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Studies of the Binding of Different Iron Donors to Human Serum Transferrin and Isolation of Iron-Binding Fragments from the *N*- and *C*-Terminal Regions of the Protein

By ROBERT W. EVANS and JOHN WILLIAMS
Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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1. Trypsin digestion of human serum transferrin partially saturated with iron(III)–nitrilotriacetate at pH 5.5 or pH 8.5 produces a carbohydrate-containing iron-binding fragment of mol.wt. 43000. 2. When iron(III) citrate, $FeCl_3$, iron(II) ascorbate and $(NH_4)_2SO_4$,$FeSO_4$ are used as iron donors to saturate the protein partially, at pH 8.5, proteolytic digestion yields a fragment of mol.wt. 36000 that lacks carbohydrate. 3. The two fragments differ in their antigenic structures, amino acid compositions and peptide 'maps'. 4. The fragment with mol.wt. 36000 was assigned to the *N*-terminal region of the protein and the other to the *C*-terminal region. 5. The distribution of iron in human serum transferrin partially saturated with various iron donors was examined by electrophoresis in urea/polyacrylamide gels and the two possible monoferric forms were unequivocally identified. 6. The site designated A on human serum transferrin [Harris (1977) *Biochemistry* **16**, 560–564] was assigned to the *C*-terminal region of the protein and the B site to the *N*-terminal region. 7. The distribution of iron on transferrin in human plasma was determined.

Studies on the relative affinities of the two metal-binding sites of transferrins for iron have produced many conflicting results that are often further confused, unnecessarily, by the lack of clarification of the source and species.

For human serum transferrin, equilibrium dialysis indicated that there was sequential occupancy of the sites ($K_1 = 400K_2$, where K_1 is the association constant for iron at the first site and K_2 that for the second site) (Davis *et al.*, 1962), although with the same technique Aasa *et al.* (1963) concluded that the sites were equivalent and independent. It was thought that in the first determination true equilibrium was never attained.

A moving-boundary-electrophoretic study (Aisen *et al.*, 1966) in which $(NH_4)_2SO_4$,$FeSO_4$ was added to human serum transferrin at pH 6.7 showed that the protein can be separated into three species, with 0, 1 and 2 atoms of iron. The distribution of iron was thought to support the theory of random iron binding, but on careful re-examination the results suggest a sequential binding process. Gel isoelectric focusing also resolves three forms of transferrin (Hovanessian & Awdeh, 1976); however, the distribution of iron, when added as iron(III)–nitrilotriacetate to the protein in 1 mM-$NaHCO_3$, agrees well with that predicted for equivalent sites. Both these electrophoretic methods fail to resolve the two possible monoferric forms of the protein, presumably because they have the same isoelectric point, as with the two monoferric species of hen ovotransferrin (Williams, 1975). However, polyacrylamide-gel electrophoresis in the presence of 6 M-urea (Makey & Seal, 1976) will resolve iron-free human serum transferrin, the two monoferric transferrins and fully saturated transferrin. Until now this technique has not been used to study in detail the distribution of iron when added to the iron-free protein.

Lane (1975) has shown that it is possible to separate two monoferric forms of human serum transferrin by chromatography on DEAE-cellulose at pH 7.9. From the distribution of iron in samples of protein that had been partially saturated with $FeCl_3$ at pH 5.0 and subsequently raised to pH 7.9 he concluded that binding had taken place randomly.

In spite of the results supporting the equivalence of the sites, other workers have now shown that the two sites exhibit different pH-dependencies. One site retains its iron down to about pH 5, whereas the other loses its iron around pH 6 (Princiotto & Zapolski, 1975; Lestas, 1976). The site that retains its iron at low pH, designated the A site, has also been shown to bind iron preferentially at pH 7.5 when iron(III)–nitrilotriacetate is used as the iron donor (Harris, 1977*a*).

In view of the conflicting reports on the binding of

iron to human serum transferrin we set out to examine the way in which various iron donors occupy the iron-binding sites on the protein. We have used a method, first reported by Williams (1974), for the isolation of the *N*-terminal fragment of hen ovotransferrin, which involves partial iron saturation of the protein, proteolytic digestion of unoccupied binding sites and identification of the iron-binding fragments remaining. We have then exploited the observed differences in the order of binding of different iron donors to isolate and characterize the two iron-binding domains. With the electrophoretic method of Makey & Seal (1976) we have been able to compare the distribution of iron when added to transferrin in different forms and under different conditions and then been able to identify the monoferric form of transferrin present in normal blood.

