Differences Between the Binding Sites for Iron Binding and Release in Human and Rat Transferrin

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Iron release from human and rat transferrin was studied using a system in which the release mediated by 2,3-diphosphoglycerate in the presence of desferrioxamine was measured by the change in absorbance at 295 nm. With both proteins iron release could be resolved into two components that represented release from the two iron binding sites at markedly different rates. The rates of iron release were directly proportional to the hydrogen ion concentrations. No difference was found between the rates of iron release from the two isotransferrins isolated from rat plasma. Measurement of the rates of iron release was used to determine at which site iron was bound after addition to the apotransferrins. When iron was added at pH 6.0, it preferentially bound to the more slowly releasing site of both species of transferrin. However, when added at pH 7.4 the iron of ferrous ammonium sulfate and ferric chloride bound equally to the two binding sites, but that of ferric nitrilotriacetate and ferric citrate bound preferentially to the slow-releasing site. Radioactive iron bound to the slow- or fast-releasing sites was taken up at similar rates by reticulocytes. It is concluded that the two sites of human and rat transferrin differ in the process of iron binding and in the rate of iron release but that this is unlikely to be of functional importance.

Transferrin contains two specific iron binding sites, each of which binds one atom of Fe$^{3+}$ along with one molecule of HCO$_3^-$ (or CO$_3^{2-}$). Elucidation of the nature of these sites and of the molecular details of the reaction with iron is a problem of importance for understanding the biologic functions of the protein. One question that has received considerable attention is whether or not the sites are identical both in terms of iron binding properties and in their capability of donating iron to tissues. Earlier work suggested that the sites were identical, but more recent studies have shown that there are small but definite differences.

Although transferrin binds iron very firmly, the iron is rapidly released to certain tissues, particularly erythropoietic tissue. In addition, the iron may be released from transferrin and transferred to other transferrin molecules or to the ferric iron chelator desferrioxamine under the action of certain organic phosphate compounds such as 2,3-diphosphoglycerate and ATP or iron chelators.

The aim of the present investigation was to study the phosphate-mediated release of iron from transferrin to determine if there were any differences between the two iron binding sites and to see whether or not such differences were of functional importance. 2,3-Diphosphoglycerate was chosen as the mediator of iron release because of its relative stability. The fact that it can cause the re-
lease of iron from transferrin does not imply that it functions in such a manner in immature erythroid cells. However, it is possible, but unproven, that other organic phosphates such as ATP or GTP may have such a function and may be the link between cellular metabolism and iron uptake.13

MATERIALS AND METHODS

Transferrin. Two preparations of human transferrin were used. One was obtained from Behring Diagnostics in the iron-free form; this was used for all of the experiments except the one in which isoelectric focusing was performed (see Results). Any residual chelator was removed before use by dialysis for 8-16 hr in turn against 1 liter 0.1 M NaClO₄, 1 liter 0.1 M NaHCO₃, and 1 liter 0.15 M NaCl.

The second preparation of transferrin was from plasma obtained from a healthy human volunteer. The serum iron was 85 µg/dl and the unsaturated iron binding capacity was 240 µg/dl. To 75 ml plasma 25 ml 1 M Tris-HCl buffer (pH 8.3) was added. Next, ferrous ammonium sulfate (280 µg Fe²⁺ dissolved in 2 ml 0.01 N HCl) was mixed with 50 µCi ⁵⁹Fe (as ⁵⁹FeSO₄, specific activity 10 µCi/µg, dissolved in 0.15 M HCl; New England Nuclear). The added iron was sufficient to oversaturate the binding capacity of the transferrins by 55%. The mixture was then incubated at 37°C for 30 min and allowed to stand overnight at 4°C. Then 100 ml of this mixture was applied to a Sephacryl S200 column (5 x 95 cm, equilibrated with 0.1 M Tris-HCl buffer pH 8.3, void volume 300 ml), and elution was performed with the same buffer at a flow rate of 180 ml/hr. Eluate was collected in 15-ml fractions. The pink-colored fractions containing ⁵⁹Fe-transferrin were pooled and applied to a column (K 5.0 x 40 cm) filled with DEAE-Sephacel (Pharmacia) equilibrated with 0.1 M Tris-HCl buffer pH 8.3. The transferrin fraction was found in the first few centimeters of the iron exchange gel. The elution was performed using a linear buffer gradient (0.1 0.5 M Tris-HCl pH 8.3, mixed volume 500 ml). The ⁵⁹Fe-containing fractions were pooled and concentrated by pressure filtration (Amicon ultrafiltration cell 202, PM 10 filter).

