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On the mechanism of citrate inhibition of ceruloplasmin ferroxidase activity

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Ceruloplasmin is a plasma protein, which oxidizes ferrous ions in a catalytic manner. It is considered to function as a ferroxidase *in vivo*. Citrate was found to inhibit the reaction. The ceruloplasmin catalyzed oxidation of *p*-phenylenediamines, however, was not affected by citrate. The inhibitory effect is proposed to be due to formation of Fe^{2+}–citrate, which does not react with ceruloplasmin. The stability constant for the Fe^{2+}–citrate complex estimated from the present inhibition study is in good agreement with previously published data.

Keywords: ceruloplasmin, citrate, ferroxidase, iron ions

Introduction

Ceruloplasmin is a plasma protein containing six or seven copper ions per molecule (Rydén & Björk 1976), with oxidase activity towards ferrous ions (Curzon & O'Reilly 1960, Osaki *et al.* 1966). A biological role for the ceruloplasmin of serum in promoting the rate of iron saturation of transferrin has been proposed (Osaki *et al.* 1966, Carver *et al.* 1982). A recent study by Harris *et al.* (1995) on aceruloplasminemia demonstrated that ceruloplasmin is involved in the metabolism of iron *in vivo*.

Organic compounds of the *p*-phenylenediamine class can also function as a substrate for ceruloplasmin (Holmberg & Laurell 1951, Curzon 1961, McDermott *et al.* 1968). Iron ions markedly stimulate the enzymic oxidation of *p*-phenylenediamines and also promote ceruloplasmin-dependent oxidation of ascorbate, by acting as a *red/ox* cycling intermediate between the enzyme and the reducing compounds (Curzon & O'Reilly 1960, McDermott *et al.* 1968). Citrate was found to reduce the ceruloplasmin-dependent oxidation of ascorbate and *N,N*-dimethyl-*p*-phenylenediamine (Osaki *et al.* 1964). The effect of citrate was not a true inhibition of the enzyme, but prevention of enhancement of activity by contaminant iron through its strong affinity for ferric ions ($\log K = 11.85$) (McDermott *et al.* 1968).

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Witwicki *et al.* (1983), studying the inhibitory effect of albumins on ceruloplasmin ferroxidase activity, concluded that this effect was caused by citrate, contaminating the various albumin preparations. The purpose of the present investigation was to establish a mechanism for the inhibitory action of citrate on the ceruloplasmin catalyzed oxidation of ferrous ions.

Materials and methods

Human ceruloplasmin (ferroxidase, EC 1.16.3.1), human apotransferrin, sodium citrate, *p*-phenylenediamine, *N,N*-dimethyl-*p*-phenylenediamine and Tris buffer were purchased from Sigma (St Louis, MO), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, sodium acetate from Merck (Darmstadt, Germany), and desferrioxamine from Ciba-Geigy (Basel, Switzerland).

The ceruloplasmin concentration was calculated from the absorption band at 610 nm ($\varepsilon = 9.45\ mM^{-1}\ cm^{-1}$), and the concentration of apotransferrin from the band at 280 nm ($\varepsilon = 91.2\ mM^{-1}\ cm^{-1}$) (Carver *et al.* 1982). Protein solutions were treated with Chelex-100 (BioRad, Richmond, California, USA) in order to remove contaminating metal ions. $Fe(NH_4)_2(SO_4)_2$ was dissolved in water immediately prior to use. All aqueous solutions were made in deionized, glass-distilled water.

