Distribution of Iron Between the Binding Sites of Transferrin in Serum: Methods and Results in Normal Human Subjects

By Adela Leibman and Philip Aisen

When it is incompletely saturated with iron, transferrin may exist in four molecular forms: apotransferrin, monoferric (A) transferrin (with iron occupying only the A site of the protein), monoferric (B) transferrin, and diferric transferrin. By combining electrophoresis in urea-polyacrylamide gels with crossed immunoelectrophoresis using specific antihuman transferrin anti-

serum, it is possible to display and estimate the concentration of each of these four forms in normal human serum. The distribution of iron between the binding sites of transferrin is neither random nor determined by the relative binding strengths of transferrin's two sites. Rather, the more weakly binding and acid-labile B site of the protein is predominantly occupied.

The biologic significance of the two-sited nature of serum transferrin has long been a subject of speculation and controversy. Recently studies from a number of laboratories on a variety of transferrins have shown that the two sites are distinguishable in their iron-binding properties in ways that may be physiologically pertinent. Most important, in human transferrin they differ in the strength with which they bind iron, in their accessibility to different chemical forms of iron, and in their susceptibility to attack by protons. A primary problem in assessing whether these differences are functionally important in the regulation of iron metabolism by transferrin is to determine the distribution of iron between the two binding sites of transferrin in circulating blood. To approach this question we have made use of the method of urea-gel electrophoresis, which has been shown to resolve the four molecular species expected from transferrin when it is less than fully saturated with iron. These are then distinguished from the myriad other serum proteins by a second electrophoresis, in a direction perpendicular to the first, into a gel containing specific antiserum to human transferrin. This makes it possible to estimate the relative concentration of each transferrin species and thus the relative occupancy of each specific binding site.

Materials and Methods

Human serum transferrin was isolated in highly purified form from Cohn fraction IV-7, and chelate-free apotransferrin was prepared from it by methods previously detailed. Both sites on transferrin so prepared are physiologically active as iron donors for the reticulocyte. Protein concentration was estimated from absorbance at 280 nm, taking the millimolar absorption coefficient as 83.8. Transferrin at varying degrees of saturation with iron was obtained by addition of appropriate amounts of ferric nitrilotriacetate (1:2, pH 4.0) to apotransferrin in 0.1-M KCl–0.05-M HEPES buffer (pH 7.4). To assure that the anion-binding site of the Fe(III)-transferrin complex was occupied by bicarbonate, a 50-fold excess of that anion was also present.

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Buffers were extracted with dithizone, and acrylamide solutions were passed through a mixed-bed ion-exchange column to minimize contaminating metal ions. To eliminate carbamate, urea solutions were taken through a column of mixed-bed ion-exchange resin (Amberlite IR 120 and BioRad AG 2-X8) just prior to incorporation into buffers and gels. Doubly distilled water, analytic-grade reagents, and acid-rinsed laboratory ware were used in all experiments. Ultra-pure urea and sucrose were supplied by Schwarz/Mann, and acrylamide and bisacrylamide were from Eastman Kodak.

For serum studies, blood was collected from normal human volunteers in acid-washed Vacutainer tubes. Determinations of serum iron followed the procedures of Olsen et al., and assays of total iron-binding capacity (TIBC) were carried out by a semimicro modification of the method of Olson and Hamlin. A Perkin-Elmer model 360 atomic absorption spectrophotometer equipped with deuterium lamp background correction and HGA-2100 graphite furnace was used for both determinations.

Specific antibody was raised in adult male rabbits by multiple subcutaneous injections of 75 μg of a transferrin preparation containing equal amounts of iron-free protein and iron-saturated protein in Freund's complete adjuvant. In some rabbits injections were repeated after 2 wk. Harvesting of antiserum was begun 3 wk after injection and continued indefinitely at intervals of 1–3 wk. The antisera gave single lines on immunoelectrophoresis with human serum. Normal human serum also yielded a reaction of identity with purified transferrin using the antisera in the double-diffusion procedure of Ouchterlony.

