Studies on the Binding of Iron to Transferrin and Conalbumin

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SUMMARY

Electrophoretic studies of the binding of iron to transferrin indicate that the stability constants of the two binding sites are approximately equal. The iron in each site acts independently in relaxing the protons of the solvent water. It appears likely, then, that the binding sites are equivalent and independent. Similar results have been obtained for conalbumin, corroborating the evidence that the two proteins have similar structure.

Although the detailed mechanism of the binding of iron to transferrin is not yet understood, general agreement exists on a number of points. The molecule is saturated when 2 iron atoms are approximately equal. The iron in transferrin and conalbumin, and the molecule (4), have similar structure.

The molecular weight of spotransferrin was taken as 88,000 (7), and the $K_2$ >> $K_1$. The molecular weight of apotransferrin was taken as 88,000 (7), and the $K_2$ as 10.4.1

Conalbumin (Nutritional Biochemicals) was purified by chromatography on DEAE-Sephadex as described for transferrin. The iron-free protein was prepared by the same procedure as apotransferrin.

Chemicals of highest purity obtainable and distilled deionized water were used throughout.

METHODS—Restoration of Fe$^{3+}$ to apotransferrin was accomplished at pH 5.73 in 0.1 M cacodylate buffer for the electrophoretic experiments and at pH 8.0 in 0.05 M Tris-HCl buffer at pH 8.0 for the PRR$^2$ experiments. Solutions for electrophoresis were maintained at 2°C, and 3.7 $\times$ 10$^{-4}$ M HCO$_3^-$ was added to hasten equilibrium (2). The PRR studies were done at room temperature, with 5 $\times$ 10$^{-4}$ M HCO$_3^-$ added. Preparations were maintained at 2°C, and 3.7 $\times$ 10$^{-4}$ M HCO$_3^-$ was added to hasten equilibrium (2). The PRR studies were done at room temperature, with 5 $\times$ 10$^{-4}$ M HCO$_3^-$ added. Preparations

1 P. Saltman, personal communication.

2 The abbreviation used is: PRR, proton relaxation rate.
for all studies were allowed to stand approximately 5 days to assure that equilibrium had been achieved.

Restoration of Fe³⁺ to apoconalbumin was done in Tris buffer, pH 8.0, for all experiments.

Proton longitudinal (spin-lattice) relaxation times ($T_1$) were measured at room temperature, the method of spin echoes as developed by Hahn (8-10) being used. The pulse sequence 90-180-$\tau$-90-180, where $\tau$ is a variable delay, was used. The echo following each 90-180 was amplified, rectified, and applied to a two-channel gated integrator. The gates are set so that the area under each echo appears as a d.c. voltage in each of the two integrators. The time between the two echoes, and the two echo voltages, are automatically recorded for subsequent computer analysis.

Data from a given sample are fitted by least squares to the analytical form $y_2 = y_1(A - C \exp(t/T_1))$, where $y_1$ and $y_2$

**Fig. 1.** Optical spectrum of $1.8 \times 10^{-4}$ m transferrin, 0.05 m Tris, pH 8.0.

**Fig. 2.** Addition of Fe³⁺ to apotransferrin. $A$ (left), absorbance at 470 nm; $B$ (right), proton relaxation rate. Total protein concentration, $5.8 \times 10^{-4}$ m.
are the two echo heights, $A$ and $C$ are instrumental constants of
order unity, and $T_1^{-1}$ is the relaxation rate to be determined.
A 1.0- or 1.5-ml sample, 20 points along the relaxation curve,
and the apparatus noise and nonlinearity result in a typical error
of approximately 1% in $T_1$. The scatter in the measured points
probably reflects sample temperature variations (not controlled).

The relaxation rate measured is that of the water solvent
protons, and is considered to be the sum of the partial rate due
to interactions with the paramagnetic iron protein and that of
water alone. The water rate has the commonly accepted value
of 0.33 sec$^{-1}$ at room temperature, while the water-iron protein
rate depends linearly on the concentration of the paramagnetic
species when only one such species is present (11). The absolute
rate due to the iron depends also on the distance of closest
approach of water, which may vary, depending on the shielding
in a particular molecule. If different species are present, the
total rate will be the sum of weighted rates due to each species
(12).

