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A kinetic study of the coupled iron–ceruloplasmin catalyzed oxidation of ascorbate in the presence of albumin

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Ascorbate is catalytically oxidized by a coupled iron–ceruloplasmin system, the iron ions functioning as a *red/ox* cycling intermediate between ceruloplasmin and ascorbate. Serum albumin, an iron binding compound, was found to stimulate the ascorbate oxidation rate. It is proposed that ferrous ions react more rapidly with ceruloplasmin when they are bound to albumin. A K_m value of 39 μM was estimated for Fe^{2+}–albumin. Citrate and urate inhibit the iron–ceruloplasmin-dependent ascorbate oxidation by chelating ferric ions. In the presence of albumin only citrate reduced the oxidation rate, the observation suggesting the following order of iron binding ability: citrate > albumin > urate. Physiological aspects of the results have been discussed.

Keywords: ascorbate, albumin, ceruloplasmin, iron ions, citrate

Introduction

Ceruloplasmin is a blue-colored α_2-globulin containing six or seven copper ions per molecule (Frieden & Hsieh 1974, Rydén & Björk 1976). The protein is considered to function as a ferrous ion oxidase in blood plasma (Osaki *et al.* 1966, Frieden & Hsieh 1974, Carver *et al.* 1982). Ceruloplasmin catalyzes the oxidation of ascorbate in the presence of iron ions, which act as a *red/ox* cycling intermediate between the enzyme and ascorbate (Curzon 1961, McDermott *et al.* 1968). During the reaction ceruloplasmin-bound cupric ions are reduced to the cuprous state. The enzyme is reoxidized by molecular oxygen, which is reduced to water in the process, accepting four electrons from ceruloplasmin (CP):

$$CP\text{–}Cu^{2+} + Fe^{2+} \rightleftharpoons \text{complex} \rightarrow CP\text{–}Cu^{+} + Fe^{3+}$$

$$Fe^{3+} + \text{ascorbate} \rightarrow Fe^{2+} + \text{asc.radical}$$

$$CP\text{–}Cu_4^{+} + O_2 + 4H^{+} \rightarrow CP\text{–}Cu_4^{2+} + 2H_2O$$

Iron ions complex with serum albumin in a non-specific manner (Anghileri 1967, Van der Heul *et al.* 1972, Smit *et al.* 1981, Løvstad 1993). The purpose of the present investigation was to establish how albumin affected the iron-dependent ceruloplasmin catalyzed oxidation of ascorbate.

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Materials and methods

Bovine serum albumin (A 7906; charcoal treated, extensively dialyzed and essentially free of low molecular weight substances), untreated bovine serum albumin (A 4503), human ceruloplasmin (ferroxidase; EC 1.16.3.1), human apotransferrin, ascorbic acid, uric acid and Tris buffer were purchased from Sigma (St Louis, MO); $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ from Merck (Darmstadt, Germany); and sodium citrate from (British Drug Houses, Poole, UK).

Protein preparations were passed through a Chelex-100 (Bio-Rad) column in order to remove contaminating metal ions. The ceruloplasmin concentration was determined from the 610 nm absorption band ($\varepsilon = 9.45\ mM^{-1}\ cm^{-1}$, Carver *et al.* 1982); the concentration of bovine albumin ($\varepsilon = 43.6\ mM^{-1}\ cm^{-1}$, Sober & Harte 1968) and apotransferrin ($\varepsilon = 91.2\ mM^{-1}\ cm^{-1}$, Carver *et al.* 1982) from the 280 nm absorption band. Stock solutions containing ferrous ions were prepared in water immediately prior to use. All aqueous solutions were made in deionized, glass-distilled water.