Electrophoresis in urea-polyacrylamide gels was carried out by methods previously described. Gels were cast in a buffer of 0.089-M TRIS-0.089-M borate (pH 8.4) containing 6-M urea. Samples containing from 6 to 12 μg of transferrin were applied to each slot of the vertical gel slab, which was 1.3 mm in thickness. A diluting buffer was prepared from 7.5 ml of 8-M urea, 0.5 ml of 1.8-M TRIS-borate at pH 8.4, and 2 g of sucrose. To 0.87 ml of this buffer was added 0.13 ml of serum or the sample to be analyzed. A 30-μl sample of this mixture was applied to a slot of the urea-polyacrylamide-gel slab and left standing for 30 min before electrophoresis. A gradient of 7.5 V/cm was employed for electrophoresis at a temperature of 4°C for 16–17 hr. In each run, one channel was cut out and stained to localize protein bands as a guide for the subsequent step of immunoelectrophoresis.

Crossed immunoelectrophoresis, based on procedures described by Weeke and Soderholm et al., was used to display selectively the transferrin bands. Strips 6.5 mm in width were cut from the centers of channels of the urea-polyacrylamide-gel slabs. These were each washed for 15 min, with shaking in an ice bath, in a test tube containing 20 ml of buffer to remove urea, and twice more in buffer containing 10% glycerol. They were then immediately taken for immunoelectrophoresis or placed in the freezing compartment of a refrigerator until needed. Frozen strips were thawed at refrigerator temperature and then brought to room temperature before being run. Gels 1.5 mm in thickness for crossed immunoelectrophoresis were cast in two sections on 8.4- X 9.4-cm glass slides. The first section, measuring 8.4 X 3.8 cm, was poured from 4.85 ml of 1% agarose (BioRad No. 14177) in 0.02-M barbital buffer (pH 8.6) and held in place by a brass barrier bar (BioRad). After cooling, the brass bar was removed, and the second section, measuring 8.4 X 5.6 cm, was poured from 7.1 ml of agarose in barbital buffer to which were added 47 μl of antitransferrin antiserum. The concentration of antiserum was experimentally optimized for the quantities of transferrin applied to the urea-polyacrylamide gel. Higher concentrations gave peaks too small for accurate measurement, whereas lower concentrations yielded peaks too faint for delineation.

The polyacrylamide-gel strip bearing the electrophoresed sample of interest was then overlaid on the nonantibody portion of the agarose gel, abutting the boundary with the gel containing antiserum. Using the LKB Multiphor apparatus, three such slides could be taken for electrophoresis at one time. A voltage gradient of 2 V/cm was employed, with each slide drawing about 2.8 mA of current. The temperature of the cooling water was 15°C. To assure reproducibility, 24 hr were allowed for each run. Gels were then washed, dried, and stained with amido black following the procedures given by Weeke. The relative area of each peak in a gel was estimated from photographic enlargements by planimetry (extrapolating the peak to baseline) or with the aid of a DuPont curve analyzer.

RESULTS

Studies With Purified Transferrin

To compare the antigenic reactivity of apotransferrin with that of diferric transferrin, mixtures containing identical amounts (5.8 μg) of purified protein
fractions were subjected to crossed immunoelectrophoresis after urea-gel electrophoresis. In three pairs of measurements the ratios of the areas of the diferric-transferrin peaks to the apotransferrin peaks in the crossed immunoelectrophoreograms (Fig. 1) were 1.17, 1.19, and 1.02, for a mean value of 1.13. The small peak of monoferric Fe(B)-transferrin present in the apotransferrin preparations, as in Fig. 1, was taken with apotransferrin for these estimations. This correction factor was used for calculating the relative amounts of apotransferrin and diferric transferrin in subsequent studies. For estimation of monoferric transferrins, a factor of 1.06, obtained by linear interpolation, was employed, assuming that the immunoreactivities of the two species are the same.

The reliability of the crossed immunoelectrophoresis method was assessed by studying purified transferrin preparations at iron saturations ranging from zero to 100% (Fig. 2). Urea-gel electrophoresis has been shown to resolve these preparations into four components, corresponding to apotransferrin, monoferric(A) transferrin (with one atom of iron at the stronger A binding site), monoferric(B) transferrin, and diferric transferrin. Agreement between the known saturation of transferrin with iron and that calculated by densitometry of the urea-gel bands has been shown to be reasonably good. In the crossed immunoelectrophoresis of the present work, peaks were assigned to correspond to the bands observed in the urea gels. The peak closest to the origin is apotransferrin; the fastest peak is diferric transferrin; the two peaks of intermediate mobility represent the two monoferric species of transferrin, with either the A site (slower moving) or B site (faster moving) selectively occupied. The percentage saturation is then given by the expression

\[
\text{percentage saturation} = \frac{A + B + 2Fe_2}{2(a apo + A + B + Fe_2)} \times 100
\]

where apo, A, B, and Fe₂ refer to the areas encompassed by the peaks ascribed to apotransferrin, Fe(A)-transferrin, Fe(B)-transferrin, and diferric transferrin. As seen in Fig. 2 and Table 1, the saturation of transferrin determined from the
Fig. 2. Crossed immunoelectrophoresis of transferrin preparations at varying degrees of saturation with iron as indicated at the side of each pattern. Analysis of the patterns is presented in Table 1. Transferrin saturations: A, 0%; B, 20%; C, 40%; D, 60%; E, 80%; F, 100%.