Electrophoretic studies were performed in a modified Perkin-
Elmer model 238 Tiselius electrophoresis apparatus. Mobilities
were calculated from measurements in the descending limbs,
with reproducibilities of ±3%. Conductance was measured at
1000 cps at $2^\circ$, the temperature of the water bath in the apparatu-

![Fig. 3](image3.png)

**Fig. 3.** Moving boundary electrophoresis diagrams of transferrin at varying degrees of saturation with Fe$^{3+}$. Cacodylate buffer, pH 6.7, ionic strength 0.1. $E = 13$ volts per cm. Numbers indicate mobilities of adjacent peaks, in units of $-1 \times 10^{-1}$ cm$^2$ volt$^{-1}$ sec$^{-1}$. Percentages indicate saturation with Fe$^{3+}$. Photographs were taken after approximately 20,000 sec, in ascending limbs.

Optical and PRR Studies of Titrations of Transferrin with Iron—The results of experiments in which increasing amount of Fe$^{3+}$
(as Fe$_2$NH$_4$(SO$_4$)$_2$) were added to a series of tubes containing 1.5
ml of 5.1% apotransferrin in 0.05 M Tris buffer are shown in Fig.
2. In both the optical and the PRR studies a sharp end point is
achieved at 2 g atoms of Fe$^{3+}$ per mole of protein. There is
also the expected linear relationship between absorbance at
470 m$\mu$, where Fe$^{3+}$-transferrin has an absorption maximum,
and the amount of Fe$^{3+}$ added. The relationship between Fe$^{3+}$
added and the measured PRR is nearly linear until the two spe-
cific Fe$^{3+}$-binding sites of transferrin are saturated. If the as-
sumptions are made that (a) iron atoms are distributed randomly
on transferrin binding sites, and (b) the contribution to the relaxa-
tion rate from each site on the molecule is independent, then the
relaxation rate will be linear with iron concentration. The
observed rate is sufficiently linear to be consistent with these
assumptions; the justification for them will be discussed.

Further addition of the paramagnetic Fe$^{3+}$ produces little
change in the relaxation rate of water protons. Evidently,
Fe$^{3+}$ rapidly and completely hydrolyzes at this pH to form ferric
hydroxide complexes (13) which do not affect the PRR of the
bulk water. This is confirmed by measurements of PRR in
Tris buffer to which increments of Fe$^{3+}$ have been added. At
concentrations less than $6 \times 10^{-4}$ M, the added iron does not
change the PRR of the buffer, which is close to that of pure
water. At higher concentrations of Fe$^{3+}$ visible precipitation
of the ferric hydroxide occurs.

The results further suggest that in these experimental condi-
tions only the specific binding sites of transferrin are occupied by
Fe$^{3+}$ until these are saturated. Hydrolysis then competes suc-
cessfully with any nonspecific binding sites of the protein for the
excess ferric ions, so that a “doping” effect of the excess iron is
not observed (12).

Electrophoretic Studies of Transferrin—Free electrophoretic
studies were made of solutions of transferrin at varying satu-
rations with Fe$^{3+}$ (Fig. 3). Although only two protein peaks were
clearly resolved in most of the preparations, three distinct ranges
of mobilities were observed. Thus, the mobility of the single
peak of apotransferrin was $-0.95$ cm$^2$ volt$^{-1}$ sec$^{-1}$. A second,
father (more anionic) peak appears when the preparation is 20%
saturated with Fe$^{3+}$, while the area under the slower peak is
diminished. The mobility of the new component is $-1.20$.
At 40% saturation most of the protein consists of the faster component. As saturation increases to 60%, another, and still faster, component appears, with a mobility of $-1.53$; the slow peak is no longer evident. At 80% saturation most of the protein exists as the fastest component, while at 100% saturation the preparations is again homogeneous, with a mobility of $-1.50$. Three distinct transferrin species are thus identified, and this is confirmed in Fig. 4, which shows a study of the 50% saturated protein. Although three distinct peaks are present, only the middle peak is sufficiently well resolved to measure mobility, and this has the expected midrange value of $-1.16 \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$.