The two transferrin preparations were examined by disc gel electrophoresis using 8.5% polyacrylamide and Tris-glycine buffer pH 8.1. The apotransferrin from Behring Diagnostics showed two bands, a major band and a minor one estimated to contain 5%-10% of the total protein. However, both bands consisted of transferrin, since they both bound iron and showed the same increase in electrophoretic mobility. It was assumed that the minor band consisted of transferrin that had lost some of its sialic acid during preparation. The transferrin prepared in this laboratory showed only one band, which stained for both protein and iron, thus confirming its purity. Control studies showed that the rate of iron release under the action of 2,3-diphosphoglycerate was the same for both proteins and could be resolved into two components, as described below.

Rat transferrin was purified from rat plasma to which sufficient ferrous ammonium sulfate had been added to saturate the iron binding capacity of the transferrin. Two forms of the transferrin were used. The first was a mixture of two isomers of transferrin present in rat plasma.14 This was obtained from the plasma by ion exchange chromatography as previously described.15 The relative concentration of the two isomers was approximately the same as in the rat plasma from which the transferrin was purified. The second form of rat transferrin consisted of purified samples of each of the isomers, called here fast and slow transferrin because of their difference in electrophoretic mobility. Their pI values were 5.7 and 5.6, respectively. They were purified by gel chromatography on Sepharose 6B, ion exchange chromatography on Sephadex A50, and isoelectric focusing procedures.15 The absorbance ratio A₄₆₅/A₂₈₀ for diferric transferrin was found to be 0.046 as reported elsewhere.16 The mixture of the two rat transferrins was used in all experiments except where specifically indicated below.

Where necessary, iron was removed from transferrin by dialysis against 50 mM EDTA-acetate buffer pH 5.5 followed by dialysis against distilled water. 0.1 M NaClO₄, 0.1 M NaHCO₃, and 0.15 M NaCl. For most experiments iron was added to transferrin as ferrous ammonium sulfate, followed by the addition of NaHCO₃. However, in certain experiments, where indicated, the iron was added as a complex with nitrolitriacetic acid (1:2 molar ratio, pH 6.0) or with citrate (1:20 molar ratio, pH 5.5). In calculating the amount of iron to be added to transferrin solution it was assumed that the molecular weight of transferrin was 80,000 daltons.

Measurement of iron release from transferrin. The rate of iron release from transferrin was measured by the change in absorbance at 295 nm that occurred when a solution of iron-transferrin
was read against a blank solution containing identical concentrations of iron-free transferrin and the other constituents of the solutions. The absorbance measurements were made in a Beckman Model DKII double-beam spectrophotometer. The solutions contained 0.025-0.05 mM transferrin, 5 mM desferrioxamine methane sulfonate (Desferal, Ciba), and 5 mM 2,3-diphosphoglycerate (Sigma) dissolved in 0.15 M NaCl buffered with 0.1 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). The pH of the incubation solution was usually 6.9-7.0, but in certain experiments, as indicated below, some incubations were performed at other pH values.

The wavelength 295 nm is one of the two absorbance maxima found in the ultraviolet difference spectrum of iron-containing transferrin versus apotransferrin. The maxima are believed to be due to the dissociation of tyrosyl residues, which results from the binding of iron, and the absorbance has been shown to increase in proportion to the amount of iron added until a level of 2 moles iron/mole protein has been reached.\(^{17}\) Hence the decrease in absorbance of the difference spectrum may be used to measure the release of iron from the specific iron binding sites. The wavelength 295 nm was chosen in preference to the absorbance maximum at 465 nm in the visible difference spectrum of iron-transferrin versus apotransferrin because iron-ferrioxamine has a much lower absorbance at 295 nm than at 465 nm.

**Isoelectric focusing procedure.** The distribution of the \(^{59}\)Fe between monoferric and diferric transferrin species was monitored by isoelectric focusing in gel columns. In these studies 2-ml samples were dialyzed against 1 mM Tris-HCl buffer (pH 7.6), and a 0.5-ml aliquot was subjected to isoelectric focusing. A 7.5\(^\circ\) polyacrylamide column (1.4 x 18 cm) was prepared according to the method of Karlsson et al.\(^{18}\) The gel contained 10\(^{-}\)ampholine (pH range 5-8), and focusing was carried out in an electrophoresis cell (Biorad Model 155) for 16 hr at 600 V and 10\(^\circ\)C. Thereafter, the gel was cut into slices of 2 mm thickness, altogether about 90 sections/gel. The radioiron content in these slices was measured in a gamma spectrometer (Packard Model 5330). The human diferric transferrin focused 3.2 cm from the cathodal end of the gel (pH about 5.3), and the monoferric species focused 6 cm from the cathodal end (pH about 5.7).

