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IRON-CHELATING AGENTS AND THE REDUCTIVE REMOVAL OF IRON FROM TRANSFERRIN*

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Abstract—2-Formylpyridine thiosemicarbazone, a strong iron (II) and iron(III)-chelating agent, removed iron from transferrin only under reductive conditions. The reaction required a bicarbonate-labilizing anion such as nitrilotriacetate in order for reduction and release of iron to the thiosemicarbazone to occur. Thiols and ascorbate were effective as reducing agents. In one example, thioglycolate served the dual roles of anion labilizer and reducing agent. These results are considered in terms of the general problem of the removal of iron from transferrin, the known property of α-*N*-heterocyclic thiosemicarbazones to remove iron from animals and humans, and the design of iron-chelating agents for use in treatment of iron-storage problems.

The removal of iron from biological systems has been of major interest because of disease states which lead to iron-storage problems directly or indirectly because of treatment [1]. Studies in the design of biological iron-chelating agents have focused on the chelation of Fe(III) from organisms, presumably because both major storage and transport proteins, ferritin and transferrin, bind iron in the 3^+ oxidation state [2]. However, it is unclear whether these are the pools of iron available to external chelation and whether such pools contain only iron in this oxidation state.

According to previous studies of transferrin, there are two conditions under which iron is released or does not bind to this protein: (a) when no bicarbonate is present to complete the ternary complex of iron with transferrin or (b) when Fe^{3+} is reduced to Fe^{2+} [3]. Studies have shown that the iron in transferrin is normally kinetically inert with ligands of large thermodynamic stability with respect to Fe^{3+}, such as EDTA and desferrioxamine [4, 5]. However, the presence of an anion such as nitrilotriacetate (NTA) or pyrophosphate in the system leads to the slow removal of the iron and its binding as Fe(III) to the competing ligand [6, 7]. It is argued that these anions are interacting with the bicarbonate binding site to labilize the iron [4, 8–10]. The alternative possibility that a redox reaction is a key to the release of iron from transferrin has received little attention. Nevertheless, a decade ago Miller and Perkins [11] reported that the iron transfer between transferrin and ferritin is facilitated *in vitro* by the presence of a reducing agent despite the Fe^{3+} oxidation state of each protein. More recently, Bates and Graham [12] have reported that very little Fe(II) is removed from transferrin by bathophenanthroline sulfonate in the presence of ascorbate. The reaction is faster when the ternary complex of apotransferrin, iron(III), and nitrilotriacetate without bicarbonate is used in place of the natural complex. However, it is most rapid when HCO_3^- is added to the system of Tr·Fe·NTA, bathophenanthroline sulfonate and ascorbate.

In the study of the pharmacology of α-*N*-heterocyclic carboxaldehyde thiosemicarbazones, it has been demonstrated that these compounds can effectively mobilize iron from animals and man [13–15]. Furthermore, it appears as if the iron is excreted as the Fe(II) complex of the drugs [14–15]. The great avidity of such materials for Fe(II) and Fe(III) has also been described [16]. Although 2-formylpyridine thiosemicarbazone does slowly extract Fe(III) from ferritin, its reaction with transferrin has not been examined previously [16].

This present study investigates this reaction and shows that iron can be mobilized from transferrin by a reductive process. It also suggests that thiosemicarbazones may be an effective class of drugs for the chelation and removal of iron from biological systems.

MATERIALS AND METHODS

Human apo·transferrin was purchased from the Calbiochem-Behring Corp. (San Diego, CA) and was saturated with iron by a published method using iron–nitrilotriacetate as the donor complex [6]. Final preparations had average 280/466 nm absorbance ratios of 22. Nitrilotriacetate (NTA) was purchased from the Aldrich Chemical Co. (Milwaukee, WI) as the Gold Label quality. Mallinckrodt (St. Louis, MO) was the source for reagent grade $FeCl_3 \cdot 6H_2O$. Reagent grade ascorbic acid was obtained from the Fisher Scientific Co. (St. Louis, MO). With the exception of dithiothreitol (DTT), purchased from P & L Biochemicals, (Milwaukee, WI), the various thiols used in this study were products of the Aldrich Chemical Co. Desferal or desferrioxamine b (H_3D) was obtained from Ciba-Geigy (Summit, NJ) and 2-formylpyridine thiosemicarbazone (HL), which binds both Fe(II) and Fe(III), was a gift of F. A. French. All other chemicals were reagent grade or the highest purity available.