The resolution of the protein components in the 50% saturated preparation was inadequate for separation of the fastest and slowest fractions which would have made possible direct determination of their iron contents. The electrophoretic patterns were obtained at more than twice the usual voltage gradient. Higher gradients were not considered feasible because of heating effects, and longer times resulted in too much diffusion. Separation is also impossible by zone electrophoresis in starch gel or filter paper since the mobilities of transferrin and apotransferrin are the same in these systems (14). The reason for the different behavior of the transferrins in moving boundary and moving zone electrophoresis is still obscure (14).

Optical and PRR Studies of Conalbumin—A linear relationship between absorbance at 470 nm and the amount of Fe$^{3+}$ added, and PRR and Fe$^{3+}$ added, was also found in the titration studies of conalbumin (Fig. 5). The slope of the PRR line was 5.6 sec$^{-1}$ mmol$^{-1}$, a value in reasonable agreement with the 6.7 sec$^{-1}$ mmol$^{-1}$ obtained by Wishnia (15) in Fe$^{3+}$-saturated conalbumin.
Electrophoretic Studies of Conalbumin—Since the isoelectric point of conalbumin is higher than that of transferrin (13, 14), cacodylate buffer at pH 7.25 and ionic strength 0.1 was chosen for electrophoretic studies of conalbumin. The mobility of Fe³⁺-saturated conalbumin in this buffer was \(-0.52 \times 10^{-5}\) cm² volt⁻¹ sec⁻¹, while apoconalbumin was virtually stationary. These results are consistent with those obtained by Warner and Weber (16) at slightly lower pH: approximately \(-0.5 \times 10^{-5}\) cm² volt⁻¹ sec⁻¹ for iron conalbumin, and near zero for the apoprotein. Our results at intermediate levels of saturation clearly indicate the presence of a third form with intermediate electrophoretic mobility, \(-0.2\) cm² volt⁻¹ sec⁻¹ (Fig. 6). The relative areas of the slow, intermediate, and fast forms are functions of the degree of saturation with iron, as in transferrin.

**DISCUSSION**

The observed linearity of the relationship between color and degree of saturation with Fe³⁺ in transferrin, and the near linearity of PRR and degree of saturation, may be due to either of two mechanisms: (a) the two iron-binding sites of the protein are equivalent and independent with respect to chromophoric properties and accessibility to water molecules, or (b) the sites are not necessarily equivalent, but exhibit a positive interaction so that ferric ions are bound to each molecule in pairs (\(K_1 > K_2\)). (A third possibility, that Fe³⁺ is randomly distributed among the two binding sites, but that the metal ions at only one of the sites is active as a chromophore and paramagnetic relaxer, seems very unlikely.) In Case a, the linear relationships would be preserved irrespective of the sites at which the ferric ions are bound, since the increment in proton relaxation or color would be the same for an ion bound to either site. In Case b, the only species of the metal-containing protein present in significant concentration would be the one with 2 ions bound, and the concentration of this form would again be a linear function of the amount of Fe³⁺ added. Thus, optical and PRR studies alone do not distinguish between Case a and Case b, since linear relationships are expected in each.

From the careful equilibrium dialysis studies of Aasa et al. (2), the stability constants of the two sites appear to be the same within very narrow limits. This implies that the distribution of Fe³⁺ on the protein molecules is essentially random, and further that the sites are equivalent with respect to chromophoric and proton-relaxing properties. The electrophoretic studies were undertaken to search for the three species of molecules which would be present if ferric ions were randomly distributed among the available binding sites in solutions of transferrin only partially saturated with iron. These species are (a) apotransferrin, with no bound iron, (b) transferrin, with 1 bound Fe³⁺, and (c) transferrin, with 2 bound Fe³⁺ ions.

