BBA 65713

ROLE OF IRON IN THE OXIDASE ACTIVITY OF CERULOPLASMIN

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(Received October 20th, 1967)

SUMMARY

Trace iron has been found to be associated with ceruloplasmin (ferroxidase)*** and the sodium acetate buffers used in test systems even after attempts to purify these substances by chromatography on Chelex-100 and Amberlite CG-50 columns. The concentration of iron eluted with ceruloplasmin from Chelex-100 columns was estimated to be as high as 10^{-8} M. The amount of ^{59}Fe eluted with ceruloplasmin increases proportionally with ceruloplasmin concentration. Ceruloplasmin, pre-equilibrated with ^{59}Fe, was dialyzed against apotransferrin, reducing the iron concentration to less than 10^{-8} M and the molecular activity for ascorbate to less than 1.

Several previously reported substrates of ceruloplasmin werereinvestigated with respect to the role of iron in the catalytic process. The reported substrates have now been classified into three groups:

1. Fe(II), which is oxidized directly by ceruloplasmin.

2. Certain aryldiamines and polyphenols; *e.g.*, *p*-phenylenediamine and its methyl derivatives, epinephrine, norepinephrine, dopamine, and serotonin, for which oxidation is not completely inhibited by iron chelators, are directly oxidized by the enzyme. However, the rates of oxidation of most of these substrates can be increased by iron *via* a Fe(II)–ceruloplasmin coupled reaction.

3. Numerous compounds which reduce Fe(III); *e.g.*, ascorbate, hydroquinone, catechol, hydroxylamine, thioglycolate, cysteine, ferro cyanide, and DOPA, for which oxidation is completely inhibited by iron chelators, appear not to be directly oxidized by the enzyme. Therefore, they must function in an iron–ceruloplasmin coupled reaction and are iron-dependent substrates. The inhibition of the oxidation of these iron coupled substrates by apotransferrin and citrate is due to their strong chelation of Fe(III).

INTRODUCTION

Ceruloplasmin has been shown to possess oxidase activity for a number of

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*** The name ferroxidase (ferro:O_2 oxidoreductase) has been suggested on the basis of its principal naturally occurring substrates[16].

substrates *in vitro*. In 1948–51, HOLMBERG AND LAURELL[1,2] firmly established the enzymic nature of ceruloplasmin and reported that *p*-phenylenediamine, hydroquinone, catechol, pyrogallol, DOPA, epinephrine, and ascorbate were substrates. In addition, they observed that hydrosulfite, ascorbate, hydroxylamine, and thioglycolate all reversibly decolorized ceruloplasmin in the presence of oxygen. This reaction was regarded as a criterion for substrates of ceruloplasmin. Subsequently, CURZON[3] found that the enzymic oxidation of *N,N*-dimethyl-*p*-phenylenediamine could be either activated or inhibited by varying the concentration of certain transition metal ions. CURZON AND O'REILLY[4] found Fe(II) to be a substrate and showed that the product, Fe(III), oxidized *N,N*-dimethyl-*p*-phenylenediamine. Thus, an activating effect of Fe(II) *via* a coupled iron–ceruloplasmin oxidation system was proposed, and it was suggested that any substance oxidizable by Fe(III) was potentially oxidizable by this coupled system[5].

Two mechanisms were proposed by CURZON[5] to explain the function of iron. First, that Fe(II) competed as a substrate with *N,N*-dimethyl-*p*-phenylenediamine for the same site on the enzyme; and, second, that there could be competition between *N,N*-dimethyl-*p*-phenylenediamine and ceruloplasmin for Fe(II). However, LEVINE AND PEISACH[6,7] reported the stimulation of the oxidase activity only by Fe(II) and postulated an Fe(II)–*p*-phenylenediamine complex as the active substrate.

In 1960, HUMOLLER *et al.*[8] reported that the oxidation of aromatic substrates and ascorbate occurred at separate sites of the enzyme involving differently bound copper atoms. WALTER[9] also proposed two sites, one which catalyzed the oxidation of both *p*-phenylenediamine and ascorbate, and one which only oxidized *p*-phenylenediamine. Subsequently, MORELL, AISEN AND SCHEINBERG[10] reported that the ascorbate oxidase activity of ceruloplasmin was due to traces of free Cu(II), removable by Chelex-100. However, OSAKI, MCDERMOTT AND FRIEDEN[11] found that the oxidation of ascorbate by ceruloplasmin and Cu(II) differed in many significant aspects and concluded that ceruloplasmin had an ascorbate-oxidizing activity independent of free Cu (II).

Many workers have reported inhibition of the oxidase activity by metal ion chelators. CURZON AND CUMINGS[12] have recently reviewed the interaction of ceruloplasmin with some of these inhibitors. LEVINE AND PEISACH[6] have reported two distinct mechanisms by which chelators inhibit: First, by removing contaminating metal ions, and, second, by an anion effect suggested earlier by HOLMBERG AND LAURELL[13] and also by CURZON[3]. In addition to typical transition metal ion chelators, OSAKI *et al.*[11,14,15] have reported a strong inhibition of the ascorbate-oxidizing activity of ceruloplasmin by the iron-binding protein, apotransferrin, and by citrate. In addition, it was found[15] that Fe(II)could activate the ascorbate-oxidizing activity of ceruloplasmin enormously, increasing the apparent molecular activity (MA) towards ascorbate from 11 to over 200.

In view of these facts, we have re-examined the oxidase activity of ceruloplasmin in an attempt to establish the catalytic specificity of this enzyme and the role of iron in its oxidase activity.

EXPERIMENTAL PROCEDURE

Materials

Ceruloplasmin

Crystalline human ceruloplasmin was obtained by a method described earlier[11].

A solution chromatographed on Chelex-100 (Bio-Rad) to eliminate non-enzymic Cu(II) gave a single peak in the ultracentrifuge with an s_{20} value of 6.3 and an absorbance ratio, $A_{280\ m\mu}/A_{610\ m\mu}$, of 22.0 ± 0.3. ε_{mM} at 610 mμ = 10.9 was used to determine concentrations.

Apotransferrin

Iron-free transferrin, purchased from Lloyd Bros., Inc., Cincinnati, Ohio was dissolved in Chelex-100-treated water. The stability and other physico chemical properties of apotransferrin have been described in a previous paper[16].

Conalbumin

Conalbumin, iron free, was purchased from Sigma Chemical Co.

