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A kinetic study on the copper-albumin catalyzed oxidation of ascorbate

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Abstract

Serum albumin can specifically bind one Cu(II)-ion, and is proposed to function as a copper transport protein *in vivo*. Cu(II)-albumin is rapidly reduced by ascorbate. A second order rate constant of 0.54 mM^{-1} min^{-1} was estimated for the reaction. The oxidation process is catalytic, the Cu(I)-albumin molecule being reoxidized by molecular oxygen. The reaction was found to follow Michaelis-Menten kinetics, characterized by an apparent K_m-value of 0.89 mM, and a catalytic constant of 0.066 μM O_2/min. An apparent inhibition of oxygen uptake was obtained with catalase (but not with superoxide dismutase), suggesting the formation of H_2O_2 in the system. Wilson's disease patients usually have increased amounts of non-ceruloplasmin copper in plasma. The low level of plasma ascorbate observed in such patients could possibly be due, at least in part, to an oxidation by Cu(II)-albumin.

Introduction

Human- and bovine serum albumins (HSA, BSA) contain a high-affinity site for Cu(II), the binding involving both histidyl and carboxyl groups (Breslow 1964; Lau & Sarkar 1971; Zgirski & Frieden 1990; Masuoka *et al.* 1993). Experiments strongly suggest that part of the non-ceruloplasmin copper fraction in blood plasma is bound to albumin, and that the protein functions as a copper transport protein *in vivo* (Owen 1965; Marceau & Aspin 1973; Wirth & Linder 1985: Gordon *et al.* 1987).

The visible absorption spectrum of the Cu(II)-BSA complex is characterized by a broad band around 525 nm (Peters & Blumenstock 1967). A study by Zgirski & Frieden (1990) suggested a log-K value of about 12 for the Cu(II)-BSA complex at physiological pH. Later intrinsic log-K values of 11.2 and 11.8 were calculated for Cu(II)-BSA and Cu(II)-HSA, respectively (Masuoka *et al.* 1993).

The Cu(II)-BSA complex has been shown to be reduced to the cuprous state by ascorbate. Ozawa *et al.* (1993) demonstrated that the Cu(I)-BSA complex thus formed is able to react with added H_2O_2 and generate hydroxyl radicals. Since ascorbate and albumin share the same environment in blood plasma, and since ascorbate has been shown to be oxidized to a free radical in human serum (Sasaki *et al.* 1982; Mouithys-

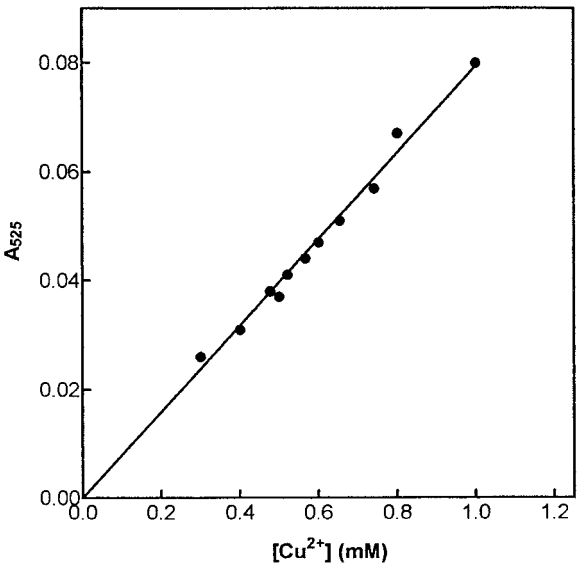


Figure 1. The 525 nm absorbance of Cu(II)-BSA as a function of Cu(II)-concentration. The solution contained 0.3–1 mM Cu(II) and 1.4 mM BSA in 0.1 M sodium acetate buffer, pH 6.0 (T = 25 °C).