The rate of the iron–ceruloplasmin catalyzed oxidation of ascorbate was measured by monitoring the oxygen consumption in the reaction mixture, using a Clark-type

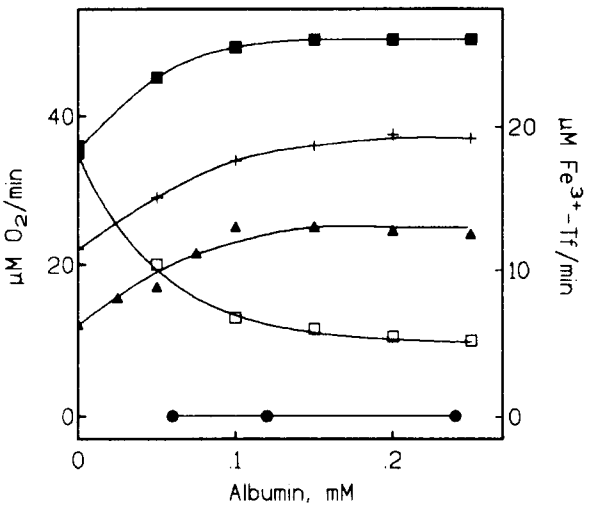

Figure 1. Effect of albumin concentration on the coupled iron–ceruloplasmin catalyzed oxidation of 1 mM ascorbate in 20 mM Tris buffer, pH 7.4 (T=30°C). Charcoal-treated, dialyzed albumin and: ■, 0.7 μM ceruloplasmin and 20 μM iron ions; ▲, 0.2 μM ceruloplasmin and 50 μM iron ions; ●, 0.7 μM ceruloplasmin, 20 μM iron ions and 72 μM apotransferrin. □, Untreated albumin, 0.7 μM ceruloplasmin and 20 μM iron ions. +, The effect of charcoal-treated, dialyzed albumin on the rate of Fe^{2+} oxidation by ceruloplasmin, using apotransferrin as a ferric ion trapping agent. The reaction mixture contained 0.35 μM ceruloplasmin, 0.12 mM ferrous ions and 45 μM apotransferrin in 0.2 M sodium acetate buffer, pH 6.0 (T=30°C).

electrode connected to a MSE-Spectroplus instrument. Spectrophotometric recordings of the ferrous ion oxidizing ability of ceruloplasmin were performed with a Pye-Unicam 8800 instrument, using a method described by Johnson *et al.* (1967).

Results and discussion

Figure 1 shows that the rate of the coupled iron–ceruloplasmin-dependent oxidation of ascorbate increased with increasing concentration of dialyzed, charcoal- and Chelex-treated albumin at pH 7.4. Eventually a steady state level was reached. Untreated albumin markedly reduced the oxidation rate (Figure 1). These preparations are usually contaminated with citrate (Witwicki *et al.* 1983), which is an effective inhibitor of iron-dependent ceruloplasmin catalyzed reactions, as demonstrated by Osaki *et al.* (1964), and also shown in Figure 5. In the following experiments only the charcoal-treated, dialyzed preparation was used. Apotransferrin, which complexes two ferric ions per molecule ($\log K_1 = 22.7$, $\log K_2 = 22.1$; Martin *et al.* 1987) completely prevented the oxidation of ascorbate (Figure 1).

Using apotransferrin as a ferric ion trapping agent and 0.12 mM ferrous ions as substrate, the rate of ferrous ion oxidation by ceruloplasmin was measured spectrophotometrically at 460 nm ($\varepsilon = 2.5\ mM^{-1}\ min^{-1}$, Johnson *et al.* 1967). A stimulatory effect of albumin was also observed in this experiment (Figure 1). It is suggested that ferrous ions are oxidized more rapidly by ceruloplasmin when they are bound to albumin.

Figure 2 shows the rate of ascorbate oxidation at different enzyme concentrations in the absence and presence of excess albumin. Sub-physiological amounts of ceruloplasmin were used. A linear relationship between rate and enzyme concentration was obtained in both cases, the activity increasing more rapidly in the albumin containing reaction system.

The rate of the coupled iron–enzyme catalyzed oxidation of ascorbate was independent of ascorbate concentration in the presence of albumin (Figure 3), suggesting that the rate determining step in the reaction system is the oxidation of Fe^{2+}–albumin to the ferric state. In the absence of albumin the oxidation rate was constant at ascorbate concentrations higher than 0.5 mM (Figure 3).