^{59}Fe

A sterile solution of $^{59}FeSO_4$ (Lot No. FS-242-7) was purchased from Abbott Laboratories, Chicago, Ill. The stock sample contained 1.2 μg Fe/ml and had a specific radioactivity of 20.9 mC/mg Fe.

p-Phenylenediamine·2 HCl

A concentrated *p*-phenylenediamine·2 HCl (Eastman Organic Chemicals) solution was chromatographed on Chelex-100 to remove contaminating heavy metal ions and recrystallized using the method previously described[16].

p-Phenylenediamine·H_2SO_4

Twice recrystallized *p*-phenylenediamine (Eastman Organic Chemicals) was dissolved in 1 M H_2SO_4 at room temperature. The solution was cooled to 5°, and the *p*-phenylenediamine·H_2SO_4 was precipitated by adding acetone.

Desferyl mesylate

N-[5-(3-[(5-aminopentyl)hydroxy-carbamoyl]propionamido)pentyl]-3-([5-(*N*-hydroxyacetamido)pentyl]carbamoyl)propionohydroxamic acid methanesulfonate was generously provided by Ciba Pharm. Co., Summit, N.J. This reagent is a specific chelator for Fe(III) with a stability constant of log K = 31.

Ascorbic acid

Ascorbic acid (U.S.P., fine crystals, Lot No. 63372) was obtained from Merck and was used without further purification. Chromatography of this material on Chelex-100 had no effect on the ascorbate oxidation.

NADH

β-Dihydrodiphosphopyridine nucleotide, disodium salt was purchased from Sigma Chemical Co. The purity was 95% by spectral analysis. The mM absorbance at 340 mμ in 0.2 M sodium acetate buffer (pH 5.2) was estimated to be 6.22. A stock solution of 0.95 mM was prepared immediately before use. The $A_{340\ m\mu}$ of the stock solution was determined immediately after being prepared and again at the end of each experiment to test for decomposition during the duration of the experiment.

Other reagents

All other reagents were of the highest analytical grade available. In those cases where metal ion contamination was suspected, the reagents were chromatographed on Chelex-100. Stock solutions of the reagents used in these experiments were prepared just prior to use. It was necessary to take this precaution since some of the compounds were not stable in aqueous solution in the presence of atmospheric O_2.

Glassware

All glassware used was washed in detergent, soaked in nitric acid, and rinsed thoroughly with Chelex-100-treated water.

Methods

Rate measurements

Reaction rates were determined by a Cary-15, Beckman DK-1, DU or DB. Oxygen-uptake measurements used a polarographic O_2-electrode. The details of these measurements have been extensively described in recent reports[16,17].

Detection of radioactivity

Radioactivity was measured in a Nuclear Chicago Model DSS scintillation detector equipped with a Tracerlab VersaMatic III scaler. The efficiency of the counter for ^{59}Fe was about 28%.

Dialysis cells

Microdialysis cells (16-A Technilab, Inc.), used in all equilibrium dialysis experiments, were washed with EDTA, thoroughly rinsed with Chelex-100-treated water, dried, and stored in the refrigerator. The Visking tubing used in the dialysis cells was treated by the method of HUGHES AND KLOTZ[18].

Fe(II) determination

Fe(II) was determined by a modified procedure of SANDELL[19] and by use of ^{59}Fe. The first method permitted detection of iron between 3.6 and 72 μM whereas nM concentrations could be detected using ^{59}Fe.

Trace iron in the reaction mixture was determined by allowing two 1.00-ml samples of 3.6 μM ceruloplasmin in 0.2 M acetate buffer (pH 5.5 and 6.5, respectively) to equilibrate with 1.1 μM ^{59}Fe for 12 h in the cold room at 3°. In addition, two 1.00-ml samples of 0.2 M acetate (pH 5.5 and 6.5, respectively) were also allowed to equilibrate with 1.1 μM ^{59}Fe for the same period of time. Each sample was then placed on a column of Chelex-100 which had been pre-equilibrated with the specified buffers (pH 5.5 and 6.5) at 25°. The column dimensions were 22 cm × 0.8 cm with flow rates of approx. 1 ml/8 min. The ^{59}Fe concentration was determined from the eluates by radioactivity measurements. Protein recovery from the columns was greater than 95%, as determined by the method of LOWRY *et al.*[20].

Dialysis of ceruloplasmin, pre-equilibrated with ^{59}Fe vs. apotransferrin

Two 1-ml aliquots of 0.15 mM ceruloplasmin, $A_{280\ m\mu}/A_{610\ m\mu}$ 21.9 ± 0.3 and a molecular activity (for ascorbate) of 11, were each dialyzed against 1 ml 0.22 mM apotransferrin in microdialysis cells for 48 h at 5°. Each system was buffered with 0.2 M acetate buffer (pH 6.5). After dialysis, the $A_{280\ m\mu}/A_{610\ m\mu}$ and molecular activity were found to be approx. 21 and 3 for one sample and 21 and 1 for the other. Aliquots from each of these 0.15 mM stock ceruloplasmin samples were diluted with Chelex-100-treated 0.2 M acetate buffer (pH 6.5) to give final concentrations of 1.5 μM.

Additional microdialysis

Cells were filled by adding 1.1 ml 1.5 μM ceruloplasmin MA-3 or MA-1 to one side of the membrane, and 1.1 ml of 57 μM apotransferrin to the other. Then, 2 μl of the stock ^{59}Fe (1.2 μg/ml, specific activity 20.9 mC/mg Fe) was added to the ceruloplasmin side. The cells were agitated at 3° on a Burrell wrist action shaker, at the slowest shaking speed to avoid denaturation of the protein. Control samples containing only ceruloplasmin and buffer were used to determine the stability of the enzyme to shaking.

At the same time, 2-μl aliquots of stock ^{59}Fe were added to each of three test tubes and diluted with 1 ml of 0.2 M acetate buffer (pH 6.5) and used as standards. Duplicate samples were removed from the cells on the shaker at fixed periods of time

(*e.g.*, 21, 35, 92, and 164 h) in one experiment and single samples at 10, 58, 106, and 202 h and a duplicate sample at 250 h in a second experiment. One-ml aliquots from each side were pipetted directly into 1-cm cuvettes, the cuvettes covered with parafilm, inserted directly into the well of the counter, and counted 3 times each for 10-min periods. The concentration of iron in the samples and the per cent recovery of ^{59}Fe were calculated from the average of the three external standards.

Immediately after the samples were counted, the ascorbate-oxidizing activity was determined. The ceruloplasmin solution was incubated for about 10 min at 30° and the reaction was started by the addition of 10 μl of $1 \cdot 10^{-2}$ M ascorbate (final substrate concentration was 0.100 mM), and the rate was followed by the decrease in $A_{265\ m\mu}$. Control rates were obtained by testing the stock ceruloplasmin and the ceruloplasmin dialyzed against buffer.

