The Transferrin-Manganese Complex: A Role for Ceruloplasmin as an Oxidant of Mn

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Abstract

Transferrin is an 80K Dalton dimeric glycoprotein known primarily for its role as a transport protein for iron in mammalian systems. Transferrin (Tf) is also known to bind a number of other ligands under physiologic conditions, including zinc, cadmium, and manganese. Manganese is a vital component of many enzyme systems. Little is known about physiologic transport and uptake of manganese. Evidence has suggested a possible relationship to transferrin (3, 4, 11), and in this experiment, I have attempted to further elucidate the transferrin-manganese relationship. Several characteristics were examined. First, the spectrophotometric properties of the transferrin-manganese complex were studied. Also, the conditions under which complex formation progressed were examined. In particular, the presence of an oxidative agent for manganese, especially the oxidase ceruloplasmin (Cp), was measured as a factor in the rate of complex formation. The overall goal became to increase understanding about the mechanism by which manganese binds to Tf, as compared to iron-transferrin, so that the process of physiologic transport and uptake of manganese may be further understood. The complex was found to have a particularly characteristic peak at 415nm, with an extinction coefficient of 1200. The presence of bicarbonate was instrumental in the formation of the complex as was an oxidative component. Oxidative components greatly increased the rate at which the complex formed. Both peroxide and permanganate were able to hasten the reaction, both

which having inherent problems in measuring the complex. Ceruloplasmin was able to catalyze the complex formation at physiologic levels. The oxidative nature of the complex formation was supported by EPR study. Also, Cp was blocked from effecting the complex by azide but not by catalase, intimating evidence that it is the oxidase core of ceruloplasmin which is essential in the complex formation, probably through the oxidation of manganese to the 3+ state.

Introduction

The primary physiologic transport protein for iron is transferrin, which binds the metal at a site on each half of the dimeric protein, giving a total of two metal ions per protein. Uptake of iron from Tf into cells is accomplished through an interaction between Tf and a Tf receptor. Turnover of this system is facilitated by the superior receptor affinity of the diferric transferrin versus apo-Tf. Iron is bound to Tf in the 3+ state. Tf has been implicated in several other roles including that of growth factor, particularly as a signal for cell proliferation (7). Manganese is a vital trace element playing crucial roles in many enzyme systems including pyruvate carboxylase, arginase, some phosphatases, and manganese superoxide dismutase. It is required for normal growth, in particular normal testicular/ovarian growth, bone development, and also for proper function of the central nervous system. Deficiencies as well as toxicities create neurologic disorders(3, 4,11, 12). Accumulating and concentrating in the liver and pancreas and brain, Mn can be found in the adult human body at a total amount of approximately 15 mg (4, 9). The need for tight regulation of such limited but vital amounts of metal is obvious, but to date the mechanisms for transport and uptake of manganese have not been elucidated. Tf has been found to bind Mn in the 3+ state

(2, 12) and manganese injected *in vivo* was identified as associating with Tf (4, 6). Such ready binding suggests transferrin may have a role in the to-date hidden mechanisms of manganese transport and uptake. Ceruloplasmin is a 132K Dalton copper-containing oxidase. The oxidase activity is a four-copper core which is evolutionarily highly-conserved (the laccase core). Cp is known as an iron oxidase in physiologic systems, and is a full oxidase, reducing oxygen to water with four electrons from the metal ions being oxidized. To date, ceruloplasmin has not been identified to oxidize manganese.

In this paper, the effort to examine the manganese-transferrin relationship was made from the standpoint of the chemical characteristics of the complex, and of the factors effecting complex formation, especially the potential oxidation of manganese by ceruloplasmin. Previous investigations have yielded a great amount of information. In 1969, Aisen prepared the Mn^{3+}-Tf complex by a seven day incubation of manganous chloride with Tf at 4°C without an oxidant (1). The visual-range peak for the complex was at 430nm with an extinction coefficient (1%/1 cm) of 1.2. This corresponds to a molar extinction coefficient of about 9600. That methodology, including the specific buffer and chelation factors for the preparation of the complex has been repeated here in order to contrast with the ceruloplasmin-catalyzed method (1). Manganese association with transferrin *in vitro* (4, 12) and *in vivo* (6, 11) have been reported previously, but the emphasis has been on the identification of the Mn associated with the transferrin-containing fraction (*in vivo* studies) or the Mn which elutes with the Tf in the *in vitro* studies. Specific complex-formation in these instances was not examined by spectrophotometric analysis or any other means. The emphasis on this paper, therefore, is to analyze the formation of the complex as specifically measured by spectrophotometry. The necessity of bicarbonate as an ancillary anion, previously reported (2), was examined as was the necessity of an oxidant.

