Requirement of intact human ceruloplasmin for the glutathione-linked peroxidase activity

In Gyu Kim*, Seon Young Park

Department of Radiation Biology, Environment Radiation Research Group, Korea Atomic Energy Research Institute, P.O. Box 105, Yusong, Taejon 305-600, South Korea

Received 19 September 1998

Abstract Structural integrity may be needed for the glutathione-linked peroxidase activity of human ceruloplasmin. Intact human ceruloplasmin has a potent peroxidase property to decompose H_2O_2 in the presence of reduced glutathione. However, the fragment of approximately 116 000 Da produced by proteolytic degradation had less than one-third of the glutathione-linked peroxidase activity of intact ceruloplasmin. When further proteolysis occurred, glutathione-linked peroxidase activity of human ceruloplasmin disappeared. In contrast, ceruloplasmin (116 000 Da and $<$ 96 000 Da) fragmented by proteolysis significantly removed H_2O_2 irrespective of the presence of reduced glutathione. Although proteolytic fragmentation of ceruloplasmin occurs, the antioxidant activity of ceruloplasmin that prevents DNA strand breaks in a metal-catalyzed reaction system was significantly maintained.

Key words: Human ceruloplasmin; Glutathione-linked peroxidase; Proteolytic fragment; Antioxidant

1. Introduction

Ceruloplasmin is an acute phase reactant protein exhibiting moderate responses to various stresses. It is thought to possess extracellular antioxidant properties in humans and mammals [1–3]. This protein is a monomeric α_2-glycoprotein that contains more than 95% of the total circulating copper in the healthy human adult. Serum ceruloplasmin levels increase with γ-irradiation [4], infection [5], age, turpentine-induced inflammation [6,7] and pregnancy [8], where it may play an important role in physiological defense. The in vivo physiological function is not known with certainty. However, it has been proposed that ceruloplasmin may have a role in copper transport, conversion of Fe^{2+} to Fe^{3+} (ferroxidase) for subsequent uptake by transferrin and exhibition of pro-oxidant activity towards low density lipoproteins under some circumstances [9–11]. Recently H_2O_2-removing activity (peroxidase) of human ceruloplasmin in the presence of reduced glutathione has also been proposed [12].

Human ceruloplasmin has a molecular weight of approximately 132 000 Da. This protein is susceptible to proteolysis and fragmentation by proteolysis occurs during purification and storage [13,14]. Because of extraordinary susceptibility to proteolytic degradation and fragmentation, an understanding of the function of ceruloplasmin has long been hindered.

In this investigation, we describe that the glutathione-linked peroxidase function of human ceruloplasmin is extremely affected by proteolytic fragmentation and that the antioxidant activity of ceruloplasmin is significantly maintained in spite of high degradation by proteolysis, and suggest that glutathione-dependent peroxidase may occur in vivo.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade. Plasmin, ε-aminocaproic acid, epichlorohydrin, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), Chelex-100 resin, agarose, trichloroacetic acid (TCA), dithiothreitol (DTT), glutathione and HEPES were purchased from Sigma (St. Louis, MO, USA) and centrifugal filter membrane was from Millipore. DEAE-Sepharose CL-6B, Sepharose CL-4B and φ X174 plasmid DNA were from Pharmacia Fine Chemicals (Uppsala, Sweden). 2-Chloroethylamine monohydrochloride was from Aldrich (Milwaukee, WI, USA). Potassium thiocyanate (KSCN) and $FeSO_4 \cdot 7H_2O$ were obtained from Waco (Japan). Because 2-chloroethylamine is a potential carcinogen, caution must be taken when handling.

2.2. Preparation of human ceruloplasmin

Human ceruloplasmin was prepared from fresh serum (Taejon National Blood Bank) using a chromatographic step on Sepharose derivatized with 2-chloroethylamine and DEAE-Sepharose by recently introduced methods [15]. All the purification steps were performed at 4°C. One of the human samples obtained from healthy donors was immediately used for the purification of intact ceruloplasmin. The other human sample was used after incubation for 2 h at 30°C in order to obtain protease-mediated fragments of 116 000 Da. Derivatized Sepharose was prepared according to the protocol described in [16]. The purity of the protein was estimated by native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis [17]. Purified 116 000-Da fragments were incubated with plasmin (plasmin/ceruloplasmin, 1:50) at 37°C. Protein estimations were carried out according to the methods of [18], using ovalbumin as the standard.