RESULTS

Trace iron associated with ceruloplasmin and sodium acetate buffer

Iron could not be detected in 35 μM Chelex-100-treated ceruloplasmin or in 97 μM non-Chelex-treated ceruloplasmin using the method of SANDELL[19]. However, when 1.1 μM ^{59}Fe was used as a tracer, 1.6 and 1.8 nM iron ion were eluted from Chelex-100 columns (pH's 5.5 and 6.5, respectively) with 3.6 μM ceruloplasmin. This corresponds to an ^{59}Fe to ceruloplasmin ratio of $4.4–5.0 \cdot 10^{-4}$. When the ceruloplasmin concentrations were increased to 9.0 and 18.0 μM at 1.1 μM ^{59}Fe tracer, the ^{59}Fe eluted with ceruloplasmin increased to between 17 and 44 nM, an increase in the ^{59}Fe to ceruloplasmin ratio to about $2 \cdot 10^{-3}$. Thus, iron in the 10^{-8} M range is eluted from the Chelex-100 columns with 9–18 μM concentrations of ceruloplasmin. ^{59}Fe equilibrated with ceruloplasmin 1 or 12 h gave identical elution patterns for ^{59}Fe.

The non-Chelex-treated 0.2 M acetate buffer used in the enzyme assay system was found to contain 10.1–27.8 μM in iron. However, only about 1 nM ^{59}Fe was eluted

TABLE I

CHROMATOGRAPHY OF CERULOPLASMIN AND ^{59}Fe ON CHELEX-100

Columns *A* and *B* were equilibrated with 0.2 M sodium acetate buffer (pH 5.5) and eluted with the same buffer. Columns *C* and *D* were at pH 6.5 in 0.2 M sodium acetate buffer. Column dimensions were 21 cm × 0.8 cm. Flow rates were 1 ml/8 min.

Expt. No.		*Columns*			
		A	*B*	*C*	*D*
	Addition to columns				
1	μM ^{59}Fe	1.1	1.1	1.1	1.1
	μM ceruloplasmin	3.6	—	3.6	—
2	μM ^{59}Fe	1.1	1.1	1.1	1.1
	μM ceruloplasmin	9.0	18.0	9.0	18.0
	Elution from columns				
1	nM ^{59}Fe	1.6	0.8	1.8	0.8
	% ^{59}Fe	0.145	0.073	0.164	0.073
	^{59}Fe/ceruloplasmin × 10^4	4.4	—	5.0	—
2	nM ^{59}Fe	25	44	19	17
	% ^{59}Fe	2.3	4.0	1.7	1.5
	^{59}Fe/ceruloplasmin × 10^3	2.8	2.4	2.1	0.9

from Chelex-100 columns with acetate buffer. Acetate buffer eluted from Amberlite CG-50 contained unabsorbed trace ^{59}Fe comparable to those eluted from Chelex-100 columns. The results of Chelex-100 chromatography of both ceruloplasmin and acetate buffer pre-equilibrated with ^{59}Fe are summarized in Table I.

Effect of iron on ascorbate oxidation by ceruloplasmin

The molecular activity of two stock 150 μM ceruloplasmin (MA-11) samples was reduced to 3 and to 1, respectively, by dialysis against 220 μM apotransferrin for 48 h at 3°. The $A_{280\ m\mu}/A_{610\ m\mu}$ ratio and the *p*-phenylenediamine oxidase activity (assayed with 30 μM EDTA) of both stock samples were unaffected by this dialysis. Electrophoresis on cellulose acetate strips at pH 6.5 in 0.05 M sodium acetate buffer indicated single bands which migrated the same distance before and after dialysis of ceruloplasmin against apotransferrin.

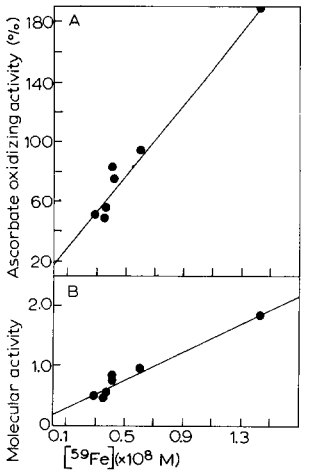
TABLE II

DIALYSIS OF CERULOPLASMIN *versus* APOTRANSFERRIN

In each experiment, 1.1 ml of 1.5 μM ceruloplasmin was dialyzed against 1.1 ml of 56 μM apotransferrin, in 0.2 M acetate buffer (pH 6.5). One-ml samples were withdrawn from each side, placed in a 1-cm quartz cell, and counted in a well-type γ-(scintillation) counter. The molecular activity of the ceruloplasmin was determined by adding 10 μl of 10^{-2} M ascorbate to the cuvette and following the decrease in absorbance at 265 mμ at 30°.

Expt. No.	*Time of dialysis (h)*	*% ^{59}Fe on ceruloplasmin side*	*Molecular activity, ascorbate*	*^{59}Fe (nM)*
1	21	36	3.0	14
	21	34	2.9	9
	35	14	1.7	6
	35	14	1.5	5
	92	10	2.4	4
	92	10	1.6	4
	164	8.5	1.4	3
	164	7.5	1.1	3
2	10	37	1.8	14
	58	15	1.0	6
	106	11	0.8	4
	154	11	0.8	4
	202	10	0.6	4
	250	8	0.5	3
	250	7	0.5	3

The results of equilibrium dialysis of 1.5 μM ceruloplasmin (MA-3 and MA-1) pre-equilibrated with 20 nM ^{59}Fe against 56 μM apotransferrin are summarized in Table II. The molecular activity for ascorbate decreases proportionally with ^{59}Fe concentration. The molecular activities calculated in Expt. 2 (Table II) were plotted as a function of ^{59}Fe concentration in Fig. 1. A plot of the data in Table II indicated the range of trace iron concentration in the stock ceruloplasmin solutions to be 21 ± 0.6 nM (MA-3) in Expt. 1 and 7.0 ± 2.6 nM (MA-1) in Expt. 2. Ceruloplasmin without added ^{59}Fe was also dialyzed against apotransferrin and the molecular activity with respect to ascorbate was observed to decrease as a function of time of dialysis. These



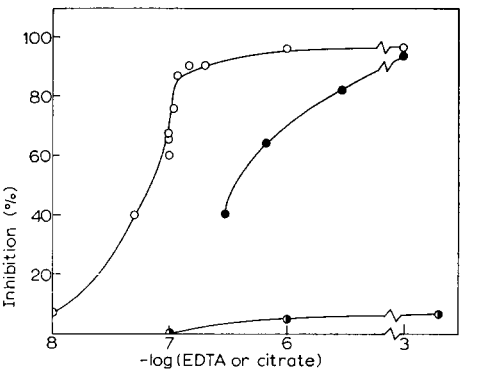


Fig. 1. Ascorbate-oxidizing activity and molecular activity as a function of ^{59}Fe. (A) The per cent ascorbate-oxidizing activity plotted as a function of ^{59}Fe in a system in which ceruloplasmin (MA-1) containing added ^{59}Fe had been dialyzed against apotransferrin in 0.2 M sodium acetate buffer (pH 6.5). (B) The molecular activity of ceruloplasmin for ascorbate plotted as a function of ^{59}Fe for the same system.