Ceruloplasmin, permanganate, and peroxide were all examined for their ability to promote complex formation. The contribution of ceruloplasmin was examined in depth, including varying time course, temperatures, and blockage by the oxidase-core inhibitor, azide. Also, to examine the possibility that ceruloplasmin acts as a half-oxidase for manganese (reducing oxygen to peroxide rather than water--the peroxide would then further the oxidation), catalase was used to destroy any peroxide formed. Electron Paramagnetic Resonance (EPR) was used to backup the oxidative nature of the Cp effect.

Materials and Methods

All spectrophotometry was performed on a Cary Model 118 Recording Spectrophotometer. Radiation measurements were made on a Beckman Gamma 5500 Gamma Counter (Beckman, Fullerton, CA). Lyophilized human apoTransferrin (Alpha Therapeutic Corp., Los Angeles) was used at a level of 55μM. Human ceruloplasmin (Vital Products, St. Louis) was used at a level of 2μM. Lyophilized bovine-liver catalase (Sigma, St. Louis) was used at a concentration of 5μg/ml. Sodium Azide was prepared in varying concentrations from a crystalline source. Manganese was used from powdered $MnSO_4$-H_20, except for the radioactive manganese which was liquid $^{54}MnCl_2$ (DuPont, Boston), and for the reproduction of Aisen's method, $MnCl_2$-$4H_2O$ (Mallinckrodt, St. Louis) was used. Mn levels, except where noted, were 110μM, giving a 2:1 molar ratio with Tf. Potassium permanganate, sodium bicarbonate, and HEPES buffer (Sigma) were all prepared from powder. Hydrogen Peroxide was obtained from a 30% (v/v) solution. All solutions were buffered in 100mM HEPES (26mg/ml) and, unless specifically indicated otherwise,

42mM bicarbonate. Incubation, unless otherwise indicated, lasted for 1 hour at 37˚C for Cp and MnO_4, and 1 hour at 20˚C for peroxide-containing solutions. Purification and resolution, where noted, was on a Sephadex G-75 column collecting 1 ml fractions. The transferrin was found to elute in the void volume, primarily. Samples prepared for EPR analysis were reaction-stopped with liquid nitrogen. EPR measurements were made under temperature controlled by liquid nitrogen.

Results

Initially, the complex was prepared without oxidant and allowed to incubate at 4˚C for seven days. Spectrophotometry revealed an absorption peak at 415nm (Fig. 1). Several repeats of the initial experiment done under varying time periods and temperatures revealed the same 415nm peak. Maximal peak formation without a catalyst was very slow in developing, requiring around seven days at 4'c, and around three days at 20'c. The peak was detrimentally effected by concentrations of Mn less than 2:1 molar with that of Transferrin (Table 1). A 2:1 molar ratio Mn:Tf solution was compared to ratio solutions both greater and lower and was found not to be superseded by higher concentration solutions in terms of final absorbance (Table 1). At 24 hours, a 2:1 molar ratio was about 95% as effective as 15:1 molar ratio in creating 415nm absorbance. However, in time-constrained experiments such as four- and eight-hours, the higher Mn:Tf ratios provided *quicker* complex-formation (Table 1).