Materials and Methods

Preparation of human serum transferrin

Human serum transferrin was prepared by a modification of the method of Roop & Putnam (1967) starting with Cohn IV fraction (Cohn *et al.*, 1946) from outdated pooled human plasma. First 3 litres of Cohn IV fraction was dialysed twice for 12h at 4°C against 10 litres of phosphate-buffered saline (Dulbecco & Vogt, 1954) and the precipitate removed by centrifugation at 6000*g* for 1h at 4°C. Iron(III)–nitriloacetate and $NaHCO_3$ were added to the supernatant to final concentrations of 0.2 and 0.5mM respectively to saturate the transferrin and the solution was then dialysed twice for 6h at 4°C against water adjusted to pH9.4 with aq. NH_3. An equal volume of 0.6% (w/v) rivanol (2-ethoxyacridine-6,9-diamine lactate monohydrate) in 5mM-Tris/HCl, pH9.4, was added dropwise overnight at 4°C to the stirred solution. The precipitate that formed was removed by centrifugation at 6000*g* for 1h at 4°C and the supernatant filtered through a pad of potato starch (BDH Chemicals, Poole, Dorset, U.K.), previously washed with 5mM-Tris/HCl, pH9.4, to remove the rivanol. Then 1.5 litres of a slurry of DEAE-Sephadex A-50 equilibrated in 5mM-Tris/HCl, pH8.8, was added to the filtrate, and after standing for 2h at 20°C the suspension was filtered through a Buchner funnel. The ion-exchange resin was washed with buffer until the eluate was protein-free and then the bound transferrin was eluted with 0.1M-NaCl. After dialysis of the eluate overnight against 10 litres of 50mM-Tris/HCl, pH8.8, the protein was adsorbed on a column (12cm×30cm) of DEAE-Sephadex A-50, in the same buffer, which was washed with buffer until the eluate was free of protein as indicated by A_{280}. The transferrin was eluted with 0.1M-NaCl, dialysed against water and freeze-dried. The yield of protein was 20g.

Iron-free transferrin

Iron was removed from transferrin by the method of Warner & Weber (1951) by using the anion-exchange resin Bio-Rad AG1 (X2, 200–400 mesh, Cl^- form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The protein was shown to be iron-free by the lack of the characteristic absorption band of iron–transferrin at 470nm [$A_{470} = 5\times10^3 M^{-1}\cdot cm^{-1}$ (Aasa *et al.*, 1963)].

Polyacrylamide-gel electrophoresis

Samples for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were dissolved in a buffer containing 5% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 1% 2-mercaptoethanol, 0.01% Bromophenol Blue and 5mM-sodium phosphate, pH7.0, and heated for 3min in a boiling-water bath. When unreduced samples were required the 2-mercaptoethanol was omitted.

Electrophoresis was carried out on gels containing 7.5% (w/v) acrylamide and 0.2% (w/v) *NN'*-methylenebisacrylamide in 0.1% sodium dodecyl sulphate and 0.05M-sodium phosphate, pH7.0.

Gels were stained for protein with Coomassie Brilliant Blue R-250 by the method of Berg (1969) and for carbohydrate with the periodic acid/Schiff's-base stain (Zacharias *et al.*, 1969). Molecular weights were determined by the use of a marker mixture that contained ovotransferrin (mol.wt. 80000), bovine serum albumin (mol.wt. 67000), lactate dehydrogenase (subunit mol.wt. 36000) and horse heart cytochrome *c* (mol.wt. 12400).

Polyacrylamide-gel electrophoresis in 6M-urea was carried out by a modification of the method of Makey & Seal (1976) as described by Williams *et al.* (1978).

Before examination of transferrin in human plasma samples the plasma was treated by the method of Matson *et al.* (1966). To 0.1ml of plasma was added 0.3ml of reservoir buffer and 0.5ml of 0.6% (w/v) rivanol in reservoir buffer, and the precipitate removed by centrifugation (1000*g* for 5min). This procedure removes all plasma proteins except the β- and γ-globulins and simplifies the gel pattern.

Starch-gel electrophoresis

Flat-bed starch-gel electrophoresis was carried out in 13% (w/v) gels in the discontinuous buffer system of Poulik (1957). Protein was stained with 1% (w/v) Naphthalene Black (12B) in methanol/acetic acid/water (5:1:5, by vol.) and destained by washing in the same solvent.