Fig. 2. Inhibition of the ascorbate-oxidizing and *p*-phenylenediamineoxidase activity of ceruloplasmin (MA-11) by EDTA and citrate. ○—○, inhibitory effect of EDTA on ascorbate oxidation measured by the decrease in absorbance at 265 mμ in a reaction mixture of 0.58 μM ceruloplasmin and 44 μM ascorbate in 0.2 M sodium acetate buffer (pH 6.3) at 30°; ●—●, effect of citrate on the same system; ◑—◑, effect of EDTA on the *p*-phenylenediamineoxidase activity in a reaction mixture of 0.58 μM ceruloplasmin, 2.5 mM *p*-phenylenediamine, 0.2 M sodium acetate buffer (pH 6.3) at 30°.

molecular activities were consistently lower than in the experiments in which ^{59}Fe was added. However, the *p*-phenylenediamine oxidase activity (assayed in 10 μM EDTA) and the $A_{280\ m\mu}/A_{610\ m\mu}$ value was unchanged before and after dialysis against apotransferrin. The enzyme activity of ceruloplasmin dialyzed against buffer did not change, indicating that the protein was not denatured during the shaking process and dialysis. Protein analysis[20] of all samples confirmed that the cells had not leaked.

Effect of EDTA, citrate, and iron-binding proteins on oxidase activity of ceruloplasmin (MA-11)

Fig. 2 shows the effect of EDTA on both the ascorbate and the *p*-phenylenediamine oxidase activities and the effect of citrate on the ascorbate-oxidizing activity of a typical ceruloplasmin preparation (MA-11). Both 0.05 μM EDTA and 0.4 μM citrate inhibit the ascorbate oxidase activity 50%, whereas 20 mM EDTA inhibits the *p*-phenylenediamine oxidase activity less than 10%. Of the known anions and organic acids present in serum (Cl^-, I^-, PO_4^{3-}, SO_4^{2-}, HCO_3^-, α-ketoglutarate, succinate, lactate, salicylate, malate, pyruvate, and citrate) only citrate showed significant inhibition of the ascorbate-oxidizing activity (0.1 mM inhibited 95%) when assayed in 0.2 M acetate buffer (pH 7.0).

As shown in Table III, apotransferrin and apoconalbumin show much less inhibitory effect on the *p*-phenylenediamine oxidase activity of ceruloplasmin after the substrate had been passed through a Chelex-100 column. Apoceruloplasmin,

TABLE III

EFFECT OF IRON-BINDING PROTEINS ON THE *p*-PHENYLENEDIAMINE OXIDASE ACTIVITY OF CERULOPLASMIN

Conditions: 0.57 μM ceruloplasmin and 35 μM *p*-phenylenediamine in 0.2 M sodium acetate buffer (pH 6.0) at 30°.

Addition	*Substrate before passing through Chelex-100 column*		*Substrate after passing through Chelex-100 column*	
	μM	*% Inhibition*	*μM*	*% Inhibition*
Apotransferrin	0.33	42	1.00	10
Apoconalbumin	1.00	31	2.50	7

0.75–46 μM, had no effect on the ascorbate or *p*-phenylenediamine oxidase activities when assayed with 0.17–0.86 μM ceruloplasmin in 0.2 M acetate buffer at pH's 5.2, 6.5, and 7.0 at 30°.

Effect of age on Fe(III) stock solutions on ascorbate oxidation by ceruloplasmin (MA-11)

The data listed in Table IV show that the age of the stock Fe(III) sample affects the rate of oxidation of ascorbate by ceruloplasmin (MA-11). The most dramatic change was found with 67 μM $Fe(NO_3)_3$. The freshly prepared $Fe(NO_3)_3$ gave 19-fold activation, whereas the week-old preparation reduced the molecular activity to 3. O_2, bubbled through a freshly prepared stock $FeCl_3$ solution, had no observable effect.

Rate of oxidation of ascorbate by Fe(III)

The pseudo first-order rate constant for the oxidation of ascorbate by Fe(III) in 0.20 M acetate buffer (pH 5.3) at 30°, was calculated to be 0.56 min^{-1}. This value was obtained by holding the ascorbate concentration constant at 100 μM and varying the Fe(III) concentration between 10 and 160 μM. Under these conditions, the reduction of Fe(III) by ascorbate is a faster reaction than the oxidation of Fe(II) by a catalytic amount of ceruloplasmin ($1–3 \cdot 10^{-7}$ M), as reported by OSAKI, JOHNSON AND FRIEDEN[16].

TABLE IV

EFFECT OF AGE OF Fe(III) SOLUTION ON ASCORBATE OXIDATION BY CERULOPLASMIN

Conditions: 0.29 μM ceruloplasmin, 100 μM ascorbate in 0.2 M sodium acetate buffer (pH 5.3) at 30°.

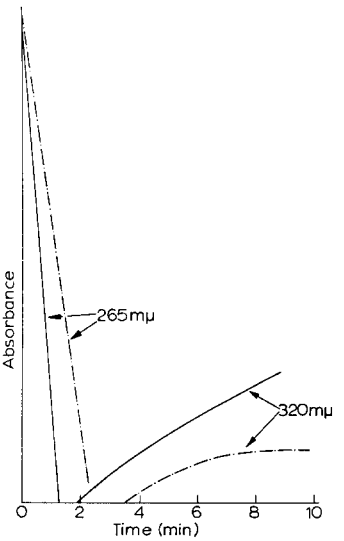
Addition	*Age of solution*	*Molecular activity*	*% Activation*
None	—	11	—
6.7 μM $FeCl_3$	1 h	93	750
6.7 μM $FeCl_3$	1 h*	93	750
6.7 μM $FeCl_3$	1 week	71	550
67.0 μM $Fe(NO_3)_3$	10 min	220	1900
67.0 μM $Fe(NO_3)_3$	1 week	3	73**

* O_2 was bubbled through $FeCl_3$ stock solution prior to addition to assay system.
** % Inhibition.

Reaction of Fe(II), ascorbate, and ceruloplasmin at 265 mμ

Fig. 3 shows the results of the reaction between Fe(II), ascorbate and ceruloplasmin at 265 mμ and 320 mμ. The appearance of Fe(III) which can be detected at 320 mμ is not observed until the ascorbate is completely oxidized in the system. If the concentration of Fe(II) is increased in the reaction mixture, the ascorbate disappears faster and the appearance of Fe(III) is observed earlier at 320 mμ. By decreasing the Fe(II) concentration, the rate of disappearance of ascorbate is reduced, delaying the appearance of Fe(III).

