The Possible Significance of the Ferrous Oxidase Activity of Ceruloplasmin in Normal Human Serum*

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SUMMARY

The oxidation of Fe(II) by serum was studied at pH 7.35 and at various oxygen concentrations which approach the physiological conditions of human serum. The nonenzymatic oxidation of Fe(II) was estimated to be insufficient to account for a rate of Fe(III)-transferrin formation necessary to provide an adequate iron supply for hemoglobin and other biosyntheses if Fe(II) is a relevant source of serum iron. The results suggested that an appreciable catalytic activity was involved in Fe(II) oxidation in serum. The ferroxidase activity of various normal human sera correlates precisely with the p-phenylenediamine oxidase activity of these sera. This catalytic activity is inhibited by azide and cyanide, and is low in sera with reduced ceruloplasmin levels. Fe(II) oxidation is also associated with the ceruloplasmin fraction on diethylaminoethyl cellulose chromatography of serum. The oxidation of Fe(II) by ceruloplasmin is zero order with respect to oxygen (10 to 200 μM), whereas the nonenzymic oxidation is first order. A biological role for the ceruloplasmin in serum is promoting the rate of iron saturation of transferrin and in stimulating iron utilization and the designation of the enzyme as serum ferroxidase is proposed.

It is generally believed that the iron entering the blood stream from the intestine is mostly in the ferrous form (1). In the plasma, Fe(II) is rapidly oxidized to Fe(III), and is incorporated into the specific iron-binding protein, transferrin, which can bind two atoms of Fe(III) per each protein molecule, forming a red Fe(III)-protein.1 When Fe(II) is added to apotransferrin, oxygen is required for the formation of the red complex, and the rate of color formation depends on the rate of Fe(II) oxidation to Fe(III) by molecular oxygen (2). In 1933, Hendrych and Mori (3) studied the rate of Fe(II) oxidation in whole blood in the presence of various amounts of oxyhemoglobin, and they found that the rate of the oxidation depended on the amount of oxyhemoglobin. They suggested a catalytic role of oxyhemoglobin on Fe(II) oxidation in vivo. However, it does not appear that they considered the high spontaneous oxidation of 0.02 M Fe(II) in whole blood. Koechlin (2), in 1952, mentioned that the rate of complex formation between ferrous iron and apotransferrin was enhanced by certain catalytic factors present in plasma. In 1961, Curzon (4), in a study of the Fe(II)-p-phenylenediamine-coupled oxidation by ceruloplasmin, discounted the possibility of significant Fe(II) oxidation by ceruloplasmin in blood. Curzon stated that "the finding that the considerable iron-oxidizing power of blood (Starkenstein & Harvalik, 1933) [5] is unaffected by cyanide (Hendrych & Mori, 1933) [3], which inhibits ceruloplasmin, suggests that the latter is unlikely to play much part in this oxidation." He mentioned further the experiments in vivo of Chase et al. (6), in which copper-deficient rats absorbed less iron than rats supplied with copper, and the report of O'Reilly that coupled iron-ceruloplasmin oxidation in the presence of p-phenylenediamine occurred in plasma at higher iron concentrations than with purified ceruloplasmin.

In 1960, Curzon and O'Reilly (7) noted the catalytic oxidation of Fe(II) by ceruloplasmin, but no further studies of Fe(II) as a substrate have been reported. In a more recent study, we noted that Fe(II) was the substrate of ceruloplasmin with the highest molecular activity at 30° of 550 at pH 6.5 in 0.20 M acetate (8). These facts led us to reinvestigate the problem of serum Fe(II) oxidation under conditions which approach the state in vivo. From these experiments, a biological role for ceruloplasmin in iron metabolism may ensue from its catalytic activity in converting Fe(II) to Fe(III), thereby promoting the rate of incorporation of Fe(III) into apotransferrin.

EXPERIMENTAL PROCEDURE

Materials

Serum—Fresh samples of 10 ml of whole blood from 19 donors (including two advanced pregnancies and fetal cord blood) were collected and allowed to clot in a cold room (4°). The serum was separated by centrifugation, stored in an ice bath, and used within 12 hours.

