Proof for the Ascorbate Oxidase Activity of Ceruloplasmin*

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The ascorbate oxidase activity of ceruloplasmin has been a subject of controversy because of (a) the relatively low oxidase activity of this serum copper protein toward ascorbic acid and (b) the different conclusions reached by several investigators. In 1951, Holmberg and Laurell (1) reported that ascorbic acid was a substrate of ceruloplasmin although it was less active as a substrate than p-phenylenediamine. Humoller et al. (2) concluded that ceruloplasmin has ascorbate oxidase activity, primarily because they had observed that ceruloplasmin and Cu(II) had a different susceptibility toward albumin. In 1962, Walter (3) described numerous kinetic features of ascorbate oxidation by ceruloplasmin which were different from the catalysis by Cu(II), and Frieden (4) suggested an authentic ascorbic acid oxidase activity of ceruloplasmin. Morell, Aisen, and Scheinberg (5) re-examined this problem with the use of a ceruloplasmin sample that was treated with a chelating resin, Chelex 100, to eliminate nonceruloplasmin copper, and concluded that ceruloplasmin had no ascorbate oxidase activity. At this point, we decided to examine this question by using all possible experimental criteria to reach a decisive conclusion.[1] The data presented here show an indisputable difference between the oxidation of ascorbate by ceruloplasmin and by Cu(II), and indicate that ceruloplasmin has definite ascorbate oxidase activity.

EXPERIMENTAL PROCEDURE

Materials

Ceruloplasmin—Partially purified human ceruloplasmin, generously supplied by Kabi, Sweden, and the American National Red Cross, was purified further by a chromatography on a DEAE-cellulose column (Bio-Rad) in the method described previously (5). A purified sample, also donated by the American National Red Cross, was dialyzed extensively against 0.1 M acetate buffer, pH 5.5, and then recrystallized twice from 0.025 M acetate buffer, pH 5.2, according to the method described by Deutsch (8). The solution of purified ceruloplasmin was ultracentrifugally homogeneous with an s_{20} value of 6.3 S, and the absorbance ratio $(A_{280}:A_{610})$ was 22.0 ± 0.3.

The ceruloplasmin solution thus obtained was then immediately treated with a column of Chelex 100 (a chelating resin of a styrene lattice with an imino-acetic acid exchange group; Bio-

Rad) as described by Morell et al. (5) to eliminate any trace amount of nonceruloplasmin copper ion. This treatment did not give any detectable change in the physicochemical properties of the enzyme such as the s_{20}, the visible and ultraviolet spectra, and $A_{280}:A_{610}$ ratio (9). Treatment with Chelex is indispensable to obtain a constant ascorbate oxidase activity per unit of absorbance at 610 mµ. In some samples, particularly with aged preparations, the original sample shows higher activity compared to the one treated by Chelex 100. This activation, however, can be restored to the normal value for Chelex-treated ceruloplasmin by adding neocuproine to the reaction mixture, suggesting that there is slight copper contamination or that, as a result of denaturation of the enzyme, free Cu(II) has separated from the protein moiety. All buffers and water used in solutions or final rinsings were also treated with Chelex 100.

Reagents—Ascorbic acid (U.S.P., fine crystals, Lot 63372) was obtained from Merck, and was used as the substrate without further purification. This ascorbic acid, dissolved in Chelex 100-treated water (5.7 mM), showed no detectable autoxidation in more than 48 hours. Neocuproine was obtained from the G. Frederick Smith Chemical Company, and was recrystallized twice from 20% aqueous alcohol. Horseradish peroxidase with an $A_{403}:A_{275}$ ratio of 3.0 was obtained from the California Corporation for Biochemical Research and was used for assay of hydrogen peroxide with ascorbic acid as a hydrogen donor. An aqueous solution of cupric sulfate pentahydrate (J. T. Baker Chemical Company; Reagent Grade purity, 99.9%) was used as the Cu(II) source.