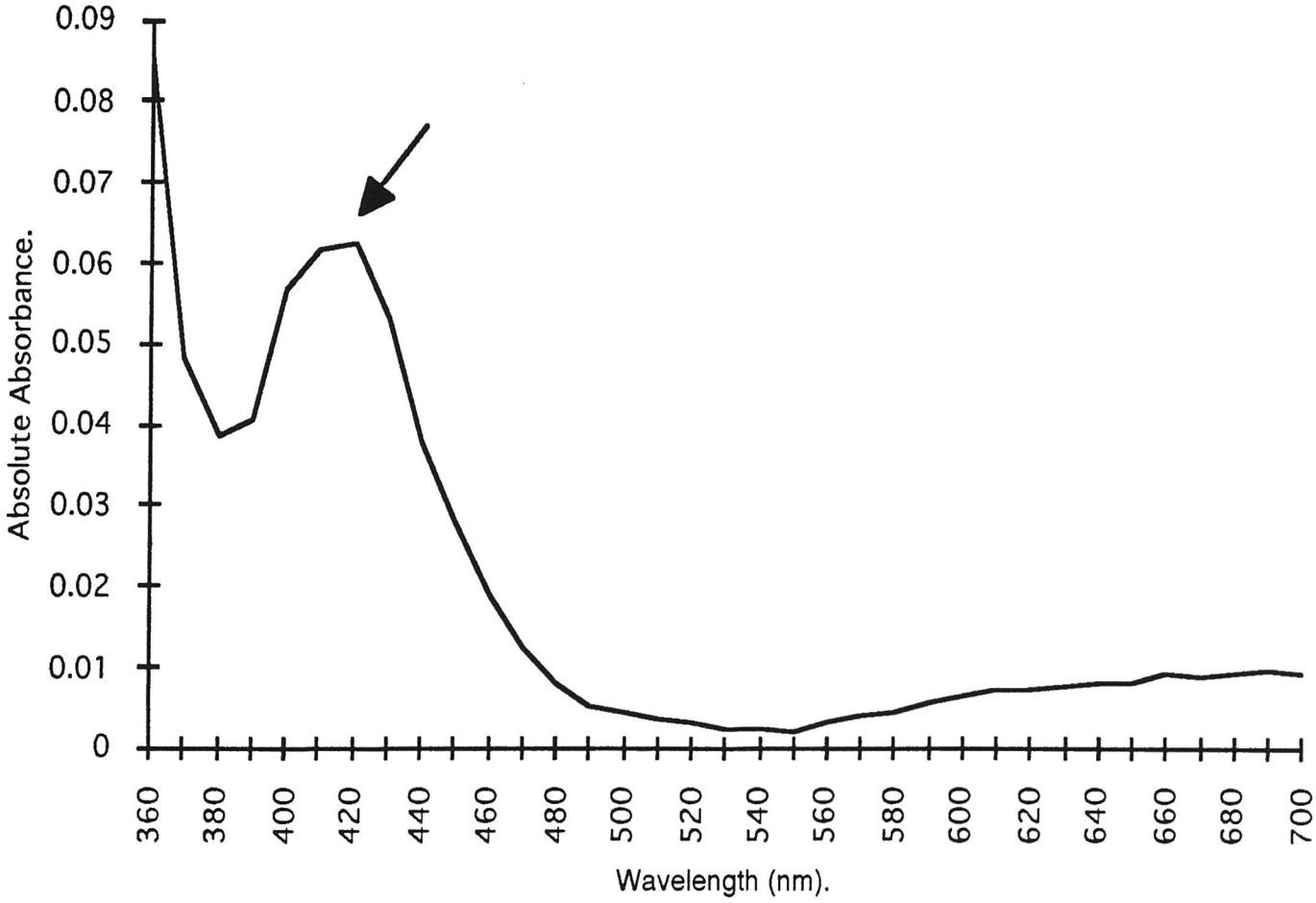

Figure 1.
Visible range absorbance of Mn+Tf solution without oxidant, 4°c, 7 days.
Arrow indicates 415 nm peak characteristic of this solution and used for as a measurement point for the Mn-Tf complex formation in later experiments.

	Absorbance over Baseline	
Molar Ratio(Mn:Tf)	8 Hours	24 Hour
.05:1	.0002	.0012
.33:1	.0004	.0038
.50:1	.0015	.0033
1.0:1	.0036	.0204
2.0:1	.0091	.0348
3.0:1	.0100	.0366
4.0:1	.0161	.0361
15.0:1	.0176	.0387

Table 1.
Effect of varying molar ratio of Mn:Tf
on rate and degree of complex-formation (measured
as absorbance over baseline at 415nm).
No oxidant was present, 4°C.

The 24 hour absorbance of non-catalyzed solution at high molar ratios (2:1 or greater) was about half the maximal absorbance over baseline recorded for Cp-catalyzed complex-formation. From the latter absorption data, a molar extinction coefficient of 1200 was determined. Repeating the methods of Aisen in 1969 (1) provided a peak at 412nm, absorbance of .0912, which converts to a molar extinction of 146 given that the solution prepared was 625μM.

