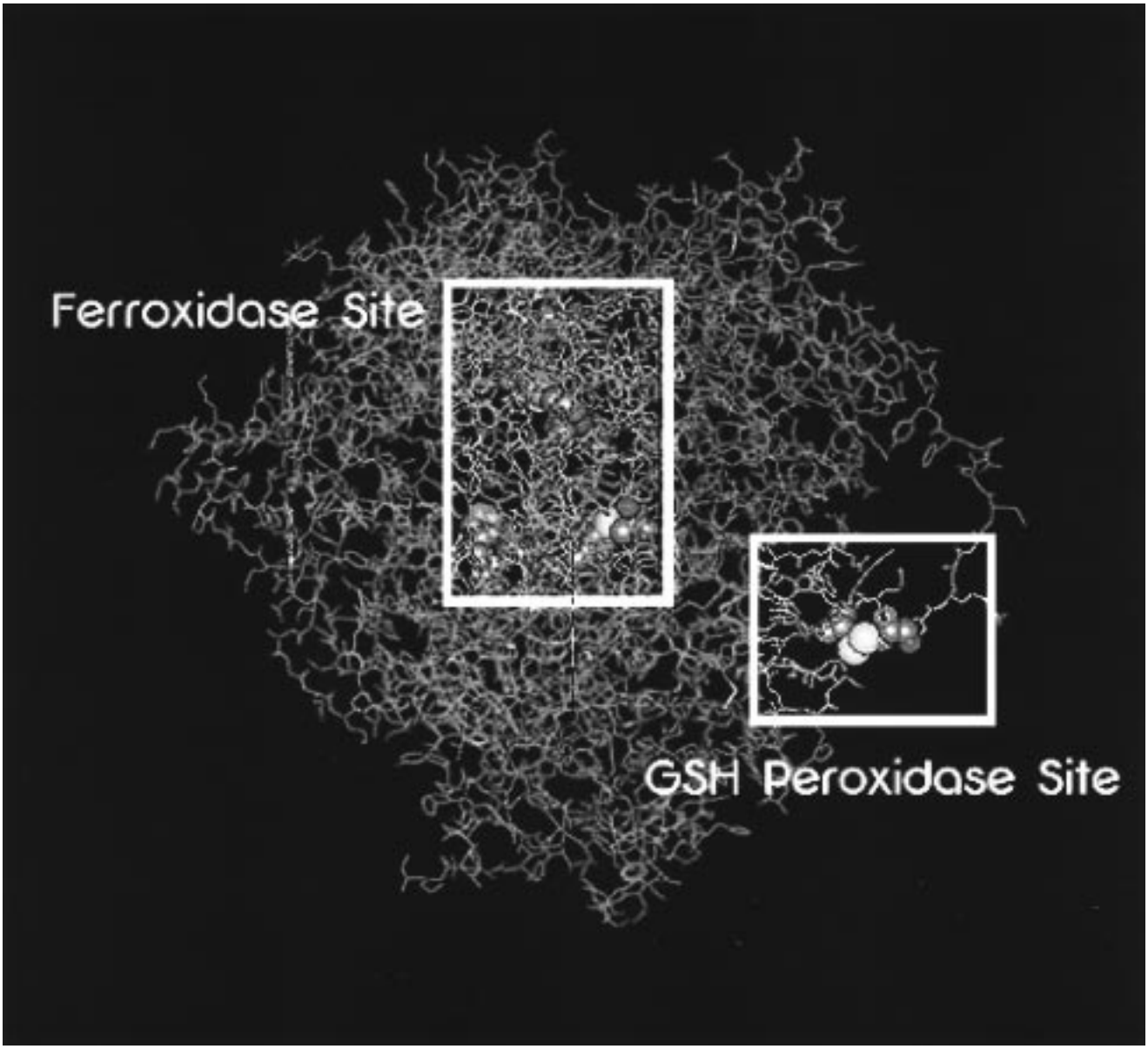

FIGURE 6: Three-dimensional structure of human ceruloplasmin determined by X-ray crystallography (PDB file name 1KCW), showing the active site for peroxidase activity. The functional cysteine at position 699 involved in the peroxidation reaction was shown in the right side of cysteine within the right lined box. The disulfide linkage between Cys-699 and its counterpart Cys-618 was represented as a spaced-filled atom model. The domains containing multi-copper complexes were boxed with a solid line (left box). The amino acid residues involved in the formation of the copper complexes were also represented as space-filled atom models.