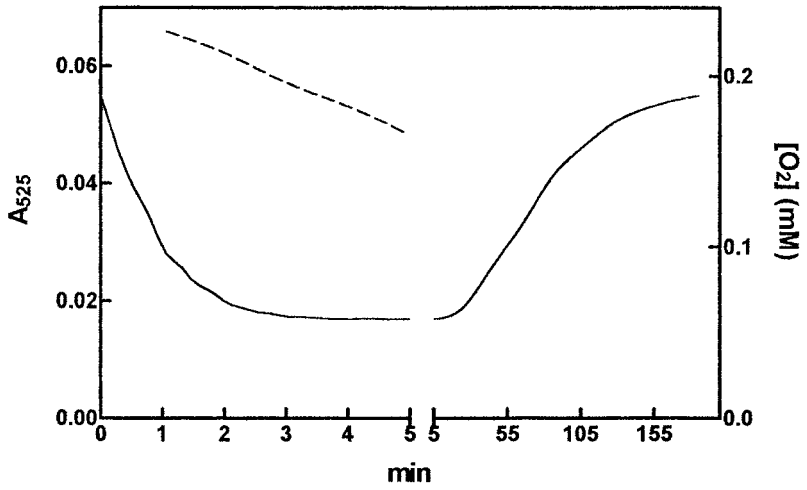


Figure 2. The change in the 525 nm absorbance of Cu(II)-BSA after addition of 1 mM ascorbate to 0.7 mM Cu(II)-BSA in 0.1 M sodium acetate buffer, pH 6.0. (T = 25 °C). The total BSA concentration was 0.93 mM. - - - - - -, Time course of oxygen consumption during the reaction.

Mickalad *et al.* 1998), it was of interest to undertake a kinetic study on the interaction of ascorbate with the Cu(II)-BSA complex.

Materials and methods

Bovine serum albumin (A 7906; charcoal treated, extensively dialyzed and essentially free of low molecular weight substances), sodium ascorbate, catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), bathocuproine disulfonate were purchased from Sigma (St. Louis, Missouri, USA), $CuSO_4$ from Riedel-de Häen AG (Seelze-Hannover, Germany), sodium azide from E.Merck AG (Darmstadt, Germany), and Sephadex G-25 (fine) from AB Pharmacia (Sweden). All aqueous solutions were made in deionised, glass-distilled water. Albumin concentration was determined from the 280 nm absorption band ($\varepsilon = 43.6\ mM^{-1}\ cm^{-1}$) (Sober & Harte 1968).

Spectrophotometric measurements were made in a Cecil 292 instrument connected to a Radiometer Rec 80 recorder. Oxygen uptake was monitored by means of a Clark-type electrode connected to a MSE Spectroplus instrument.

Results and discussion

In the presence of excess BSA the 525 nm absorption band of the Cu(II)-BSA complex was found to increase linearly with the Cu(II) concentration (Figure 1). An absorption coefficient , ε, of 0.079 $mM^{-1}\ cm^{-1}$ was estimated from this plot at pH 6.0, and used in the present study. However, as shown by Ivanov *et al.* (2000), the intensity of the 525 nm band depends markedly on pH, increasing with a rise in pH from 5.2 to 8.1.

The time course curve in Figure 2 shows that the 525 nm absorbance rapidly decreases initially, when ascorbate is added to a solution of Cu(II)-BSA. After a while a steady-state level is reached. The process consumes oxygen for the reoxidation of cuprous ions generated by ascorbate, suggesting that the metal complex oxidizes ascorbate in a catalytic manner. On prolonged standing, allowing oxygen to be reabsorbed after being depleted, the 525 nm absorbance slowly increases until the initial level is reached.