Methods

Rate Measurements—The oxidative activity of ceruloplasmin and Cu(II) was measured by two different methods at 30.0 ± 0.1°. The 3.0-ml reaction mixture contained 0.02 M acetate buffer, an aliquot of ascorbate, ceruloplasmin or Cu(II), and inhibitor where indicated.

The disappearance of ascorbate was measured spectrophotometrically at 265 mµ in a 1-cm quartz cuvette with a Beckman DK-1 instrument equipped with a constant temperature cell. Oxygen uptake was also observed in triplicate by the conventional Warburg manometric technique (6). The use of phosphate buffer was avoided because of its inhibitory effect on the ceruloplasmin-catalyzed oxidation (10, 11). When sufficient buffering action on the acetate could not be guaranteed, the pH of each reaction mixture was determined before and after the rate measurements with a Beckman pH meter equipped with a microelectrode. No variation in pH was noted over the relatively wide range of pH 5.2 to 7.5.

[1] Preliminary reports of various parts of this work have been reported by Osaki et al. (6, 7).

RESULTS AND DISCUSSION

Nonstoichiometric Oxidation of Ascorbate by Ceruloplasmin

Oxidation of ascorbate by ceruloplasmin was observed both spectrophotometrically and manometrically as shown in Fig. 1. All the experiments were carried out with great care to avoid contamination with Cu(II). In the oxygen uptake measurements, 16 μM neocuproine, which is an effective copper ion chelator but unreactive toward ceruloplasmin (3, 6), was added to the reaction mixture to eliminate the possibility of oxygen consumption by any unforeseen inorganic copper contamination within the 3- to 4-hour testing period. The results shown in Fig. 1 clearly indicate that the activity of ceruloplasmin on ascorbate oxidation is catalytic, not stoichiometric as suggested by Morell et al. (5). As discussed previously (6), 836 mμmoles of ascorbate were oxidized by 0.369 mμmole of ceruloplasmin in the experiment shown in Fig. 1a. On the assumption that all 8 copper atoms in ceruloplasmin are active, the results obtained here indicate that 280 times as many ascorbate molecules as all the copper atoms present in ceruloplasmin are oxidized. On the basis of the assumption that 2 copper atoms are used for each molecule of ascorbate, the turnover of ascorbate for each copper atom is thus increased to 560.

The nonstoichiometric nature of ascorbate oxidation by ceruloplasmin was also revealed in oxygen uptake experiments (Fig. 1b). Here again, 5880 mμmoles of the substrate were oxidized by 2.25 mμmoles of ceruloplasmin, or 323 moles of ascorbate oxidized per mole of all the copper atoms present in ceruloplasmin. On the assumption that 1 molecule of oxygen is consumed in the oxidation of every 2 molecules of ascorbic acid by ceruloplasmin, the theoretical amount of oxygen which should be taken up in this manometric measurement is 72 μl, shown by the *horizontal broken line* in Fig. 1b. The experimental value obtained here agrees with the theoretical value. The validity of the assumption mentioned above is supported further by the results on hydrogen peroxide formation (discussed later).

Previously, Morell et al. (5) studied the catalytic activity of ceruloplasmin at an ascorbate concentration of 5.7×10^{-3} M, which is slightly higher than that used in the manometric measurements described here. They observed the rate for the first 20 or 25 minutes and reported that the oxidation of ascorbate was not appreciably greater than the rate in the absence of the protein. Fig. 1b shows that any conclusion derived from an observation of less than 30 minutes at this higher ascorbate concentration is not meaningful.

Difference between Susceptibility of Ceruloplasmin and Cu(II) toward Various Inhibitors

Conclusive proof of the ascorbate oxidase activity of ceruloplasmin was obtained from the experiments comparing the effects of various compounds on ascorbate oxidation, as catalyzed by both ceruloplasmin and Cu(II), and on the products formed during the oxidation of ascorbate by both catalysts. The results are summarized in Table I and Fig. 2.

