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A study on ascorbate inhibition of ceruloplasmin ferroxidase activity

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The ferroxidase activity of ceruloplasmin is often determined according to the method of Johnson *et al.* (1967), using apotransferrin for trapping ferric ions generated by the enzyme; spectrophotometrically monitoring the Fe^{3+}–transferrin formation at pH 6.0. Reports have shown that ascorbate inhibits this reaction, and it is hypothesized that the effect could be of physiological significance in individuals with a high ascorbate to ceruloplasmin ratio in plasma (e.g. premature babies).

The present study shows that the inhibitory effect of ascorbate rapidly decreases with increasing pH. At pH 7.4 no significant effect was observed, the result suggesting that ascorbate is not a physiological inhibitor of ceruloplasmin. Furthermore, experiments demonstrate that at acidic pH the inhibitory effect of ascorbate on the rate of Fe^{3+}–transferrin formation is not primarily due to an interaction with ceruloplasmin, but to a reduction of enzymically generated ferric ions before they are bound to apotransferrin.

Keywords: ascorbate, ceruloplasmin, conalbumin, iron ions, transferrin

Introduction

Ceruloplasmin is a copper containing plasma protein, which catalytically oxidizes ferrous ions and certain aromatic diamines and diphenols (for a review, see Frieden & Hsieh 1976; Gutteridge & Stocks 1981). The protein is considered to function as a ferroxidase *in vivo* (Osaki *et al.* 1966, Carver *et al.* 1982, Harris *et al.* 1995). Ceruloplasmin is also proposed to be an important extracellular antioxidant, its effect mainly ascribed to the oxidation of ferrous ions, preventing them from stimulating lipid peroxidation and Fenton chemistry (Gutteridge 1978, 1985, Gutteridge *et al.* 1980, Gutteridge & Stocks 1981). During the catalytic action enzyme cupric ions are reduced by substrate. Molecular oxygen reoxidizes the cuprous ions formed, and is reduced to water by accepting four electrons from the enzyme (Osaki 1966, Carver *et al.* 1982). Formation of reactive oxygen intermediates is thus prevented.

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Ascorbate is reported to inhibit the ferroxidase activity of ceruloplasmin (Gutteridge 1991, Powers *et al.* 1995). The degree of inhibition was dependent on the ratio of ascorbate to ceruloplasmin (Gutteridge 1991). In plasma of premature babies high concentrations of ascorbate, and low concentrations of ceruloplasmin, were measured (Powers *et al.* 1995). At these high ascorbate to ceruloplasmin ratios a substantial inhibition of the ferroxidase activity was observed (Powers *et al.* 1995). It was hypothesized that this effect might compromise antioxidant mechanisms and exacerbate oxidant damage *in vivo*.

In the ascorbate inhibition studies (Gutteridge 1991, Powers *et al.* 1995) the ceruloplasmin ferroxidase activity was measured according to the method of Johnson *et al.* (1967) at pH 6.0 In the present communication, the possible role of ferroxidase inhibition by ascorbate under physiological conditions was evaluated by studying the pH dependence. The mechanism of ascorbate inhibition is discussed.

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Materials and methods

Materials

Human ceruloplasmin (E.C. 1.16.3.1), human apotransferrin, apoconalbumin, sodium ascorbate, promazine and Tris buffer were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Chelex 100 from BioRad (Richmond, CA, USA); $Fe(NH_4)_2(SO_4)_2.6H_2O$ and sodium acetate from E. Merck AG (Darmstadt, Germany). Ceruloplasmin was treated with Chelex 100 in order to remove contaminating copper ions associated with the enzyme preparation. The concentration of ceruloplasmin was calculated from the 610 nm absorption band ($\epsilon = 9.45$ mM^{-1} cm^{-1}) (Carver *et al.* 1982).

Methods

The ability of ceruloplasmin to oxidize ferrous ions was determined by the method of Johnson *et al.* (1967), using apotransferrin (or apoconalbumin (Gutteridge 1991)) for trapping the ferric ions generated by the enzyme. The formation of Fe^{3+}–transferrin (Fe^{3+}–conalbumin) was followed spectrophotometrically at 460 nm.