2.3. Measurement of free sulfhydryl group by chemical modification

Purified human ceruloplasmin (200 μg) was incubated with 500 μM DTNB in 100 mM HEPES buffer (1 ml), pH 7.0 at 25°C. Free sulfhydryl residues of protein were determined by the measurement of the release of 2-nitro-5-mercaptobenzoic acid at 412 nm [19].

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 the 50 μl of reaction mixture containing 500 μM GSH, 50 mM HEPES buffer at pH 7.0, and an appropriate amount of sample, and then incubated at 37°C. After 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 [20].

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

Three hundred nanograms of φ X174 plasmid DNA was mixed with 3 μM $FeCl_3$, 10 mM DTT and 20 μg ceruloplasmin in 20 μl of 50 mM HEPES (pH 7.0) and the reaction mixtures were incubated for 20 min at 37°C for the analysis of DNA strand breaks by hydroxyl radicals (•OH). Separation of different conformations of φ X174 was performed by electrophoresis using 0.8% agarose gel. Electrophoresis

*Corresponding author. Fax: (82) (42) 861-9560.
E-mail: igkim@nanum.kaeri.re.kr

PII: S0014-5793(98)01253-8

was carried out in 80 mM Tris-acetate (8 mM) buffer, pH 8.0, containing 2 mM EDTA. The DNA band on agarose gel was stained with ethidium bromide (0.5 μg/ml). After gel electrophoresis, the fractions of the three different forms were estimated by densitometric analysis using a Bio-profile image analysis system (Vilber Lourmat, France).

3. Results

Intact human ceruloplasmin has a molecular weight of 132 000 Da. All the purification steps were performed using the phosphate buffer containing 20 mM ε-aminocaproic acid to inhibit the appearance of proteolytic fragments (64 000–96 000 Da). The absorbance ratio (A_{610nm}/A_{280nm}) of the purified protein is greater than 0.060. Although the proteolytic fragments with an apparent molecular weight of 116 000 Da were produced (< 5%), further fragmentation did not occur. The fragment with 116 000 Da was further degraded and was not detected in the plasmin treated ceruloplasmin (Fig. 1). In this investigation, it was demonstrated that only intact human ceruloplasmin might have complete glutathione-linked peroxidase activity. Conversely, the 116 000-Da ceruloplasmin produced by proteolytic degradation had less than one-third of the glutathione-dependent H_2O_2 removing activity of intact proteins. However, this protein removed H_2O_2 significantly in the absence of reduced glutathione (> 75 μM). When 116 000-Da ceruloplasmin was not detected as a result of further fragmentation by proteolysis (digested with plasmin for 4 h), glutathione-linked peroxidase activity of human ceruloplasmin disappeared completely. On the other hand the glutathione-independent H_2O_2 removing ability (> 250 μM) was significantly increased (Fig. 2).

We examined ceruloplasmin for antioxidant activity by measuring the extent of strand breaks to inhibit the DNA damage by a DTT metal-catalyzed oxidation reaction system. When the antioxidant activity of fragmented ceruloplasmin was compared with that of intact proteins, the 116 000-Da ceruloplasmin maintained a similar antioxidant activity that blocks DNA strand breaks (> 79%). In addition, regardless of the disappearance of a glutathione-linked peroxidase activity of human ceruloplasmin by further proteolysis, the conversion of supercoiled DNA to the open circular and linear form was significantly prevented by ceruloplasmin (> 50%) although the antioxidant activity of proteolyzed ceruloplasmin (< 116 000 Da) was different from that of intact proteins (Fig. 3). The reaction of sulfhydryl groups with DTNB in proteins results in the release of 2-nitro-5-mercaptobenzoic acid. This reaction of ceruloplasmin may be dependent on the extent of proteolysis. The release of 2-nitro-5-mercaptobenzoic acid (absorbance at 412 nm) was not observed in intact ceruloplasmin. On the other hand ceruloplasmin fragmented by proteolysis was reacted with DTNB and we observed the significant release of 2-nitro-5-mercaptobenzoic acid (Fig. 4).