Spectrophotometry of non-complex solutions (solution in which transferrin and manganese are not both present) revealed that HEPES, bicarbonate, azide, permanganate, peroxide, and manganese provide no absorption peaks in the visible region. ApoTransferrin and ceruloplasmin have minimal visible band peaks, but neither provide interference with measurement at the 415nm region. Catalase, on the other hand, has a very strong peak at about 410nm due to its heme group. However, at a level of 5 μg/ml, contribution to 415nm measurement was negligible. Except when manganese and transferrin were present together no combination of any solution material produced significant 415nm absorption (except, as mentioned, the catalase, which was kept negligible by using catalytic amounts). This indicated the reliability of 415nm absorbance as a measure of complex formation. Binding of radioactive manganese ($^{54}MnCl_2$) to transferrin in the presence of Cp, 24 hour incubation at 4°C, yielded a strong peak in the transferrin fraction (Fig. 2). Bicarbonate levels were shown to effect rate-of-formation for the complex. Specifically, exclusion of bicarbonate from the solution prevented all but minimal amounts of complex-formation even over one week periods. Excess bicarbonate (greater than the normal 42mM used in all other experiments) provided slightly increased rate of formation, but not significant differences in absorption after long periods (no elevation of final absorbance).

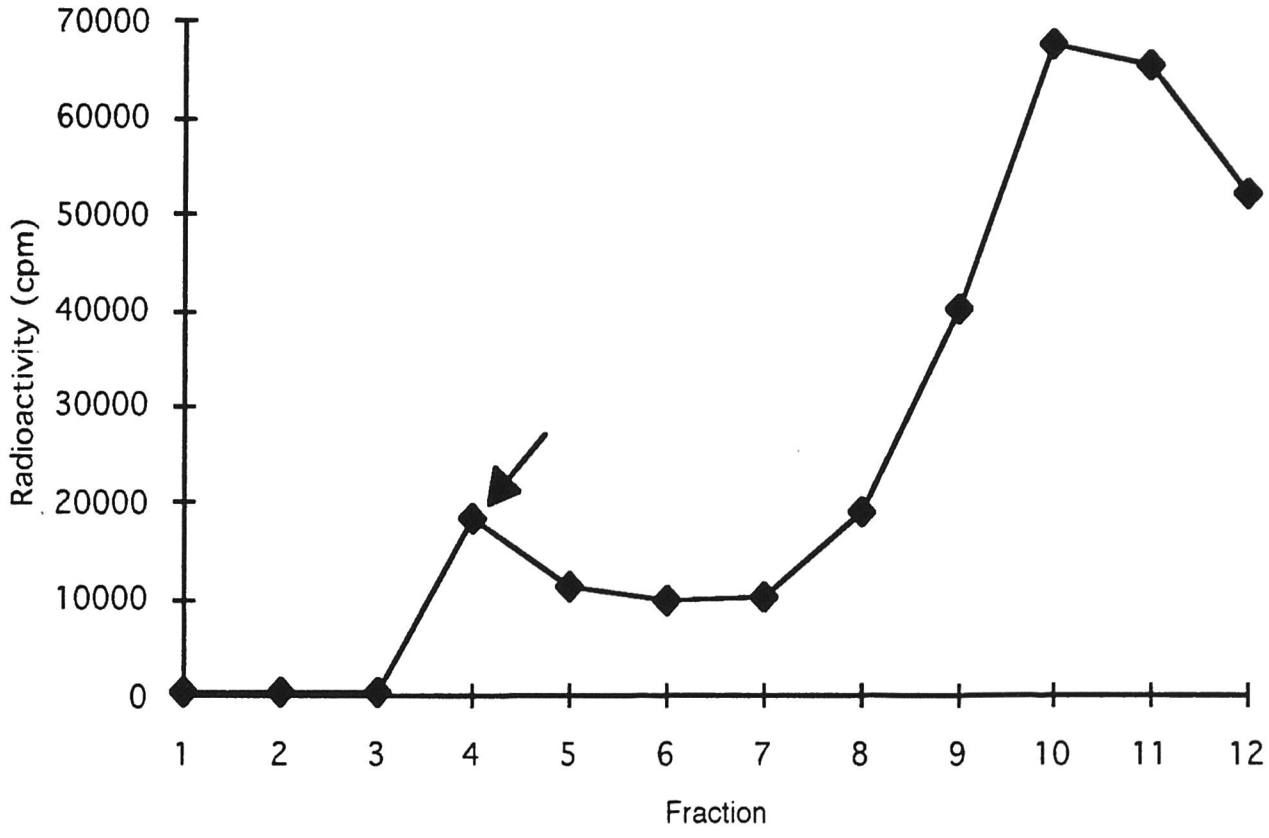

Figure 2.
Association of 54Mn with the transferrin fraction (arrow).
Mn and Tf incubated with Cp, 4˚c, 24 hours.

