THE ASCORBATE OXIDASE ACTIVITY OF CHELEX TREATED CERULOPLASMIN*

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The question of the ascorbate oxidase activity of ceruloplasmin (CP) remains unsettled. Initially, Holmberg and Laurell (1951) in their early work on the oxidase activity of this important serum Cu-protein, reported that ascorbate was a substrate though less active than p-phenylendiamine. The work of Humoller, et. al. (1960) supported the existence of the ascorbate oxidase activity of CP and noted the different susceptibility of CP and Cu(II) toward albumin. Walter and Frieden (1962) presented evidence for the existence of ascorbate oxidase activity and this enzyme. More recently, Morell, Aisen and Scheinberg (1962) studied the ascorbate oxidase activity of CP which was treated with Chelex-100 to eliminate non-protein Cu ion and concluded that ascorbate was not a substrate for CP. Since it is generally agreed that the Cu(II) of CP is reversibly reduced by ascorbate and that the reduced form of the protein can react directly with oxygen, this controversial problem has been reexamined by a comparison of the ascorbate oxidation catalyzed by Chelex-100 treated CP with that catalyzed by Cu(II). As with CP which has not been treated with Chelex-100 (Walter 1962, Walter and Frieden 1962), the results indicate that the reaction between CP and ascorbate is catalytic and not only stoichiometric. Moreover, the CP reaction is not inhibited by reagents which inhibit Cu(II) catalysis and has numerous kinetic characteristics different from the Cu(II) catalyzed oxidation of ascorbate.

CERULOPLASMIN - Commercially purified human CP (KABI, Sweden) was chromatographed at 2° on a DEAE - cellulose column (Bio-Rad. Lab.)

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using a linear gradient elution from 0.05M acetate buffer at pH 5.7 to the same buffer containing 0.15M sodium chloride. The absorbance ratio, ($A_{280m\mu}/A_{610m\mu}$) of the sample was 21.7. The ultracentrifugal analysis gave only one peak with an S_{20} of 6.3 extrapolated to zero conc. The CP solution thus obtained was treated with Chelex-100 column according to the method described by Morell et. al. (1962) to eliminate any trace amount of inorganic non-CP copper ion. All buffers, reagents and water used in solutions or final rinsings were also treated with Chelex-100.

ASCORBATE OXIDASE ACTIVITY OF CERULOPLASMIN - The oxidative activity of CP and Cu(II) was measured by two different methods at 30.0± 0.1° described below. The 3.0 ml. reaction mixture contained 0.2M acetate buffer of pH 5.2, an aliquot of the ascorbate solution, CP or inorganic copper, and in some cases, inhibitor. The disappearance of ascorbic acid was measured spectrophotometrically at 265 mμ in a 1 cm Quartz cuvette using a Beckman Spectrophotometer Model DK-1, equipped with a constant temperature cell. Oxygen uptake was also observed in triplicate using conventional Warburg manometric technique.

IS THE REACTION BETWEEN ASCORBATE AND CERULOPLASMIN-Cu STOICHIOMETRIC?

The fact that many times the number of ascorbate molecules as CP-Cu can be oxidized by this protein can be shown using both spectrophotometric and manometric methods. In the data shown in Fig. 1, at zero time, 20 microliters of a stock solution of ascorbate was added to the 3.00 ml reaction mixture to bring the final ascorbate concentration to 3.84×10^{-5}M. No measurable oxidation of ascorbate was observed during an initial 10 minute standing period. When 1.23×10^{-7}M CP was added, a rapid reduction of the ascorbate proceeded, resulting in a decrease of absorbance at 265 mμ of approximately 0.5 units. When the absorbance reached a value below 0.10, an additional aliquot of 20 μl ascorbate was added to the same reaction mixture, producing an initial elevation of absorbance at 265 mμ followed by its disappearance as the ascorbate was oxidized. This procedure was repeated six times with the continuous recording of the absorbance changed as shown in Fig. 1. There was no reason to believe that an indefinite number of ascorbate aliquots could have been added. The results shown in Fig. 1 indicate that a solution of ascorbate acid of 2.7×10^{-4}M was oxidized