Immunoelectropherograms is in reasonable agreement with the saturation calculated from the amount of iron added to each preparation.

In these experiments incompletely saturated transferrin preparations showed a preponderance of iron at the A site. This is consistent with the previously reported observation that iron presented to the protein as its complex with nitrilotriacetate is preferentially directed at pH 7.4 toward the A site when an excess of bicarbonate is present. Under the conditions of our experiments the transferrin-iron bond is so strong that redistribution of the metal after initial binding should not occur, so that true equilibrium is not attained during the time course of the experiments.

Table 1. Distribution of Iron Between Binding Site A of Transferrin in Purified Preparations

<table>
<thead>
<tr>
<th>Percentage Saturation</th>
<th>Relative Concentrations</th>
<th>Percentage Saturation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>apo Fe(A)</td>
<td>Fe(B)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>20</td>
<td>26</td>
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</tr>
<tr>
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<td>60</td>
<td>&lt;2</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
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</table>
Studies With Serum

In Fig. 3 the immunoelectrophoretic patterns obtained from sera of three normal male and three normal female subjects are presented. Table 2 presents the percentage saturation of serum transferrin estimated from these patterns along with that calculated from the serum iron studies. The agreement between estimated saturation and calculated saturation tends to be less than satisfactory when the serum iron is low, but otherwise it seems to be in a reasonable range. Most samples were run in duplicate, with the estimated saturations within ±7% of the mean.

Despite the precautions of using acid-washed glassware and dithizone-extracted buffers, it is conceivable that the manipulations involved in the electrophoresis exposed the small amounts of serum to contamination with extraneous iron. However, this is not likely to be a major problem, since iron salts bind to the specific sites of transferrin slowly in the absence of appropriate iron-complexing agents. Furthermore, purified apotransferrin, in quantities comparable to the transferrin contained in the serum samples and exposed to the same manipulations, does not exhibit appreciable iron-bearing components by crossed immunoelectrophoresis (Fig. 1). However, at the present stage in the development of the crossed immunoelectrophoresis technique for displaying transferrin species the procedure is

![Fig. 3. Crossed immunoelectrophoresis of transferrin in normal human sera. Samples 1–3 from females; samples 4–6 from males. Analysis of the patterns is presented in Table 2.](from www.bloodjournal.org by guest on August 30, 2017. For personal use only.)
Table 2. Distribution of Iron Between Binding Sites of Transferrin in Serum

<table>
<thead>
<tr>
<th>Subject</th>
<th>Serum [Fe] (µg/dl)</th>
<th>TIBC (µg/dl)</th>
<th>Percentage Saturation</th>
<th>Areas (% of total)</th>
<th>Percentage Saturation (calculated)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>apo Fe(A)</td>
<td>Fe(B)</td>
</tr>
<tr>
<td>1</td>
<td>86</td>
<td>334</td>
<td>26</td>
<td>41</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>290</td>
<td>21</td>
<td>34</td>
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<tr>
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<td>110</td>
<td>322</td>
<td>34</td>
<td>34</td>
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</table>

probably best considered to be qualitative. Edge effects were often noted in the immunoelectropherograms, with outside peaks appearing less symmetric and less sharp than inner peaks. This distressing phenomenon is well known and is probably caused by irregularities in the voltage gradients near the sides of the gels. Also, the peak due to Fe(A)-transferrin is often poorly separated from the peak of Fe(B)-transferrin, so that estimation of its area is difficult and subject to judgmental error. Finally, when peaks were markedly asymmetric, or exhibited appreciable trailing, estimation of peak areas became difficult, so that it was sometimes necessary to repeat a run. Despite these limitations, we believe the insights offered by the crossed immunoelectrophoretic method are unique and valuable.