In order to find out whether ascorbate was able to remove copper from BSA, a solution of Cu(II)-BSA was mixed with ascorbate and then administered on a Sephadex G-25 column. The ascorbate concentration was kept much higher than the concentration of Cu(II)-BSA, so although a fraction of ascorbate was oxidized during the experiment, most of it remained in the reduced form. After separation all the copper was associated with the BSA fraction (Figure 3). The experiment suggests that the ascorbate induced reduction of the 525 nm band could not be attributed to a removal of copper from the protein molecule. The initial rate of reduction of the Cu(II)-BSA complex ,V, by ascorbate was determined at various ascorbate con-

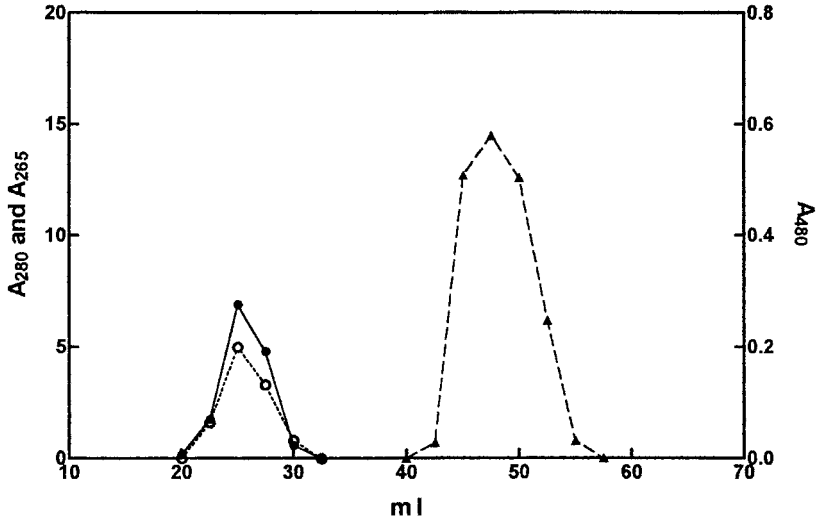
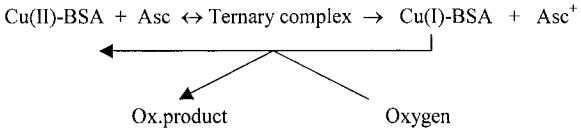


Figure 3. Separation of BSA (•) and ascorbate (▲) on a Sephadex G-25 (fine) column (1.2 × 50 cm) after mixing 70 μM Cu(II)-BSA (total BSA concentration = 0.93 mM) with 8 mM ascorbate in 0.1 M sodium acetate buffer, pH 6.0 (T = 22 °C). Volume = 1.0 ml. Eluant: 0.1 M sodium acetate buffer, pH 6.0. Flow rate: 0.5 ml/min. BSA and ascorbate were detected by the absorbance at 280 nm and 265 nm, respectively, and copper (o) as Cu(I)-bathocuproine at 480 nm by adding 0.1 ml 5 mM bathocuproine to 0.9 ml of each fraction collected, and grains of ascorbate as reducing agent in the fractions not containing ascorbate.

centrations. As shown in Figure 4 a linear correlation was obtained between the rate, V, and the concentration of ascorbate. From the plot a second order rate constant for the reaction, k = V/([Cu(II)-BSA][Asc]), was estimated to 0.54 mM^{-1} min^{-1}.

The ascorbate 'oxidase' activity of Cu(II)-BSA was studied by monitoring the consumption of oxygen used for reoxidizing cuprous ions during the process. Figure 5 shows the relationship between activity and ascorbate concentration. The hyperbolic curve obtained can be explained in terms of a complex formation between Cu(II)-BSA and ascorbate; the latter gradually 'saturating' the metal complex.