The data in Fig. 4 show that if trace iron is the rate-limiting factor in the reaction mixture, the concentration of apotransferrin-treated ceruloplasmin (MA-1) is no longer rate determining. Above 10^{-7} M ceruloplasmin, the rate of oxidation of Fe(II) by ceruloplasmin approaches the rate of reduction of Fe(III) by ascorbate. As expected, an increase in the ascorbate concentration resulted in an increase in the limiting rate of this cycle of reactions.



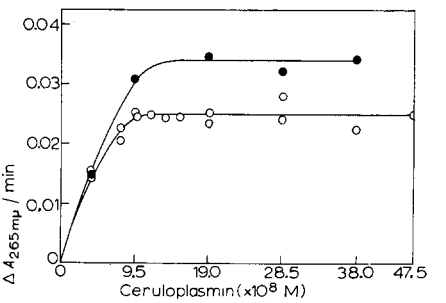


Fig. 3. Plot of absorbance *versus* time for the Fe(II)–ascorbate system observed at two different wavelengths. ———, two identical experiments followed at two different wavelengths. The oxidation of ascorbate is followed by the decrease in absorbance at 320 mμ, in a reaction mixture containing 0.26 μM ceruloplasmin, 100 μM ascorbate and 670 μM Fe(II) buffered by 0.2 M sodium acetate buffer (pH 5.3) at 30°. ·—·—·, two identical experiments in which the Fe(II) concentration is 67 μM.

Fig. 4. Rate of oxidation of ascorbate as a function of ceruloplasmin (MA-1) concentration. ○—○, 0.1 mM ascorbate; ●—●, 0.15 mM ascorbate. Conditions: 0.2 M sodium acetate buffer (pH 5.5) at 30°.

Other compounds as substrates for ceruloplasmin

The oxidase activity of ceruloplasmin toward a number of previously reported substrates[2] was investigated by following the rates of oxidation in the O_2-electrode at 30°. The experiments were designed to accomplish the following:

(a) to distinguish the substrates for which ceruloplasmin has oxidase activity, even when the iron present in the system is chelated;

(b) to compare iron-stimulated rates of ceruloplasmin-catalyzed oxidation of various substrates under uniform conditions;

(c) to ascertain the effect of the order of addition of iron upon the iron-stimulated rates.

To prevent stimulation of ceruloplasmin's oxidase activity by iron ion, EDTA was added to the reaction mixture before ceruloplasmin was injected. If oxidase activity was found for a particular substrate in the presence of EDTA, a substrate concentration was used at which v_{max} was obtained under these conditions, providing an estimate of molecular activity. If no activity could be observed in the presence of EDTA, a substrate concentration of 1.0 mM was used for these experiments.

The iron-stimulated rates were measured after injection of more $FeNH_4(SO_4)_2$ than the EDTA present (either 34.6 μM or 44.9 μM Fe(III) was added to the reaction mixture which contained 30 μM EDTA). Ethylenediamine was added (final concentration = 30 μM) to prevent complicating effects due to non-enzymic Cu(II) which might be displaced from its EDTA complex by injected Fe(III). Ethylenediamine does not chelate Fe(II) or Fe(III) appreciably at pH 5.2 (ref. 21).

TABLE V

MOLECULAR ACTIVITY AND OTHER KINETIC DATA FOR SUBSTRATES OF CERULOPLASMIN

Oxidation rate in μM $O_2 \cdot min^{-1} \cdot (\mu M$ ceruloplasmin$)^{-1}$. Conditions: 30°, 0.2 M acetate (pH 5.2), 0.72 μM ceruloplasmin. Under these conditions, the molecular activity of Fe(II) was 34.6. Order of additions was as follows: (a) Buffer, substrate and chelators (if appropriate) were added first in all experiments. (b) In Column *D*, Fe^{3+} (denoted as Addition *2*) was added 4–7 min prior to the addition of enzyme (denoted as Addition *3*). (c) In Column *E*, ceruloplasmin (denoted as Addition *2*) was added 2–3 min before the addition of Fe^{3+} (denoted as Addition *3*). (d) All additions are numbered consecutively. (e) Columns *C*, *D*, and *E* for epinephrine represent the standard deviation of the individual values of 7 experiments. All other ± values represent the range of values obtained in 3 experiments.

Compound	*Concn. (mM)*	*A* *No chelator* *(1) 0.72 μM ceruloplasmin*	*B* *(1) 30 μM ethylenediamine* *(2) 0.72 μM ceruloplasmin*	*C** *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 0.72 μM ceruloplasmin*	*D* *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 44.9 μM Fe^{3+}* *(3) 0.72 μM ceruloplasmin*	*E* *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 0.72 μM ceruloplasmin* *(3) 44.9 μM Fe^{3+}*
p-Phenylenediamine·H_2SO_4	10	21 ± 3	17 ± 1	12 ± 1	25 ± 1	22 ± 2
Serotonin creatinine sulfate	22	8.5 ± 1.7	5.7 ± 0.7	5.1 ± 0.6	15 ± 1	9.1 ± 2.8
Dopamine**	22	6.8 ± 1.1	7.7 ± 0.3	3.4 ± 0.6	12 ± 1	13 ± 1
Norepinephrine**	23	5.5 ± 0.3	5.7 ± 0.3	4.7 ± 0.6	9.1 ± 0.6	9.6 ± 1.1
L-Epinephrine	22	5.7 ± 0.6	5.2 ± 0.3	3.2 ± 0.2	4.9 ± 0.5	4.3 ± 0.6

* Saturating substrate concentrations were determined from *v vs.* [*S*] plots for each substrate. Since each substrate was determined at its maximum velocity, this number represents the molecular activity of ceruloplasmin with respect to each substrate.

** Dopamine and norepinephrine were prepared from the hydrochlorides by exchanging the chloride for sulfate on Dowex 1-X 8 anion-exchange columns in which the resin was precharged with sulfate.

TABLE VI

OXIDATION OF Fe-DEPENDENT "SUBSTRATES" IN THE PRESENCE OF CERULOPLASMIN

All conditions as stated in the text of Table V, except for different Fe^{3+} concentration. All "substrate" concentrations were 1.0 mM. 'None' represents an oxidation rate of < 0.2 μl O_2/min.