The ferroxidase activity of ceruloplasmin was determined according to the method of Johnson *et al.* (1967). It was corrected for autoxidation of ferrous ions. The *p*-phenylenediamine and *N,N*-dimethyl-*p*-phenylenediamine oxidase activity of ceruloplasmin was measured spectrophotometrically by monitoring the formation of colored

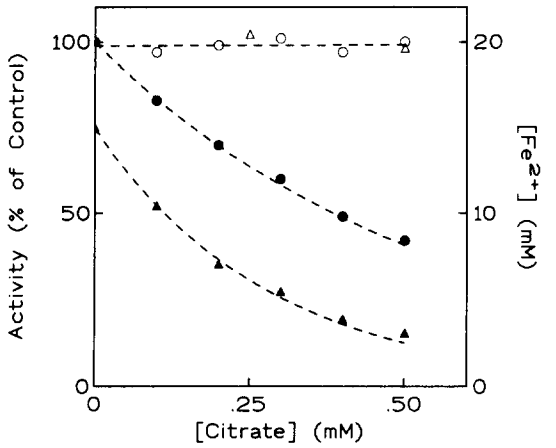

Figure 1. Ceruloplasmin oxidase activity against ferrous ions (●), *N,N*-dimethyl-*p*-phenylenediamine (○) and *p*-phenylenediamine (△) as a function of citrate concentration (0.1–0.5 mM). The reaction solutions for ferroxidase activity determination contained 0.16 μM ceruloplasmin, 15 μM ferrous ions and 25 μM apotransferrin in 40 mM Tris buffer, pH 7.4 (T=30°). ▲, Concentration of free ferrous ions in the reaction solution, calculated from the standard curve in Figure 2. The reaction solutions for determining the rate of the catalytic oxidation of *p*-phenylenediamines contained 0.3 μM ceruloplasmin, 0.1 mM desferrioxamine and 0.5 mM *N,N*-dimethyl-*p*-phenylenediamine in 40 mM Tris buffer, pH 7.4, or 0.5 mM *p*-phenylenediamine in 0.2 M sodium acetate buffer, pH 7.0 (T=30°C).

oxidation products (Curzon 1961, Peisach & Levine 1963, Løvstad 1969). Desferrioxamine was added to the reaction solutions in order to prevent trace iron from interfering with the reaction. It was corrected for autoxidation of *N,N*-dimethyl-*p*-phenylenediamine.

A Pye Unicam 8800 instrument was used for spectrophotometric measurements.

Results and discussion

In order to determine the ceruloplasmin ferroxidase activity, apotransferrin was used for trapping ferric ions generated by the action of ceruloplasmin on Fe^{2+} (Johnson *et al.* 1967). The protein binds two ferric ions per molecule ($\log K_1 = 22.7$, $\log K_2 = 22.1$; Martin *et al.* 1987). The complex is characterized by an absorption band at 465 nm ($\varepsilon = 2.3\ mM^{-1}\ cm^{-1}$; Carver *et al.* 1982). Figure 1 shows that citrate reduced the rate of ferrous ion oxidation. Excess citrate (10 mM) was found to completely prevent the ceruloplasmin catalyzed reaction. However, citrate did not affect the iron-independent enzymic oxidation of *p*-phenylenediamine and *N,N*-dimethyl-*p*-phenylenediamine (Figure 1), suggesting that the inhibitory effect on ferroxidase activity is not due to a direct interaction with the enzyme. Since citrate does not compete with apotransferrin for ferric ions (Carver *et al.* 1982, McGregor & Brock 1992), and is able to bind Fe^{2+} ($\log K = 3.08$, Sillén & Martell 1964), it is proposed that citrate inhibits the ferroxidase activity by forming an Fe^{2+}–citrate complex, which does not react with ceruloplasmin.

Figure 2 shows the initial rate of ferrous ion oxidation by the enzyme plotted against substrate concentration at pH 7.4. An apparent K_m value of 8.9 μM (SE = 0.8 μM) was calculated by means of a computer (Cleland 1967). Since the kinetics of Fe^{2+} oxidation is characterized by two K_m values (0.6 and 50 μM at pH 6.5; Osaki 1966), the K_m obtained represents an overall value. From this standard curve the amount of free ferrous ions in the different citrate-containing reaction solutions was determined. In Figure 1 the Fe^{2+} concentration is plotted against the concentration of citrate, when the reaction solution contained a total of 15 μM iron. The equilibrium constant for the reaction between Fe^{2+} and citrate,

$$K = \frac{[Fe^{2+}\text{–Cit}]}{([Cit_t]-[Fe^{2+}\text{–Cit}])([Fe^{2+}_t]-[Fe^{2+}\text{–Cit}])}$$

where t is the total concentration, could then be estimated. Table 1 lists the $\log K$ values obtained at different iron