The inhibitors of ceruloplasmin or Cu(II) can be divided into two classes: (a) those which inhibit ceruloplasmin at concentrations that do not affect Cu(II), e.g. citric acid, and (b) those which inhibit Cu(II) but do not affect ceruloplasmin
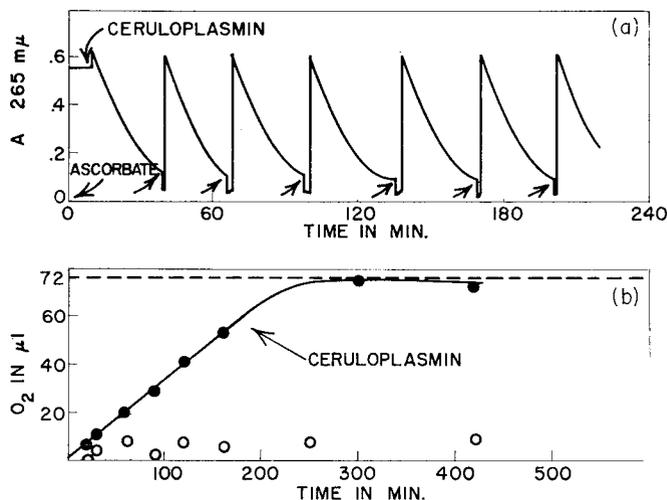

Fig. 1. The nonstoichiometric nature of the ceruloplasmin-catalyzed oxidation of ascorbate. *a*, the time course of the absorbance change at 265 mμ, reproduced from a continuous recording chart of a Beckman recording spectrophotometer, model DK-1. An ascorbic acid solution (20 μl) sufficient to produce a final concentration of 38 μM was added at the time indicated by the *arrow*. The reaction mixture contained 1.23×10^{-7} M ceruloplasmin and 0.2 M acetate buffer, pH 5.2, at 30°. *b*, microliters of oxygen consumed, plotted against time. The reaction mixture contained 7.5×10^{-7} M ceruloplasmin, 2×10^{-3} M ascorbic acid, and 1.65×10^{-5} M neocuproine in 0.2 M acetate buffer, pH 5.2, at 30°. The total volume of the reaction mixture was 3.00 ml. ● and ○, value of oxygen taken up with and without ceruloplasmin, respectively.

activity, e.g. neocuproine and serum albumin.[2] Citric acid, an example of the first class, was recently identified as the principal serum inhibitor of ceruloplasmin for both *p*-phenylenediamine and ascorbate oxidation (7). The inhibition by citrate was competitive for both ascorbate and *N,N*-dimethyl-*p*-phenylenediamine with K_I values of 4×10^{-7} and 7×10^{-7} M, respectively (9). A concentration of 5.0 μM citrate gave over 80% inhibition of ceruloplasmin, whereas no effect of 5.0 μM citrate was observed on the Cu(II) catalysis of ascorbate. On the other hand, 3.3 μM neocuproine or 0.2% bovine albumin inhibits Cu(II) more than 99%, whereas the ceruloplasmin-catalyzed oxidation of ascorbate is not affected (Table I).

Difference in Products Formed

The difference between the catalytic actions of ceruloplasmin and Cu(II) was also demonstrated in hydrogen peroxide formation during ascorbate oxidation. The amount of hydrogen peroxide formed was measured by peroxidase with the use of ascorbic acid as a hydrogen donor. The results are shown in Fig. 2. The first step of the reaction sequence is the ascorbate oxidation by Cu(II) or ceruloplasmin. Near the end of the reaction, another aliquot of ascorbate was added together with an inhibitor for Cu(II) or ceruloplasmin (*a* and *a'*). The oxidation of ascorbate by Cu(II) was completely inhibited by 6 μM neocuproine, and ceruloplasmin-catalyzed oxidation was inhibited more than 90% by 80 μM citrate. At this point, 4 μg of horseradish peroxidase were added to each reaction mixture

[2] Some of the early kinetic studies in which ascorbate was used as a ceruloplasmin substrate were reported by Walter and Frieden (3, 12).

