Ferroxidase activity of rat ceruloplasmin

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Williams, D. M., G. R. Lee, and G. E. Cartwright. Ferroxidase activity of rat ceruloplasmin. Am. J. Physiol. 227(5): 1094-1097. 1974.—The ferroxidase activity of rat ceruloplasmin was studied under various assay conditions. The ferroxidase activity was optimal at pH 6.0-6.2 in acetate buffer in the absence of ascorbate, and under these conditions the activity was about 1/3rd that of human ceruloplasmin and 1/10th that of porcine ceruloplasmin. However, hypoceruloplasminemic rats developed hypoferraemia, and the administration of ceruloplasmin was followed by a prompt increase in plasma iron. Thus, ceruloplasmin appears to be essential for the flow of iron from reticuloendothelial cells to transferrin in the rat as well as the pig.

Previous studies in hypoceruloplasminemic swine indicated that ceruloplasmin is essential for the flow of iron from reticuloendothelial cells to plasma transferrin (17). Cell-to-plasma flow of iron became sufficiently impaired in such animals as to cause hypoferraeemia when the ceruloplasmin concentration decreased to less than 1/5 of the normal value. The administration of small amounts of ceruloplasmin (0.5-10% of that normally present) was followed by a prompt increase in plasma iron.

Based on the studies in vitro demonstrating that ceruloplasmin (ferroxidase) catalyzes the oxidation of iron (14) and on the observations that ferreic but not ferrous iron is bound by apotransferrin (4), it was proposed that ceruloplasmin functions in iron metabolism by oxidizing iron, thereby enhancing the rate of transferrin formation.

This concept of the role of ceruloplasmin in iron metabolism was difficult to reconcile with observations in the rat. Rat ceruloplasmin, when studied at pH 6.7 in phosphate buffer in the presence of ascorbate, possessed very little ferroxidase activity (17). Therefore, the present study was undertaken to define the ferroxidase activity of rat plasma and purified rat ceruloplasmin under various assay conditions, to compare the ferroxidase activity of rat ceruloplasmin to the ferroxidase activity of porcine and human ceruloplasmin, and to ascertain if ceruloplasmin is required in the rat for the flow of iron from reticuloendothelial cells to transferrin.

Materials and Methods

All blood specimens were collected in specially cleaned glassware (3) with heparin as an anticoagulant. Human apotransferrin was obtained from Certified Blood Service, Inc., Woodbury, N. Y. and dialyzed against 0.15 M sodium chloride before use (8). Porcine, rat, and human ceruloplasmin were prepared by a method reported elsewhere (13). The final preparations were dialyzed against 0.15 M sodium chloride before use. Plasma iron and copper were determined by atomic absorption spectroscopy after deproteinization by the method of Trinder (18). β-Phenylenediamine (βPD) oxidase activity was assayed by the method of Ravin (16), modified by the use of up to 1 ml of plasma when ceruloplasmin levels were low.

Ferroxidase activity was measured by the method of Johnson et al. (7). The reaction was carried out in sodium phosphate buffer, final concentration 0.0133 M, or in acetate buffer, final concentration 0.2 M, at the pH specified. The 1.0-ml reaction mixture consisted of 0.2 ml of appropriately diluted plasma (1:5-1:10), apotransferrin (30 μM), ferrous ammonium sulfate (30 μM), and buffer. Ascorbic acid, when used, was added to the iron solution to make a final concentration in the reaction mixture of 300 μM. The final concentration of sodium azide, when added, was 100 μM. After the addition of the iron or iron-ascorbate solution, the rate of increase in absorbance at 460 nm was measured at room temperature in a Cary recording spectrophotometer, model 15. Determined values were corrected by subtracting the nonenzymatic rate of transferrin formation, which was measured in the appropriate buffer containing all the above agents except plasma. The nonenzymatic rate was approximately one-sixth the enzymatic rate.