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* S. O'Reilly, personal communication to G. Curzon (4).

1 Apotransferrin, transferrin, and Fe(III)-transferrin are defined as the iron-free transferrin, the iron-saturated transferrin, and the ferric iron bound to transferrin, respectively.

2746
Ceruloplasmin—Crystalline ceruloplasmin was obtained by the method described earlier (9), and treated by a column of Chelex 100 (Bio-Rad) to eliminate nonenzymic copper ion from ceruloplasmin (10). A solution of crystalline ceruloplasmin was ultracentrifugally homogeneous with an s_{20,w} value of 6.3 S and an absorbance ratio (A_{280}:A_{410}) of 22.0 ± 0.3 (8).

Ferrous Iron—Crystalline ferrous ammonium sulfate hexahydrate (Fe(NH₄)₂(SO₄)·6H₂O; J. T. Baker, Lot 25899) was used as the ferrous ion source. The reagent was dissolved in Chelex 100-treated water immediately before use.

p-Phenylenediamine-2HCl—A concentrated p-phenylenediamine-2HCl (Eastman) solution, dissolved in 0.05 M acetate buffer of pH 5.2, was treated by a Chelex 100 column, pre-equilibrated to the same buffer, to remove contaminating heavy metals (mostly copper and iron). Recrystallization of the reagent was then carried out according to the method reported by Henry et al. (11). The crystals thus obtained were stored in a brown bottle and dissolved in 0.3 M acetate buffer, pH 5.2, just before the activity measurement of sera. The concentration of the stock solution was 2.76 × 10^{-2} M.

Apotransferrin—Iron-free transferrin (Lloyd Brothers, Inc., Cincinnati) was dissolved in Chelex 100-treated water at 2.2 × 10^{-4} M and stored in an ice bath. Spectrophotometric analysis showed that the stock solution contained more than 98% apotransferrin. Electrophoresis of the apotransferrin on cellulose acetate strips, with two different buffers (pH 7.0, 0.05 M acetate; pH 8.8, high resolution buffer, Gelman Instrument Company), produced only a single band.

**Methods**

\[
\begin{align*}
\text{H₂O} & \xrightarrow{\text{ceruloplasmin}} \text{2 Fe(II)} \\
\overline{\text{I₂O₃}} & \xrightarrow{\text{reduced ceruloplasmin}} \text{2 Fe(III)} \\
& \xrightarrow{\text{apotransferrin}} \text{transferrin}
\end{align*}
\]

Two methods were used to study Fe(II) oxidation in this work. As indicated in the above diagram, 0.5 mole of oxygen is consumed and 1 molecule of transferrin is formed. Both measurements have been made as described below.

**Rate of Fe(III)-Transferrin Formation**

In the spectrophotometric method, the 1.00-ml reaction mixture contained 0.0135 M phosphate or 0.20 M acetate buffer: 55 μM apotransferrin, ceruloplasmin, or serum; an aliquot of ferrous ammonium sulfate; and 100 μM ascorbate, where indicated. The amount of Fe(III)-transferrin formed was calculated from the absorbance change at 460 μM for each Fe(III) in transferrin, based on the molar absorbance constant per Fe(III), ε_{460} = 2500 M⁻¹ cm⁻¹ for Fe(III)-transferrin, reported by Assa et al. (12).

The formation of Fe(III)-transferrin was measured at 460 μM in a 1-cm quartz cuvette with a Cary 15 spectrophotometer equipped with 0.1 absorbance expansion and a constant temperature cell at 30.0°C ± 0.1°C. The reaction mixture of 0.85 ml, containing all components except Fe(II), was deoxygenated by bubbling N₂ gas (prepurified N₂ gas, typical O₂ content, 8 ppm, Matheson) for 3 min. The substrate, dissolved in water which was air-equilibrated at 0°C, was transferred to a syringe and equilibrated at room temperature (27°C). Then, 150 μl were injected rapidly into the reaction mixture through a 20-gauge hypodermic needle with a flow rate of approximately 5 ml per sec, with rapid and sufficient mixing.