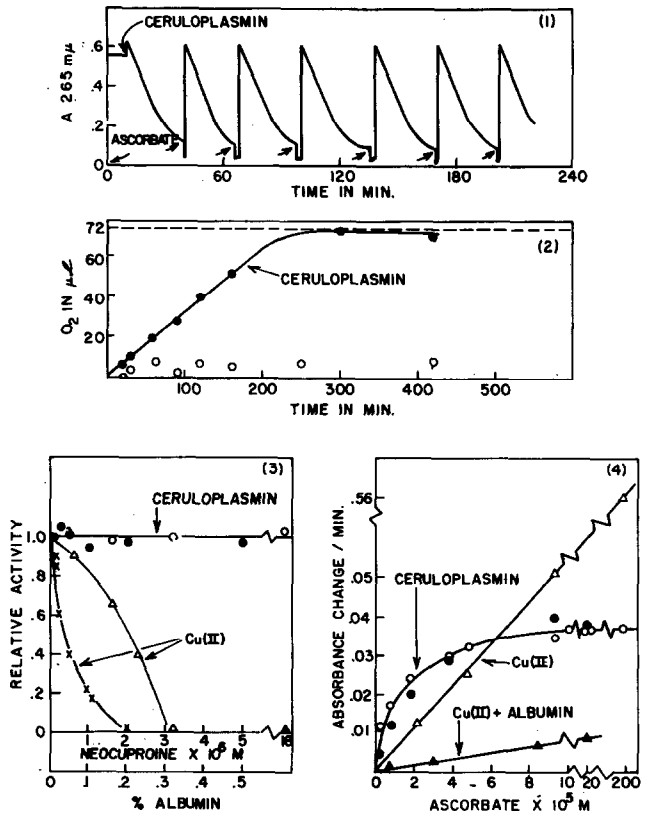

Fig. 1 The time course of the absorbance change at 265 mu was reproduced from a continuous recording chart of Beckman Recording Spectrophotometer Model DK-1. 20 ul of an ascorbic acid solution sufficient to produce a final concentration of 3.84×10^{-5}M was added at the time indicated. The reaction mixture contained 1.23×10^{-7}M ceruloplasmin, .2M acetate buffer, pH 5.2 at 30°.

Fig. 2 Microliters of oxygen consumed were plotted against time. 7.5×10^{-7}M CP and 1.65×10^{-5}M Neocuproine were used. The closed and the open circles represent the value of oxygen taken up with and without CP, respectively.

Fig. 3 The relative activities of CP and Cu(II) at various concentration of inhibitors are plotted against the concentration of Neocuproine and bovine serum albumin. The concentration of ascorbic acid, CP and Cu(II) used were 3.6×10^{-5}M, 1.5×10^{-7}M, and 1.6×10^{-6}M, respectively. —○—○—; CP and Neocuproine. —●—●—; CP and bovine serum albumin. —▲—▲—; Cu(II) and Neocuproine. —X—X—; Cu(II) and bovine serum albumin.

Fig. 4 The rate of ascorbate oxidation as the absorbance change at 265mu was plotted against substrate concentration. 1.95×10^{-7}M CP or 1.6×10^{-6}M Cu(II) was used with or without 0.15% bovine serum albumin as indicated.

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by 1.23×10^{-7}M CP. Even assuming all the Cu in CP is Cu(II), this represents 270 times as many ascorbate molecules oxidized as all the atoms

of Cu present in CP. Corrected for one electron change per Cu and assuming that less than one-half of the Cu of CP is Cu(II) (Morell et. al., 1963), the turnover of Cu(II) is increased to over 1080.

The non-stoichiometric nature of the ascorbate oxidation catalyzed by CP was also shown in oxygen uptake experiments using conventional Warburg technique. In these experiments, 1.65×10^{-5}M Neocuproine was used to completely inhibit any unforseen contamination by Cu(II) in the 3-4 hour test period. It was reported earlier and confirmed in this work that Neocuproine inhibits the Cu(II) catalyzed oxidation of ascorbate (Wahlborg, 1957) but does not affect the catalytic oxidation of either p-phenylenediamine or ascorbate by CP (Walter, 1962). The data shown in Fig. 2 indicate that a solution of 7.5×10^{-7}M CP catalyzed the oxidation of 1.96×10^{-3}M ascorbate in about 200 min., over 320 times the number of total Cu atoms, or, corrected as before, closer to 1280 atoms of Cu. Assuming one-half mole of oxygen is consumed per mole of ascorbate, the oxygen uptake approaches the theoretical value as shown by the dashed line in Fig. 2. It is emphasized that both these data are based on total extent of reaction and not on any initial rates of ascorbate oxidation.

IS THE ASCORBATE OXIDASE ACTIVITY OF CERULOPLASMIN DUE TO NON-CERULOPLASMIN Cu ION?

As described earlier, CP and all solutions were passed through a Chelex-100 column to eliminate trace contamination with Cu ion. In subsequent experiments, Chelex-100 purified CP retained its ability to uniquely catalyze the oxidation of ascorbate, even in the presence of reagents which inhibit the Cu(II) catalyzed oxidation. Thus while we believe that the evidence presented in this paper suggest that CP and the reagents used here were free of trace contamination of Cu ion, many of the arguments are valid even if traces of Cu ion were present. For example, it has been observed that ascorbate catalysis by CP proceeds despite the addition of an excess of Neocuproine, bovine serum albumin, or thyroxine (Walter, 1962). Traces of non-CP copper ion, even if present, would be accessible to chelation by these reagents and thus could not account for the CP activity.