The reactions of iron-transferrin with ligands were

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followed spectrophotometrically over the wavelength range of 400–600 nm as a function of time. When HL served as the ligand for iron, changes in the absorbance at 600 nm, the absorbance maximum of $Fe(II)L_2$, were used to follow the reaction. When HL was not present, the reaction was monitored at the absorbance maximum of iron transferrin, 466 nm.

RESULTS

Past work has shown that desferrioxamine (H_3D) can remove iron(III) from transferrin only when a *labilizing* anion is present which interacts with bound bicarbonate [9]. There are many such molecules, one being nitrilotriacetate. Figure 1 shows the kinetics of iron-transfer from the protein to receiving chelating agents as a function of the presence of NTA and reducing agents. As seen by others, H_3D slowly sequesters Fe(III) when NTA is also in solution [9]. However, 2-formylpyridine thiosemicarbazone is ineffective as a receiving ligand for Fe(III) in the presence of NTA, possibly because the log stability constant of 21 for $Fe(III)L_2$ at pH 7.4 ($Fe^{3+} + 2\ HL_{\text{all forms}} \rightleftharpoons FeL_2$) is not as large as the value of 25.7 for Fe(III)D at this pH [17, *].

This thiosemicarbazone is also an excellent chelator of Fe(II). Thus, the removal of iron from transferrin was examined under reductive conditions. Table 1 and Fig. 2 summarize the basic observations made when ascorbate served as the reducing agent. According to Table 1a, ascorbate alone or in combination with HL had no effect on the visible absorbance band of transferrin. However, when NTA was present, ascorbate very slowly reduced the iron centers. Upon the further addition of HL to this reaction mixture, the characteristic spectrum of $Fe(II)L_2$ with its band maximum ot 600 nm developed over time, as illustrated in Fig. 2, showing that iron was being

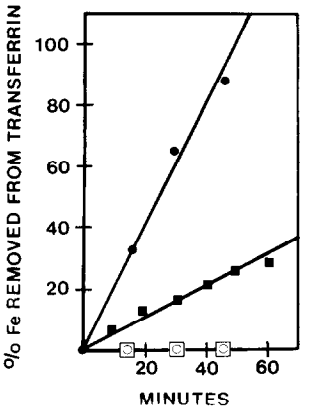


Fig. 1. Kinetics of transfer of iron from transferrin to other ligands. In each run 0.1 mM iron saturated transferrin was used. Key: (○) 4 mM H_3D; (□) 4 mM HL and 15 mM NTA; (■) 4 mM HL, 15 mM NTA and 50 mM ascorbate; and (●) 4 mM H_3D and 15 mM NTA. All reactions were carried out in 0.15 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) buffer, pH 7.4, at 20°.

removed from the protein. The rate of removal was comparable but somewhat less than that achieved by H_3D in the presence of NTA (Fig. 1). Thus, iron can be reduced and taken out of transferrin but only if the anion labilizer, nitrilotriacetate, participates in the reaction.

In Fig. 2 it can be seen that the rate of iron transfer from transferrin to HL is dependent upon NTA, ascorbate and HL concentrations. The initial, observed rates were taken from plots of primary data in which a single reactant was varied over a range of concentrations, holding the others constant. As others have found using H_3D and labilizing anions, the overall reaction does not follow pseudo first-order kinetics, even under conditions of large excess NTA, ascorbate and HL [9]. Instead, the figure indicates that all three substances participate in the rate-determining steps of the reaction mechanism

* W. E. Antholine and D. H. Petering, unpublished information.

Table 1. Reaction of Fe-transferrin under reducing conditions*

	NTA	Reductant	Competing ligand	% Change in absorbance†
(a)	+			0 (466 nm)
	−	Ascorbate		0 (466 nm)
	+	Ascorbate		11 (466 nm)
	−		HL	0 (466 nm)
	+		HL	0 (466 nm)
	−	Ascorbate	HL	3 (600 nm)
	+	Ascorbate	HL	20 (600 nm)
(b)	−	Dithiothreitol		0 (466 nm)
	−	Thioglycolate		0 (466 nm)
	+	Dithiothreitol		14 (466 nm)
	−	Dithiothreitol	HL	0 (600 nm)
	−	Thioglycolate	HL	16 (600 nm)
	+	Dithiothreitol	HL	14 (600 nm)

* Reaction conditions: final concentrations in reaction mixture: 0.1 mM Fe-transferrin, 4 mM HL (except 0.8 mM HL with dithiothreitol), 50 mM ascorbate, 20 mM NTA, 10 mM dithiothreitol and 40 mM thioglycolate. All solutions were made up in 0.2 M Hepes buffer, pH 7.4. Temperature 20°.