TABLE I

Effects of various compounds on ascorbate oxidation by ceruloplasmin and Cu(II)

All experiments were carried out at pH 5.2 and 30° unless otherwise stated. In the citrate inhibition experiments 38 μM ascorbate, and in the neocuproine and albumin inhibition experiments 36 μM ascorbate, were used.

Inhibitor	Concentration[a]	Inhibition of ascorbate oxidation by		
		Ceruloplasmin[b]		Cu(II),[c] pH 5.2
		pH 5.2	pH 7.0	
		%	%	%
Citrate[d]	0.2	42		0
	0.33		40	
	0.5	62		0
	1.67		53	
	2.0	81		0
	5.0	88		0
	10.0	94		0
	16.7		87	
	84.0		97	
	100.0[e]	95		18
Neocuproine	1.6	0		37
	2.3	0		60
	3.3	0		100
	16.0	0		100
Bovine serum albumin	0.01	0		12
	0.03	0		40
	0.05	0		60
	0.10	0		80
	0.20	0		99

[a] Citrate and neocuproine concentrations are micromolar; bovine serum albumin concentration is given as per cent.

[b] Ceruloplasmin concentration was 1.5×10^{-7} M except in citrate inhibition, in which 1.6×10^{-7} and 2.7×10^{-7} M ceruloplasmin were used at pH 5.2 and 7.0, respectively.
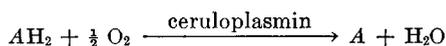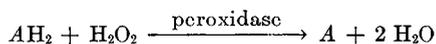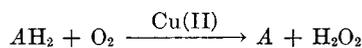
[c] Cu(II) concentration was 1.6×10^{-6} M.

[d] McDermott (9).

[e] Corresponds to the normal range of citrate concentration in human plasma.

as indicated by the *arrow* designated *Pox.* in Fig. 2. A significant difference was observed. A stoichiometric amount of ascorbate was oxidized when peroxidase was added to the Cu(II)-ascorbate system. However, none of the ascorbate was oxidized by peroxidase in the ceruloplasmin-ascorbate system.

These results clearly show that the oxidation catalyzed by Cu(II) results in hydrogen peroxide formation, whereas the ceruloplasmin-catalyzed reaction does not produce hydrogen peroxide, as indicated by the equations

$$AH_2 + O_2 \xrightarrow{\text{Cu(II)}} A + \underline{H_2O_2}$$

$$AH_2 + \underline{H_2O_2} \xrightarrow{\text{peroxidase}} A + 2 H_2O$$

$$AH_2 + \tfrac{1}{2} O_2 \xrightarrow{\text{ceruloplasmin}} A + H_2O$$

where AH_2 and A denote ascorbate and dehydroascorbate, respectively. These results are in agreement with those of

Powers and Dawson (13) and Dawson and Tokuyama (14), who showed that no hydrogen peroxide was formed during ascorbate oxidation by a plant ascorbic acid oxidase.

Numerous Kinetic Differences between Two Reactions

pH Dependence—Another marked difference between ascorbate oxidation by ceruloplasmin and by Cu(II) is the dependence on pH. As shown in Fig. 3, Cu(II) possess greater activity at the higher pH values within a pH range of 5.0 to 7.5. Ceruloplasmin gives maximum activity around pH 6.0; the activity gradually declines on both sides of this optimum pH, yielding a typical enzymic pH dependence curve as shown in Fig. 3. Holmberg and Laurell in 1951 (10), and more recently Curzon (11), reported a similar pH dependence for the activity of ceruloplasmin with *p*-phenylenediamine or *N*,*N*-dimethyl-*p*-phenylenediamine as substrate. The pH dependence curves for the oxidation of these three substrates by ceruloplasmin thus have similar characteristics.