**Rate of Fe(II) Oxidation Measured by Oxygen Electrode**

The change in oxygen concentration was measured in a 4.2-ml reaction mixture with a Beckman polarographic oxygen sensor, model 39065, polarized at −0.80 volt and connected to a Sargent recorder, model S.R., equipped with a range switch between 1.25 and 125 mv. The oxygen concentration of the reaction mixture in an air-tight chamber was directly recorded on a chart. The minimum change in oxygen concentration detectable is 0.01 μM at maximum sensitivity. The oxygen sensor and reaction chamber were carefully thermostated at 30.00°C ± 0.01°C. To the reaction mixture containing oxygen of the desired concentration, 5 to 10 μl of a deoxygenated Fe(II) solution were injected by a microsyringe. Each micromole of oxygen was used to oxidize 4 μmoles of Fe(II) to Fe(III). No H₂O₂ formation was detected in either the enzymic or the nonenzymic oxidation of Fe(II).4

**p-Phenylenediamine Oxidation Measured Spectrophotometrically**

The change in absorbance at 540 μm due to the catalytic activity of human serum was measured in a 1-cm quartz cuvette with a Cary 15 spectrophotometer at 30°C. The reaction mixture contained 200 μl of serum, 9.2 mM p-phenylenediamine, and 0.20 M acetate buffer of pH 5.2. Total volume of the reaction mixture was 0.60 ml.

**RESULTS AND DISCUSSION**

**Incorporation of Fe(II) into Apotransferrin by Different Sera**

Since the principal form of absorbed iron is believed to be Fe(II), the rate of incorporation of Fe(II) into transferrin was examined. In the data summarized in Fig. 1, various concentrations of the indicated sera, supplemented with 55 μM apotransferrin and 100 μM ascorbate, were studied for the rate of Fe(III)-transferrin formation. In human serum, this reaction is inhibited almost 100% in the presence of 1 mM azide or cyanide. Chicken serum, which is low in ceruloplasmin, shows a greatly reduced Fe(III)-transferrin formation rate. The rate is also appreciably less in young Rana pipiens tadpole serum, which is also low in ceruloplasmin (13). The estimated rate of Fe(III)-transferrin formation rate of five human male sera in the presence of 100 μM ascorbate at pH 7.35 is 300 ± 60 μM per min, compared to a nonenzymic rate of 24 μM per min.

**Correlation between p-Phenylenediamine and Fe(II) Oxidation by Normal Human Sera**

The correlation between p-phenylenediamine and Fe(II) oxidase activity by different human sera was tested. Fig. 2 indicates that the correlation between these two activities is excellent, with a correlation coefficient of r = 0.725. This value of r is significant at least at the 98% probability level, indicating a strongly positive correlation of these two activities. The elevated level of human ceruloplasmin in advanced pregnancy and the reduced level in cord blood are well known (14).

4 S. Osaki, unpublished data.
Chromatographic Identification of Serum Protein Fraction Which Accelerates Rate of Fe(III)-Transferrin Formation

Column chromatography with DEAE-cellulose was used to locate the serum protein fraction responsible for Fe(II) oxidation and subsequent incorporation of Fe(III) into apotransferrin.

Fresh human sera from five healthy men were combined, and 5 ml were dialyzed overnight against 2 liters of 0.05 M acetate buffer at pH 5.5. A small amount of a white precipitate was obtained after centrifugation and was discarded since it had no activity. The supernatant, containing most of the serum proteins, was applied to a small DEAE-cellulose column (5 x 50 mm) equilibrated with the same buffer. A major portion of the serum protein (Fraction I) came through the column without adsorption. The column was then washed with 50 ml of the same buffer, followed by successive elution with 5 ml of 0.1 M, 0.2 M, 0.3 M, and 0.6 M acetate buffer (pH 5.8), resulting in fractions designated II, III, IVa, IVb, and V. Fraction IVa was the first 2.5 ml eluted by 0.3 M buffer, and IVb was a remaining 2.5-ml eluent. The absorbance at 280 nm, Fe(III)-transferrin formation from Fe(II), and p-phenylenediamine oxidase activity of each fraction are shown in Table I. These results show that Fraction IV contained both the p-phenylenediamine oxidase activity and Fe(III)-transferrin formation activity. These data, together with the data shown in Fig. 2, clearly indicate that the oxidase activity which can be attributed to ceruloplasmin is also associated with the ability to convert Fe(II) to Fe(III)-transferrin.