In the absence of apotransferrin, and presence of ascorbate, ferric ions are reduced by ascorbate; the iron ions thus acting as a *red/ox*-cycling intermediate between enzyme and ascorbate. In this system the ferroxidase activity was determined by monitoring the oxygen uptake by means of a polarographic oxygen electrode.

Instruments

Spectrophotometric measurements were performed in a Pye-Unicam 8800 instrument. A Clark-type oxygen electrode, connected to an MSE Spectroplus instrument, was used for measuring oxygen uptake.

Results and discussion

Powers *et al.* (1995) observed that 50 μM to 0.3 mM ascorbate inhibited ceruloplasmin ferroxidase activity, measured at pH 6.0 as the rate of Fe^{3+}–transferrin formation. The present study confirms this observation (Figure 1). However, at pH 7.0 the inhibitory effect was markedly reduced, and at pH 7.4 no significant inhibition was observed (Figure 1). The ascorbate concentrations reducing the reaction rate by 50% (I_{50}) were 58 μM and 0.18 mM at pH 6.0 and 7.0, respectively.

Oxygen uptake measurements, performed in the absence of transferrin at pH 7.4 (Figure 2), showed that an increase in ascorbate concentration from 0.2 mM to 1.0 mM did not affect the ferroxidase activity. At pH 6.0 the reaction rate was somewhat reduced by ascorbate. The I_{50} value of 3 mM, calculated from the semilogarithmic plot in Figure 2, was,

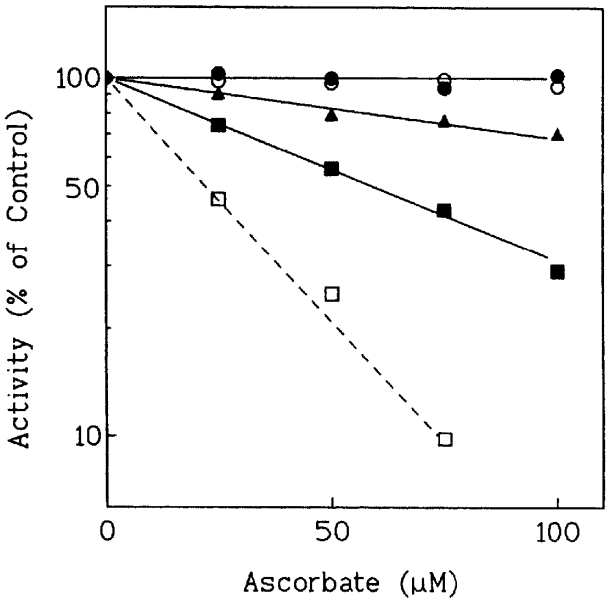

Figure 1. Effect of ascorbate concentration on ceruloplasmin ferroxidase activity, measured as the rate of Fe^{3+}–transferrin formation at pH 6.0 (■), 7.0 (▲) and 7.4 (●), or as Fe^{3+}–conalbumin formation at pH 6.0 (□) and 7.4 (○). The reaction mixture contained 0.07 μM ceruloplasmin, 50 μM ferrous ions, 25 μM apotransferrin (or apoconalbumin), and 25–100 μM ascorbate in 0.2 M sodium acetate buffer, pH 6.0 and 7.0, or in 40 mM Tris buffer at pH 7.4 ($T = 30°C$).

however, considerably higher than the value estimated by monitoring Fe^{3+}–transferrin formation (Figure 1). The results suggests that, even at high ascorbate to ceruloplasmin ratios, ascorbate is not a ceruloplasmin inhibitor at physiological pH. Consequently, it seems unlikely that ascorbate contributes to the low plasma antioxidant level of premature babies through an interaction with ceruloplasmin, as proposed by Powers *et al.* (1995).

In Figure 3 the ferroxidase activity in the presence of 0.2 mM ascorbate, determined as the rate of Fe^{3+}–transferrin and Fe^{3+}–conalbumin formation, is directly compared with the activity determined by monitoring oxygen uptake in the transferrin-free reaction system. At pH 7.2 the values were in good agreement. At pH 6.5 the rate of Fe^{3+}–transferrin formation was only about 30% (with apoconalbumin about 24%) of the rate measured from oxygen uptake, and at pH 6.0 only 12%. Thus in the presence of ascorbate, only a fraction of ferroxidase activity is detected with the method of Johnson *et al.* (1967).

