THE ROLE OF CERULOPLASMIN IN Fe(III)–TRANSFERRIN FORMATION IN VITRO

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1. Introduction

The demonstration of iron mobilization by ceruloplasmin (EC 1.12.3.1) in perfused dog liver [1] and studies on copper-deficient animals [2] strongly suggest that ceruloplasmin is a direct molecular link between copper and iron metabolism.

The ferroxidase activity of ceruloplasmin was first observed in [3] with the confirmation [4] that the oxidase activity of ceruloplasmin was greater for iron than for any other substrate. Thus, it was suggested that the ceruloplasmin catalyzed oxidation of iron and was an essential step on the pathway of Fe(III)–transferrin formation. However, several observations may be cited against this theory:

(i) There is no evidence for defective iron mobilization in patients with Wilson's disease [5];
(ii) Rat ceruloplasmin exhibits a relatively low ferroxidase activity when compared to the corresponding human or pig proteins [6];
(iii) The ferroxidase activity of human and rat ceruloplasmin at physiological pH is only ~1/7th of the activity, that it has at pH 6.7 and 6.0, respectively [7].

According to [8] transferrin binds ferrous iron at the specific metal binding sites and the resulting Fe^{2+}–transferrin complex is highly susceptible to oxidation. In plasma, transferrin also reacts very poorly with iron which has been oxidized to Fe^{3+} by an agent such as ceruloplasmin [8].

Here, we have attempted to clarify the role of ceruloplasmin in transferrin formation in vitro, using ferrous iron and ferritin as sources of iron.

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2. Materials and methods

Human apotransferrin was obtained from Behringwerke AG, Marburg. Ceruloplasmin was purified from human plasma by the method in [9] and the enzymatic activity measured using paraphenylenediamine as substrate [10]. ATP, 2,3-diphosphoglycerate and horse spleen ferritin were from Boehringer, Mannheim. All reagents used were of analytical grade and solutions were made with iron-free distilled water. Iron content in ferritin was estimated as in [11].

Transferrin saturation was determined at 37°C in 1 ml cuvettes containing Hepes buffer, 0.1 M (pH 6.7 or 7.4); either 0.6 μmol horse spleen ferritin (3500 iron atoms/ferritin molecule) or 0.13 mM ferrous ammonium sulphate and 62 μmol apotransferrin. Ceruloplasmin was added at 6 μmol final conc. and in some experiments, 1 mM final conc. in ascorbate, citrate, ATP or 2,3-DPG was used. Transferrin saturation was monitored at 466 nm using a DW-2a Aminco spectrophotometer (Beun-de Ronde, Amsterdam). The amount of Fe(III)–transferrin formed was calculated based on ϵ_{mM} 4.56 and mol. wt 80 000 [12].

3. Results and discussion

At physiological pH, in vitro, apotransferrin can be fully saturated in <5 min using a stoichiometric amount of ferrous ammonium sulphate as the source of iron (fig.1). In the presence of 1 mM ascorbate, transferrin saturation occurs in 1 min. If ceruloplasmin and apotransferrin are incubated for 1 min prior to addition of ferrous iron, a maximum of 80% saturation is attained in 30 s. However, if ferrous iron and ceruloplasmin are mixed and then apotransferrin added 1 min later, saturation is very slow and is attained only after 24 h. Under these experimental conditions,

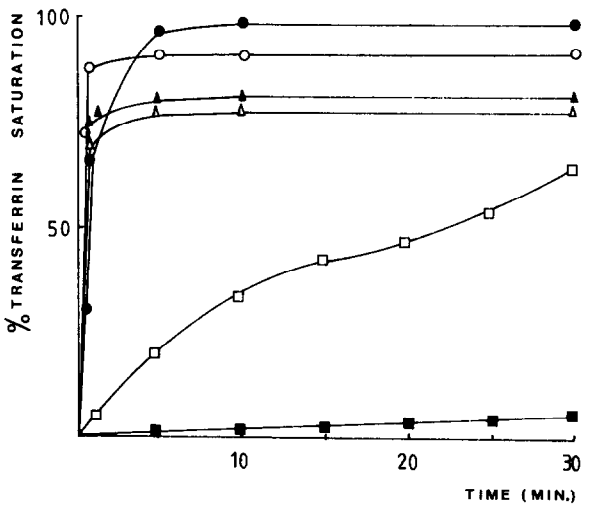

Fig.1. Influence of ceruloplasmin in transferrin formation using ferrous iron. Transferrin saturation was followed at 37°C in 1 ml cuvettes containing 0.1 M Hepes buffer (pH 7.4). Reagents were: 62 μmol apotransferrin (Apotf); 0.13 mM ferrous ammonium sulphate (Fe^{2+}); 6 μmol human ceruloplasmin (Cp); 1 mM ascorbate. The reagents were added at 1 min intervals as follows: (■) Fe^{2+} + Cp + Apotf; (▲) Apotf + Cp + Fe^{2+}, (●) Apotf + Fe^{2+}. The open symbols indicate the same additions in the presence of ascorbate.