maleimide without preincubation with DTT (eluting profile 3 of Figure 2). To rule out the possibility that the single fluorescent peak came from incomplete and nonspecific modification of CP by *N*-(1-pyrene)maleimide, CP was reacted with an excess amount of *N*-(1-pyrene)maleimide under a reductive denaturation condition after modifying CP with iodoacetamide in the presence of DTT, and then the resulting CP was completely digested with trypsin. In that case, the corresponding fluorescent peptide eluting at 26.2 min was not eluted (eluting profile 1 of Figure 2). This result indicates that a cysteine within the peptide eluting at 26.2 min is competitively modified by *N*-(1-pyrene)maleimide and iodoacetamide, resulting in the complete loss of antioxidant activity. The peptide eluting at 26.2 min was sharply collected directly from the outlet of the fluorescence detector, and twice rechromatographed under the same condition for obtaining the purified single peptide. The peak purity was confirmed by the exact overlap of multichromatography traces detected at 210, 254, and 280 nm by using a UV−visible diode array detector with the chromatography detected by a fluorescence detector. The amino acid sequence of the peptide that eluted at 26.2 min was determined to be YTVNQXR. The repetitive yield of five Edman cycles (YTVNQ) was calculated to be 87.4% (Y_0 = 1872.3 pmol). From a database analysis, this sequence was identified as ^{694}YTVNQCR^{670} in the amino acid sequence of human CP. Taken together, these results evidently indicate that Cys-699 has a role in essential nucleophilic catalysis in the antioxidant reaction.

Identification of Peroxidase Activity of Ceruloplasmin. Now, it is very important to identify an enzyme or in vivo thiol-reducing equivalent capable of supporting the antioxidant activity of CP. The enzymatic thiol-regenerating systems such as thioredoxin and glutaredoxin did not provide CP with

the antioxidant activity (data not shown). Glutamine synthetase was also inactivated by a thiol-MCO system, in which DTT was replaced by GSH or *N*-acetyl-L-cysteine as a reducing agent (data not shown), but the inactivation was completely prevented by CP (Figure 3). To investigate GSH as a candidate for an in vivo hydrogen donor to CP, we compared the efficiency of GSH as an electron donor to CP with that to TPx. As shown in Figure 3b, GSH and *N*-acetyl-L-cysteine did not act as an effective electron donor to TPx. On the other hand, both GSH and *N*-acetyl-L-cysteine exhibited a similar ability to support the antioxidant activity of CP to that of DTT (Figure 3a). We also examined an inhibitory effect of GSH derivatives on the GSH-linked antioxidant activity of CP. Figure 3c shows a competitive inhibition of the GSH-linked antioxidant activity of CP by various GSH derivatives. These results suggest that CP may act as a GSH-linked antioxidant enzyme in vivo.