In contrast to the studies with purified transferrin loaded with iron presented as its complex with nitrilotriacetate, transferrin in serum exhibits a preponderant occupancy of the B site (Fig. 3). In the serum samples tested, the ratio of monoferric Fe(B)-transferrin to monoferric Fe(A)-transferrin ranged from 2.6 to over 30. Since at the pH of blood the A site binds iron six times more strongly than the B site, it is clear that there is neither a random nor an equilibrium distribution of iron between the two binding sites of transferrin in serum.

**DISCUSSION**

The two sites of human transferrin are inequivalent and are distinguishable by a variety of techniques. Thus the site designated the A site binds iron more strongly than the B site, is more resistant to removal of iron by acid, and has different spectroscopic properties than the B site when occupied by Fe(III), VO²⁺, or Cu(II). Interestingly, however, the sites appear to be equivalent in their susceptibility to reducing agents and in their ability to donate iron to human reticulocytes. Because transferrin in serum is normally less than saturated with iron, the relative occupancies of the two sites of the protein in serum are of interest and may have bearing on the physiologic role of the protein.

In a previous approach to this question by Hovanessian and Awdeh, gel isoelectric focusing was applied to the study of iron distribution in serum transferrin. The bulk of serum iron was found in diferric transferrin at saturations varying from 20% to 39%. However, in these studies isoelectric focusing did not discriminate between the two types of monoferric transferrin, so that no statement about the relative occupancy of each site could be made. Furthermore, the transferrins focus at pH 5.2–5.6, where acid-promoted scrambling of iron between the sites and among transferrin molecules may occur and where one site is particularly vulnerable to attack by protons. The effects of metal-binding ampholytes in the
distribution of iron are also difficult to assess. In our studies, serum was never exposed to lower than physiologic pH or to iron-complexing agents that might mediate exchange of iron between binding sites. Since the binding of iron to transferrin is then too tight for spontaneous dissociation of iron from the protein to occur, the likelihood of redistribution of transferrin-bound iron seems negligible. It is known that spontaneous iron exchange among transferrin molecules under conditions that prevail in serum does not occur.22

The finding that apotransferrin and iron-saturated transferrin differ in their reactivities with specific antiserum on crossed immunoelectrophoresis is not surprising. Immunochemical differences between iron-saturated and iron-free transferrins have long been recognized.23 To minimize the effects of these differences, our crossed immunoelectrophoretic studies were carried out with antiserum from rabbits immunized with equal amounts of diferric transferrin and apotransferrin. Nevertheless, the reactivity with antiserum of the iron-bearing protein was slightly but consistently greater than the reactivity of the iron-free protein. In our studies, estimations of the proportions of apotransferrin, the two monoferric transferrins, and diferric transferrin were corrected for this effect.

Agreement between the known saturations of purified transferrin preparations and the saturations estimated by crossed immunoelectrophoresis is reasonably satisfactory (Table 1). Results of quantitative studies of transferrin species in serum are less agreeable (Table 2). In part, this may reflect the inherent inaccuracies in determination of serum iron and TIBC24 or interference of other serum proteins with the electrophoretic separation of transferrin species. However, it is also likely that the crossed immunoelectrophoresis method is not yet sufficiently refined for precise and reliable quantitation, and we believe that the present method is best considered to offer qualitative information.

Despite this limitation, the patterns shown in Fig. 3 make it clear that iron in serum is not randomly distributed among the binding sites of transferrin molecules. Indeed, the more weakly binding B site of the protein was occupied to a substantially greater degree than the A site in the six samples studied. Since the binding of iron to A and B sites alike is so strong under physiologic conditions that iron is not redistributed once bound at either site,3 it is not surprising that an equilibrium distribution is not observed. Rather, our results may indicate that the physiologic donors of iron to transferrin preferentially recognize the B site, a property shared by a variety of iron-complexing agents capable of functioning in vitro as iron donors for transferrin.3

Since its enunciation over a decade ago, the Fletcher-Huehns hypothesis suggesting that the two sites of human transferrin have distinctive physiologic roles has been the subject of continuing speculation and experimentation.3 Our observation that one site of the protein is selectively occupied in serum is compatible with but not truly confirmatory of this hypothesis. Studies of ferrokinetic and iron-donating properties of transferrins in which one or the other site is selectively occupied by iron may be needed for a definitive judgment of the validity of the Fletcher-Huehns model.

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DISTRIBUTION OF IRON IN TRANSFERRIN


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