Ceruloplasmin and the reactions forming diferric transferrin

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The rate of formation of diferric-transferrin has been studied using various combinations of Fe(II), Tf, Cp and h serum. When the reactants were added in a correct physiological sequence, ceruloplasmin and diluted human serum showed the fastest rate of saturation of transferrin.

Transferin, diferric *Ceruloplasmin* *Human serum* *Saturation rate* *Reaction sequence* *Physiological conditions*

1. INTRODUCTION

Ceruloplasmin (Cp, ferroxidase; iron (II):O_2 oxidoreductase, EC 1.16.3.1) is the blue copper protein of vertebrate plasma [1]. The multifunctional nature of this protein is now well documented [2]. Cp has been shown in vivo to be a direct molecular link between iron and copper metabolism [3,4]. It has been proposed that the ferroxidase activity of Cp promoted the rate of formation of $Fe(III)_2$–transferrin (Tf) in serum [3].

In [5,6] it has been attempted to discount the role of Cp in several in vitro experiments utilizing $Fe(III)_2$–Tf formation. Authors in [7] have described a reaction leading to the formation of $Fe(III)_2$–Tf using Fe(II) ions as the source of iron; ambient oxygen along with HCO_3^- (5.0 or 10 mM) was shown to be a rate enhancement factor. However, they did not use Cp. We wish to provide evidence that these in vitro experiments do not provide data appropriate for conditions which approach the in vivo fate of Fe(II) as it enters the plasma from the liver or other tissues. Under conditions approaching physiological Cp has a marked acceleratory effect on the rate of formation of $Fe(III)_2$–Tf. We will use the set of reactions in table 1 to illustrate and identify Fe(II) oxidation reactions under consideration.

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Abbreviations: Cp, Ceruloplasmin; h serum, human serum; Tf, Apo-transferrin; $Fe(III)_2$-Tf, diferric transferrin

2. MATERIALS AND METHODS

Cp was isolated from human plasma by a modified method [8]. The absorbance ratio A_{610}/A_{280} for human Cp was 0.045. Human Tf was obtained from Behringwerke (maximum iron content 20 μg/g). All solutions were prepared in chelexed water and further purified by using Chelex-100 resin to remove trace metal contamination. Tf saturation was determined at 37°C as in [9]. To the buffer, reagents were mixed in the order represented by the sequence of reactants in table 1 at 1-min intervals. The final concentration of the reagents were as follows: 100 mM HEPES (pH 7.5); 55 μM Tf; 109 μM Fe (II) or Fe(III) in the form of ferrous or ferric ammonium sulfate; and 1.88 μM Cp. Reactions (a) through (j) contained ambient oxygen estimated to be 216 μM at 37°C and ambient CO_2 concentrations at 300 μM, except reaction (j) which contained 42 mM HCO_3^-. The (f) and (g) experiments contained 72 mM HEPES.

In reactions (f) and (g), the 27% fresh human serum used contained, in addition to 675 nM Cp, the following concentrations: 165 μM albumin; 11 μM ascorbic acid: 7.3 mM bicarbonate; 135 μM phosphate; 8.1 μM (1/3) ferric-saturated Tf. These serum concentrations were estimated from standard tables [10]. The amount of $Fe(III)_2$–Tf formed was calculated using ϵmM = 4.56; a M_r of 80000 was assumed for Tf [11]. The pH of the final reaction mixtures ranged from 7.35–7.40.

It is recognized that the complexity of these reactions did not permit a precise measure of rate con-

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Table 1
Sequence of reactions leading to the formation of diferric transferrin

	Reactants	Comments
(a)	Tf + Fe(II)	Auto-oxidation of Fe(II). Slower than (c) or (d)
(b)	Fe(II) + Tf	Auto-oxidation of Fe(II); rate and saturation less than (a)
(c)	Tf + Cp + Fe(II)	Cp catalyzed; rate ≅ (d) (in vivo sequence)
(d)	Fe(II) + Tf + Cp	Auto-oxidation followed by Cp catalysis = (c)
(e)	Fe(II) + Cp + Tf	Cp catalyzed, Fe(II) oxidation rapid; Tf saturation low
(f)	Tf + h serum + Fe(II)	Fast at 27% h serum (in vivo conditions)
(g)	Fe(II) + h serum + Tf	Rate <(f) >(e)
(h)	Tf + Fe(III)	Slowest rate due to $[Fe(OH)_x]_n$
(i)	Fe(III) + Tf	Similar to (h)
(j)	Fe(III) + Tf	With 42 mM HCO_3^- faster than (i)

Each reactant was added at 1-min intervals. All reactions contained ambient O_2 (216 μM), ambient HCO_3^- (300 μM) and HEPES buffer (pH 7.5): Fe(II), Fe(III) = 109 μM; Tf = 55 μM; Cp = 1.88 μM

stants. Thus our data are confined to relative rates of $Fe(III)_2$–Tf formation.