Dependence of Activity on Ascorbate Concentration—The saturation of activity is also clearly illustrated in Fig. 4. The logarithm of percentage of substrate remaining plotted against time shows that Cu(II) oxidation is linear from zero time, as predicted for a first order reaction. However, ceruloplasmin oxidation is initially nonlinear, but becomes first order after the concentration of ascorbate declines to less than 2×10^{-5} M. The initial nonlinear portion of the ceruloplasmin oxidation curve represents a mixed order of both first and zero order rates. The difference in the rate dependence between ceruloplasmin and Cu(II) is also
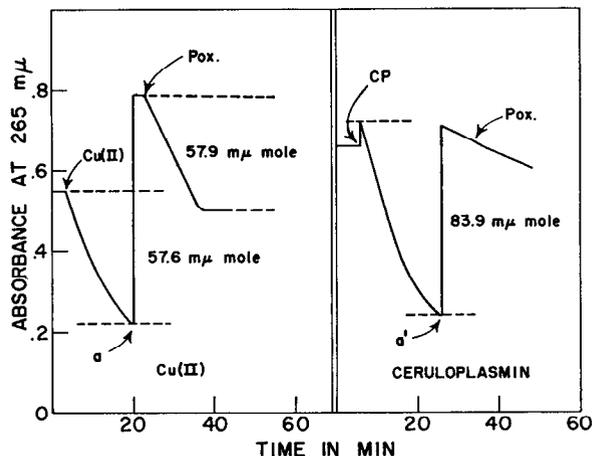

FIG. 2. Difference in peroxide formation in the Cu(II)- and ceruloplasmin-catalyzed oxidation of ascorbate. The time course of the absorbance change at 265 mμ is reproduced from a recording chart. At zero time, the reaction mixture contained 4×10^{-5} M ascorbic acid in 0.2 M acetate buffer, pH 5.2, at 30°. When ceruloplasmin (CP) was used as catalyst, 5 μM neocuproine was added to the reaction mixture at the beginning to block any effect due to contamination by Cu(II). Then ascorbate was oxidized either by Cu(II) (1.6×10^{-6} M) or by ceruloplasmin (2.7×10^{-7} M). At the points indicated by *a* and *a'*, a mixture of neocuproine and citric acid, or citric acid by itself, together with 20 μl of 5.7 mM ascorbic acid solution, was added to each reaction mixture. The final concentrations of neocuproine and citrate in the reaction mixture were 6 and 80 μM, respectively. The oxidation by Cu(II) was stopped completely by neocuproine, and ceruloplasmin oxidation was inhibited up to 90% by citrate. Peroxidase (4 μg) was then added to each reaction mixture to detect hydrogen peroxide with ascorbic acid as the hydrogen donor at the point indicated by the *arrow* designated *Pox.*
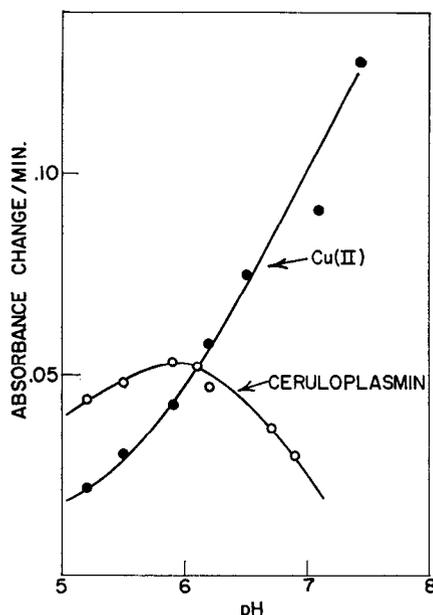
Fig. 3. The activities of ceruloplasmin and Cu(II) plotted against various pH values between 5 and 7.5. Acetate was used as a buffer throughout this experiment, and no pH change was observed at the higher pH values due to insufficient buffering. The concentrations of the substrate, ceruloplasmin, and Cu(II) were 4×10^{-5}, 2.7×10^{-7}, and 1.5×10^{-6} M, respectively.