Reliance on the presence of an oxidizing agent was strongly demonstrated. Peroxide and permanganate both greatly enhanced the rate-of-formation for the complex (fig. 3). However, peroxide was associated with a decline in absorption after 15 minutes at 37˚C and after 1 hour at 20˚C. Permanganate had similar results under short periods but a similar, although lesser, decrease in success was demonstrated over increasing time and temperature. Slight 415nm absorption was created by permanganate and transferrin without manganese being present.

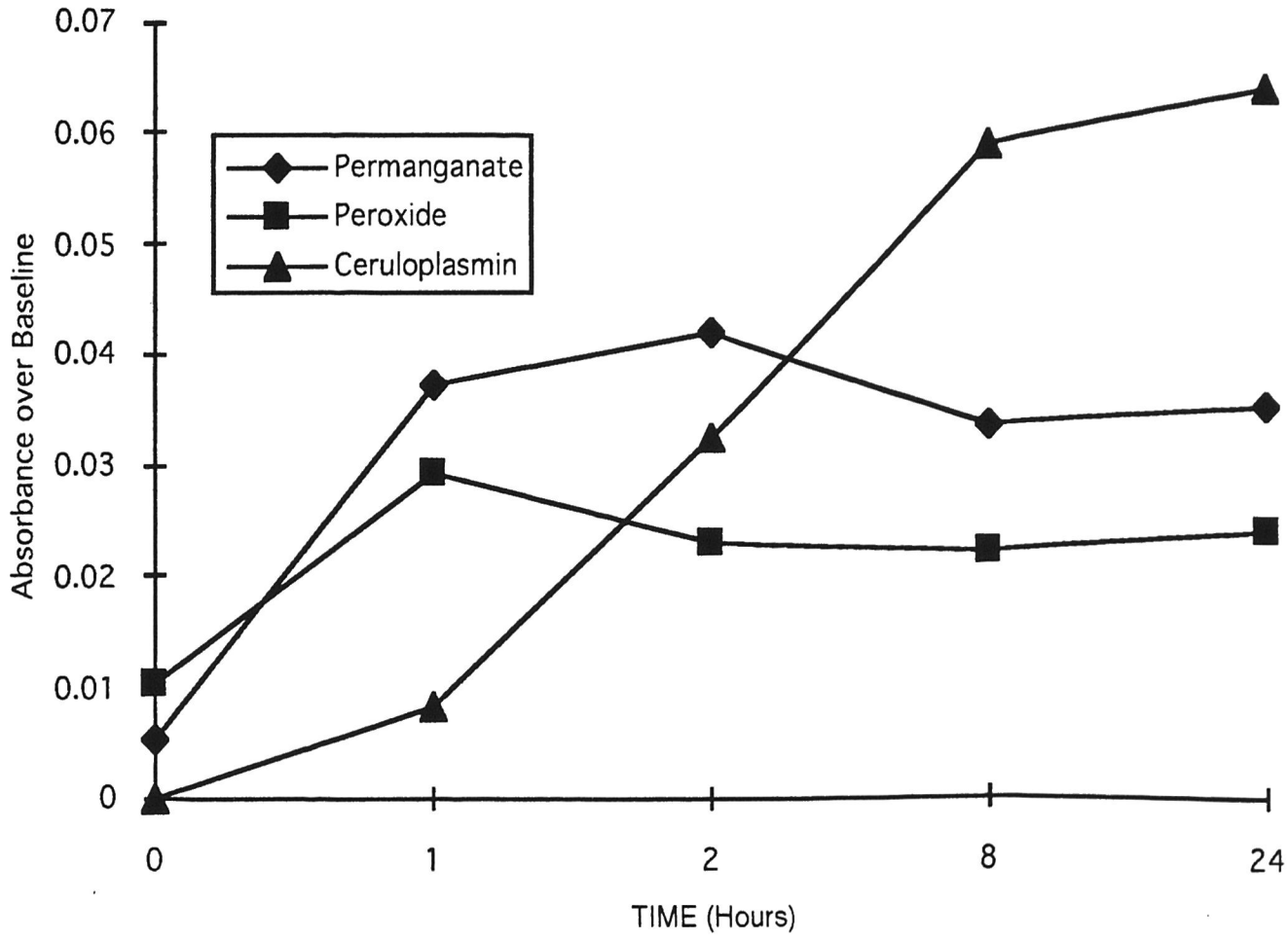

Figure 3.
4°C incubation. Effects of oxidizing agents on complex formation.
2:1 Mn and Tf.