3. RESULTS

The various reactants, their order of addition and $Fe(III)_2$–Tf formation are shown in table 1 and fig. 1 and 2. Each reagent was mixed at 1-min intervals (except for a 5-min interval in the reaction represented by curve b′, fig. 1). In fig. 1, the percent Fe(III) saturation of Tf is shown at 1-min intervals for 30 min. Fig. 2 shows the formation of $Fe(III)_2$–Tf in several reactions for an expanded time scale for the first 3 min. The comments included in table 1 compare the relative rates of these reactions.

Reactants (a) (fig. 1,2) show the rates of $Fe(III)_2$–Tf formation by Fe(II) and Tf. This was assumed to be the rate of auto oxidation of Fe(II) and was relatively slow compared to (c) which corresponds to a physiological sequence. The rate of (a) was less than that of (d) as shown by the immediate increase in rate when 1.88 μM Cp was added after 1 min (arrow, fig. 2).

Fe(II) ions kept in buffer (pH 7.5) for 1 min and 5 min before the addition of Tf showed considerable reduction in the rate of Tf saturation (fig. 1, curves (b) and (b′)). The results indicate that Tf and Fe(II) ions should be present in the reaction mixture to achieve maximum extent of saturation.

Fig. 1, curve (c) shows that iron saturation after oxidation of Fe(II) to Fe(III) was instantaneous. In fig. 2 a 10-fold increase in the rate is seen between

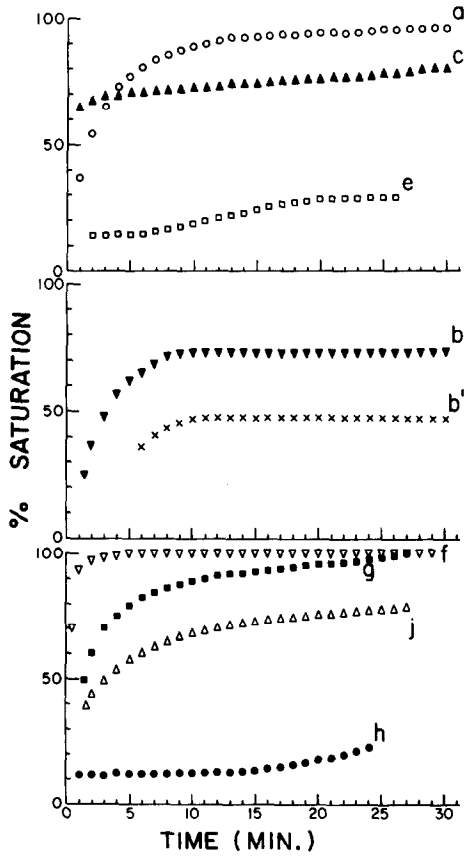

Fig. 1. The percent of $Fe(III)_2$–Tf formation was studied for 30 min at 37°C in 1-ml cuvettes containing HEPES buffer (pH 7.5). The reagents were added at 1-min intervals. For procedures and sequence of additions see section 2 and table 1. The identification of the curves is given in table 1.

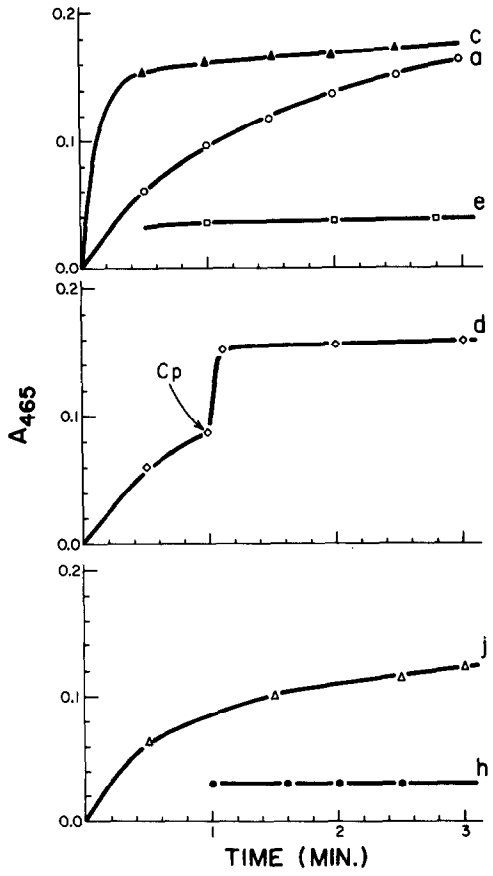

Fig. 2. Absorbance at 465 nm during the formation of $Fe(III)_2$–Tf of an expanded time scale for the first 3 min of the reactions indicated in table 1.

(a) and (c). Since Fe(II) ions were added last, there is little, if any, evidence for Fe(II)–Tf interaction.