Comparison of Rates of Fe(II) Oxidation with and without Ceruloplasmin

In order to determine the comparative rates of enzymic and nonenzymic oxidation of Fe(II) under approximately physiological conditions, Fe(II) oxidation was studied under a variety of circumstances. It was shown that the nonenzymic rate of Fe(II) oxidation is first order with respect to both Fe(II) and O_2 throughout a wide range of concentration. In contrast, Fe(II) oxidation catalyzed by ceruloplasmin is zero order at
**TABLE I**

DEAE-cellulose chromatographic fractionation of human serum for p-phenylenediamine oxidase activity and Fe(III)-transferrin formation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>(A_{280})</th>
<th>Ratio of activity to (A_{280})</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td>(\Delta A_{280}/\text{mL})</td>
</tr>
<tr>
<td>I</td>
<td>5.0</td>
<td>32.2</td>
<td>0.0073</td>
</tr>
<tr>
<td>II</td>
<td>5.0</td>
<td>2.32</td>
<td>0.0026</td>
</tr>
<tr>
<td>III</td>
<td>5.0</td>
<td>2.07</td>
<td>0.0031</td>
</tr>
<tr>
<td>IVa b</td>
<td>2.5</td>
<td>2.70</td>
<td>0.0053</td>
</tr>
<tr>
<td>IVb*</td>
<td>2.5</td>
<td>1.10</td>
<td>0.100</td>
</tr>
<tr>
<td>V</td>
<td>5.0</td>
<td>0.80</td>
<td>0.053</td>
</tr>
</tbody>
</table>

* See text for the details of the fractionation.

* IVa was the only observable blue fraction (\(A_{280} = 0.027\)). IVb was too dilute.

Inhibition of Fe(II) Oxidation in Serum by Azide and Cyanide

Certain earlier data suggested that serum enzymes catalyzing the oxidation of Fe(II) may not have been involved in the rate-limiting step in Fe(III)-transferrin formation. These data (3, 5) involved the use of Fe(II) concentrations of 0.2 \(\mu M\). As suggested by the data in Fig. 4, the nonenzymic oxidation rate of Fe(II) would be so great at this Fe(II) concentration that enzymic oxidation would be unimportant. Therefore, since the non-enzymic oxidation is not appreciably inhibited by either azide or cyanide, the role of the enzyme would be discounted. However, when more physiological conditions are approached, the data in Fig. 1 and in Table III show that strong inhibition of Fe(II) oxidation in serum by azide and cyanide is observed.
Effects of Ascorbate on Enzymic and Nonenzymic Oxidation of Fe(II) at pH 7.4

Since appreciable amounts of reducing substances (mostly ascorbate at about 40 μM) may be present in human sera, the effects of ascorbate were studied on both enzymic and non-enzymic Fe(II) oxidation. The results shown in Table IV represent data obtained with ceruloplasmin or sera at about one-tenth their undiluted level. The over-all rates decrease markedly in the presence of ascorbate, apparently because of the decrease in the non-catalyzed oxidation of Fe(II). However, the net enzymic rates are not reduced even in the presence of 300 μM ascorbate, which is approximately 7 times more concentrated than the usual serum ascorbate concentration. Thus, the ceruloplasmin-catalyzed oxidation of Fe(II) is not affected by an excess of ascorbate, the principal reducing substrate in plasma.

Possible Significance of Ceruloplasmin and Transferrin in Iron Metabolism

Under normal conditions, almost all of the iron bound to transferrin is rapidly taken up by the marrow (15-18). The observed half-life of Fe(III)-transferrin in vivo was reported to be 1.32 hours, whereas the half-life for the protein moiety was much slower, 16 hours (19).

Any erythroid stimulation leads to a faster turnover of iron in vivo, and erythroid suppression has the opposite effect (19). It should be emphasized here that only the reticulocytes are capable of utilizing the Fe(III) bound to transferrin, although both the reticulocytes and matured red cell can take up free Fe(III). Katz and Jandl (19) stated: “Thus, transferrin, in some manner, directs the entry of iron into those cells which are still actively synthesizing hemoglobin and prevents its presumably needless accumulation by the matured cell.”