Ceruloplasmin was also effective at promoting rapid formation of the complex (Figs. 3,4), and did so without harming the complex over long periods. In summary of the differing effects, permanganate and peroxide acted to provide rapid complex-growth but with ensuing deleterious effects, whereas the Cp-effect was one of a lag period but eventually followed by more significant peak absorbance. The ability for Cp to facilitate complex formation was completely blocked by azide, which is known to irreversibly block the oxidase core of Cp and related enzymes (Fig. 4). Azide failed to inhibit either peroxide or permanganate. In addition, no detriment to Cp-catalyzed complex-formation was seen in the presence of catalase (Fig. 4).

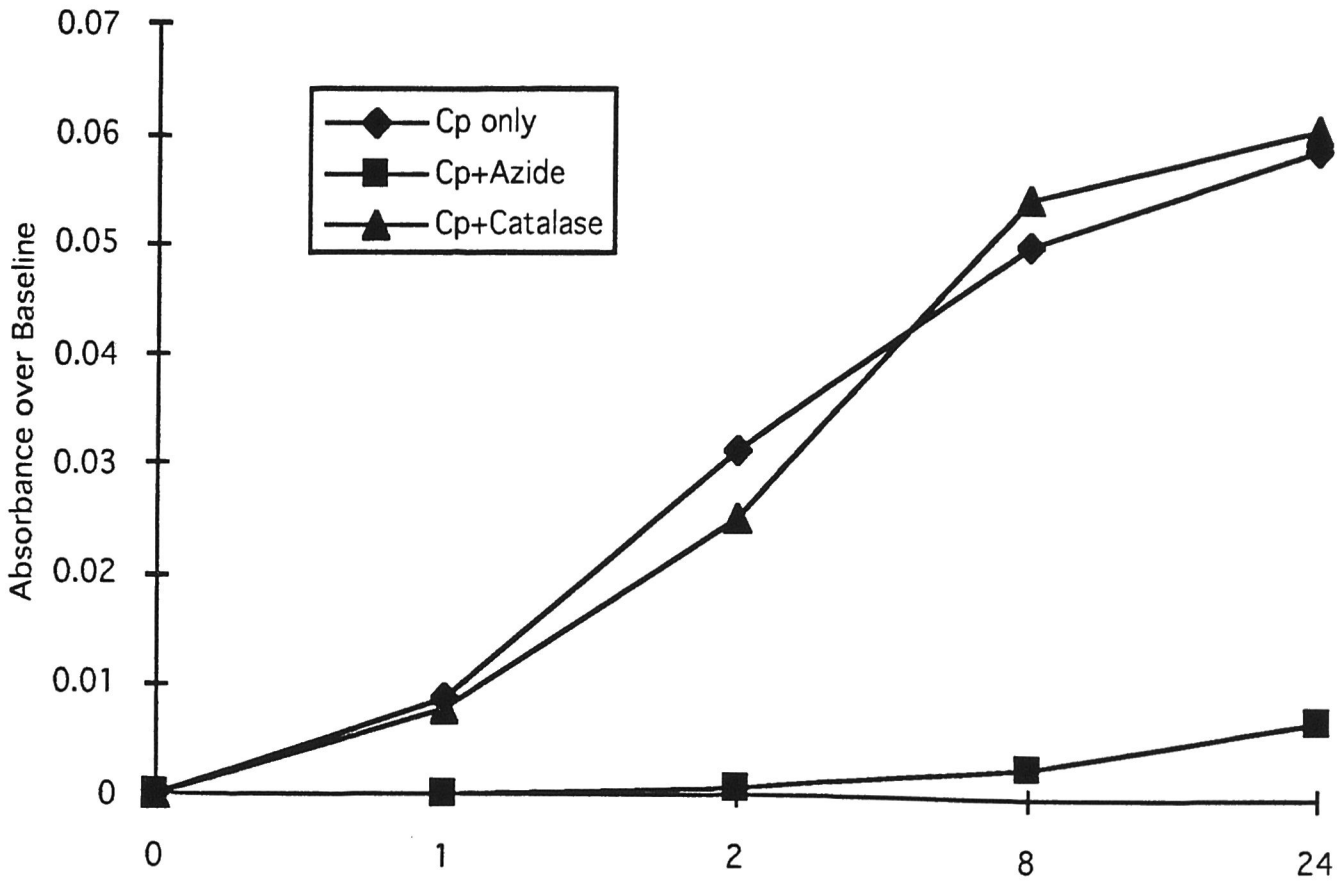

Figure 4.
Effect on Cp-enhanced complex formation by azide and catalase.
2:1 Mn and Tf.