illustrated by a plot of the rates at various concentrations of ascorbate shown in Fig. 5.[2] Also included in Fig. 5 is the effect of albumin on the ceruloplasmin and Cu(II) reaction (cf. Table I). Similar data showing different substrate curves for Cu(II) and ceruloplasmin were reported previously by Walter (3), who used ceruloplasmin that had not been treated with Chelex 100.

The first order rate constants calculated from the data in Fig. 1a were 0.023 per minute per μM Cu(II) and 0.053 per minute per μM ceruloplasmin copper at pH 5.2 and 30°. The values at pH 5.5 and 37° presented by Morell et al. were 0.033 per minute per μM Cu(II) and 0.00038 per minute per μM ceruloplasmin copper. The constant obtained for the Cu(II)-catalyzed reaction estimated from our data is 0.031 per minute per μM Cu(II), which agrees well with the value of Morell et al. (5) if we consider the pH and temperature difference. However, there is a large difference between the first order rate constant of the ceruloplasmin reaction reported by the two laboratories, because Morell et al. did not estimate the value of the constant at an ascorbate concentration where the rate is truly first order.

As reported previously, the rate of ascorbate oxidation by ceruloplasmin approaches a maximum at an ascorbate concentration of 5×10^{-5} M (Fig. 5), which corresponds to the average human plasma ascorbate concentration (6). The rate constant calculated from spectrophotometric measurements at a substrate concentration of 1×10^{-4} M was 11.9 μM ascorbate per minute per μM ceruloplasmin (6) whereas the value obtained from the manometric method at 1.95×10^{-3} M ascorbate was 11.4 μM ascorbic acid per minute per μM ceruloplasmin. These two values agree with each other within experimental error. The higher ascorbate concentration in the manometric method should give a rate constant at least 10 times greater than that at a lower ascorbate concentration if this oxidation were due to nonenzymic Cu(II) rather than ceruloplasmin. The zero order rate constant

of ceruloplasmin-catalyzed oxidation, however, can be estimated from the data presented by Morell et al. They observed a 10% decrease of ascorbate concentration, which initially was 5.7×10^{-3} M, in the presence of 1 μg of ceruloplasmin copper within approximately 20 minutes (5). The calculated zero order rate constant based on this observation is 14.4 μM ascorbate per minute per μM ceruloplasmin at pH 5.5 and 37°. This value is in reasonable agreement with the zero order rate constant of 11.4 μM ascorbate per minute per μM ceruloplasmin mentioned above at pH 5.2 and 30°. The difference between a zero order and a first order reaction is not readily distinguishable experimentally within the first 10% of substrate change, even when the experimental values are plotted semilogarithmically.

Differences of Activation Energy in Ascorbate Oxidation Catalyzed by Ceruloplasmin and Cu(II)—It was of interest to determine whether there was a difference in activation energies between the ceruloplasmin- and Cu(II)-catalyzed reactions. When
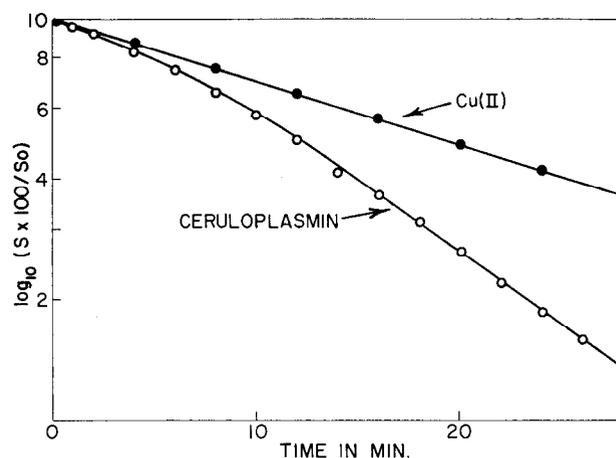