A typical enzyme saturation curve was obtained when the rate of albumin stimulated ascorbate oxidation was plotted against Fe^{2+}–albumin concentration, giving rise to a straight line in a double reciprocal plot (Figure 4). A K_m value of 39 ± 4 μM (SE) and a catalytic constant of $890 \pm 41\ min^{-1}$ (SE) were calculated for Fe^{2+}–albumin by means of a computer program published by Cleland (1967). A biphasic curve was obtained when free ferrous ions acted as substrate; the kinetics characterized by two K_m constants: 0.6 and 50 μM (Osaki 1966).

Citrate and urate are iron binding compounds, proposed to complex ferric ions in plasma of iron overloaded patients, when the transferrin molecules are saturated with iron (Davies *et al.* 1986, Grootveld *et al.* 1989). They both inhibit the coupled iron–ceruloplasmin catalyzed oxidation of

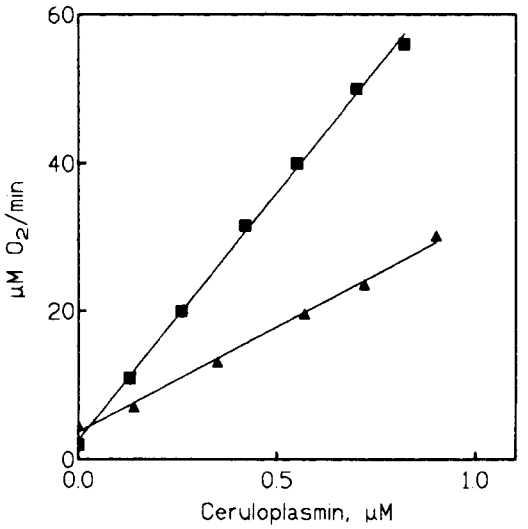

Figure 2. Effect of ceruloplasmin concentration on the coupled iron–ceruloplasmin catalyzed oxidation of ascorbate in the absence (▲) and presence (■) of 0.2 mM albumin (charcoal-treated, dialyzed). The reaction mixture contained ceruloplasmin (0.13–0.9 μM), 20 μM iron ions and 1 mM ascorbate in 20 mM Tris buffer, pH 7.4 (T=30°C).

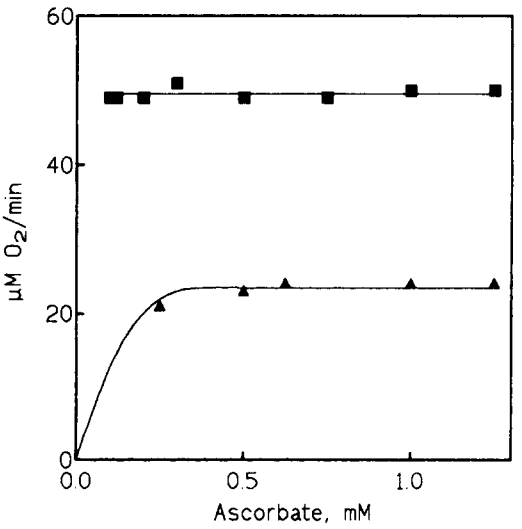

Figure 3. Effect of ascorbate concentration on the coupled iron–ceruloplasmin catalyzed oxidation of ascorbate in the absence (▲) and presence (■) of 0.2 mM albumin (charcoal-treated, dialyzed). The reaction mixture contained 0.7 μM ceruloplasmin, 20 μM iron ions and ascorbate (0.1–1.25 mM) in 20 mM Tris buffer, pH 7.4 (T=30°C).