Ceruloplasmin ferroxidase activity was calculated by subtracting the ferroxidase activity measured in the presence of azide from the ferroxidase activity measured in the absence of azide. Since only about 78% of the ferroxidase activity of ceruloplasmin is inhibited by 100 μM azide (9, 14, 19), this calculation gives an underestimate of the ceruloplasmin ferroxidase activity measured in plasma.

Copper deficiency was produced in weanling female rats by feeding a condensed milk diet low in copper. A similar diet has been used to produce copper deficiency in swine and is described elsewhere (10). All animals were given 5 mg of iron in the form of iron dextran (Imferon) intramuscularly daily for the first 6 days of the experiment.

Results

Comparison of ferroxidase activity of rat plasma with that of porcine and human plasma. As judged by the plasma copper concentration and the βPD oxidase activity, the ceruloplasmin concentration of rat plasma was intermediate between pig and man (Table 1). However, the ceruloplasmin ferroxidase activity of rat plasma, when measured in phosphate buffer at pH 6.7 in the presence of ascorbate, was less than that of man and pig.

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The PPD oxidase : copper ratio was similar in the plasma of all three species (Table 1). The ceruloplasmin ferroxidase : copper ratio and the ceruloplasmin ferroxidase : PPD oxidase ratio were greater in pig plasma than in the plasma from human subjects and lowest in the rat, indicating the relative ferroxidase activities per unit of ceruloplasmin when measured at pH 6.7 in phosphate buffer in the presence of ascorbate.

Ferroxidase activity of purified ceruloplasmins. The ferroxidase activity of purified ceruloplasmin from all three species was studied as a function of pH in phosphate and in acetate buffer. However, the ferroxidase activity of rat ceruloplasmin was 1/3rd that of human ceruloplasmin and plasmin. The ferroxidase activity of porcine ceruloplasmin was 1/10th that of porcine ceruloplasmin. Thus, as in the pig (17), the flow of iron was not impaired until after the PPD oxidase activity decreased to less than 1% of the normal value.

Role of ceruloplasmin in iron metabolism in copper-deficient rats. If ceruloplasmin is required for the optimal movement of iron from reticuloendothelial cells to transferrin in the rat, as it is in the pig (17), it follows that 1) hypoferremia should develop during the course of copper deficiency in the rat, 2) hypoceruloplasminemia should precede the development of hypoferremia, and 3) the administration of ceruloplasmin should be followed by a prompt increase in plasma iron. Accordingly, copper deficiency was induced in rats and these events were monitored.

The PPD oxidase activity and the concentration of iron and copper in the plasma of rats during the development of copper deficiency are shown in Fig. 1. The plasma iron remained above 200 μg/100 ml for the first 36 days on the deficient diet and then decreased to 133 μg/100 ml over the next 14 days. The decline in ceruloplasmin concentration, as measured by the PPD oxidase activity and the plasma copper concentration, preceded the development of hypoferremia. On the 22nd day of the copper-deficient diet, at which time the mean plasma iron value was 227 μg/100 ml, the mean plasma copper was 7% of the control value (11 μg/124 μg). The PPD oxidase activity was 6% of the control value (.0042/.360). On the 36th day of the experiment, at which time the plasma iron was 235 μg/100 ml, the plasma copper was 9% of the control value (11 μg/124 μg). Thus, as in the pig (17), the flow of iron was not impaired until after the PPD oxidase activity decreased to less than 1% of the normal value.

Purified rat ceruloplasmin was infused rapidly intravenously into a group of copper-deficient rats. Purified pig ceruloplasmin was infused into a second group of animals. The amount of ceruloplasmin PPD oxidase activity infused was calculated to represent 20% of the amount in the circulation of the normal rat. Physiologic saline was infused into a third group of animals as a control, and copper in the form of copper sulfate was infused intravenously into a fourth group of rats. The amount of copper infused was calculated to be the same as the amount of copper contained in the ceruloplasmin which was infused into the first two groups. The animals in all four groups were sacrificed by exsanguination 3 hr after the completion of the infusion, and plasma was obtained for the measurement of iron, copper,
and p/II oxidase activity. The plasma iron was appreciably greater in the two groups receiving ceruloplasmin than in saline- and copper-injected control groups (Table 3), indicating that the ceruloplasmin mobilized iron into the plasma.