The inhibitory effect of ascorbate was found to be dependent on the ratio of ascorbate to apotransferrin, the rate of Fe^{3+}–transferrin formation

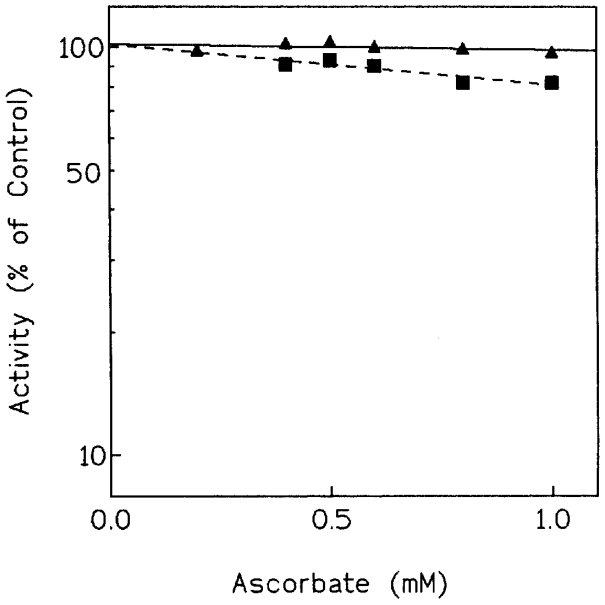

Figure 2. Effect of ascorbate concentration on ceruloplasmin ferroxidase activity, measured as the rate of oxygen uptake in the absence of apotransferrin (apoconalbumin). The reaction mixture contained 0.9 μM ceruloplasmin, 20 μM ferrous ions, and 0.2–1 mM ascorbate in 0.2 M sodium acetate buffer, pH 6.0 (■), or in 40 mM Tris buffer, pH 7.4 (▲) (T = 30°C).

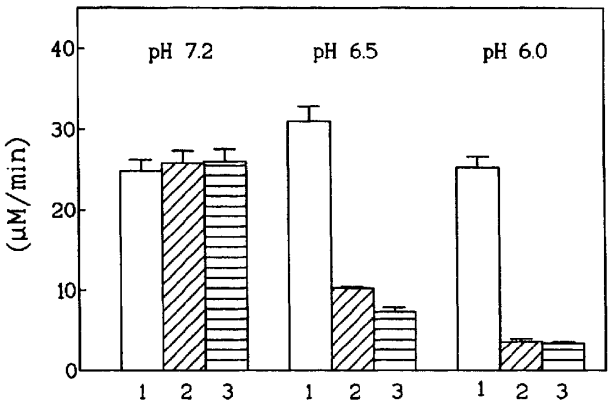

Figure 3. Ceruloplasmin ferroxidase activity, measured as the rate of oxygen uptake (1) in the absence of apotransferrin (apoconalbumin), compared with activity measured as the rate of Fe^{3+}–transferrin (2) and Fe^{3+}–conalbumin (3) formation at pH 7.2, 6.5 and 6.0, in the presence of 25 μM apotransferrin (conalbumin). The reaction mixture contained 0.2 μM ceruloplasmin, 30 μM ferrous ions, and 0.2 mM ascorbate in 0.2 M sodium acetate buffer, pH 7.2, 6.5 and 6.0 (T = 30°C). Mean values ± SEM (n = 3) are presented.