**Iron uptake by reticulocytes.** Reticulocyte-rich blood was obtained from a patient with sickle cell anemia who was subjected to weekly venesections and from rats and rabbits with hemorrhagic anemia. The cells were washed with 0.15 M NaCl and incubated in Hanks and Wallace balanced salt solution with transferrin preparations labeled with \(^{59}\)Fe (see Results). The methods of incubation, washing the cells, and counting radioactivity were the same as previously described.\(^{19}\)

## RESULTS

When diferric human or rat transferrin was incubated with desferrioxamine plus 2,3-diphosphoglycerate, the absorbance of the solution at 295 nm decreased in a curvilinear fashion. With both species of transferrin the absorbance measurements when plotted semilogarithmically could be resolved graphically into two linear components, each of which accounted for 50% of the iron released (Fig. 1). The slopes of both components varied with the pH of the incubation solution, increasing in direct proportion to the H\(^+\) concentration. The results for rat transferrin are shown in Fig. 2. In the case of human transferrin the equations for the regression lines for the fast and slow rates of release, respectively, were:

\[
y = (12.25 \times 10^7) x - 0.0026 \quad \text{(correlation coefficient} \gamma = 0.978), \quad (1)
\]

\[
y = (2.70 \times 10^7) x - 0.0144 \quad \text{(} \gamma = 0.992), \quad (2)
\]

where \(y\) is the rate of iron release in (percent/min) and \(x\) the H\(^+\) concentration (moles/liter).

The above results suggested that the two iron atoms bound by human or rat transferrin were released at different rates under the conditions of the experiment. That is, each iron binding site has an intrinsic rate constant for iron release and the constants for the two sites are very different. An alternative ex-
Iron release from diferric human transferrin (A) and from diferric rat transferrin (B) mediated by 5 mM 2,3-diphosphoglycerate at pH 7.1 and 38°C. See text for details of method. Curves for change in absorbance at 295 nm were resolved graphically into two exponentials.

Fig. 2. Effect of hydrogen ion concentration on rates of iron release from rat transferrin mediated by 2,3-diphosphoglycerate. Regression lines were calculated by method of least squares. ●, values for fast iron release rate; ○, slow release rate.
of transferrin are present until the protein is saturated with iron.\textsuperscript{20} In a second experiment samples of human transferrin labeled with \textsuperscript{59}Fe were incubated with 2,3-diphosphoglycerate and desferrioxamine until approximately 30\%, 50\%, and 75\% of the radioiron had been released from the transferrin. The samples were then dialyzed against two changes of 0.15 M NaCl and two changes of distilled water and were subjected to isoelectric focusing in polyacrylamide gels. Following this the gels were sliced and the radioactivity counted. As shown in Fig. 4 the starting material contained both diferric transferrin and the two forms of monoferric transferrin. The release of iron from transferrin was associated with a decrease in the concentration of iron on diferric transferrin and a relative increase in only one of the two forms of monoferric transferrin. This conclusion was shown to be correct in a subsequent experiment in which the different forms of transferrin were separated by electrophoresis in 6 M urea using the method
described by Makey and Seal. It was shown that incubation of diferric human transferrin with 2,3-diphosphoglycerate and desferrioxamine resulted in the appearance of only one of the two monoferric transferrin bands.

In the case of rat transferrin it was necessary to consider a third possible explanation for the different rate constants for iron release in addition to the two mentioned above, that the results were due to differences between the two transferrin isomers present in rat plasma. However, when the rates of iron release were measured using purified samples of diferric transferrin-fast and transferrin-slow, identical results were obtained (Fig. 5). Monoferric forms of the fast and slow transferrin types in which the iron was bound to the acid-stable binding site were then tested. It was found that iron release could be described by a first-order plot and that at any given pH the release rate was identical for slow and fast transferrins and the same as the slow-release rate found with diferric transferrin (Fig. 5).

