DOES TRANSFERRIN EXHIBIT FERROXIDASE ACTIVITY ?*

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SUMMARY

The oxidation of Fe^{2+} to Fe^{3+} by oxygen at pH 7.45 is a first order reaction with a 25 minute half life. In the presence of apotransferrin the oxidation rate is greatly enhanced and Fe^{3+}-transferrin is formed. The apotransferrin mediated reaction reaches 50% completion in one minute; it does not follow simple first order kinetics. Iron-saturated transferrin does not exhibit the rate enhancement effect suggesting that the specific metal binding sites are the loci of the iron oxidation. Addition of H_2O_2, an agent which rapidly oxidizes Fe^{2+} to Fe^{3+}, during the reaction of Fe^{2+} with apotransferrin greatly decreases the yield of Fe^{3+}-transferrin. It is postulated that the basis of the rate enhancement effect is the binding of Fe^{2+} to the metal binding site of the transferrin molecule, followed by a rapid oxidation of the iron to the trivalent form.

Transferrin, the plasma iron transport protein, binds Fe^{3+} under physiological conditions. Transferrin also binds several other di- and trivalent metal ions (1). Fe^{2+} binding is uncertain, but is of special interest since there is evidence that iron is more readily assimilated by the intestine and may become available to the blood in the ferrous form (2). Indeed, Frieden and coworkers (3,4) have postulated that the role of ceruloplasmin (ferroxidase) in iron metabolism is catalyzing the oxidation of "free" Fe^{2+} to Fe^{3+} so that the latter might become bound to transferrin. The binding of Fe^{2+} to transferrin has not been demonstrated. Gaber and Aisen (5) found no evidence for the formation of an Fe^{2+}-transferrin complex. Ross et al. (6) investigated the formation of Fe^{3+}-trans-

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ferrin from Fe^{2+} salts in the presence of dissolved oxygen, and discussed two possible reaction sequences: 1) the oxidation of Fe^{2+} to Fe^{3+} followed by a rapid reaction of the latter with apotransferrin; and 2) the formation of an Fe^{2+}-transferrin complex followed by oxidation of the iron to the trivalent form. The authors did not determine which sequence is operative. Koechlin (7) suggested that prior oxidation of iron is rate limiting (sequence 1). This observation is a fundamental tenet in using Fe^{3+}-transferrin formation as a basis for the spectrophotometric assay of ceruloplasmin activity (3,4).

EXPERIMENTAL AND RESULTS

Rate of Oxidation of Fe^{2+} in Buffer - $Fe(NH_4)_2(SO_4)_2$ was dissolved in oxygen free distilled water and taken to pH 7.45 (under nitrogen) by passing a stream of ammonia over the surface while monitoring the pH potentiometrically. It is important that the pH be adjusted under anaerobic conditions. An 0.1 ml aliquot of the Fe^{2+} solution is removed and added to 9.9 ml of air equilibrated buffer containing 5 mM Tris-HCl and 20 mM NaCl at pH 7.45. An aliquot of the Fe^{2+}-buffer solution is taken immediately upon mixing and at various times thereafter and analyzed for Fe^{2+} using o-phenanthroline (8). Control experiments indicate the o-phenanthroline test is linear with Fe^{2+} and not affected by the presence of Fe^{3+}. The oxidation of Fe^{2+} in buffer solution is plotted in first order form in Fig. 1. The reaction follows first order kinetics with a half life of 25 minutes.

Rate of Oxidation of Fe^{2+} in the Presence of Apotransferrin - Human apotransferrin was obtained from Behring Diagnostics, and made chelate free by an adaptation of published methods (9). In an experiment exactly parallel to the above, Fe^{2+} was added to an air equilibrated apotransferrin solution. Aliquots of the reaction mixture were taken at various times and Fe^{2+} determined by the o-phenanthroline method. The reaction was

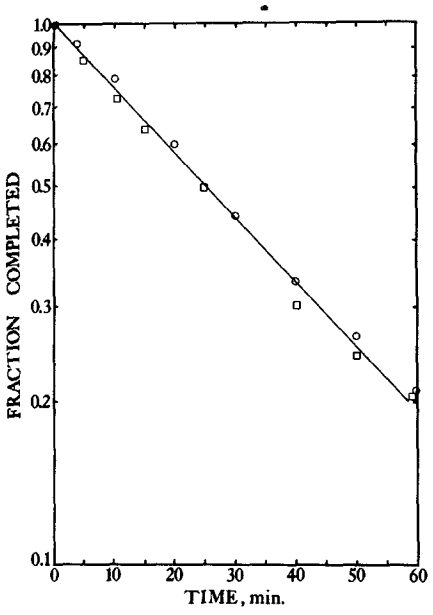

Fig. 1.