† Per cent change in absorbance for either iron transferrin or for the calculated maximum absorbance of FeL_2. The wavelength at which the measurement was made is in parentheses. All reactions were run for 30 min.

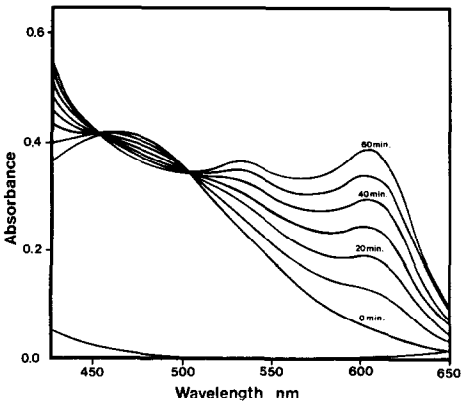


Fig. 2. Visible spectral changes during removal of iron from transferrin. Conditions: 0.1 mM Fe-transferrin, 10 mM NTA, 4 mM HL and 50 mM ascorbate.

and that saturating concentrations of each can be reached. A detailed study and analysis of the reaction kinetics are now in progress.

The reduction is not unique to ascorbate (Table 1b). Dithiothreitol can also serve this role but only in the presence of a labilizing anion such as NTA. Following the report that thioglycolate can act as a labilizing anion in the reaction of transferrin with desferal, a similar reaction was studied in which HL replaced H_3D [6]. In contrast to ascorbate or dithiothreitol, thioglycolate required no other labilizing anion to participate in the reductive transfer of iron to HL. Thus, this thiol appears to serve the dual role of reducing agent and competing anion.

DISCUSSION

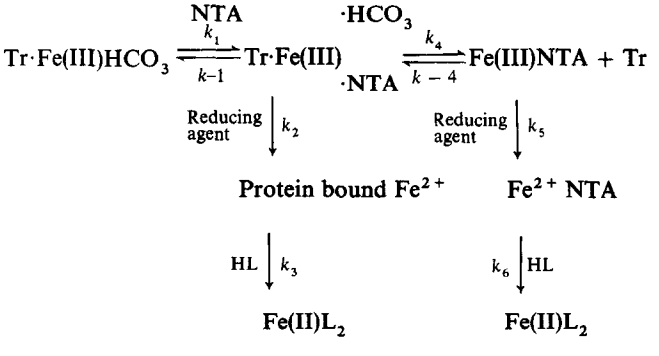
These results demonstrate that a reductive mode of iron release is possible in model systems and is comparable in efficiency to the ligand substitution process. In both types of reactions a labilizing ligand, such as NTA, is a necessary component. A minimal reaction pathway for the reduction mechanism based on previous studies and the present results would be steps 1–3. Here NTA interacts with the bicarbonate binding site. The efficacy of thioglycollate in replacing NTA as well as in reducing the Fe(III) supports this model, for previous studies by Bates and coworkers showed that thioglycollate has the steric requirements to bind in the bicarbonate binding site [18].

The alternative route, steps 1, 4–6, has been postulated by Carver and Frieden for the reaction of transferrin with PP_i at pH 6.1 in the presence of bicarbonate [19].

Here the labilizing ligand NTA serves as an intermediate chelator of Fe(III). Under conditions of our study, NTA in the presence of bicarbonate cannot compete thermodynamically with apotransferrin for Fe^{3+}. The log conditional stability constant for Fe NTA at pH is orders of magnitude less than the value for Tr·Fe·HCO_3 of 10^{24} M^{-1} [20, 21]. Consistent with this, we see no alteration of the visible or e.p.r. spectrum of transferrin in the presence of NTA. Thus, $k_1k_4/k_{-1}k_{-4}$ is very small. Given the rapidity with which Fe(III) NTA can donate iron to transferrin in the standard method for the reconstitution of the protein with iron, it is difficult to imagine the rate-limiting step in this second mechanism being other than either step 1 or 4 [6]. If this is so, the overall rate would not depend upon either the concentration of reducing agent or receiving ligand for Fe(II). Because there is a dependence on each of these reagents as shown in Fig. 3, the pathway, 1, 4–6, is not an attractive mechanism. However, the schemes for both mechanisms are qualitative because of the complexity of the kinetics and the inability at present to define intermediate species in the pathway. In either case, however, the empirical observation remains that iron can be labilized in transferrin by common physiological ligands such as ascorbates or thiols.