The above experiments indicated that the iron atoms bound at the two sites of both human and rat transferrin were released at different rates. Hence by measuring the rates of release of iron under known conditions of pH and 2,3-diphosphoglycerate concentration it should be possible to determine how much of the iron was present on each site of the transferrin molecule. This approach was applied to a study of the binding of iron in different chemical forms by the transferrin molecule. The iron was added to transferrin as FeCl₃ in 0.05 M HCl, ferrous ammonium sulfate in 0.05 M HCl, iron-nitrilotriacetic acid (1:2 molar ratio, pH 6.0), and iron-citrate (1:20 molar ratio, pH 5.5). NaHCO₃ was then added at 50 times the molar ratio of iron. The transferrin solution was buffered with 0.1 M HEPES at pH 6.0 or pH 7.3. Sufficient iron was added to achieve degrees of saturation of the transferrin varying from 25% to 90%. Addition of the iron and the NaHCO₃ did not alter the pH of the transferrin solution by more than 0.1 unit. After the solutions had stood at room temperature for 1 hr and at 4°C overnight the pH values were adjusted to 6.9 with 0.1 N NaOH or HCl and the solutions dialyzed against 0.15 M NaCl-0.1 M HEPES, pH 6.9. Iron release mediated by 2,3-diphosphoglycerate was then measured. The results are summarized in Table 1. When the iron was added in any chemical form at pH 6.0 up to 50% saturation, it bound almost completely to the slow-releasing site and only at higher degrees of saturation to the other

![Fig. 5. Iron release from fast and slow forms of rat transferrin.](image)
Table 1. Effect of Form of Iron, Percentage Saturation, and pH of Addition to Transferrin on Fraction Subsequently Released at the Slow Rate in the Presence of 2,3-Diphosphoglycerate

<table>
<thead>
<tr>
<th>Form of Iron Added to Transferrin</th>
<th>Saturation of Transferrin (%)</th>
<th>Iron Released at Slow Rate (Percentage of Total)</th>
<th>Human Transferrin</th>
<th>Rat Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 6.0* pH 7.4</td>
<td>pH 6.0* pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Fe^{2+} (as ferrous ammonium sulfate)</td>
<td>50</td>
<td>96 53</td>
<td>97 49</td>
<td></td>
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<tr>
<td></td>
<td>90</td>
<td>53 51</td>
<td>49 51</td>
<td></td>
</tr>
<tr>
<td>Fe^{3+} (as FeCl₃)</td>
<td>25</td>
<td>100 51</td>
<td>51</td>
<td></td>
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<td></td>
<td>50</td>
<td>99 53</td>
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<tr>
<td></td>
<td>90</td>
<td>58 50</td>
<td>50</td>
<td></td>
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<tr>
<td>Nitrilotriacetate</td>
<td>25</td>
<td>98 99</td>
<td>99</td>
<td></td>
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<td></td>
<td>50</td>
<td>100 94</td>
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<tr>
<td></td>
<td>90</td>
<td>58 52</td>
<td>52 54</td>
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<tr>
<td>Citrate</td>
<td>50</td>
<td>98 93</td>
<td>95 79</td>
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</tr>
<tr>
<td></td>
<td>90</td>
<td>57 63</td>
<td>58 55</td>
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Each value is the mean of two or three measurements.

*pH at which iron was added.

site. However, at pH 7.3 different results were obtained using the various chemical forms of the iron and between human and rat transferrin. Ferrous and ferric iron appeared to bind randomly to the two sites, although with ferric iron only 70-80% was bound to the specific binding sites, as indicated by the absorbance at 465 nm. With iron-nitrilotriacetate and iron-citrate at saturations of 50% or less the iron bound preferentially to the slow-releasing site. In the case of human transferrin the degree of preference for this site was almost complete, but with rat transferrin some iron bound to the fast releasing site so that at 50% saturation about 75% of the iron was on the slow site and 25% on the fast site (Table 1).

The ability of reticulocytes to take up iron bound to either binding site of transferrin was tested in the following experiment: Diferric human and rat transferrin containing ^{59}Fe at one site or the other were prepared. First apo-transferrin was 50% saturated by adding iron complexed with nitrilotriacetic acid at pH 5.9. Then NaHCO₃ was added. With one sample of transferrin from each species the iron was labeled with ^{59}Fe, and in another it was unlabeled. After the solution stood at 20°C for 4 hr, the pH was adjusted to 7.2 with 0.1 M NaOH. Then the transferrin samples were saturated with Fe-nitrilotriacetic acid, NaHCO₃ was added, and the solutions were incubated at 37°C for 1 hr before being dialyzed against 0.1 M NaHCO₃ and two changes of 0.15 M NaCl. For the second iron addition ^{59}Fe was used with the samples that previously had unlabeled iron and unlabeled iron was used with the samples containing ^{59}Fe. The specificity of labeling with ^{59}Fe at the two binding sites was tested by measuring ^{59}Fe release at pH 7.0 using the 2,3-diphosphoglycerate and desferrioxamine system. It was found that with human transferrin 88% of the iron added at pH 5.9 was at the slow-releasing site and with rat transferrin 81% of the iron was at that site. The transferrin samples were then incubated with human, rat, and rabbit reticulocytes. As shown in Fig. 6 no difference was observed between iron uptake from the two sites using human transferrin and human reticulocytes or rat transferrin and rat reticulocytes. Similarly, no differ-
Fig. 6. Iron uptake by human reticulocytes from human transferrin (A) and by rat reticulocytes from rat transferrin (B). Human transferrin was labeled to 50% saturation at pH 6.0 with $^{55}$Fe (e, o) or $^{56}$Fe (a, i) and then saturated with iron at pH 7.4 with $^{56}$Fe (e, o) or $^{59}$Fe (a, i). Iron concentration of incubation solution was 0.5 μg/ml (e, a) or 0.25 μg/ml (o, i). Rat transferrin was labeled to 50% saturation at pH 6.0 with $^{55}$Fe (e) or $^{56}$Fe (o) and then saturated at pH 7.4 with $^{56}$Fe (e) or $^{59}$Fe (o). Incubations were performed at iron concentrations of 0.5 and 0.25 μg/ml, but no differences were found with the two concentrations; hence mean results for the two concentrations are shown.

