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Antioxidant Activity of Ceruloplasmin in Muscle Membrane and in Situ Lipid Peroxidation

Joseph Kanner,* Frida Sofer, Stella Harel, and Linda Doll

Ceruloplasmin acts as ferroxidase, catalyzing the oxidation of ferrous ions to the ferric state and reducing oxygen to water. Membranal lipid peroxidation initiated by an enzymic ADP–iron or by nonenzymic iron redox cycle systems was inhibited by ceruloplasmin. However, membranal lipid peroxidation initiated by H_2O_2-activated MetMb is not inhibited by ceruloplasmin. In the presence of iron ascorbate, ceruloplasmin inhibited lipid peroxidation initiated by H_2O_2-activated MetMb at a concentration one-tenth of that which inhibits iron ascorbate membrane lipid peroxidation. This synergistic effect seems to be derived from the possible hydroperoxidase activity of MetMb in the presence of ascorbic acid and ceruloplasmin. In situ minced turkey muscle lipid peroxidation was inhibited by ceruloplasmin, which indicates that most of this peroxidation is catalyzed by free iron ions.

Ceruloplasmin (EC 1.16.3.1) is the major copper-containing protein of extracellular fluids. It has a molecular weight of approximately 134 000 with six or seven copper ions per molecule. Three biological functions are known for ceruloplasmin: (i) copper storage and supply within cells for incorporation into other copper proteins such as superoxide dismutase (SOD) or cytochrome oxidase (Marceau and Aspin, 1973a,b); (ii) maintenance of copper homeostasis in the tissues; (iii) in vivo ferroxidase enzyme, first proposed by Osaki et al. (1966). The enzyme catalyzed the oxidation of ferrous ions in the presence of oxygen to ferric ions and water by eq 1 and 2.

$$E\text{–}Cu^{2+} + 4Fe^{2+} \rightarrow E\text{–}Cu^{+} + 4Fe^{3+} \quad (1)$$

$$E\text{–}Cu^{+} + O_2 \xrightarrow{+4H^+} E\text{–}Cu^{2+} + 2H_2O \quad (2)$$

Human blood serum has been known for several years to be a powerful lipid inhibitor (Barber, 1961; Vidlakova et al., 1972). This was shown later to be dependent on ceruloplasmin activity.

The aim of this study was to identify the antioxidant effect of ceruloplasmin in three different radical-generating systems and in situ minced muscles of turkey.

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MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis), ascorbic acid, and trichloroacetic acid were purchased from Merck (Darmstadt, FGR). Myoglobin type I from equine skeletal muscle, potassium chloride, L-histidine-free base, thiobarbituric acid (TBA), bovine serum albumin (BSA), and β-nicotinamide adenine dinucleotide disodium salt reduced from type X (NADPH), and ceruloplasmin type VII, bovine (CP), were obtained from Sigma Chemical Co. (St. Louis, MO). Ferric chloride was purchased from BDH Chemicals Ltd. (Poole, England), and adenosine 5′-diphosphate salt (ADP), from Boehringer-Mannheim GmbH (Mannheim, FGR).

Methods. Isolation of the microsomal fraction from fresh turkey muscle tissue was done by a procedure described previously (Apgar and Hultin, 1982; Kanner and Harel, 1985). The enzymatic microsomal model system contained KCl (0.12 M), NADPH (0.4 mM), ADP (0.4 mM), $FeCl_3$ (0.01 mM), histidine (5 mM), and microsomes (0.5 mg of protein/mL at pH 7.3). Microsomal nonenzymic lipid peroxidation initiated by the iron redox cycle system contained ascorbic acid (0.2 mM), $FeCl_3$ (0.01 mM), acetate buffer (50 mM at pH 7.0), and microsomes (1.0 mg/mL of protein). Microsomal nonenzymic lipid peroxidation initiated by activated metmyoglobin contained MetMb (0.03 mM) and H_2O_2 (0.03 mM), preincubated for 3 min, microsomes (1 mg of protein/mL), and acetate buffer (50

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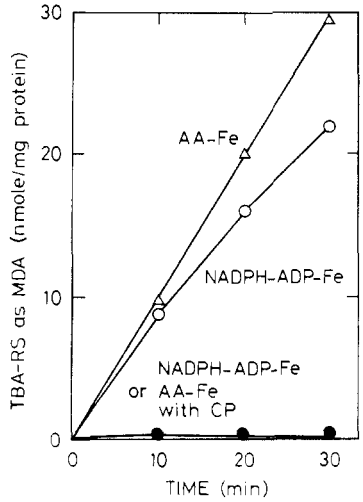

Figure 1. Inhibition of enzymic and nonenzymic membrane lipid peroxidation by ceruloplasmin (CP): ○, enzymic system [membranes (0.5 mg of protein/mL), NADPH (0.4 mM), ADP (0.4 mM), $FeCl_3$ (0.01 mM at pH 7.3)]; △, nonenzymic system [membranes (1 mg of protein/mL), ascorbic acid (0.2 mM), $FeCl_3$ (0.01 mM), at pH 7.0]; ●, enzymic and nonenzymic systems in the presence of 10 units of ceruloplasmin.