The ability of certain compounds to inhibit the Cu(II) catalyzed oxidation of ascorbate has been mentioned previously. If these same compounds do not block the CP catalyzed oxidation of ascorbate, then the CP catalysis cannot

be due to Cu(II) contamination. The data in Fig. 3 affords a convincing demonstration of the effect of two such compounds, Neocuproine and bovine serum albumin. Neither compound, up to 1.6×10^{-5}M, Neocuproine and 0.5% bovine serum albumin affected the CP action. But at 3.0×10^{-6}M Neocuproine and 0.2% bovine serum albumin, all the Cu(II) catalyzed oxidation was blocked, presumably due to Cu(II) complex formation. In the oxygen uptake experiments shown in Fig. 2, Neocuproine also did not affect the CP catalyzed reaction. This data confirms the earlier observations of Humoller et. al. (1960) who also reported that serum albumin did not inhibit the ascorbate oxidation by partially purified CP that had not been treated with Chelex-100.

KINETIC DIFFERENCES BETWEEN CERULOPLASMIN AND Cu(II)

The CP catalyzed reaction differs from the Cu(II) catalyzed oxidation in numerous kinetic features. At low ascorbate concentrations, as shown by Walter and Frieden (1962), the CP reaction shows a typical enzymic velocity vs. substrate curve as in Fig. 4. In this same range of ascorbate concentration the Cu(II) reaction is essentially pseudo first order with respect to ascorbate concentration. A first order plot for Cu(II) is linear, but the CP catalyzed reaction obviously deviates from first order kinetics being pseudo-zero order at 10^{-4}M ascorbate. The rate of the CP reaction vs. ascorbate concentration is also not affected by 0.5% bovine serum albumin. The Cu(II) reaction is, of course, greatly depressed by this reagent. When the respective reaction rates were measured at 6 different temperatures between 5° and 35°, the calculated activation energy for the CP catalyzed reaction was 12.5 kcal./mole and that for Cu(II) was 16.3 kcal./mole. While a difference of about 3.8 kcal./mole is small, the measurements are believed to be sufficiently accurate to justify the conclusion that the activation energy of CP is significantly lower than the Cu(II) reaction under identical conditions.

The data in Fig. 4 may well account for some of the controversy regarding the catalytic nature of CP towards ascorbate. At a low ascorbate concentration, 2.0×10^{-5}M, the rate of ascorbate oxidation is .025 absorbance units per min. for 1.95×10^{-7}M CP or 1.3×10^{-5} absorbance units per min. per mole of protein. For 1.60×10^{-6}M Cu(II) at 2.0×10^{-5}M, the relative rate is .011 absorbance units per min, or 6.9×10^{3} absorbance units per atom of Cu(II). Thus, at 2.0×10^{-5}M ascorbate, the CP molecule

is 19 times as active as Cu(II). Even if all 8 Cu atoms of CP were active, CP would still be about 2 1/2 times as active as Cu(II). At lower ascorbate concentrations, the ratio of activities would be somewhat larger.

The data of Morell et. al. (1962) can be reinterpreted in terms of this present work. Morell et. al. (1962) used an ascorbate concentration of 5.7×10^{-3}M which is 100 times the saturation level for CP but below the saturation level for the Cu(II) reaction. Thus it would be expected from the data in Fig. 4 that at equivalent Cu, the Cu(II) catalyzed reaction would be many times as fast as the CP reaction when the ascorbate concentration is saturating both systems and is much higher than the typical plasma ascorbate level of 4×10^{-5}M.

It is of interest that CP is fully saturated just in this range of ascorbate concentration, 5×10^{-5}M, which corresponds to the average human plasma ascorbate value of about 0.8 mg. %. As shown in Fig. 4 at 4×10^{-5}M ascorbate, the CP catalyzed oxidation proceeds at a faster rate than an equivalent amount of Cu(II) even in the absence of plasma proteins. The K_m for the CP catalyzed reaction is 1.3×10^{-5}M and the zero order rate constant is 15 moles of ascorbate per min. per CP molecule. For the Cu(II) reaction, the K_m is 1000 times as great (in the 10^{-2}M range) and the zero order rate constant is estimated to be about 500 moles of ascorbate per min, per Cu(II). The catalytic advantages of CP-Cu over free Cu(II) is that the binding of ascorbate is increased about 1000 fold and that the catalytic activity with respect to ascorbate is now fully protected against inhibition by the most prominent protein of the plasma, albumin (Frieden, 1962).

SUMMARY - Chelex treated CP has been shown to have ascorbate oxidase activity. The reaction catalyzed is at least many hundred times that predicted from a stoichiometric reaction between ascorbate and the Cu(II) of CP. The CP catalyzed oxidation of ascorbate is not affected by bovine serum albumin and Neocuproine, both of which completely inhibit the Cu(II) catalyzed reaction. Numerous significant kinetic differences have also been observed between the ceruloplasmin and Cu(II) catalyzed reactions.

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