At present, there is no direct evidence defining the oxidation state of iron during the physiological removal or iron from transferrin *in vivo*. However, studies do suggest a reductive step at some point in the process of iron uptake by reticulocytes from transferrin, after which the iron is available to Fe(II) specific chelating agents [22]. Similarly, the present work with HL suggests that iron(II) may be available to this ligand *in vivo* via reduction of transferrin. That is, because the log binding constant for $Fe(III)L_2$ at pH 7.4 is 21, some three orders of magnitude less than that for iron-transferrin, the ligand apparently is thermodynamically unable to react with the iron centers of transferrin even in the presence of NTA [18, 20]. However, in a reducing environment at pH 7.4 HL is both thermodynamically (log $K^{Fe}_{Fe(III)L_2} = 15.8$) and kinetically competent to sequester Fe(II) [16, *]. The blue Fe(II) complex

* W. E. Antholine and D. H. Petering, unpublished information.



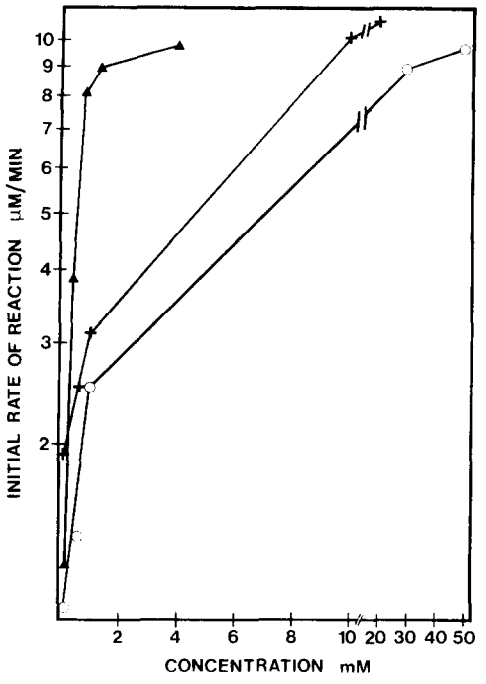


Fig. 3. Concentration dependence of kinetics of iron removal. Iron-transferrin concentration: 0.1 mM; Key: (○–○) ascorbate varied, 4 mM HL and 10 mM NTA; (▲–▲) HL varied, 50 mM ascorbate and 10 mM NTA; (+-+) NTA varied 50 mM ascorbate and 4 mM HL. Reactions were carried out in 0.15 M Hepes buffer, pH 7.4, at 20°.

thus formed is then presumably excreted in the urine of animals and patients, giving rise to the peculiar green–brown urine seen in such studies [14–15].

According to human studies utilizing 5-hydroxy-2-formylpyridine thiosemicarbazone, the amount of iron excreted is 6–100 times the control levels in cancer patients [14]. This is comparable to the quantity of iron which can be mobilized with the clinically used iron-chelating agent, desferrioxamine [14]. Hence, as suggested earlier, it would be of interest to examine selected α-*N*-heterocyclic carboxaldehyde thiosemicarbazones as agents for the removal of iron from individuals with problems of iron-overload [15].

In one study, 5-hydroxy-2-formylpyridine thiosemicarbazone was tested as an iron chelator [23]. However, as seen in cancer chemotherapy studies, the drug was too toxic for use. It has also been suggested elsewhere that some iron complexes of ligands showing cytotoxicity may themselves be the active forms of the complexes *in vivo* [24]. Thus, some of the iron complexes may produce significant host toxicity. Nevertheless, the extensive work of French and coworkers shows that there are a number of thiosemicarbazones such as 2-formyl-3-hydroxy-4-methylpyridine thiosemicarbazone and 1-formyl-5-sulfonate-isoquinoline thiosemicarbazone, which contain the basic metal-binding structure. These are tolerated in animals at concentrations much higher than the 5-hydroxy compound and yet have little or no antineoplastic activity [25]. Thus, there are reasonable candidates for examination among this class of compounds.

More generally, this study points out that the iron in transferrin can be removed by a reductive mechanism. Therefore, in the design of potential iron-chelating agents for clinical use, there is a chemical basis for constructing Fe(II)-specific ligands to complement the efforts to date which have centered on ligands which preferentially bind Fe(III) [2].

In summary, this study demonstrates there are conditions under which iron may be reductively removed from transferrin. An anion to labilize the transferrin-bound bicarbonate is needed. This type of reaction is a plausible way by which α-*N*-heterocyclic carboxaldehyde thiosemicarbazones can bind iron in organisms. These findings suggest new approaches to the design of iron-chelating agents useful in removing iron from humans with iron-storage problems.

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Note added in proof—Kojima and Bates [*J. biol. Chem.* **254**, 8847 (1979)] have recently reported on the reductive removal of iron from transferrin.

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