Thiol-linked peroxidase activity of human ceruloplasmin

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Received 29 June 1998

Abstract Human ceruloplasmin exhibited different antioxidant effects according to the electron donors in a metal-catalyzed oxidation system. Purified ceruloplasmin did not play a significant role in the protection of DNA strand breaks in the ascorbate/Fe^{3+}/O_2 system. However, when ascorbates were replaced with a thiol-reducing equivalent such as dithiothreitol, DNA strand breaks were significantly prevented by the same amount of ceruloplasmin. Ceruloplasmin did not catalyze the decomposition of H_2O_2 in the absence of reduced glutathione. On the contrary, ceruloplasmin showed a potent peroxidase ability to destroy H_2O_2 in the presence of reduced glutathione. In conclusion, the removal of H_2O_2 by human ceruloplasmin is not simply stoichiometric but thiol-dependent.

Key words: Ceruloplasmin; Metal-catalyzed oxidation system; Ascorbate; Dithiothreitol; Reduced glutathione; Peroxidase

1. Introduction

The ceruloplasmin is a normal, copper-containing plasma protein that has extracellular antioxidant properties in humans and mammals. Gene expression and oxidase activity levels of ceruloplasmin increase with turpentine-induced inflammation [1,2] and fetal development [3]. Ceruloplasmin is synthesized mainly in the liver in response to humoral factors such as interleukin-6 [4] and is secreted into the blood plasma. Recent studies demonstrate that ceruloplasmin genes are also predominantly expressed in extrahepatic cells such as lung and liver [5,6]. The in vivo physiological function has not yet been clarified, however, it has been proposed that ceruloplasmin may have a role in oxidation of Fe^{2+} to Fe^{3+} (ferroxidase) and promotion of lipoprotein oxidation under some circumstances [7–9]. The ferroxidase activity of ceruloplasmin prevents formation of metal-catalyzed hydroxyl radicals ($^{\bullet}OH$) from hydrogen peroxide (H_2O_2) and so inhibits iron and ferritin-dependent lipid peroxidation [10,11].

It has also been reported that ceruloplasmin reacts with superoxide radicals ($^{\bullet}O_2$) and H_2O_2. However, because both reactions appear to be stoichiometric, ceruloplasmin has practically no functions as superoxide dismutase and catalase. This stoichiometric reaction with physiological concentration of H_2O_2 does not lead to formation of $^{\bullet}OH$ and is not associated with inactivation of the protein [12,13]. Until now, a peroxidase function of human ceruloplasmin has not been suggested. In this investigation, in addition to the many already known functions of ceruloplasmin, we would like to propose that ceruloplasmin has a thiol-linked peroxidase function.

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2. Materials and methods

2.1. Materials

All reagents were of analytical grade. Sepharose CL-4B, DEAE-Sepharose CL-6B and pBR322 plasmid DNA were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): agarose, Chelex-100 resin, epichlorohydrin, trichloroacetic acid (TCA), dithiothreitol (DTT), glutathione (GSH), HEPES. 2-Chloroethylamine monohydrochloride was from Aldrich Chemical Co. (Milwaukee, WI, USA). Potassium thiocyanate (KSCN) and $FeSO_4 \cdot 7H_2O$ were supplied by Waco Chemical Co. (Japan). It should be noted that chloroethylamine is a potential carcinogen and presents an inhalation and skin contact hazard.

2.2. Preparation of human ceruloplasmin

Fresh serum was supplied by Taejon National Blood Bank and immediately used for purification. Human serum ceruloplasmin was purified by using chromatography on Sepharose derivatized with chloroethylamine and DEAE-Sepharose by the methods previously described [14]. Derivatized Sepharose was prepared according to the protocol described in [15]. The purity of the protein was estimated by native and sodium dodecyl sulfate polyacrylamide gel electrophoresis [16]. Protein concentrations were determined according to the methods of [17], using ovalbumin as the standard.

2.3. Analysis of DNA breaks induced by metal-catalyzed oxidation (MCO) system

A 20-μl reaction mixture containing 0.3 μg of pBR322 plasmid DNA, 3 μM $FeCl_3$, 10 mM reducing equivalents (DTT or ascorbate) and 20 μg ceruloplasmin in 50 mM HEPES (pH 7.0) was incubated for 60 min at 37°C for the analysis of DNA strand breaks by $^{\bullet}OH$. In metal-catalyzed Fenton reaction, 10 μM $FeSO_4$ and 500 μM H_2O_2 were used and the reaction mixture was incubated for 30 min at 37°C. These DNA samples were applied to 0.8% agarose gel to examine DNA strand cleavage. Electrophoresis was carried out in TAE (80 mM Tris, 8 mM acetate, 2 mM EDTA, pH 8.0). The DNA band on agarose gel was stained with ethidium bromide (0.5 μg/ml). The fractions of the three forms in each sample were estimated by densitometric analysis using a Bio-profile image analysis system (Vilber Lourmat, France).