Analysis of the Cp effect by EPR is based on the fact that Mn^{2+} gives a strong EPR signal, whereas Mn^{3+} does not give an EPR signal. Therefore, a loss of signal from a prepared Mn^{2+} solution can indicate conversion to Mn^{3+}. EPR analysis of solutions of Mn^{2+} with and without Ceruloplasmin demonstrated a loss of the 2+ signal in the presence of Cp, and no such loss without it (Fig. 5).

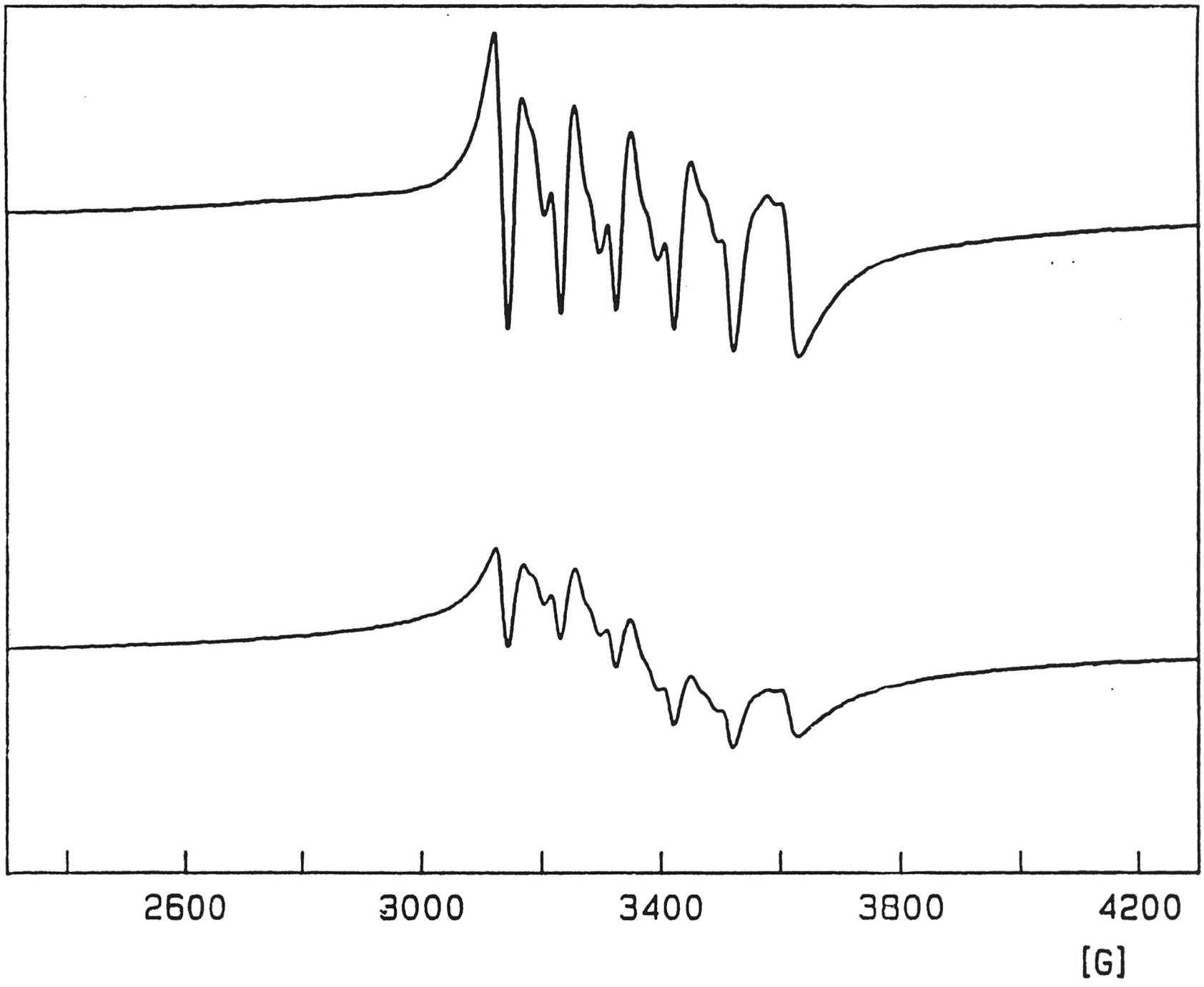

Figure 5.