(Asc^+ = ascorbate radical) An apparent Michaelis' constant, K_m, of 0.089 mM, and a maximum activity of 4.6 μM O_2/min (turnover constant = 0.066 min^{-1}) was calculated by means of a computer program, which enables a computer to make least square fits of data to the enzymic rate equation, ($V = V_{max}[S]/(K_m + [S])$) (Cleland 1967). The curve drawn by the computer fits well with the experimental data (Figure 5). The rate of oxygen uptake was independent of the

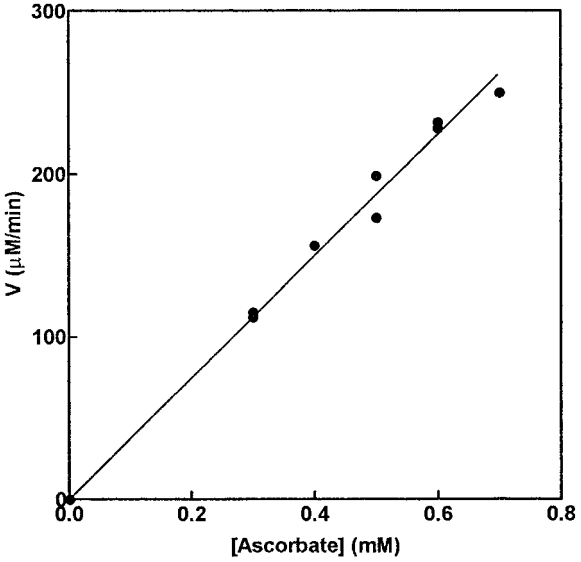


Figure 4. Initial rate of Cu(II)-BSA reduction by ascorbate. The solution contained 0.7 mM Cu(II)-BSA (total BSA concentration = 0.93 mM) and 0.3–0.7 mM ascorbate in 0.1 M sodium acetate buffer, pH 6.0 (T = 25 °C).

BSA concentration as long as it exceeded that of copper.

Since the reoxidation of Cu(I)-BSA to the cupric state is a one-electron transfer, one would expect that the superoxide radical is formed. However, superoxide dismutase, transforming superoxide radicals to H_2O_2

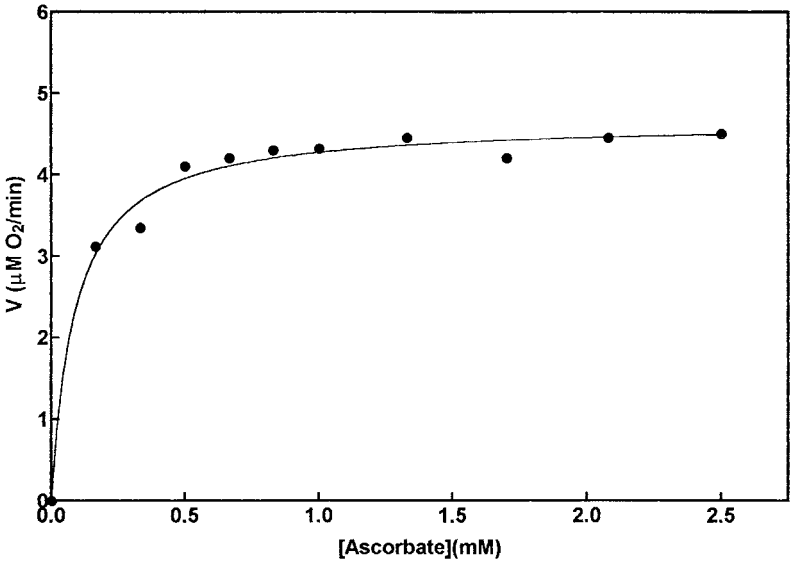


Figure 5. Effect of ascorbate concentration on the rate of oxygen consumption during the copper-BSA catalyzed oxidation. The solution contained 70 μM copper-BSA (total BSA-concentration = 0.93 mM) and 0.33–2.5 mM ascorbate in 0.1 M sodium acetate buffer, pH 6.0 (T = 25 °C).