2.4. Determination of peroxidase activity of ceruloplasmin in the presence of GSH

To determine the peroxidase activity of ceruloplasmin, the reaction was started by the addition of 500 μM H_2O_2 into 50 μl reaction mixture containing 500 μM GSH, 50 mM HEPES buffer, pH 7.0, and an appropriate amount of sample, and then incubated at 37°C. After 20 and 40 min, TCA solution (0.8 ml, 12.5%, w/v) was added to the reaction mixture to stop the reaction, followed by the addition of 200 μl of 10 mM $FeSO_4$ and 100 μl of 2.5 N KSCN to develop a purple color. The measurement of the remaining H_2O_2 was performed by monitoring the decrease of absorbance at 480 nm [18].

3. Results and discussion

It has been shown that human ceruloplasmin is more sensitive to plasmin-mediated proteolysis than rat ceruloplasmin [19]. Human ceruloplasmin (130 000 Da) was cleaved initially to a product with an apparent molecular weight of 116 000 Da and further proteolysis occurred during extraction. Therefore

PII: S0014-5793(98)00817-5

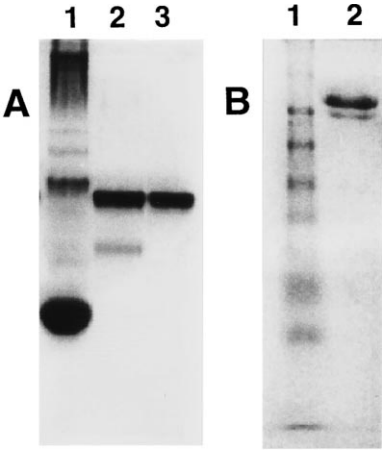

Fig. 1. Native (A) and sodium dodecyl sulfate (B) polyacrylamide gel electrophoresis analysis of ceruloplasmin at different stages of purification (7.5% and 10% acrylamide, respectively). A: Lane 1: sample from plasma; lane 2: sample from chloroethylamine-Sepharose chromatography; lane 3: sample from DEAE-Sepharose chromatography. B: Lane 1: sample from DEAE-Sepharose chromatography; lane 2: standard markers. The molecular weights are 36.3, 42.7, 55.3, 75.2, 85.3, 116.5 and 198 kDa from the bottom.

all the purification steps were performed using the buffer containing 20 mM ε-aminocaproic acid to minimize proteolytic cleavage. In this experiment, the purified protein has an A_{610nm}/A_{280nm} ratio of 0.063. The purity of ceruloplasmin was also identified by polyacrylamide gel electrophoresis. Although a proteolytic fragment with apparent molecular weight of 116 000 Da was produced ($<10\%$), further fragmentation did not occur (Fig. 1).