...ences were observed when all of the heterologous systems of transferrin and cells were tested (data not shown).

DISCUSSION

The results of these experiments show marked differences in the properties of the two iron binding sites of human and rat transferrins but no differences between the two isotransferrins present in rat plasma. The results with human transferrin confirm other observations based on physicochemical studies of iron-transferrin interaction.38 Hence there are clearcut differences between the sites with regard to the rates of iron release to 2,3-diphosphoglycerate and desferrioxamine, the effect of pH on iron binding, and the rate of binding of iron-nitrilotriacetate and iron-citrate.

The experiments with nitrilotriacetate and citrate are of interest because these compounds are commonly used to chelate iron before addition to transferrin for studies of cellular uptake or ferrokinetics. It has usually been assumed that the iron bound randomly to the two sites, and the results of the experiments have been interpreted based on this assumption. Clearly this is not valid, and it will be necessary to reevaluate such experiments. The present results with human transferrin and nitrilotriacetate confirm those of Harris21 that iron in this form binds preferentially to one binding site even at neutral pH. They are also comparable to similar observations made with conalbumin.22-24

The fact that under the action of 2,3-diphosphoglycerate iron is released more rapidly from one iron binding site than the other even at physiologic pH values could lead to a functional difference between the sites and could represent the basis of the hypothesis proposed by Fletcher and Huehns25 that such a difference is of physiologic importance. However, this is unlikely for the following two reasons: First, one assumption of the Fletcher and Huehns hypothesis is that one site donates its iron preferentially to immature erythroid cells and the other to liver parenchyma cells. The more rapid release of iron from one site could not form the basis for such a behavior. Second, the results of the reticulo-
cyte experiments failed to show any difference in iron uptake from either site. It must therefore be concluded that the difference in iron-releasing properties of the two sites of transferrin is not of functional importance.

The above conclusion is at variance with that of several workers who have presented results supporting the concept of functional heterogeneity of the two iron binding sites of transferrin (see review by Aisen and Brown). However, other investigators found no evidence of such heterogeneity when they studied iron uptake by cells or tissues of the same species as that from which the transferrin was obtained. Possible reasons for the conflicting results in the literature include failure to ensure that all of the radioiron was bound by the specific iron binding sites of transferrin, contamination of transferrin solutions with radioiron-labeled hemoglobin, and the use of heterologous systems, all of which could lead to results that appear to indicate functional heterogeneity of the iron binding sites.

The last of these reasons is illustrated by the experiments presented by Harris and Aisen. They found no difference in the iron-donating properties of the two binding sites when human or rabbit transferrin doubly labeled with $^{59}$Fe and $^{55}$Fe was incubated with homologous reticulocytes. However, one iron binding site of human transferrin appeared to be a better donor of iron to rabbit reticulocytes than the other site. This is in contrast to the results obtained in the present work. The reason for this discrepancy is uncertain, but one possible explanation (Harris DC: Personal communication) is that the human transferrin used by Harris and Aisen became contaminated with rabbit transferrin during the initial incubation with rabbit reticulocytes that they performed to remove some of the $^{59}$Fe from transferrin, after which $^{55}$Fe was added to replace the $^{59}$Fe removed and the transferrin was reincubated with a fresh sample of cells. Some of the $^{55}$Fe would bind to the contaminating rabbit transferrin, which would donate its iron to rabbit reticulocytes more rapidly than the human transferrin. This could explain the results obtained and could lead to the conclusion that the two sites of human transferrin were functioning differently when no such difference existed.

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