"Substrate"	*A* *No chelator* *(1) 0.72 μM ceruloplasmin*	*B* *(1) 30 μM ethylenediamine* *(2) 0.72 μM ceruloplasmin*	*C* *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 0.72 μM ceruloplasmin*	*D* *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 34.6 μM Fe^{3+}* *(3) 0.72 μM ceruloplasmin*	*E* *(1) 30 μM ethylenediamine, 30 μM EDTA* *(2) 0.72 μM ceruloplasmin* *(3) 34.6 μM Fe^{3+}*
Ascorbate	15 ± 4	16 ± 2	—*	18 ± 3	20 ± 3
L-Cysteine·HCl	1.5 ± 0.6	1.1 ± 0.3	None	26 ± 1	23 ± 3
L-DOPA	1.1 ± 0.3	0.7 ± 0.3	None	5.1 ± 0.6	8.5 ± 2.9
Hydroquinone	5.1 ± 0.6	3.1 ± 0.6	None	18 ± 1	15 ± 2
Catechol	3.0 ± 0.8	1.7 ± 0.3	None	11 ± 1	12 ± 3
Hydroxylamine·HCl	20 ± 1	18 ± 2	None	30 ± 2	26 ± 2
Thioglycolic acid	14 ± 2	11 ± 1	None	19 ± 2	19 ± 2
$K_4Fe(CN)_6\cdot(H_2O)_3$	13 ± 1	11 ± 1	None	10 ± 1	12 ± 4

* A rate of 0.5 ± 0.2 μM O_2/min was obtained upon addition of 0.72 μM ceruloplasmin. Addition to the chelated ascorbate system of less than μM Fe^{3+} (no enzyme present) produced the same rate. The same rates were observed if 114 μM EDTA was present. It is presumed that this small rate is due to the Udenfriend system composed of ascorbate, O_2, and EDTA in the reaction mixture, together with a small amount of Fe^{3+} added with the enzyme.

Since iron could conceivably form complexes, with some of the substrates, iron-stimulated rates in which the iron was pre-incubated with the system before 0.72 μM ceruloplasmin was added, were compared to those iron-stimulated rates in which the iron was added after the enzyme was injected.

The results of these experiments are summarized in Tables V and VI. An EDTA concentration of 30 μM was shown in all cases to be sufficient to eliminate the activating effect of iron, since addition of more EDTA or addition of other chelators such as desferyl mesylate and 1,10-phenanthroline did not further diminish the chelated rates. It should be noted that the stock $FeNH_4(SO_4)_2$ solution was pH 1.7 to avoid formation of iron polymers. When an equal volume (8.3 μl) of chelexed H_2O adjusted to pH 1.7 with H_2SO_4 was injected in place of the iron solution, no effect was observed, and the pH of the buffered reaction mixture was not changed.

To eliminate the possibility of inhibition due to high concentrations of chloride, *p*-phenylenediamine·H_2SO_4 was prepared. For the same reason, norepinephrine and dopamine were prepared from the hydrochlorides by exchanging chloride for sulfate on a Dowex 1 -X 8 anion-exchange column in which the resin was pre-charged with sulfate.

Comparison of test results from the O_2-electrode with spectrophotometric results indicated that the response time of the O_2-electrode was not a source of error below 30 μM O_2/min. Care was taken to keep all data within this limit. For Tables V and VI, the linear part of the rate following an initial lag was measured. At least three runs were made to establish the variation of each result.

The results separate the substrates into two groups: (1) a group of substrates for which ceruloplasmin has activity even when the iron in the system is chelated by EDTA, and (2) a group for which ceruloplasmin possesses oxidase activity only in the presence of unchelated iron. Substrates belonging to the first group include L-epinephrine, *p*-phenylenediamine·H_2SO_4, norepinephrine, dopamine, and serotonin. Substrates belonging to the second group include ascorbate, hydroquinone, $K_4Fe(CN)_6$, catechol, L-DOPA, hydroxylamine·HCl, thioglycolic acid, and L-cysteine·HCl.

The data in Columns *D* and *E* in Tables V and VI indicate that there are no significant differences due to the order of addition, with the possible exception of serotonin, but they show that iron activates, even though the effect is small for epinephrine. Columns *A* and *B* in Tables V and VI show that ethylenediamine which chelates Cu(II) in strong preference to Fe(III) has little effect on the system.

Metal ions as substrates and/or activators

Millimolar Fe(II), Co(II), Ni(II), Mn(II), Mg(II), Zn(II), Cu(II), and Fe(II) were tested as possible substrates and/or activators of the *p*-phenylenediamine oxidase activity of ceruloplasmin by measuring O_2 consumption. Fe(II) was found to be the only substrate among the metals tested. Pre-incubation of ceruloplasmin with mM concentrations Co(II), Ni(II), Mn(II), Mg(II), Zn(II), Cu(II) and Fe(II) had no effect on the oxidation of 13.7 μM Fe(II) by the enzyme. Only Fe(III) reacted with *p*-phenylenediamine before the addition of enzyme. This reaction was observed both spectrophotometrically and by O_2 consumption.

At 77 μM metal ion, the order of stimulating effect determined spectrophotometrically was Fe(II) > Fe(III) > Ni(II) > Zn(II) > Co(II) in a test system containing 23 mM *p*-phenylenediamine, 0.35 μM enzyme and 0.15 M sodium acetate buffer (pH 5.2) at 30°. O_2-uptake assays indicated the order of stimulating effect as Fe(II) > Ni(II) > Co(II) > Zn(II) in a system containing 41 μM metal ion, 4.6 mM *p*-phenylenediamine, 0.56 μM enzyme, and 0.2 M sodium acetate buffer (pH 5.2) at 30°. The order of stimulating effect reported by CURZON[3] was Fe(II) > Ni(II) > Zn(II) > Co(II) > Fe(III) in a system containing 2 μM metal ion, 4.6 mM *p*-phenylenediamine, 46 nM enzyme and 0.08 M acetate buffer (pH 5.5) at 25°. CURZON[3] also noted that at 20 μM, Fe(III) had a greater stimulating effect than all other metal ions investigated with the exception of Fe(II).

In every case, Fe(II) gave the greatest stimulation. Also, none of the metal ions investigated, with the exception of Fe(II), had an effect on the absorbance of ceruloplasmin at 610 mμ. Thus, it appears that Fe(II) is the only metal ion of those reported as activators which is a true substrate for ceruloplasmin.

An investigation of some cations, at their concentrations in serum, indicated that Al(III), Ca(II), Mg(II), and Mn(II) had no detectable effect on the ascorbate-oxidizing action of ceruloplasmin. However, 55 μM Zn(II) inhibited 55% while 17 μM Fe(III) activated 200%. The Cu(II) oxidation of ascorbate was inhibited by the addition of ceruloplasmin as reported earlier[11].