In the MCO system composed of Fe^{3+}, O_2 and reducing equivalents as an electron donor, oxidation of a reducing equivalent such as DTT produces H_2O_2 in a reaction catalyzed by iron ($2RSH+O_2+Fe^{3+} \rightarrow RSSR+H_2O_2+Fe^{2+}$). The H_2O_2 then reacts in a metal-catalyzed Fenton reaction to produce the ultimate toxic species, $^{\bullet}OH$ ($H_2O_2+Fe^{2+} \rightarrow Fe^{3+}+{}^{\bullet}OH+OH^-$). It is now well understood that the reducing equivalents/Fe^{3+}/O_2 system induces strand breaks in plasmid DNA and mammalian DNA as well as the inactivation of many enzymes [20,21]. To evaluate whether purified human ceruloplasmin is effective at protecting against $^{\bullet}OH$ mediated DNA strand breaks, the MCO system has been used as a convenient model. The extent of strand breaks was measured by the loss of supercoiled DNA coupled to an increase in the open circular form and linear form in agarose. Purified ceruloplasmin significantly prevented DNA strand breaks in the DTT/Fe^{3+}/O_2 system ($>95\%$). However, DNA strand breaks were not prevented as much in the ascorbate MCO system as in the DTT MCO system by the same amount of ceruloplasmin ($<25\%$). Where DTT is added to the ascorbate/Fe^{3+}/O_2 system, DNA strand breaks were diminished by ceruloplasmin (Fig. 2A). This protein inhibited DNA strand breaks to the extent of 65% in the 10 μM Fe^{2+}/500 μM H_2O_2 system. When DTT (0.5 mM) was added to the Fe^{2+}/H_2O_2 system, DNA strand breaks occurred severely. However, DNA strand breaks were significantly prevented by addition of ceruloplasmin (Fig. 2B). This probably means that the catalytic activity of human ceruloplasmin is closely related to thiol-reducing equivalents. We also examined ceruloplasmin for peroxidase activity by measuring the decrease of H_2O_2 in the presence of reduced GSH instead of DTT. When reduced GSH was absent in the reaction mixture, human ceruloplasmin hardly catalyzed decomposition of H_2O_2. Only a slight amount of H_2O_2 was removed by ceruloplasmin (<20 μM). However, the ceruloplasmin of the reaction mixture, to which reduced GSH was added, significantly removed H_2O_2 (Fig. 3A). In serum, few proteins showing an enzymatic mechanism to remove oxygen free radicals and their reactive oxygen derivatives have been identified [22,23]. However, catalase or selenium-linked glutathione peroxidase contributes little to the removal of H_2O_2 in extracellular fluids including blood plasma because of no or low activity [24]. Therefore, it may be that human ceruloplasmin, which is present in much higher concentration in plasma than the above enzymes, can be a potentially important enzymatic antioxidant in extracellular fluid.

It has been previously shown that chloride ions dramatically enhance the oxidase activity of human ceruloplasmin at neutral pH and strongly inhibited the oxidase activity at $pH<6$ [25]. In this investigation, we found that thiol-depend-

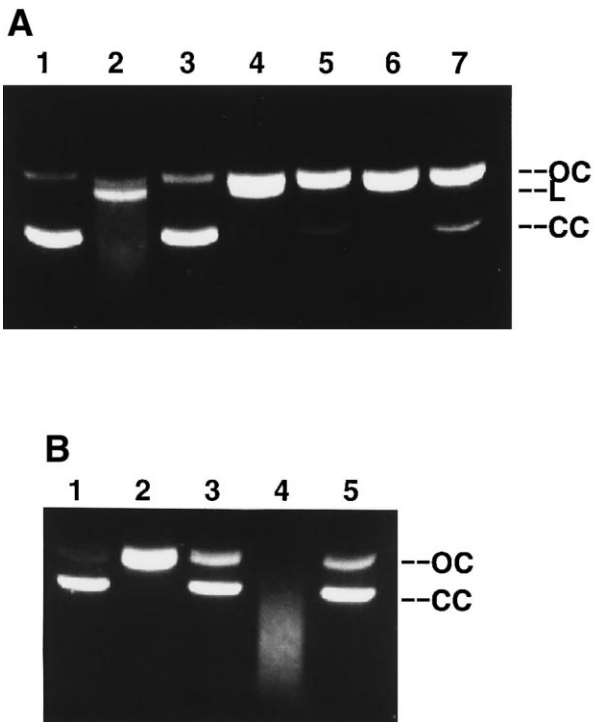

Fig. 2. A: Effect of human ceruloplasmin on DNA strand breaks induced by reducing equivalent/Fe^{3+}/O_2 system. Lane 1: 0.3 μg of pBR322 plasmid; lane 2: 0.3 μg of pBR322 plasmid in DTT/Fe^{3+}/O_2 system; lane 3: 0.3 μg of pBR322 plasmid in DTT/Fe^{3+}/O_2 system containing 20 μg of ceruloplasmin; lane 4: 0.3 μg of pBR322 plasmid in ascorbate/Fe^{3+}/O_2 system; lane 5: 0.3 μg of pBR322 plasmid in ascorbate/Fe^{3+}/O_2 system containing 20 μg of ceruloplasmin; lane 6: 0.3 μg of pBR322 plasmid in 5.0 mM ascorbate+5.0 mM DTT/Fe^{3+}/O_2 system; lane 7: 0.3 μg of pBR322 plasmid in 5.0 mM ascorbate+5.0 mM DTT/Fe^{3+}/O_2 system containing 20 μg of ceruloplasmin. B: Effect of human ceruloplasmin on DNA strand breaks induced by Fe^{2+}/H_2O_2 system. Lane 1: 0.3 μg of pBR322 plasmid; lane 2: 0.3 μg of pBR322 plasmid in 10 μM $FeSO_4$/500 μM H_2O_2 system; lane 3: 0.3 μg of pBR322 plasmid in 10 μM $FeSO_4$/500 μM H_2O_2 system containing 10 μg of ceruloplasmin; lane 4: 0.3 μg of pBR322 plasmid in 0.5 mM DTT/10 μM $FeSO_4$/500 μM H_2O_2 system; lane 5: 0.3 μg of pBR322 plasmid in 0.5 mM DTT/10 μM $FeSO_4$/500 μM H_2O_2 system containing 10 μg of ceruloplasmin. CC: closed circular; OC: open circular; L: linear form.

