Mobilization of Liver Iron by Ferroxidase (Ceruloplasmin) *

(Received for publication, July 3, 1969)

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SUMMARY
Iron was mobilized by human ferroxidase from dog liver into the perfusate under simulated physiological conditions. The rate of mobilization was estimated to be 1.8 μM per min per 4.9 μM ferroxidase. Human serum albumin with trace copper was found to be inactive in iron mobilization. The low Kₐ and high k values of ferroxidase toward ferrous ion enabled us to deduce that the elimination of free iron in the perfusate by ferroxidase would generate a very steep concentration gradient between iron storage cells and the capillary system. This concentration gradient would promote a rapid iron out-flux from the cells by diffusion.

Although copper deficiency causes a defect in iron metabolism of animals (1–4) the direct relationship between these two metals has not been demonstrated in vitro. However, it has been well established in vivo that copper deficiency causes a rapid decrease of both plasma iron and copper levels (2, 5). Since a decrease of serum copper correlates closely with the p-phenylenediamine oxidase activity, another enzymic activity of ferroxidase (ferro-O₂-oxidoreductase, EC 1.12.3), and more than 93% of serum copper is ferroxidase-bound (6), a hypocupremia may be regarded as hypoceruloplasminemia as far as serum copper is concerned (2).

The demonstration of iron oxidase activity of ceruloplasmin in our laboratory led to a hypothesis of the possible importance of ferroxidase activity of ceruloplasmin in normal iron metabolism (7). As a test in v i t r o for the hypothesis of O s a k i, Johnson, and F r i e d e n (7), R a g a n et a l. (8) have studied the effect of injected copper and homologous ceruloplasmin into copper-deficient swine and fructose (10 mg/100 ml). The extremely low K₃ and high rate constant values of ferroxidase in isolated systems enabled us to deduce that the elimination of free iron in the perfusate by the catalytic activity of ferroxidase would generate a very steep concentration gradient between the inside of iron storage cells and the outside, and hepatic cells to the plasma, was stimulated by injection of ceruloplasmin (8). They concluded that the results strongly support the role of ceruloplasmin as a serum ferroxidase (7).

These observations prompted us to study the mobilization of the stored iron into the plasma by perfusing a normal dog liver with apotransferrin dissolved in 0.01 M Tris-HCl buffer, pH 7.4, + 0.9% NaCl solution. The oxygen partial pressure of the perfusing solution was kept low (PO₂ = 30 to 40 mm Hg) to simulate the venous plasma. The PO₂ was monitored with an oxygen electrode system (7). The perfusing solution was allowed to pass through a flow cuvette with a 10-mm optical path. The iron transferrin formation in the perfusate was observed spectrophotometrically at 460 μM with the use of a Cary model 15 spectrophotometer equipped with 0.1 absorbance expansion (7, 9). The recirculation time of the perfusate was approximately 100 sec and its total volume was 120 ml at the end of the experiment. As shown in Fig. 1, 31 μM spotransferrin labeled (A) in the figure, infused at the beginning, picks up iron from liver slowly at the rate of 0.36 μM/100 sec. The additional infusion of either 0.0035 N HCO₃⁻ (B) or 800 μM ascorbate (C) did not affect Fe(III)-transferrin formation. However, the infusion of 4.9 μM human ceruloplasmin (D) showed an instantaneous and dramatically rapid formation of Fe(III)-transferrin with a rate of 3 μM Fe(III)-transferrin per 100 sec.

In other experiments, aliquots of the perfusate were taken during the same interval as the spectrophotometric monitoring at 400 μM. These aliquots were acidified and the iron was allowed to react with α,α-dipyridyl in the presence of 7 mM ascorbate. The iron-α,α-dipyridyl complex was measured spectrophotometrically at 550 μM.

Table I indicates that the two independent methods of iron measurement produced equivalent results.

The mobilization of iron from ferritin by chelating agents has been extensively studied by P a p e et al. (11). However, the chelators used in their experiments (such as 0.1 M nitrilotriacetic acid, 0.1 M EDTA, or 0.1 M sodium citrate) were either not physiological or were not used at physiological concentrations. Moreover, the mobilization rate into the outer chelating solution was slow with a maximum rate of 0.2 ~ 0.07 μM of iron per liter per hour in the presence of 0.1 M nitrilotriacetate compared to the present observation, 6 μM of iron per liter per hour, under closely physiological conditions except the ascorbate concentration which was somewhat higher than normal. The infusion of the following compounds to the dog liver system, in the absence of ferroxidase, did not mobilize iron from the liver: 220 μM human transferrin, 3 μM human serum albumin + 21 μM CuSO₄, 36 μM bovine serum albumin, 5 μM CuSO₄, 120 μM citrate, glucose (90 mg/100 ml), and fructose (10 mg/100 ml).

The extremely low K₃ (0.2 to 0.0 μM) value of crystalline ferroxidase toward ferrous ion has been observed (12). A significantly high value, 1.2 × 10⁶ M⁻¹ sec⁻¹, was obtained as a second order rate constant for the reaction between ferrous ion and ferroxidase (13). These low K₃ and high rate constant values of ferroxidase in isolated systems enabled us to deduce that the elimination of free iron in the perfusate by the catalytic activity of ferroxidase would generate a very steep concentration gradient between the inside of iron storage cells and the outside.
Acknowledgments—We are indebted to Dr. E. Frieden for his generous support and discussion, and to Dr. H. Lipner for his valuable advice and technical assistance.

Table I
Comparison between two different iron assay methods

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Absorbance change</th>
<th>Iron concentration change in perfusate</th>
<th>Total iron released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. a</td>
<td>0.016</td>
<td>7.2</td>
<td>120</td>
</tr>
<tr>
<td>1. b</td>
<td>0.020</td>
<td>7.0</td>
<td>115</td>
</tr>
<tr>
<td>2. a</td>
<td>0.020</td>
<td>9.1</td>
<td>150</td>
</tr>
<tr>
<td>2. b</td>
<td>0.027</td>
<td>9.6</td>
<td>158</td>
</tr>
</tbody>
</table>

* The enzyme concentration was 0.6 to 0.9 µM.
* The spectrophotometric method for Fe(III)-transferrin formation (9).
* Chemical determination of iron using α,α-dipyridyl (10).

REFERENCES

A New Spin Label Specific for the Active Site of Serine Enzymes*

(Received for publication, July 28, 1969)

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SUMMARY

A new spin label has been synthesized which reacts specifically with the active site serine residue of esteratic enzymes. The conformation of the active site of acetylcholinesterase bound to human erythrocyte membranes, ox erythrocyte membranes, and rat brain nerve ending particles has been studied using this new reagent. In all three systems, the active site region appears to be exposed and readily accessible to small molecules. These results are in marked contrast to those obtained with spin-labeled α-chymotrypsin which appears to have a much more closed and inaccessible active site region.

The rapid inactivation of acetylcholinesterase (acyethylcholine acetyl-hydrolase, EC 3.1.1.7) by organophosphonates has been studied extensively (2) and has been attributed to the highly specific reaction of the reagent with the serine residue located at the active site of the enzyme (3). Our interest in the microstructure of this enzyme has led us to take advantage of this very selective reaction. By combining the reactive phosphono-fluoridate moiety with a nitroxyl radical we have obtained a molecule capable of selectively spin-labeling the reactive serine residue of AC-esterase. Thus, one may study the conformation of the active site region by comparing the line shapes of the electron paramagnetic resonance spectra of the free unbound radical and the covalently attached radical. The unbound spin...
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J. Biol. Chem. 1969, 244:5757-5758.

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