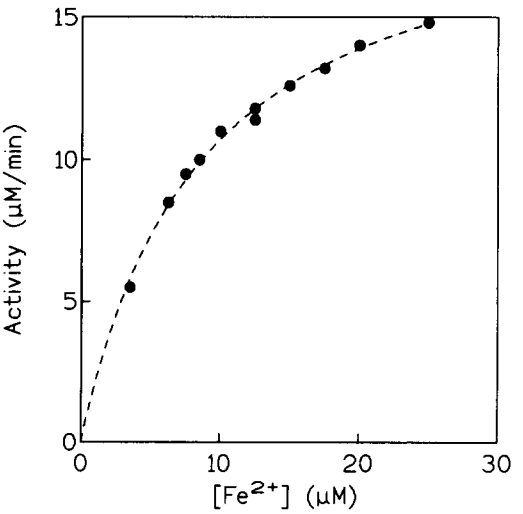

Figure 2. Ceruloplasmin ferroxidase activity plotted against substrate concentration. The reaction solutions contained 0.16 μM ceruloplasmin, ferrous ions (3.5–25 μM) and 25 μM apotransferrin in 40 mM Tris buffer, pH 7.4 (T=30°C).

Table 1. Equilibrium constant logarithms

$[Fe^{2+}]$ (μM)	log K[a]	
	pH 7.4[b]	pH 7.0[c]
15	3.7	
20	3.7	3.9
25	3.5	3.6
50		3.8
60		3.9
75		3.9

[a] Published value = 3.08 (Sillén & Martell 1964).
[b] Experimental conditions as described in the legend to Figure 1.
[c] The reaction solutions contained 0.07 μM ceruloplasmin, ferrous ions, citrate (0.5–2 mM) and 25 μM apotransferrin in 0.2 M sodium acetate buffer (T=30°C).

concentrations at pH 7.4 and 7.0. (The standard curve (V versus $[Fe^{2+}]$) obtained at pH 7.0 was characterized by an apparent K_m of 12.5 μM (SE = 1.2 μM).) The mean log K value, 3.8, is in good agreement with the published value of 3.08 (Sillén & Martell 1964) and supports the proposition that citrate reduces the activity by binding ferrous ions. The normal plasma concentration of citrate (0.067–0.143 mM) inhibited the reaction less than 25%.

Citrate-contaminated bovine serum albumin (A 4903 Type V; Sigma) has been reported to be without inhibitory effect on ceruloplasmin ferroxidase activity at pH 7.4 (Witwicki *et al.* 1983), while another study demonstrated a marked inhibition by Type V albumin at physiological pH (Løvstad 1995). This discrepancy cannot be accounted for at present. Possibly the latter preparation was more contaminated with citrate.

Plasma from hemochromatosis patients contains iron–citrate complexes when the iron-binding capacity of transferrin is exceeded (Grootveld *et al.* 1989). Increased levels of plasma citrate were also observed in these patients (up to 0.43 mM) (Grootveld *et al.* 1989). Miller & Aust (1989) reported that Fe^{3+}–citrate can be reduced by ascorbate. Fe^{2+} citrate is rapidly oxidized by molecular oxygen, the reaction generating the intermediate active oxygen species, $\dot{O}_2^-$ and H_2O_2 (Carver *et al.* 1982, Miller & Aust 1989). When ceruloplasmin functions as a *red/ox* cyclic intermediate between ferrous ions and oxygen, the latter is reduced to water by accepting four electrons from the enzyme (Osaki *et al.* 1966, Carver *et al.* 1982). The present study suggests that ceruloplasmin is unable to prevent a possible Fe^{2+}–citrate-dependent generation of potentially harmful oxygen derivatives through an interaction with Fe^{2+}–citrate.

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