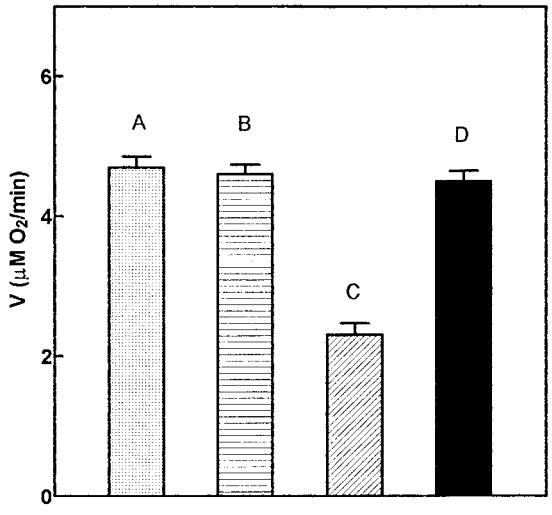


Figure 6. Rate of oxygen consumption during the copper-BSA catalyzed oxidation of ascorbate, in the absence (A), and presence of 318 U/ml superoxide dismutase (B), 295 U/ml catalase (C), or 295 U/ml catalase + 0.33 mM azide (D). The solutions otherwise contained 70 μM Cu(II)-BSA (total BSA concentration = 0.8 mM) and 1.7 mM ascorbate in 0.1 M sodium acetate buffer, pH 6.0 (T = 25 °C).

and oxygen, did not cause an apparent inhibition of oxygen consumption, when present in the reaction solution (Figure 6). Also the commonly used nitro-blue tetrazolium test (Halliwell & Gutteridge 1989) failed to detect superoxide radicals, although they were produced during the copper catalyzed oxidation of ascorbate in the absence of BSA. The failure to detect superoxide radicals could be due to an immediate dismutation of the newly formed radicals. In the presence of catalase, however, an apparent reduction of the rate of oxygen consumption was detected, the effect involving the catalytic activity of the enzyme, since azide, a potent inhibitor, markedly reduced the effect (Figure 6). Azide did not affect the reaction in the absence of catalase. The result suggests that $H_2O_2$is formed in the reoxidation process, the apparent inhibition being due to the catalytic conversion of H_2O_2 to H_2O and oxygen. The observation that the process generates H_2O_2, which can react with Cu(I)-BSA and produce the highly reactive hydroxyl radicals (Ozawa *et al.* 1993), suggests the possibility that the reoxidation process may have a damaging effect on biomolecules. H_2O_2 was also formed when cysteine and reduced glutathione were used as reducing agents for Cu(II)-albumin (R.A.Løvstad, unpublished data).

Ascorbate radicals have been detected in blood and plasma/serum of normal individuals by means of eletron-spin-resonans (ESR) technique. The radicals could be formed spontaneously or after administration of ascorbate (Lohmann *et al.* 1981, Sasaki *et al.* 1982; Mouithys-Mickalad 1998). Sasaki *et al.* (1985) suggested that albumin and ceruloplasmin could be involved in this phenomen. However, in a thorough study Curzon and Young (1972) concluded that ceruloplasmin in serum at physiological pH did not oxidize

ascorbate, even if purified ceruloplasmin possessed some activity at acidic pH. Mouithys-Mickalad *et al.* (1998) reported that addition of ascorbate to a preparation of crude HSA produced an ESR-signal characteristic of the ascorbate radical. This could possibly be attributed to the fact that commercial albumin preparations often are contaminated with copper, presumably as a result of the procedure used to isolate albumin from plasma (Quinlan *et al.* 1992).

The possibility exists that the copper-albumin complex may react with ascorbate in the blood, creating harmful reactive oxygen species during the reoxidation process. Altough the concentration of copper-albumin normally is low in human plasma, it has been demonstrated that plasma from Wilson's disease patients have a high level of non-ceruloplasmin copper. In these patients the level of plasma ascorbate is also markedly reduced (Ogihara *et al.* 1995). An interaction between Cu(II)-HSA and ascorbate might, at least in part, account for this.

Acknowledgements

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