Fig. 4. Kinetic differences between ceruloplasmin and Cu(II) catalysis of ascorbate oxidation. The percentage of ascorbate remaining is plotted against time. The concentrations of ceruloplasmin, Cu(II), and initial ascorbate were 1.93×10^{-7}, 1.6×10^{-6}, and 4.8×10^{-5} M, respectively.
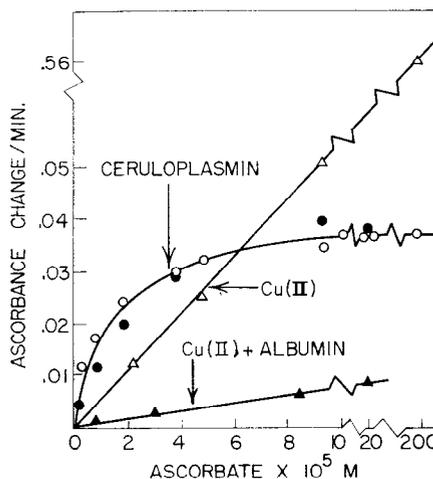

Fig. 5. Kinetic difference between catalysis of ascorbate oxidation by ceruloplasmin and Cu(II). The rate of ascorbate oxidation as the absorbance change at 265 mμ per minute is plotted against substrate concentration. Ceruloplasmin $(1.95 \times 10^{-7}$ M) or Cu(II) $(1.6 \times 10^{-6}$ M) was used with or without 0.15% bovine serum albumin as indicated.

the respective reaction rates were measured at six different temperatures between 5° and 35°, the calculated activation energy for the ceruloplasmin-catalyzed reaction was 12.5 kcal per mole and that for the Cu(II) reaction was 16.3 kcal per mole. The data obtained are shown in Fig. 6. While a difference of 3.8 kcal per mole is small, the measurements are believed to be sufficiently accurate to justify the conclusion that the activation energy of ceruloplasmin is significantly lower than the Cu(II) reaction under identical conditions.

The many kinetic differences between the ceruloplasmin- and the Cu(II)-catalyzed reactions discussed here and obtained by other workers are summarized in Table II, together with some qualitative differences. It is clear that the oxidation of ascorbate
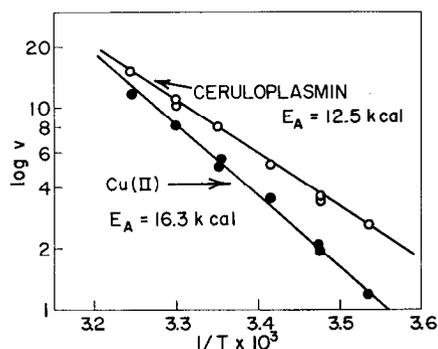

FIG. 6. The rates of ascorbate oxidation catalyzed by 3.9 × 10^{-7} M ceruloplasmin or 3.2 × 10^{-6} M Cu(II) at various temperatures between 5° and 35° are plotted against the reciprocal of the absolute temperature. The reaction mixture contained 3.8 × 10^{-5} M ascorbic acid in 0.2 M acetate buffer, pH 5.2, with each catalyst. The respective activation energies estimated were 12.5 kcal per mole for ceruloplasmin and 16.3 kcal per mole for Cu(II).

TABLE II

Summary of differences between ceruloplasmin- and Cu(II)-catalyzed oxidation of ascorbate

Kinetic parameters were obtained at pH 5.2 and 30° unless otherwise stated.