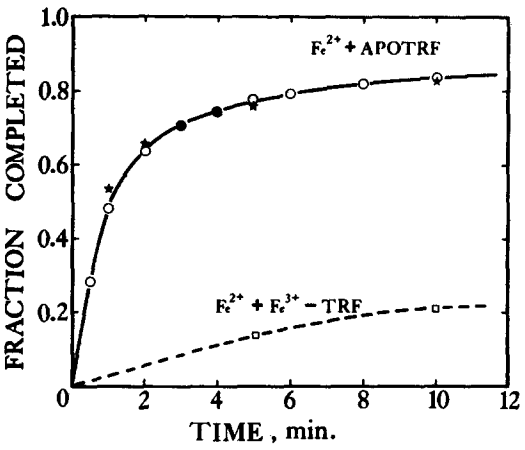

Fig. 2.

Figure 1. The oxidation of Fe^{2+} to Fe^{3+} by dissolved oxygen in buffer alone (o) and in 2 x 10^{-4}N Fe^{3+}-transferrin (□). Both solutions were pH 7.45 and contained 5 mM Tris-HCl and 20 mM NaCl. The Fe^{2+} concentration was 2 x 10^{-4}M. Both reactions were monitored by the o-phenanthroline method.

Figure 2. The oxidation of Fe^{2+} to Fe^{3+} by dissolved oxygen in the presence of apotransferrin (solid line). The reaction was monitored by the o-phenanthroline method (★) and the increase in absorbancy at 470 nm (o). The reaction mixtures initially contained 2 x 10^{-4}N apotransferrin, a saturating amount of Fe^{2+}, 5 mM Tris-HCl, and 20 mM NaCl. The protein solution was air equilibrated. For comparison the initial phase of the oxidation reaction in the presence of 2 x 10^{-4}N Fe^{3+}-transferrin is shown (dashed line).

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also monitored by the increase in absorbancy at 470 nm due to the formation of Fe^{3+}-transferrin. The results are shown by the upper curve in Fig. 2. The two methods of monitoring give equivalent results. The oxidation rate is greatly increased. The time required to reach 50% completion is reduced from 25 minutes in the buffer alone, to 1 minute in the presence of apotransferrin. The reaction does not follow simple first order kinetics.

Rate of Oxidation of Fe^{2+} in the Presence of Iron Saturated Transferrin - Factors which might be involved in the rate enhancement effect observed

with apotransferrin include: 1) catalysis by trace contaminants; 2) non-specific action by the transferrin molecule; or 3) binding of Fe^{2+} to the specific metal binding site of transferrin with an increased susceptibility of the iron to oxidation. To investigate these alternatives, we carried out the Fe^{2+} oxidation in the presence of chelate free iron saturated transferrin. In Fig. 2 is shown the initial portion of the reaction. It is much slower than the apotransferrin mediated reaction. In Fig. 1, the long term data is presented in first order form. It is apparent that transferrin has no effect on the oxidation process unless vacant metal binding sites are present, pointing to these specific sites as the loci of the rate enhancement effect.

Contamination Considerations - We do not believe contaminants are a factor for the following reasons. 1) Behringwerke AG transferrin is 99% pure, containing traces of hemopexin (0.45%) and β_2 glycoprotein II (0.3%). 2) Further purification by SE Sephadex C-50 chromatography (10) eliminated the hemopexin and had no effect on the reactivity of apotransferrin. 3) Iron-saturated transferrin, containing the trace macromolecular impurities, has no effect on the iron oxidation rate in buffer or on the rate enhancement effect in the presence of apotransferrin. 4) Azide, a strong ceruloplasmin inhibitor, has no effect on the apotransferrin reactivity. 5) Low molecular weight impurities are eliminated by gel filtration first in 0.1 M $NaClO_4$ and then in buffer. 6) Our data indicates that the oxidation reaction must take place at the metal binding site by molecular oxygen.