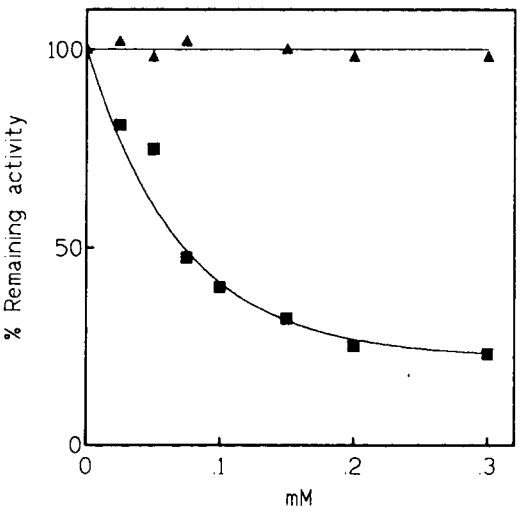

Figure 5. Plot of percentage ascorbic acid oxidase activity of control against concentration of citrate (■) and urate (▲) in the presence of 0.5 mM albumin (charcoal-treated, dialyzed). The reaction mixture contained 0.7 μM ceruloplasmin, 20 μM iron ions and 1 mM ascorbate in 20 mM Tris buffer, pH 7.4 (T=30°C).

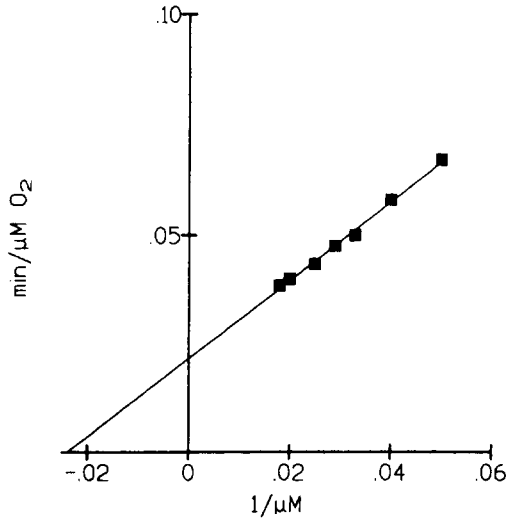

Figure 4. Reciprocal rate of Fe^{2+}–albumin oxidation plotted against the reciprocal Fe^{2+}–albumin concentration. The reaction mixture contained 0.2 μM ceruloplasmin, iron ions (20–55 μM), 0.2 mM albumin and 1 mM ascorbate in 20 mM Tris buffer, pH 7.4 (T=30°C).

ascorbate in the absence of albumin. According to Witwicki *et al.* (1983) citrate does not exert a significant inhibitory effect on the ferroxidase activity of ceruloplasmin at pH 7.4. Thus the observed inhibition of the *red/ox* cycling system is probably due to a slow reduction of Fe^{3+}–citrate by ascorbate. In the presence of 0.5 mM albumin (approximately physiological concentration), however, only citrate inhibited the reaction (Figure 5). The lack of effect of urate suggests that albumin is a better iron chelating agent than urate, which probably plays a minor role as a chelator of non-transferrin-bound (NTB) iron in iron overloaded patients. At normal physiological concentrations (about 0.1 mM) citrate inhibited the ascorbate oxidation about 60% (Figure 5), suggesting that albumin competes effectively with citrate for iron, in accordance with a previous chromatographic study (Løvstad 1993). A report on NTB iron in plasma from hemochromatosis patients concluded that 50–70% was bound to citrate, the rest probably to proteins (Grootveld *et al.* 1989). Albumin is proposed as a likely candidate (Gutteridge *et al.* 1985). Interestingly, Minetti *et al.* (1992), studying NTB iron stimulated oxidation of ascorbate in iron loaded plasma, concluded that the effect was not caused by iron–citrate, but by other, unidentified iron complexes.

Iron overloaded individuals are characterized by a decreased level of ascorbate in plasma (Lynch *et al.* 1967, Jacobs *et al.* 1971). The possibility exists that iron–albumin complexes act as a *red/ox* cycling intermediate between ascorbate and ceruloplasmin *in vivo*, contributing to the increased consumption of ascorbate observed in these individuals.

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