Parameter or property	Ascorbate oxidation catalyzed by	
	Ceruloplasmin	Cu(II)
K_m (μM)	13[a]	7000[b]
Zero order constant (μM min^{-1} μM^{-1})	14.4[c]	150[b]
As measured at 265 mμ	11.9	
As measured by O_2 uptake	11.4	
First order rate constant (minutes/ μM copper)	0.053	0.023
		0.033[d]
Reaction order at 100 μM ascorbate	Zero order	First order
Activation energy (kcal/mole)	12.5	16.5
Optimum pH	6.0	7.5
H_2O_2 formation	None	Stoichiometric[e]
Inhibition (%) by		
10 μM citrate	94	3
16 μM neocuproine	0	100
0.2% albumin	0	99

[a] Osaki *et al.* (6).

[b] Walter (3).

[c] Estimated from the data at pH 5.5 and 37° by Morell *et al.* (5).

[d] At pH 5.5 and 37° (Morell *et al.* (5)).

[e] (H_2O_2)/(ascorbate) = 1.

catalyzed by ceruloplasmin is greatly different from that catalyzed by Cu(II), and that there is no reason to believe that any Cu(II) contamination is present in the enzyme preparation. Thus, it can be concluded that ceruloplasmin has an authentic ascorbate oxidase activity.

Possible Significance of Ascorbate Oxidase Activity of Ceruloplasmin

Does the ascorbate oxidase activity of ceruloplasmin have any significance *in vivo*? To answer this question, we estimated the half-life of ascorbate, assuming that serum is a closed system with respect to ascorbate (*i.e.* ascorbate is moving neither in nor out), and that zero order kinetics applies at 5 × 10^{-5} M ascorbate (50 μM). As shown in Table II, the zero order rate for ascorbate oxidation is 11.9 μM ascorbate per minute per μM ceruloplasmin at pH 5.2. From Fig. 3 we can correct this rate downward at pH 7.4 to about 6 μM ascorbate per minute per μM ceruloplasmin, since the rate at serum pH is about one-half the rate at pH 5.2. The average concentration of ceruloplasmin in normal human serum is 2 × 10^{-6} M or 2 μM. Thus we can expect the oxidation of 12 μM ascorbate per minute. In slightly over 2 minutes, approximately 25 μM ascorbate will be oxidized. Therefore on this basis, the half-life of ascorbate in serum is conservatively estimated to be slightly over 2 minutes. A similar value is obtained if the appropriate calculation is made from the data in Fig. 4.

This unbelievably short half-life of serum ascorbate appeared to be inconsistent with the expected stability of ascorbate in normal human sera. Therefore, we sought an alternative explanation for the stability of serum ascorbate. We found evidence for a dialyzable serum inhibitor which proved to be citrate (7). The high concentration of citrate and its powerful inhibition of ceruloplasmin suggest that the ascorbate oxidase activity of ceruloplasmin in serum is held in check by the common metabolite, citrate. Similar arguments might also be extended to the other oxidase activities of ceruloplasmin. Because of its high concentration, ceruloplasmin, despite its low molecular activity, might be expected to oxidize appreciable fractions of serum epinephrine, serotonin, and their analogues if the activity of the enzyme were not effectively blocked by citrate. Thus the control by citrate of the stability in serum of epinephrine, serotonin, and their analogues, as well as ascorbate, may be of considerable importance in maintaining normal serum levels of these important compounds with their far reaching pharmacological activities.

SUMMARY

The catalyses of ascorbate oxidation by ceruloplasmin and Cu(II) have been compared and found to be different in every property tested. Numerous kinetic differences such as the dependence on pH, the effect of ascorbate concentration, and the relative activation energy have been found. Perhaps the most important difference is the specific sensitivity of the ceruloplasmin reaction to inhibition by citrate, and the specific inhibition of the Cu(II) reaction by neocuproine and serum albumin. The Cu(II) reaction has also been shown to produce a stoichiometric amount of hydrogen peroxide, whereas the enzyme produces none. It is concluded that ceruloplasmin has an authentic and distinctive ascorbate oxidase activity.

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