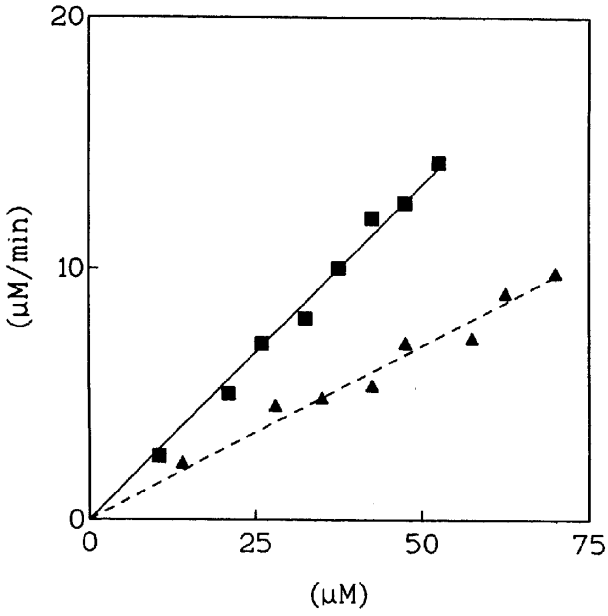

Figure 4. Effect of apotransferrin (■) and apoconalbumin (▲) on ceruloplasmin ferroxidase activity, measured as the rate of Fe^{3+}–transferrin and Fe^{3+}–conalbumin formation, respectively, in the presence of 50 μM ascorbate. The reaction mixture contained 0.14 μM ceruloplasmin, 50 μM ferrous ions, and 10.5–52.5 μM apotransferrin (14–70 μM apoconalbumin) in 0.2 M sodium acetate buffer, pH 6.0 (T = 30°C).

increasing with increasing amounts of apotransferrin (Figure 4), approaching the activity measured in the absence of ascorbate. A similar result was obtained when apotransferrin was exchanged with apoconalbumin (Figure 4). Figure 5 shows that ascorbate is consumed in the apotransferrin-containing reaction system at pH 6.0. In the absence of enzyme, or when enzyme activity is inhibited by azide, the rate of ascorbate oxidation was low (Figure 5).

The results suggest that at acidic pH the inhibitory effect of ascorbate is not primarily due to a binding to ceruloplasmin, but to a competition with apotransferrin (or apoconalbumin) for ferric ions generated by the enzyme; ascorbate reducing them back to the ferrous state before they are complexed to apotransferrin (apoconalbumin). The affinity of transferrins for ferric ions decreases with decreasing pH (Princiotto & Zapolski 1975, Carver & Frieden 1978, Morgan 1979, Baldwin *et al.* 1982); a fact which may explain why the degree of ascorbate inhibition is increased when the pH is lowered (Figure 1). The weak inhibitory effect (I_{50} = 3 mM), observed at pH 6.0 in the oxygen uptake study (Figure 2), could possibly be explained in terms of an interaction between ascorbate and ceruloplasmin.

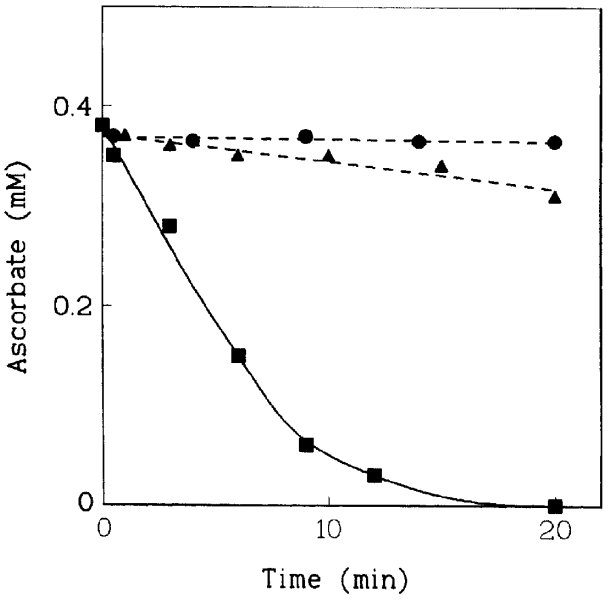

Figure 5. Time course of ascorbate oxidation in a reaction mixture containing 0.38 mM ascorbate, 50 μM ferrous ions, 50 μM apotransferrin and: (1) 0.14 μM ceruloplasmin (■); (2) 0.14 μM ceruloplasmin + 0.23 mM azide (▲); or (3) no ceruloplasmin (●), in 0.2 M sodium acetate buffer, pH 6.0 ($T = 30°C$). Ascorbate concentration was determined as described in a previous communication (Løvstad 1974).

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