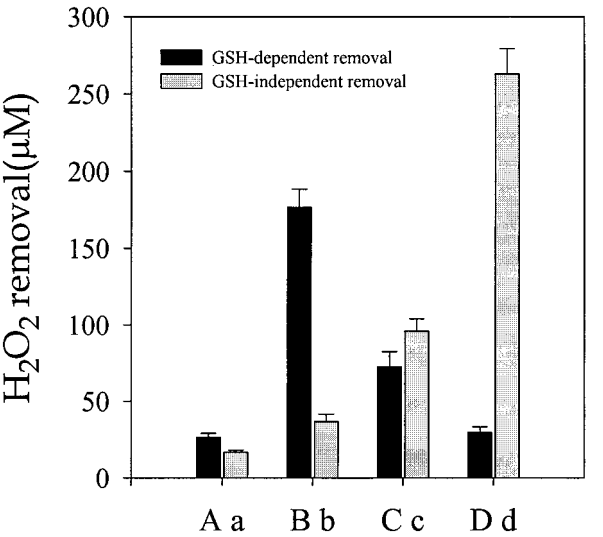

Fig. 2. Glutathione-dependent and -independent H_2O_2 removing activity of human ceruloplasmin. A: 500 μM glutathione+500 μM H_2O_2; a: 500 μM H_2O_2; B: 50 μg purified intact human ceruloplasmin (132 000 Da)+500 μM glutathione+500 μM H_2O_2; b: 50 μg human ceruloplasmin (132 000 Da)+500 μM H_2O_2; C: 50 μg ceruloplasmin produced by protease-mediated proteolysis (116 000 Da)+500 μM glutathione+500 μM H_2O_2; c: 50 μg human ceruloplasmin (116 000 Da)+500 μM H_2O_2; D: 50 μg ceruloplasmin digested with plasmin for 4 h (< 116 000 Da)+500 μM glutathione+500 μM H_2O_2; d: 50 μg human ceruloplasmin (< 116 000 Da)+500 μM H_2O_2. Data indicates the means of triplicate analyses.

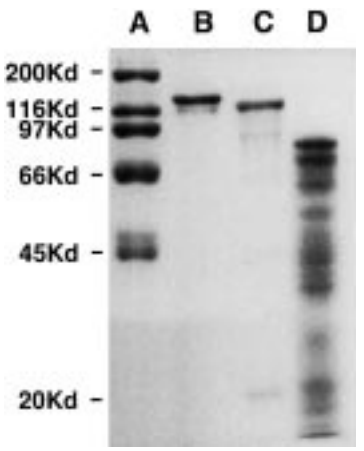

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of human ceruloplasmin (10%). Lane A: Standard markers; lane B: purified intact human ceruloplasmin (132 000 Da); lane C: 116 000-Da ceruloplasmin produced by proteolytic degradation; lane D: ceruloplasmin digested with plasmin for 4 h (< 116 000 Da).

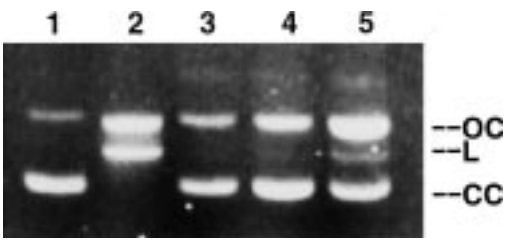

Fig. 3. Antioxidant effect of human ceruloplasmin on DNA strand breaks induced by DTT/Fe^{3+}/O_2 reaction system. Lane 1: 0.3 μg of ϕ X174 plasmid; lane 2: 0.3 μg of ϕ X174 plasmid in DTT/Fe^{3+}/O_2 system (reaction mixture); lane 3: reaction mixture+5 μg purified intact human ceruloplasmin (132 000 Da); lane 4: reaction mixture+5 μg ceruloplasmin produced by protease-mediated proteolysis (116 000 Da); lane 5: reaction mixture+5 μg ceruloplasmin digested with plasmin for 4 h (< 116 000 Da).