Role of Fe(II) in the NADH-coupled system

When *p*-phenylenediamine was used as a substrate in the NADH-coupled system described by WALAAS AND WALAAS[22], the reaction observed at 540 mμ was inhibited from 26 to 40% by μM concentrations of the iron chelators EDTA, desferyl

mesylate, citrate, and 1,10-phenanthroline and activated 80% by 42 μM Fe(II). However, this coupled reaction was not effectively inhibited by these chelators as in the ascorbate system. When DL-norepinephrine was used as the substrate in the NADH-coupled system, there was a slight activating (1–6%) effect observed by 42 μM Fe(II) and 40–50 μM concentrations of chelators with the exception of 8.3 mM EDTA which showed an activation of 20%. This unexpected observation may be explained by a spectroscopically compensating phenomenon near 340 mμ due to the formation of colored product(s) from DL-norepinephrine oxidation[23].

DISCUSSION

Determination of trace iron in the components of the reaction mixture

The 50% activation of the *N,N*-dimethyl-*p*-phenylenediamine and *p*-phenylenediamine oxidase activities by 0.1 μM Fe(II)[3,7] made it essential to determine the amount of trace iron in the test system. The data presented in Table I indicate that iron in the 10^{-8} M range is eluted from Chelex-100 columns with 9–18 μM ceruloplasmin concentrations. This concentration of iron is 2–5% of the lowest K_{m1} value calculated by OSAKI[24] for the oxidation of Fe(II) by ceruloplasmin, and, therefore, could allow a measurable rate of oxidation.

Effect of iron on ascorbate oxidation by ceruloplasmin

Since iron was found in the components of the test system, its effect on the ascorbate-oxidizing activity of ceruloplasmin was investigated. Dialysis of ceruloplasmin against apotransferrin caused a decrease in the molecular activity with respect to ascorbate, with no other detectable effect on the ceruloplasmin. This suggested that the decrease in ascorbate oxidation was the result of the removal of trace iron from the test system by apotransferrin. Additional dialysis experiments on the MA-3 ceruloplasmin indicated that the molecular activity in one case was decreased from 3.0 ± 0.2 to 1.0 ± 0.4. The concentration of trace iron which produces this molecular activity was calculated to be 7.0 ± 2.6 nM. Thus, it was estimated that the original ceruloplasmin sample before dialysis against apotransferrin contained about 72 nM iron and that the ceruloplasmin sample after dialysis against apotransferrin but before the addition of ^{59}Fe contained about 21 nM iron.

Data in Fig. 1 show that the ascorbate-oxidizing activity remaining after 10 h to be greater than the control. This results from the fact that the control (MA-1) contained less iron than the 19.5 nM ^{59}Fe initially added to each of the dialysis cells. Although the molecular activity for ascorbate decreased proportionally with ^{59}Fe concentration, the *p*-phenylenediamine oxidase activity of ceruloplasmin is not completely dependent on iron since even the addition of mM EDTA showed less than 10% inhibition of *p*-phenylenediamine oxidase activity.

Effect of chelators on oxidase activity

Several interpretations have been offered to explain the effect of chelators, particularly EDTA, on the *p*-phenylenediamine oxidase activity of ceruloplasmin from several different sources[3,6,25].

BROMAN[25] has suggested that EDTA binds the copper in ceruloplasmin and thus inhibits the enzyme activity. However, BLUMBERG *et al.*[26] have demonstrated that

EDTA has an insignificant effect on the ceruloplasmin copper. CURZON[3] suggested that the EDTA removes iron which activates the *p*-phenylenediamine–oxidase activity. LEVINE AND PEISACH[6] have postulated an Fe(II)–*p*-phenylenediamine complex as the active substrate for ceruloplasmin and have suggested that EDTA caused inhibition by competing with the substrate for Fe(II).

While we do not doubt the existence of an Fe(II)–*p*-phenylenediamine complex at sufficiently high ligand concentrations[27], compelling evidence for a role of this complex in ceruloplasmin activity is lacking. The observation that Fe(II) appears not be oxidized by the enzyme in the presence of *p*-phenylenediamine is due to rapid reduction of Fe(III) by *p*-phenylenediamine. This can be explained by the fact that Fe(II) is the best substrate for ceruloplasmin[15,16] and will compete with *p*-phenylenediamine for the active site. This is supported by CURZON[5] who postulated a competition between Fe(II) and *p*-phenylenediamine for the active site, and OSAKI[24] who reported kinetic evidence for the competition of the two substrates. Therefore, both these substrates might be oxidized at the same site(s). Since LEVINE AND PEISACH[6] followed the reaction by observing the increase in absorbance of the oxidation product of *p*-phenylenediamine at 490 mμ, the inhibition observed might have resulted from the preferred oxidation of Fe(II). Recently, OSAKI[24] has shown that by increasing the Fe(II) concentrations from 10 to 50 μM, the apparent *p*-phenylenediamine oxidase activity recorded at 540 mμ is inhibited. Thus, the preferential oxidation of Fe(II) by ceruloplasmin could explain the apparent inhibition of the *p*-phenylenediamine oxidation measured spectrophotometrically. In addition, no inhibition by Fe(II) on the *p*-phenylenediamine oxidase activity in this system was observed in O_2-uptake measurements.

BLUMBERG *et al.*[26] have demonstrated from proton relaxation and electron spin resonance studies that chelators such as EDTA do not react directly with the ceruloplasmin copper. OSAKI *et al.*[11,14,15] have reported a strong inhibition by apotransferrin and citrate on the ascorbate-oxidizing activity of ceruloplasmin. However, no complex could be demonstrated between apotransferrin and ceruloplasmin. Therefore, the inhibition by apotransferrin and other iron chelators probably involves the removal of trace iron.

Stimulation of the ascorbate oxidase activity of ceruloplasmin was approximately the same with both Fe(II) and Fe(III). This is in contrast to the data on *N*,*N*-dimethyl-*p*-phenylenediamine presented by CURZON[3], who found Fe(II) to give faster rates than Fe(III), and LEVINE AND PEISACH[6,7] who found no stimulation by Fe(III). These observations may be explained by comparing the enzymic rate of oxidation of ascorbate with fresh Fe(III) solutions with Fe(III) solutions more than a week old, which showed less activation than those freshly prepared. This is believed to be the result of the formation of iron polymers at pH's above 5 which have recently been reported by SALTMAN[28].