Effect of Cp on EPR signal of Mn after 6 hours incubation at 20'C.
Mn^{2+} generates an EPR signal, Mn^{3+} does not.
The upper signal is non-Cp and is about identical to
a time zero signal (not shown). The lower signal is the
Cp-catalyzed Mn and shows considerable decay.

Discussion

When manganese and transferrin are allowed to incubate together, a 415nm peak arises with a molar extinction coefficient of around 1200. The peak is dependent upon both Mn and Tf being present and resembles the 460nm peak of diferric-Tf. This data indicates that the peak is representative of Mn-Tf complex formation. The 415nm peak reported in this paper differs slightly from the 430nm of Aisen in 1969. The spectrophotometer used in these experiments were checked with known absorbances, and shown to be correctly calibrated to within 1 nm across the visible range. This indicates the reliability of the instrument and lends credit to the significance of the 415nm peak when combined with the multiply-repeated (>30 times) identification of the peak. Repeating the methods of Aisen produced a peak comparable to the other methods in this paper. The differences could arise from the fact that the original transferrin used in 1969 was prepared from a blood-fraction method, possibly including interfering or contributing components.

The reliance on bicarbonate and on an oxidizing agent indicate that the complex forms analogous to that of diferric-Transferrin. Bicarbonate functions as an ancillary anion, necessary for the ligand interaction with the protein. Reports have suggested that both phosphate and borate may function in this manner in the transferrin reactions, as well (10). Both peroxide and permanganate hasten the reactions, but with apparent side-reactions. Peroxide seems to interfere with long-term stability of the complex, probably from actual degradation of the protein through oxidation of side groups (the peroxide did not cleave the protein because Tf still eluted

normally after peroxide treatment [data not shown]). Permanganate has been demonstrated previously to interfere with transferrin and partially inactivate it (10). Thus, permanganate may contribute manganese to the complex formation as well as inactivate the protein to some degree. This combination of interference along with the fact that permanganate is not physiologic relegates its use in this procedure to the trivial.

Ceruloplasmin catalyzes this reaction as do permanganate and peroxide but at a slower rate and with out deleterious or interfering side-effects. The slow pace could be explained by a slightly lower affinity for Mn^{2+} as compared to Fe^{2+}, a metal which Cp oxidizes rapidly. Evidence for the oxidation of manganese by Cp is further strengthened by the EPR studies. Mn^{2+} creates the signals seen in the EPR plot (fig), whereas Mn^{3+} has no signal. The loss of signal from the manganese in the presence of Cp, but not without it, implies oxidation from 2+ to 3+. Furthermore, blockade by azide lends plausibility to the role of Cp in this system, because it indicates the mechanism involves the oxidase core of the protein, the core which is known to be the critical component in some physiologic metal oxidations and which is specifically inactivated by azide. The specific involvement of the core is also supported by the catalase experiment, which showed catalase to be ineffective at blockade of the Cp effect, thereby suggesting that the protein is acting as a full-oxidase and not as a half-oxidase producing its metal-oxidizing effect through the production of peroxide. This data may implicate a physiologic role for ceruloplasmin as a manganese oxidant, a parallel to its role as iron oxidant.

Conclusion

Evidence for the binding of manganese to transferrin has accumulated to a sparse and non-coalescing degree for the past 25 years. Much of the previous data occurred under non-physiologic conditions or were acquired in non-specific (associational relationship for Mn and Tf) parameters. This paper was an attempt to link those previously-reported relationships into a physiologically-correct analysis of the manganese-transferrin complex. Evidence was collected supporting the complex-formation of dimanganic-transferrin under physiologic conditions. The evidence included evidence that oxidation of manganese to the 3+ state was necessary, highlighted by a potential physiologic oxidant: ceruloplasmin. Permanganate is completely non-physiologic, and the levels of peroxide used to create oxidation were, as well. However, ceruloplasmin demonstrated its effects under physiologic concentrations and conditions. Further study is necessary to determine if transferrin has a role in the transport and uptake of manganese in living systems, and if indeed ceruloplasmin is the manner in which manganese is oxidized prior to the formation of the complex.

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