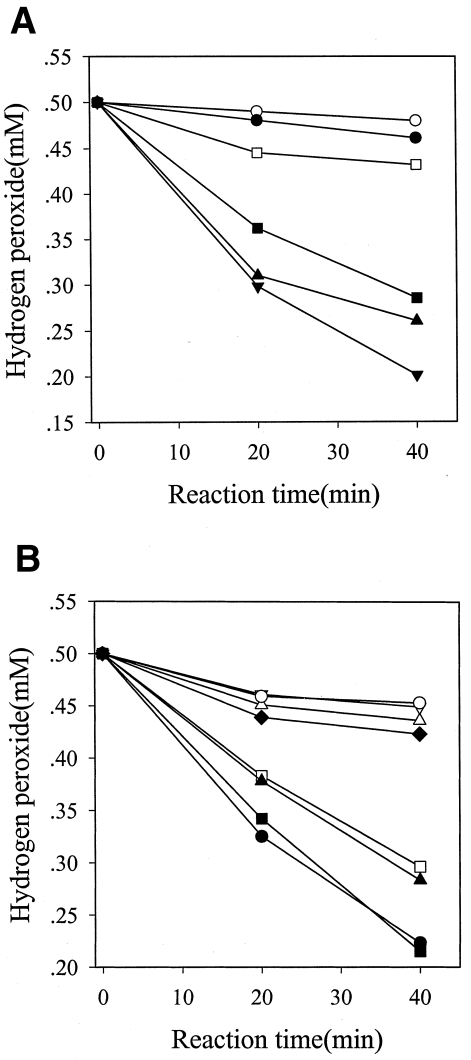

Fig. 3. A: H_2O_2-removal activity of human ceruloplasmin supported by glutathione. Peroxidase reaction was carried out in a 50-μl reaction mixture according to the methods described in Section 2. Open circle: 500 μM H_2O_2; closed circle: 50 μg ceruloplasmin+500 μM H_2O_2; open square: 500 μM H_2O_2+500 μM GSH; closed square: 50 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH; closed triangle: 75 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH; closed triangle down: 100 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH. Data represent the means of triplicate analyses. B: Changes of peroxidase activities of human ceruloplasmin by NaCl. Peroxidase reaction was carried out in a 50-μl reaction mixture containing NaCl. Open circle: 50 μg ceruloplasmin+500 μM H_2O_2+150 mM NaCl; open triangle down: 50 μg ceruloplasmin+500 μM H_2O_2+150 mM KCl; open triangle: 50 μg ceruloplasmin+500 μM H_2O_2+150 mM sodium acetate; closed diamond: 500 μM H_2O_2+500 μM GSH+150 mM NaCl; open square: 50 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH; closed triangle: 50 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH+150 mM sodium acetate; closed circle: 50 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH+150 mM KCl; closed square: 50 μg ceruloplasmin+500 μM H_2O_2+500 μM GSH+150 mM NaCl. Data represent the means of triplicate analyses.

ent peroxidase activity of ceruloplasmin was increased by chloride ions. The rate of peroxidase activity was 1.8-fold higher in the presence of 150 mM NaCl or KCl than in the absence of salt. However, sodium acetate lacking chloride ion did not affect the peroxidase activity of ceruloplasmin (Fig. 3B).

In conclusion, human ceruloplasmin inhibits the formation of $^{\bullet}OH$ by the enzymatic decomposition of H_2O_2 as well as by oxidation of Fe^{2+} to Fe^{3+} and the H_2O_2-removing function of ceruloplasmin is not simply stoichiometric but thiol-dependent.

Acknowledgements: The authors wish to acknowledge the financial support of the Ministry of Science and Technology (MOST) of the Republic of Korea.

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