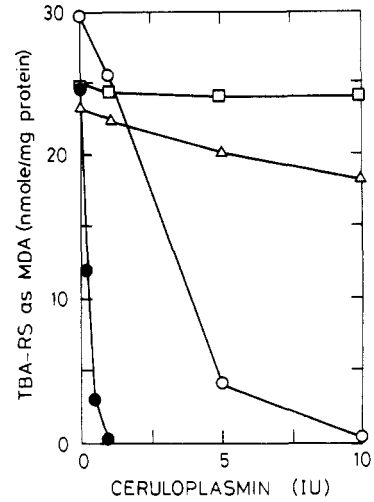

Figure 2. Effect of ceruloplasmin (CP) concentration on muscle membrane lipid peroxidation initiated by H_2O_2-activated MetMb and iron ascorbate. The concentration of the membranes in all experiments was 1 mg of protein/mL. Key: ○, ascorbic acid (0.2 mM), $FeCl_3$ (0.01 mM); △, MetMb and H_2O_2 (30 μM each); ●, MetMb and H_2O_2 plus ascorbic acid and $FeCl_3$; □, MetMb and H_2O_2 plus ascorbic acid and $FeCl_3$ in the presence of autoclaved ceruloplasmin.

mM at pH 7.0). All model systems were incubated in air in a shaking water bath at 37 °C.

Fresh or frozen (−20 °C for 7 days) turkey dark muscle (after thawing) was ground in a Moullinex food processor with a plastic work bowl and stainless steel blades for 60 s. One part of the sample was mixed with ceruloplasmin (150 U/g of tissue). The samples were stored at 4 °C and tested periodically for lipid peroxidation. Thiobarbituric acid reactive substances were determined in model systems by a procedure of Bidlack et al. (1973) and in muscle tissues by a method developed by Witte et al. (1970) and calculated as malonodialdehyde (MDA) using an extinction coefficient of $\epsilon_{532} = 1.55 \times 10^5$ M m^{-1} (Buege and Aust, 1978).

The results are means of triplicates of one microsomal preparation; in the figures, each bar (I) denotes the standard deviation.

RESULTS AND DISCUSSION

Membranal lipid peroxidation initiated by an enzymic ADP–Fe or by nonenzymic iron redox cycle systems was inhibited by ceruloplasmin (Figure 1). Enzymic microsomal NADPH and ADP–Fe-dependent lipid peroxidation were found in our model system to derive from a hydroxyl radical site-specific attack or by ferryl ions uninhibitable by hydroxyl radicals scavengers (Hazan, 1986). Similarly, it was found that membranal lipid peroxidation stimulated by iron redox cycle seems to be derived from hydroxyl radical site-specific attack or from ferryl ions uninhibitable by hydroxyl radicals scavengers (Kanner and Harel, 1985). It seems that in both systems the stimulation of lipid peroxidation was also derived from the catalytic reduction of hydroperoxides to free radicals by ferrous ions generated by NADPH cytochrome 450 reductase and ascorbic acid. Both reactions are dependent on the presence of ferrous ions (eq 3 and 4).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^\bullet \quad (3)$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + HO^- + RO^\bullet \quad (4)$$

Ceruloplasmin, which acts as ferroxidase, catalyzed the oxidation of ferrous to ferric ions without the production of oxygen free radicals. This reaction could explain the inhibitory effect of ceruloplasmin toward membranal lipid peroxidation in both model systems. Recently, it was found that ceruloplasmin could inhibit enzymic liver microsomal lipid peroxidation (Yamashoji and Kajimoto, 1983), the oxidation of liposomes (Nakano et al., 1984; Gutteridge, 1985a), and the Fenton reaction which decomposes DNA (Gutteridge et al., 1980) and deoxyribose (Gutteridge, 1985b). The inhibition of membranal lipid peroxidation by ceruloplasmin in the system containing ascorbic acid and ferrous ion seems to be derived primarily from the capability of the protein to chelate ferrous ions into a complex of enzyme–Fe^{2+}. Such a complex, determined by electron spin resonance, was reported by Cannistraro et al. (1981).

The physical state of membrane lipids seems to affect both the penetration of exogenously added enzymes such as SOD and catalase into the membrane and their effectiveness in preventing lipid peroxidation in our iron ascorbate model system (Kanner et al., 1986). For the same reason, exogenously added ceruloplasmin is not expected to penetrate into the membrane; however, because of its chelating properties, the enzyme seems to remove iron from these binding sites and to oxidize it to ferric ion in the free solution.