The Peroxide Effect - Addition of H_2O_2 to Fe^{2+} in buffer leads to the rapid oxidation of the bulk of the iron. To test the effect this would have on the rate and extent of Fe^{3+}-transferrin formation, we added Fe^{2+} to apotransferrin containing H_2O_2. The results are shown in Fig. 3. Spectrum #2 is Fe^{3+}-transferrin, formed by the addition of Fe^{3+}-NTA (nitrilotriacetate) to apotransferrin (11). Spectrum #3 is obtained one

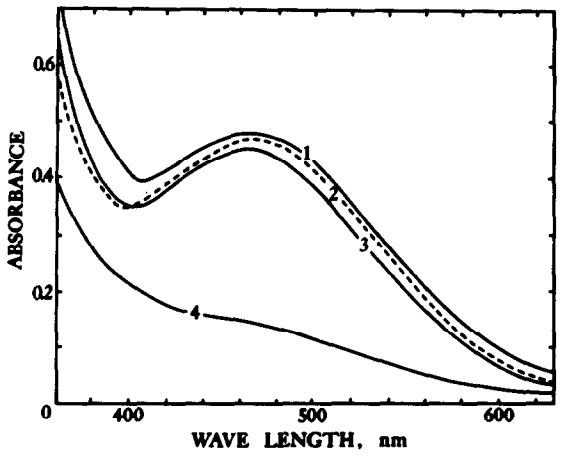

Figure 3. Spectra obtained upon the reaction of 2 x 10^{-4} N apotransferrin with a saturating amount of Fe^{3+}-NTA (#2) or $Fe(NH_4)_2(SO_4)_2$ (#3). Spectrum #4 was obtained after addition of the Fe^{2+} salt to apotransferrin containing 2 x 10^{-4} M H_2O_2. Upon addition of a saturating amount of Fe^{3+}-NTA to the mixture of spectrum #4, Fe^{3+}-transferrin was formed and spectrum #1 was recorded. Spectra were obtained with a Cary 15 spectrophotometer with the slight hemopexin shoulder (0.03A) at 415 nm being deleted.

hour after the addition of Fe^{2+} to apotransferrin. Spectrum #4 results when Fe^{2+} is added to apotransferrin containing H_2O_2. There is a marked decrease in the yield of Fe^{3+}-transferrin when H_2O_2 is present. Spectrum #4 is also obtained if H_2O_2 is added quickly after mixing Fe^{2+} and apotransferrin. To test if the H_2O_2 might be acting on the protein, rather than simply oxidizing free Fe^{2+}, we added a saturating amount of Fe^{3+}-NTA to the mixture of spectrum #4. Immediately the orange-red color of Fe^{3+}-transferrin appeared and spectrum #1 was recorded. The peroxide effect suggests that if the iron is oxidized to Fe^{3+} prior to binding to the protein it becomes essentially unavailable. The observation that Fe^{3+} in the *absence of chelating agents* is poorly sequestered by transferrin is in agreement with the finding of Ross et al. (6) and data from our laboratory (12). The addition of one equivalent of Fe^{2+} to apotransferrin yields from 85 to 94% saturation in agreement with Ross et al. (6). Some iron would be expected to oxidize during the reaction time course without

association with transferrin.

DISCUSSION

Our interpretation of these observations is that transferrin binds Fe^{2+} at the specific metal binding site and the resultant Fe^{2+}-transferrin complex is highly susceptible to oxidation. The postulated reaction sequence is:

$$Fe^{2+} + TRF \rightleftharpoons Fe^{2+}\text{-}TRF \xrightarrow{O_2} Fe^{3+}\text{-}TRF$$

A fascinating aspect of bioinorganic chemistry is the assimilation of specific metals at biological metal binding sites. The process must be mediated by specific ligand exchange reactions. The results presented here emphasize that transferrin would react only partially with "free" Fe^{2+} in plasma and very poorly with iron which had been oxidized to Fe^{3+} by an agent such as ceruloplasmin. In contrast, the iron of monomeric Fe^{3+}-chelate complexes is rapidly and stoichiometrically sequestered by the protein (11,13). We feel that these observations lend support to the view of Mitchell et al. (14) that transferrin assimilates iron from specific donor-receptor sites on cellular surfaces. The role of redox equilibria and perhaps ceruloplasmin in the mobilization of iron from these sites will provide for exciting experimentation.

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