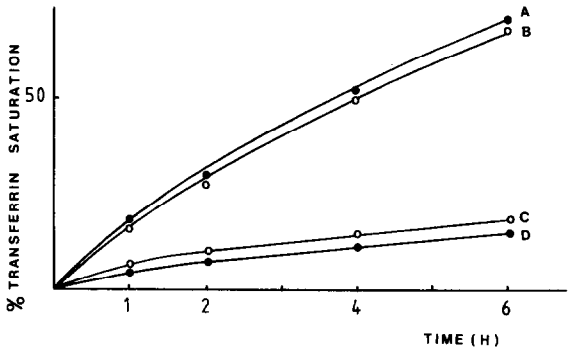

Fig.2. Influence of ceruloplasmin in transferrin formation using ferritin as source of iron. Transferrin saturation was followed at 37°C in 1 ml cuvettes containing 0.1 M Hepes buffer (pH 7.4). Reagents were: 62 μmol apotransferrin; 0.6 μmol horse spleen ferritin; in (A,B) ascorbate was present at 1 mM final conc. The A_{466} was measured as a function of time. The closed symbols indicate the presence of ceruloplasmin (final conc. 6 μmol). There was no ascorbate in (C,D).

in the presence of ascorbate, iron saturation is achieved in 40 min. Similar observations have been made at pH 6.7, which is the optimum pH for the ferroxidase activity of human ceruloplasmin. These data suggest that apotransferrin can readily react with ferrous iron, this reaction being enhanced by ascorbate. However apotransferrin reacts very slowly with iron which has been oxidized to Fe^{3+} by ceruloplasmin or with ferric iron in the absence of citrate or other complexing agents. This observation is in good agreement with the Bates' hypothesis [8]. According to our results, ceruloplasmin seems to catalyse the formation of transferrin when ferrous iron has been complexed with apotransferrin.

When ferritin is used as source of iron, in the presence of ascorbate, citrate, 2,3-DPG, ATP or without mediator, ceruloplasmin does not exert any positive effect in transferrin saturation at pH 7.4 or 6.7 (fig.2) and (table 1). As reported in [13], we observed that there is a direct transfer of iron between ferritin and apotransferrin in the absence of any mediating agent, whereas in the presence of ascorbate, the velocity of iron deposition in transferrin is ~5-times higher.

Citrate shows a slight positive effect but ATP and 2,3-DPG do not enhance iron exchange.

Further studies on the role of these mediators will be reported later.

To explain the direct transfer of iron between ferritin and transferrin, transferrin may pick up iron made available at the surface of ferritin [13]. This hypothesis is quite reasonable since iron from the ferritin core is in equilibrium with iron bound to the protein on the catalytic sites of iron oxidation [14]. The mobilization of iron from the ferritin core could be due to the presence of a specifically bound flavin cofactor.

In summary, apotransferrin has a low affinity for

Table 1
Percentage of transferrin saturation after 6 h of incubation

Mediator	With ceruloplasmin	Without ceruloplasmin
Ascorbate	71.8	66.4
Citrate	16.7	20.4
ATP	14.4	13.2
2,3-DPG	12.0	15.8
No mediator	13.8	15.2

Experiments were carried out at 37°C in 1 ml cuvettes containing 0.1 M Hepes buffer (pH 7.4). Reagents were: 62 μmol apotransferrin; 0.6 μmol horse spleen ferritin; 6 μmol human ceruloplasmin; either ascorbate, citrate, ATP or 2,3-DPG, 1 mM. The A_{466} was measured in function of time. These values are mean values of 3 independent experiments

iron that has been oxidized by ceruloplasmin. Moreover, ceruloplasmin has no effect on iron transfer between ferritin and apotransferrin. Based on these observations, we suggest that if ceruloplasmin plays any role in transferrin formation, it could catalyze transferrin formation when ferrous iron has first been complexed by apotransferrin.

In thus seems reasonable to suppose that transferrin formation from 'free' iron in plasma might involve ceruloplasmin but only if the iron is in the reduced state and bound to the apotransferrin molecule prior to the action of ceruloplasmin; these are conditions unlikely to be attained in vivo at physiological pH and in the presence of potential iron chelators. The absence of any effect of ceruloplasmin on iron exchange between ferritin and apotransferrin, even in the presence of mediators, is consistent with this conclusion. If ceruloplasmin has an effect on iron incorporation into transferrin, it could be at the site of iron donation to transferrin either at the plasma membrane or in the interior of the cell, if we assume that iron is supplied in the ferrous form. Experiments to test this hypothesis are under way. It should be added as a caution that clear-cut evidence that iron is incorporated into transferrin as Fe(II), then oxidized either by the protein itself or by an external agent such as ceruloplasmin, is at best equivocal [8,15].

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