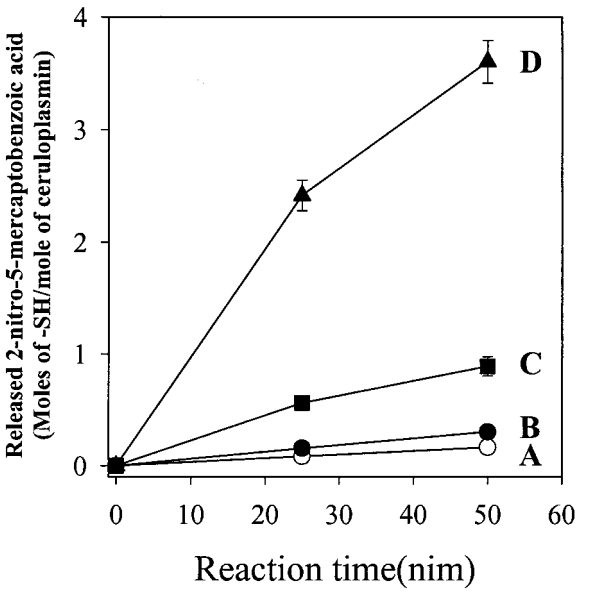

Fig. 4. Analysis of free sulfhydryl groups by measurement of the release of 2-nitro-5-mercaptobenzoic acid at 412 nm. Ceruloplasmin (200 μg) was incubated with 500 μM DTNB in 100 mM HEPES buffer (1 ml), pH 7.0 at 25°C. A: 500 μM DTNB in 100 mM HEPES buffer; B: intact ceruloplasmin+500 μM DTNB in 100 mM HEPES buffer; C: 116 000-Da ceruloplasmin produced by proteolytic degradation+500 μM DTNB in 100 mM HEPES buffer; D: ceruloplasmin digested with plasmin for 4 h (< 116 000 Da)+500 μM DTNB in 100 mM HEPES buffer. Data indicate the means of triplicate analyses.

4. Discussion

During purification and storage, the 132 000-Da ceruloplasmin was partly replaced by a product with an apparent molecular weight of 116 000 Da by proteolysis and further fragmentation occurred [21]. The protease responsible for ceruloplasmin degradation has not been identified, but a plasma metalloproteinase may be responsible for degradation of ceruloplasmin and incubation of intact ceruloplasmin with plasmin or trypsin yields fragments similar in size to those observed in highly degraded ceruloplasmin [14,22]. Human ceruloplasmin was more susceptible to proteolytic degradation than was rat ceruloplasmin [13]. This proteolytic cleavage with an apparent molecular weight of 116 000 Da did not induce profound structural alterations and change the copper contents of the molecule, and consequently the oxidase activities of ceruloplasmin were not changed [23]. In contrast to the above results, it has also been proposed previously that human ceruloplasmin may exhibit potent pro-oxidant activity in vitro, cause oxidative modification of low density lipoproteins and proteolytically modified ceruloplasmin (116 000 Da) has less than one-fourth of the pro-oxidant activity of intact protein. Therefore Fox et al. suggested that the seventh copper of human ceruloplasmin (loosely bound copper) or structural integrity is essential for the expression of ceruloplasmin pro-oxidant activity in vitro since the pro-oxidant action of ceruloplasmin was not consistent with previous reports of antioxidant activity [23,24]. In these studies, we would like to propose that the glutathione-linked peroxidase activity of ceruloplasmin may be strictly dependent on the structure of the molecules in vitro: specific proteolytic cleavage induces the complete loss of the glutathione-dependent peroxidase properties.

On the contrary, highly fragmented ceruloplasmin (< 116 000) removed a large amount of H_2O_2 in the absence of reduced glutathione. The reason may be that the functional groups responsible for the sacrificial antioxidant activity including free sulfhydryl residues were exposed to the solvent because of the profound conformation changes by proteolysis and these residues may play an important role in the sacrificial antioxidant function [25]. It is likely that copper ions are not related with H_2O_2 removing since copper that may be released was eliminated by using centrifugal filter membrane (Ultrafree-15, MW 5000). Its amino acid sequence contains a total of 14–15 cysteine residues per molecule and the number of the free sulfhydryl group as determined by radiolabeled iodoacetate was 3.0–4.0 [26,27]. The fact that only highly fragmented ceruloplasmin by proteolysis reacted with DTNB means that severe structural changes might occur by proteolysis. Coinciding with glutathione-independent H_2O_2 removing of fragmented ceruloplasmin (< 116 000 Da) was significant in the prevention of DNA strand breakage. This result means that in a metal-catalyzed Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$), the H_2O_2 or ${}^{\bullet}OH$ may be effectively removed by ceruloplasmin irrespective of the presence of reduced glutathione.