Taken together, the results previously mentioned suggest that CP may be an antioxidant enzyme exhibiting a GSH-linked peroxidase activity because CP has an essential Cys-699 for the nucleophilic catalysis in the antioxidant reaction supported by GSH. We examined CP for a peroxidase activity toward *tert*-butyl hydroperoxide and H_2O_2 by monitoring the decrease in the amount of an alkyl hydroperoxide in the presence of GSH. Within a reaction time of 30 min, the amount of hydroperoxide remaining decreased as a function of time (Figure 4a). Figure 4b shows the alkyl hydroperoxidase activity is linearly proportional to the concentration of CP. The peroxidase activity increased as the saturation function with respect to the amount of GSH (Figure 4c) and *tert*-butyl hydroperoxide (Figure 4d). The apparent V_{max} value in the presence of 5 mM *tert*-butyl hydroperoxide was calculated to be 156 ± 3 nmol min^{-1} mg^{-1} and in the presence of 5 mM H_2O_2 to be 49 ± 5 nmol min^{-1} mg^{-1} (data not shown). The apparent K_m value for GSH was 1.57 mM, for *tert*-butyl hydroperoxide was 0.63 mM, and for H_2O_2 was 0.87 mM. The V_{max} values are comparable to those of mammalian GSH peroxidases (i.e., several hundred nanomoles per minute per milligram) (*19*, *20*). The V_{max}/K_m value for *tert*-butyl hydroperoxide was calculated to be about 4 times higher than that for H_2O_2. These *tert*-butyl hydroperoxide- and GSH-dependent kinetic results clearly indicate that CP has the activity for GSH-dependent alkyl hydroperoxide peroxidase.

To compare the antioxidant activity of CP with that of other peroxidases, we measured the protection activities of CP and bovine erythrocyte GSH peroxidase against the inactivation of glutamine synthetase caused by the MCO system. Figure 5 shows that the protection activity of CP is comparable to that of bovine erythrocyte GSH peroxidase, which suggests that CP has an antioxidant activity comparable to that of bovine erythrocyte GSH peroxidase.

DISCUSSION

In summary, our results suggest that CP is a multifunctional enzyme exhibiting both activities of ferroxidase and GSH-dependent peroxidase. The redox-active sulfhydryl group of cysteine at position 699 in CP may act as a functional nucleophile to reduce hydroperoxide, which is supported by GSH as an electron donor. Our suggestion is supported by recent reports that CP has an antioxidant activity against the oxidative cleavage of plasmid DNA by a MCO system in the presence of DTT (*7*), and against the oxidation of protein in vivo (*21*).

As shown in Figure 6, the functional Cys-699 was located on the outside of CP. This domain for the peroxidase activity exists apart from the sites for the multi-copper complexes responsible for the ferroxidase activity. The classical GSH peroxidase, found in the cytoplasm of various eukaryotic cells, reduces H_2O_2 and organic hydroperoxides such as *tert*-butyl hydroperoxide. The GSH peroxidase is more active toward H_2O_2 (*22*). In contrast to classical GSH peroxidase, CP is more active toward *tert*-butyl hydroperoxide. Therefore, CP has an alkyl hydroperoxide peroxidase activity rather than the classical GSH peroxidase activity. The higher reactivity toward *tert*-butyl hydroperoxide suggested that the active site for peroxidase activity of CP might be designed to remove organic hydroperoxides such as lipid hydroperoxide in extracellular fluids.

A combination of two activities of CP in extracellular fluids containing Fe^{2+} and hydroperoxides would make CP a very efficient extracellular antioxidant enzyme due to removal of the two reactants (i.e., hydroperoxides + Fe^{2+} → hydroxyl radical + Fe^{3+}). Because of the low level of GSH in human plasma ranging from 5 to 20 μM (*23*, *24*), the physiological role of CP as a GSH peroxidase in human plasma remains debatable. If oxidative stress and oxidative stress-related pathology such as inflammation induce the secretion of GSH into a specific site of plasma, it is possible that more GSH is available at that site, and CP detoxifies the reactive oxygen species. This assumption may be supported by recent observations such as an oxidative stress-induced expression of hepatic γ-glutamylcysteine synthetase (*25*), which is a key enzyme for biosynthesis of GSH, an increase in the level of GSH efflux by oxidative stress (*26*), and hormonal regulation of glutathione efflux (*27*−*32*).

The data reported in this study suggest that further analysis of the function of CP as a GSH-linked alkyl hydroperoxide peroxidase may be of value in elucidating the mechanism of the clinical and pathological features of aceruloplasminemia.

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BI990444B