**TABLE 3. Effect of intravenous infusions of ceruloplasmin on plasma iron and copper of copper-deficient rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>pII Oxidase Activity, A550 mm</th>
<th>Plasma Copper, µg/100 ml</th>
<th>Plasma Iron, µg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>16</td>
<td>0.003±0.0010</td>
<td>19±3.6</td>
<td>165±13.6</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>14</td>
<td>0.004±0.0004</td>
<td>18±2.9</td>
<td>192±11.0</td>
</tr>
<tr>
<td>Rat ceruloplasmin</td>
<td>14</td>
<td>0.005±0.0036</td>
<td>35±0.0</td>
<td>303±10.4</td>
</tr>
<tr>
<td>Pig ceruloplasmin</td>
<td>9</td>
<td>0.030±0.0065</td>
<td>25±2.7</td>
<td>328±28.8</td>
</tr>
</tbody>
</table>

Values represent means ± SE.

and p/II oxidase activity. The plasma iron was appreciably greater in the two groups receiving ceruloplasmin than in saline- and copper-injected control groups (Table 3), indicating that the ceruloplasmin mobilized iron into the plasma.

**DISCUSSION**

As previously reported (17), the ceruloplasmin ferroxidase activity of rat plasma is negligible as compared with human and porcine plasma when measured at pH 6.7 in phosphate buffer in the presence of 300 µM ascorbate. The ferroxidase activity of rat ceruloplasmin is maximal at pH 0.0-6.2 in acetate buffer in the absence of ascorbate. Under these conditions, the ferroxidase activity is about 1/3rd that of human ceruloplasmin and 1/10th that of porcine ceruloplasmin.

In spite of the relatively low iron-oxidizing activity of rat ceruloplasmin, hypoceruloplasminemic rats developed hypoferrremia, and the administration of ceruloplasmin was followed by a prompt increase in plasma iron. As in the pig (17), hypoferrremia did not develop until after the ceruloplasmin concentration had been reduced to less than 1 % of the normal concentration. Thus, ceruloplasmin is essential for the flow of iron from reticuloendothelial cells to transferrin in the rat as well as in the pig.

The manner in which ceruloplasmin functions in the transfer of iron from reticuloendothelial cells to transferrin remains to be determined. Bates and Schlabach (1) have presented cogent arguments against the concept that ceruloplasmin functions physiologically by oxidizing iron in plasma. The relatively low ferroxidase activity of rat ceruloplasmin also raises the question as to whether the iron-oxidizing activity of this protein is the major or only function in enhancing the movement of iron. Finally, the ferroxidase activity of ceruloplasmin at physiologic pH is only about one-seventh of the activity at pH 6.7 (19).

One possible explanation for the role of ceruloplasmin in the movement of iron from reticuloendothelial cells to transferrin is that ferrous iron occupies specific iron-binding sites on the membranes of reticuloendothelial cells and that ceruloplasmin is required to remove iron from these sites, first by entering into a reaction with the site itself and then by the formation of a ceruloplasmin-iron intermediate which then transfers iron to apotransferrin by a specific ligand exchange reaction. Evidence for such a ceruloplasmin-iron intermediate has been presented (12, 13).

It is also possible that the role of ceruloplasmin in iron metabolism is even more indirect. Ceruloplasmin may not act at the membrane-plasma interface but may correct the deficiency of some other copper-containing enzyme. In this regard, it has recently been shown in our laboratory (20) that cytochrome oxidase, a copper-containing mitochondrial enzyme, is required for the intracellular reduction of iron. This enzyme is known to be severely depleted in copper deficiency (5, 11). Ceruloplasmin may function by regenerating cytochrome oxidase (2) and thereby enhances the formation of the membrane-bound ferrous iron pool which is available to transferrin. Studies are now underway in our laboratory to define more specifically the manner in which ceruloplasmin is involved in the flow of iron.

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