The facts described above support the generally accepted idea that any Fe(II) absorbed from the intestine is oxidized, incorporated into apotransferrin in the plasma, and transferred to the marrow. Transferrin appears to be the protein which exclusively supplies iron to the marrow.

There is no detectable amount of free iron ion in plasma. From the normal urinary excretion (20, 21), it is estimated that the free iron ion concentration in plasma could be about 0.1 μg/100 ml or 0.02 μM. It would be reasonable to consider that Fe(II) is oxidized to Fe(III) in plasma under these conditions. With 0.02 μM as the free Fe(II) concentration in dynamic equilibrium in plasma, 3.3 liters as the plasma volume, and the experimentally obtained maximum first order rate constant for nonenzymic oxidation of Fe(II) as 1.12 min⁻¹, the maximum amount of Fe(II) oxidized during 24 hours, without ceruloplasmin, is estimated to be 94 μmoles/24 hours.

It has been recognized that the average daily turnover of iron in the plasma is 30 to 40 mg per day or about 540 to 720 μmoles per day, including 1 to 3 mg of iron absorption from the intestine in the form of Fe(II) (1, 21). There are many potent reducing substances, such as ascorbate, in plasma. The ascorbate reduces Fe(III) rapidly to produce Fe(II), but this also reduces the overall oxidation, as seen in Tables II and IV. Thus, the rate of nonenzymic oxidation is below the actual requirement for the conversion of Fe(II) to Fe(III). The enzymic oxidation by ceruloplasmin, on the other hand, is sufficient: 1025 μmoles per day, or approximately 11 times more activity than the nonenzymic oxidation. This high rate of Fe(II) oxidation may also be required to assimilate Fe(II) when a large amount of iron is taken orally. Otherwise, a substantial amount of iron might be excreted in the urine, but this is not observed (20).

Fig. 5 represents a diagram of some of the currently accepted principal features of iron metabolism, although many of these proposals have been strongly challenged by Saltman (22). While it is not our purpose here to evaluate the various theories of iron absorption and transfer, none of these proposed mechanisms excludes the possibility of the conversion of Fe(II) or Fe(II) chelates into Fe(III)-transferrin in the serum. Moreover, the most recent information suggests that the availability of iron to the reticulocytes of the bone marrow can be rate-limiting. Our contention is that the rate of formation of Fe(II) from Fe(III) in plasma and of transferrin formation could play a significant role in the over all turnover of iron. The fact that ceruloplasmin can increase this rate under physiological conditions suggests that this is a possible biological function for ceruloplasmin.

The proposed role of ceruloplasmin in promoting the iron...
saturation of transferrin might also be extended to other iron-containing proteins and enzymes. Broman (23) has proposed that ceruloplasmin is involved in the biosynthesis of cytochrome oxidase through the transfer of a copper-containing prosthetic group. Our data suggest that the biosynthesis of those proteins which incorporate iron either from transferrin, ferritin, or some other Fe(III) carrier, or directly from the Fe(II) state, may be stimulated by the catalytic action of ceruloplasmin on Fe(II) oxidation.

In view of the data presented in this paper, we propose that the enzymic activity of ceruloplasmin permits its classification as a ferro-O₂ oxidoreductase (Main Class I) with a new subclass (e.g. Subclass 12), based upon Fe(II) as its likely substrate in vivo, and a second subclass, 3, usually reserved for O₂ as acceptor. If p-phenylenediamine, an unnatural but popular substrate, is used for its classification, it would be numbered 1.7.3.2 (24).

The fact that Fe(II) is also the substrate of highest molecular activity, 550 Fe(II) per min per enzyme molecule, justifies the designation of this enzyme as serum ferroxidase. While the name ceruloplasmin, coined by its discoverers, Holmberg and Laurel1 (25), will always retain its historical importance, we anticipate that the name ferroxidase may be more useful than designating this enzyme as a sky blue substance from plasma.

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Shigemasa Osaki, Donald A. Johnson and Earl Frieden


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