In conclusion, this study supports that structural integrity of a molecule is needed for the glutathione-linked peroxidase activity of ceruloplasmin in vitro: fragmentation of ceruloplasmin by proteolysis results in the loss of the glutathione-linked peroxidase function. On the other hand the antioxidant activity that blocks DNA damage was sustained. Since the main form of circulating human ceruloplasmin is the intact (about 90%) form, we can speculate that human cerulopalsmin may have a potent glutathione-linked peroxidase in vivo.

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

References

[1] Halliwell, B. and Gutteridge, J.M.C. (1990) Arch. Biochem. Biophys. 280, 1–8.
[2] Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1980) FEBS Lett. 112, 269–272.
[3] Krsek-Staples, J.A. and Webster, R.O. (1993) Free Radic. Biol. Med. 14, 115–125.
[4] Kim, I.G., Park, S.Y. and Kim, K.C. (1998) Biochem. Mol. Biol. Int. 45, 599–608.
[5] Cousins, R.J. (1985) Physiol. Rev. 65, 238–239.
[6] Semsei, I. (1991) Gerontology 37, 199–207.
[7] Giltin, J.D. (1988) J. Biol. Chem. 263, 6281–6287.
[8] Fleming, R.E. and Giltin, J.D. (1990) J. Biol. Chem. 265, 7701–7707.
[9] Osaki, S., Johnson, A. and Frieden, E. (1966) J. Biol. Chem. 241, 2746–2757.
[10] Halliwell, B. and Gutteridge, J.M.C. (1986) Arch. Biochem. Biophys. 221, 332–332.
[11] Swain, J.A., Darley-Usmar, V. and Gutteridge, J.M.C. (1994) FEBS Lett. 342, 49–52.
[12] Kim, I.G., Park, S.Y., Kim, K.C. and Yum, J.J. (1998) FEBS Lett. 431, 473–475.
[13] Ryan, T.P., Grover, T.A. and Aust, S.D. (1992) Arch. Biochem. Biophys. 293, 1–8.
[14] Ehrenwald, E. and Fox, P.L. (1994) Arch. Biochem. Biophys. 309, 392–395.

[15] Musci, G., Patti, M.C.B., Fagiolo, U. and Calabrese, L. (1993) J. Biol. Chem. 268, 13388–13395.
[16] Calabrese, L., Carbonaro, M. and Musci, G. (1988) J. Biol. Chem. 263, 6480–6483.
[17] Laemmli, U.K. (1970) Nature 227, 680–683.
[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[19] Lundbald, R.L. (1995) in: Techniques in Protein Modification, The Modification of Cysteine, pp. 63–89, CRC Press, Boca Raton, FL.
[20] Tappel, A.L. (1978) Methods Enzymol. 52, 506–513.
[21] Kingston, I.B., Kingston, B.L. and Putnam, F.W. (1977) Proc. Natl. Acad. Sci. USA 74, 5377–5381.
[22] Moshkov, K., Lakatos, S., Hajdu, J., Zavodsky, P. and Neifakh, S.A. (1979) Eur. J. Biochem. 94, 127–134.
[23] Ehrenwald, E., Chisolm, G.M. and Fox, P.L. (1994) J. Clin. Invest. 93, 1493–1501.
[24] Fox, P.L., Mukhopadhyay, C. and Ehrenwald, E. (1995) Life Sci. 56, 1749–1759.
[25] Halliwell, B. (1988) Biochem. Pharmacol. 37, 569–571.
[26] Lyden, L. (1984) in: Copper Proteins and Copper Enzymes, Vol. III: Ceruloplasmin (Lontie, R., Ed.) pp. 37–100, CRC Press, Boca Raton, FL.
[27] Ortel, T.L., Takahashi, N. and Putnam, F.W. (1984) Proc. Natl. Acad. Sci. USA 81, 4761–4765.