In reaction (d), since Cp was added last, there is a possibility of Fe(II)–Tf interaction. Subtracting the auto-oxidation for the first min gave a 10-fold increase in the enzymic oxidation and in $Fe(III)_2$–Tf formation (fig. 2, curve d).

When Cp and Fe(II) ions were mixed, Fe(III) ions were formed instantaneously because of ferroxidase activity. When Tf was added after 1 min, no specific saturation of Tf took place. Less than 15% saturation was observed in the first 5 min (fig. 1 curve (e)).

In reaction (f), human serum diluted to 27% was used and 97% saturation of Tf was obtained in 1 min. This reaction corresponds to an in vivo sequence.

When serum and Fe(II) ions were mixed (g), Fe(III) ions were formed almost instantaneously primarily because of the ferroxidase activity of Cp in the serum (estimated to be 0.68 μM). When Tf was added after 1 min, 90% saturation was seen after 10 min. Thus (g) is much faster than (e) which had a similar sequence but no serum.

Ferric ions showed the slowest rate of iron transfer to Tf (reactions (h), (i), not shown). However, in the presence of 42 mM HCO_3^- (reaction (j)), Fe(III) gave a much faster rate than reaction (e).

4. DISCUSSION

Our results support a role of Cp in promoting the saturation of Tf when the reactants were added in a correct physiological sequence. The fastest reactions shown in fig. 1 and 2 are (c), (d) after Cp addition, and (f). Reactions (c) and (f) occur under conditions similar to those expected in serum. In (c), Fe(II) is added to a mixture of Tf and Cp and in (f) Fe(II) confronts a mixture of Tf and 27% h serum. Reaction (e) is of no physiological significance.

Reactions (a), (c) and (e) show a relative rate similar to the corresponding reactions reported in [6]. On the basis of reactions similar to (a), (c) and (e), they concluded that 'Tf reacts with iron which has been oxidized to Fe(III) by Cp'. While this conclusion might fit reactants in (e), it is not consistent with the high rate observed for reactants (c), (d) and (f). They also contended that 'Cp seems to catalyse the formation of $Fe(III)_2$–Tf when Fe(II) has been complexed with Tf'. This is not in accord with the fact that (d) is no faster than (c) in which Tf is mixed with Cp before Fe(II).

The slower reactions (b), (b'), (e) and (h) involve delayed mixing of Tf with Fe(II) or Fe(III) ions at pH 7.5. These rates are due to hydrolysis [12] and polymerization of Fe(III) or Fe(II) which is auto-oxidized to Fe(III). The rate of the auto-oxidation of Fe(II) in the presence of Tf, reaction (a), is believed to reflect a balanced oxidation and Tf reaction. Thus, there is little chance for Fe(III) to be hydrolysed before it is complexed by Tf. The fact that reaction (g) is considerably faster than (e) suggests that serum components, other than Cp, maintain Fe(II) or Fe(III) in a more available form.

A comparison of (j) with (h) and (i) suggests the formation of a Fe(III)–HCO_3^- complex which can transfer Fe(III) to Tf before extensive hydrolysis. High concentration of HCO_3^- (27 mM) found in serum may also prevent iron polymerization to form a reactive Fe(III)–HCO_3^- complex and subsequently Tf saturation, in agreement with an earlier report from this laboratory [13].

The use of serum and the appropriate order of additions in our experiments in table 1 were of a more realistic design than other published studies of the formation of diferric–Tf from Fe(II). However, because of experimental limitations we could not match in vivo conditions precisely. All experiments used dissolved oxygen at ~216 μM, higher than found in the plasma (53–120 μM). However, the reduction in oxygen concentrations would not affect the rate of the Cp-catalyzed reactions, since Cp is saturated at low oxygen levels, ~10 μM [3]. In contrast, the rate of Fe(II) oxidation is likely to be first order with respect to low oxygen concentrations [3]. The physiological concentration of Fe(II) is another example. When $Fe(III)_2$–Tf is used as the end point, Fe(II) must be $\geqslant$109 μM. Yet it is estimated that free Fe(II) concentration in the plasma is $<1\mu$M [3]. Again this would lower the rates with Fe(II) alone by factor of 100, but would not affect the Cp oxidation, since it has a K_m of 0.6 μM for Fe(II) [14]. We also note that the concentration of ascorbate (1 mM) used in [6] was 20–30-times greater than typical serum levels. Other constituents of serum also have been shown to have a marked effect on $Fe(III)_2$–Tf formation [15]. Neither in [6] nor in [7] have these facts been taken into account if the design of their reactions was intended to represent in vivo conditions.

Finally, we note that Cp offers a distinct qualitative advantage in Fe(II) oxidation since it produces no superoxide, peroxide nor hydroxyl radicals [15]. These oxygen by-products have been reported for the auto-oxidation of Fe(II) [16].

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