Membrane lipid peroxidation initiated by H_2O_2-activated MetMb is not inhibited by ceruloplasmin (Figure 2). However, ceruloplasmin, in the presence of iron ascorbate, inhibits lipid peroxidation initiated by H_2O_2-activated MetMb at a concentration one-tenth of that which inhibits iron ascorbate membranal lipid peroxidation alone. Ceruloplasmin in this system works to decrease the prooxidative effect of ascorbic acid (e.g., ferrous ions and oxygen free radicals) and enhances its antioxidative effect. It is well-known that MetMb can interact with hydroperoxides, and this reaction seems to activate MetMb to a possible oxyferryl ion. Ascorbic acid in this system reduces the oxyferryl ion and thereby prevents the oxidation of an unsaturated fatty acid. It is possible to describe this reaction by eq 5 and 6, where AH_2 = ascorbic acid and A = dehydroascorbic acid.

$$(Fe^{III} + ROOH \rightarrow (Fe^{IV}{=}O + RO^- \quad (5)$$

$$(Fe^{IV}{=}O + AH_2 \rightarrow (Fe^{III} + A + H_2O \quad (6)$$

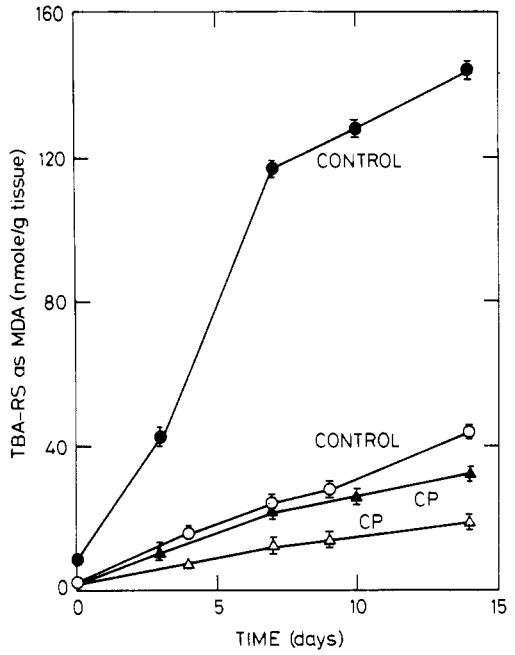

Figure 3. Effect of ceruloplasmin (150 U/g) on in situ lipid peroxidation in minced turkey muscle tissues: ●, control, minced muscle (run 1); ▲, minced muscle (run 1) in the presence of ceruloplasmin; ○, control, minced muscle (run 2); △, minced muscle (run 2) in the presence of ceruloplasmin.

Reactions 5 and 6 resemble the activity of a hydroperoxidase like glutathione peroxidase, which decomposes hydroperoxides to nonradical compounds. The decomposition of hydroperoxides by heme proteins in the presence of ascorbic acid has been documented (O'Brien, 1969).

In situ minced turkey muscle lipid peroxidation was inhibited by ceruloplasmin (Figure 3). This result was obtained with two different sources of turkey muscles, one of which tends to oxidize rapidly, the other more stable. In both samples, ceruloplasmin prevents between 76% and 56% of the lipid peroxidation process. As a great part of the oxidation is prevented by low concentration of EDTA (Kanner et al., 1988) and ceruloplasmin, we conclude that "free" iron ions are the major prooxidant factor in minced turkey muscles. Our results open a new way for the utilization of ceruloplasmin, a natural byproduct of animal blood, as a natural antioxidant for muscle foods.

Registry No. Fe, 7439-89-6; ceruloplasmin, 9031-37-2; ascorbate, 50-81-7.

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Limited Proteolysis of Ovalbumin by Pepsin

Naofumi Kitabatake,* Kaoru Indo, and Etsushiro Doi

The hydrolysis of hen egg white ovalbumin by porcine pepsin was examined at various pHs. Changes in molecular size of the ovalbumin occurring during hydrolysis were investigated by SDS–polyacrylamide gel electrophoresis. A strictly limited hydrolysis was observed at pH 4. Only a single peptide bond in the original ovalbumin (MW 45 000) was cleft, and a peptide with a molecular weight of about 3000 was released. Both the released peptide and the residual protein (MW 42 000) were resistant to further hydrolysis by prolonged incubation or addition of more pepsin. The cleavage site was between His-22 and Ala-23 of the ovalbumin amino acid sequence.

Enzymatic modification is a useful method to improve and upgrade the functional and nutritional properties of food protein; in particular, proteolytic hydrolysis is widely applied for such modification (Yamamoto, 1975; Kay, 1982; Adler-Nissen, 1986). Kitabatake and Doi (1985) prepared a transparent gel from pepsin-treated ovalbumin and egg white by heating although nonproteolyzed proteins gave turbid gels. However, it is known that an extensive hydrolysis of protein gives deteriorative effects: formation of a bitter peptide or loss of a functional property. Proteolytic activities are required to be controlled in some cases. For this purpose limited proteolysis is available.

The transparent gel obtained from pepsin-treated ovalbumin, described above, gave no bitterness and a

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