Since 3 protons are displaced and 1 bicarbonate ion is bound for the binding of a ferric ion (2), at a pH higher than the isoelectric point the net anionic charge of transferrin is increased by 1 for each bound metal ion. Electrophoretic studies were performed at pH 6.7, close to the isoelectric pH 5.5 (14), where the relative charge difference (ratio of charge difference to total charge) is sufficient to be detectable electrophoretically, while the metal-protein complex is still stable. The existence of the expected three protein species is clearly confirmed (Figs. 3 and 4). The increase in net negative charge of the protein as iron is bound is reflected in the increased anionic mobility of the iron-bearing species. Furthermore, the observed relative concentration of each species at varying Fe³⁺ saturations roughly corresponds to that predicted from a random distribution. It seems clear, then, that the intrinsic association constants of the iron-binding sites of transferrin are the same, and that the sites are equivalent with respect to proton-relaxing and chromophoric properties, and do not interact. This conclusion is also consistent with preliminary electron paramagnetic resonance studies of a single crystal of Fe³⁺-saturated transferrin, which indicate that the two sites have identical resonances.

The slight deviation from linearity of the relationship between PRR and relative saturation of transferrin may be due to experimental error (temperature variation), protein-protein interaction from the relatively high protein concentration (approximately 5%), or to a real but slight difference in the relaxing properties of the sites. Whatever the mechanism responsible, the observed magnitude of the deviation is small enough so that the argument presented above is substantially valid.

We also conclude from the electrophoretic studies that the time for exchange of a ferric ion between binding sites on different molecules is long compared to the time required for the separation of different species in the electric field, thus making electrophoretic resolution possible. In the opposite case, where the exchange time is short compared to the separation time, the protein would be expected to behave electrophoretically as a single species at all degrees of saturation; the anionic mobility of this species would increase as the degree of saturation increases. The long exchange time is consistent with the finding that days to weeks may be required for equilibrium to be established between free and protein-bound Fe³⁺ in transferrin preparations (2).

The behavior of conalbumin in the spectrophotometric, PRR, and electrophoretic studies at varying degrees of saturation with Fe³⁺ is substantially the same as that of transferrin, indicating that the distributions of Fe³⁺ at equilibrium are probably very similar in the two proteins. This is consistent with the finding that the proteins (from the same species) differ only in their carbohydrate prosthetic groups (4), but it is at variance with the equilibrium binding studies of Warner and Weber (5). The results of their work indicate that \(K_2 \gg K_1\), so that the protein complex with a single bound ferric ion would be present in negligible concentration. Only two protein species should then be detected on electrophoresis. Interestingly, however, Warner and Weber (16) had earlier reported the presence of a third protein component in electrophoretic studies of incompletely saturated conalbumin, with a mobility between that of the iron-free and iron-saturated forms. The reason for the apparent discrepancy between the result of electrophoretic and equilibrium binding studies is still not clear. In view of the findings by Aasa et al. (2) that days to weeks are required to reach equilibrium in transferrin, it may be that true equilibrium had not yet been attained in the 16- to 24-hour intervals in the experiments of Warner and Weber (5). In any case, the present studies indicate the existence of a third species in solutions of conalbumin incompletely saturated with iron (Fig. 6). This species has an electrophoretic mobility intermediate between that of apoconalbumin and Fe³⁺-saturated conalbumin, and in all probability represents protein with a single bound ferric ion. Accordingly, the values of \(K_2\) and \(K_1\) for conalbumin must be approximately equal.

The difference in the accessibility of solvent water to the iron bound by each protein as reflected in the PRR, 2.6 sec⁻¹ mm⁻¹

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R. Title, and P. Aisen, work in progress.
[Fe^{3+}] for transferrin and 6.6 \times 10^{-1} \text{mM}^{-1} [Fe^{3+}] for conalbumin, may be due to a species difference or to a conformational change induced by the difference in carbohydrate residues.

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