Role of iron in the catalytic process

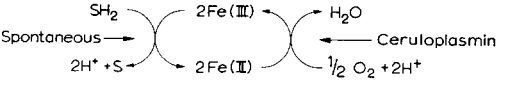
Recently, OSAKI *et al.*[15,16,24] have investigated the kinetic parameters of the Fe(II) oxidation by ceruloplasmin and found it to be the best substrate for this enzyme. When the rate of oxidation of Fe(II) by ceruloplasmin in the presence of ascorbate was measured, the appearance of Fe(II) was not observed spectrophotometrically until essentially all of the ascorbate had been oxidized. If Fe(III) was reduced by

ascorbate as fast as it was produced by the enzymic oxidation, one would not expect to observe its formation in the presence of ascorbate. Thus, if ascorbate has the same role as *N*,*N*-dimethyl-*p*-phenylenediamine in CURZON AND O'REILLY's system[4], Fe(III) should oxidize ascorbate. The pseudo first-order rate constant for this reaction was calculated to be 0.558 min^{-1}. From the data presented by OSAKI, JOHNSON AND FRIEDEN[16], the pseudo first-order rate constant for the oxidation of Fe(II) by ceruloplasmin appears to be the rate-limiting step. This is also indicated by the data of Fig. 4, in which increasing ceruloplasmin showed no effect on the rate of oxidation of ascorbate. Thus, a coupled iron ceruloplasmin reaction similar to the one described by CURZON AND O'REILLY[4] for *p*-phenylenediamine oxidation could be involved in the oxidation of ascorbate.

The evidence reported by OSAKI, MCDERMOTT AND FRIEDEN[11] that ceruloplasmin has ascorbate oxidase activity independent of Cu(II) is explained by the data presented here indicating that iron at a concentration sufficient to give the observed ratios is eluted with ceruloplasmin from Chelex-100. OSAKI, MCDERMOTT AND FRIEDEN[11] demonstrated that this activity differs from the Cu(II)-catalyzed oxidation in numerous key features. Kinetic differences were noted for pH dependence, the effect of ascorbate concentration and the relative activation energies. These differences can be explained by the fact that Fe(II) is the substrate rather than ascorbate. The differences in effects observed for the inhibitors can also readily be interpreted. KLOTZ AND CURME[29] have demonstrated the copper is bound rather strongly by albumin. The preferential affinity of neocuproine for Cu(I) relative to iron is also well known[30]. Studies on the Cu(II)-catalyzed oxidation of ascorbate showed that citrate had little effect on this oxidation, whereas citrate inhibited the enzymic oxidation more than 95% (ref. 11). This is probably an indication of the relative stability constants of Fe(III) citrate ($\log K = 11.85$) and Cu(II) citrate ($\log K = 5.2$) complexes[31]. It had been demonstrated previously that citrate is a potent inhibitor of the ascorbate-oxidizing activity of ceruloplasmin[14] probably due to the binding of traces of iron. Apotransferrin could inhibit in the same way. Finally, no hydrogen peroxide is formed by the enzymic oxidation of Fe(II) to Fe(III) by ceruloplasmin[24], whereas a stoichiometric amount of H_2O_2 was observed in the Cu(II)-catalyzed oxidation of ascorbate. Thus, if ceruloplasmin could be freed of trace iron ion, we conclude that it would have no ascorbate oxidase activity.

Other compounds as substrates for ceruloplasmin

In light of the previously reported data showing that the ascorbate-oxidizing and other oxidase activities of ceruloplasmin are dependent on iron, other substrates were studied to determine if they were involved in an iron–ceruloplasmin coupled reaction as illustrated below (SH_2 represents an oxidizable substrate):



From the previous discussions and data presented in Tables V and VI the following generalizations have been made about the substrate specificity of ceruloplasmin:

1. Fe(II) is oxidized directly by the enzyme.

2. Certain aryldiamines and polyphenols; *e.g.*, *p*-phenylenediamine, epinephrine, norepinephrine, dopamine, and serotonin, for which oxidation is not completely inhibited by iron chelators, are directly oxidized by the enzyme. However, their oxidation can be activated by Fe(III), and it is likely that the iron–ceruloplasmin coupled reaction is responsible for at least part of the activation. Since the iron-activated rate with epinephrine is still relatively slow, this substrate might be an exception.

3. Numerous compounds which reduce Fe(III); *e.g.*, ascorbate, hydroquinone, catechol, hydroxylamine, thioglycolate, ferrocyanide, and DOPA are not oxidized when traces of iron are removed by selected chelators. It is probable that the iron–ceruloplasmin coupled reaction is responsible for their oxidation as with ascorbate, and, thus, they are iron-dependent substrates.

The data presented in this paper are consistent with the coupled iron–ceruloplasmin oxidation mechanism presented by CURZON AND O'REILLY[4]. Fe(II) is oxidized to Fe(III) by ceruloplasmin, and any compound which reduces Fe(III) is potentially oxidizable *via* the coupled iron–ceruloplasmin system.

From the data available on the Fe-independent substrates, it appears that there must be at least two functional groups in the ring of aromatic substrates which increase the ring electron density. If one of these groups is an $-NH_2$, the compound is a substrate. However, if there is no $-NH_2$ group present, there have to be three groups appropriately substituted in the aromatic ring for the compounds to be substrates. This is borne out by the fact that the aryldiamines, *p*-aminophenol, serotonin, and the catacholamines except DOPA are all directly oxidized by the enzyme, whereas catechol and hydroquinone are not.

Metal ions as substrates and/or activators

Transition metal ion activation of the *p*-phenylenediamine oxidase activity reported by CURZON[3] has subsequently been attributed to contamination by trace amounts of Fe(II) by PEISACH AND LEVINE[7]. If Fe(II) were responsible for this stimulatory effect observed by other metal ions, it would have to be present in contaminating concentrations of 5–30% to produce the stimulation observed by 1 μM Fe(II).

In addition, 30 μM Zn (II) has been shown to have an inhibitory effect on the ascorbate oxidase activity of ceruloplasmin, while 30 μM Ni(II) has no observable effect on this activity. If these metal ions which stimulate *p*-phenylenediamine oxidase activity have a direct effect on the enzyme, one would expect to see a similar effect on the ascorbate-oxidizing activity which is actually a measure of the Fe(II) oxidation by the enzyme. Also, none of the metal ions investigated as substrates, with the exception of Fe(II), had an effect on the absorbance at 610 mμ which has been observed for all true substrates. Therefore, it appears that Fe(II) is the only metal ion of those reported as activators which is a true substrate for ceruloplasmin. These results suggest that activation by other metal ions does not involve a mechanism analogous to that occurring with iron. Since the oxidation of *p*-phenylenediamine by ceruloplasmin goes through several free radical intermediates resulting in a mixture of products, it is possible that the effect of metal ions other than Fe(II) results from reaction with one of these intermediates. CURZON AND CUMINGS[12] have reported inhibition by one of the products of *p*-phenylenediamine oxidation by ceruloplasmin. The effect of these other metal ions may be to complex and/or react with an inhibitory

product in some way so that it can no longer inhibit the enzyme. The net result would be an apparent activation.

ACKNOWLEDGEMENTS

This investigation was supported in part by Grant HE 08344, U.S. Public Health Service, and by Contract AT-(40-1)2690, Division of Biology and Medicine, U.S. Atomic Energy Commission.

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