Preparation of iron chelates

Iron(III)–nitrilotriacetate and iron(II) ascorbate were prepared by the method of Woodworth (1966).

Iron(III) citrate was prepared with an iron/citrate molar ratio of 1:20. Under these conditions the predominant species in solution is thought to be iron(III) dicitrate (Spiro *et al.*, 1967).

Immunological methods

An antiserum to human serum transferrin was raised in a sheep. Antigen–antibody reactions were observed by the Ouchterlony (1958) method with undiluted antisera. Antigen solutions were either prepared by dissolving protein (1 mg/ml) in 0.1 M-sodium phosphate, pH 7.0, or taken directly from column fractions.

Peptide 'maps'

Protein was taken up in 5% (v/v) formic acid and digested with pepsin (from pig stomach mucosa; Sigma Chemical Co., St. Louis, MO, U.S.A.) at an enzyme/substrate ratio of 1:30 (w/w) for 15 h at 37°C. One-dimensional electrophoretic patterns were run at pH 6.5 and 3.5. A sample (25 nmol) of each digest was applied directly to 2.54 cm (1 in) of starting line on Whatman 3MM paper and electrophoresis was carried out for 1 h at 60 V/cm. Peptides were stained with the cadmium acetate/ninhydrin reagent of Heilmann *et al.* (1957) and in addition tyrosine- and tryptophan-containing peptides were detected by the specific stains of Jepson & Smith (1953).

Amino acid analysis

Protein samples were hydrolysed at 105°C with 5.7 M-HCl in sealed evacuated tubes, after flushing several times with N_2, for 24 h and analysed on a Rank Hilger Chromaspek J180 instrument.

Preparation of human serum transferrin partially iron saturated at pH 8.5

Samples of human serum transferrin in 0.1 M-$NaHCO_3$, pH 8.5, partially saturated with iron(III)–nitrilotriacetate, iron(II) ascorbate or $(NH_4)_2SO_4$,-$FeSO_4$ were prepared by addition of the calculated amount of iron donor after titration of the iron-free protein by monitoring the change in A_{470}. As the uptake of iron by transferrin is a slow process when iron(III) citrate and $FeCl_3$ are used as iron donors (Bates *et al.*, 1967; Bates & Schlabach, 1973) samples of protein partially saturated by them were obtained by addition of an amount based on the values for the other iron chelates. In all cases the partially iron-saturated samples were left for 15 h at 4°C to ensure a quantitative binding of the iron, which was confirmed by measurements of A_{470}.

Preparation of human serum transferrin partially iron-saturated with iron(III)–nitrilotriacetate at pH 5.5

Iron-free protein (300 mg) in 10 ml of 0.1 M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/0.01 M-$NaHCO_3$, pH 5.5, was 40%-iron-saturated with iron(III)–nitrilotriacetate, as judged by A_{470}. After standing at 20°C for 45 min the solution was passed through a column (1 cm × 10 cm) of Bio-Rad AG 1-X2, which had been previously equilibrated with the same buffer, to remove any unbound iron. The pH was then adjusted to 7.45 by addition of 0.1 M-NaOH.

Digestion of partially iron-saturated human serum transferrin

Partially iron-saturated samples of protein (50 mg/ml) were digested with trypsin (bovine trypsin type XI treated with diphenylcarbamoyl chloride; Sigma) for 6 h at 37°C at an enzyme/protein ratio of 1:30 (w/w), followed by a second addition of the same amount of enzyme and incubation for a further 15 h. To check that this procedure digested away all iron-free sites a sample of iron-free protein was treated in the same way and shown to be unable to bind iron.

Results

Susceptibility of iron-free and iron-saturated human serum transferrin to proteolytic digestion

Treatment of iron-free transferrin with trypsin at pH 8.5 results in complete loss of its iron-binding ability, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis reveals that the protein has been digested to low-molecular-weight peptides. Under the same conditions the fully-saturated protein retains its bound iron, as judged by its A_{470}, and moves as a single band of mol.wt. 80000 on sodium dodecyl sulphate/polyacrylamide gels. However, when the fully saturated/protein was examined by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol, several bands were observed, suggesting that the protein had undergone some internal cleavage but was still held together by disulphide bridges. This suggestion is confirmed by starch-gel electrophoresis, which can detect charge heterogeneity in a given protein. Untreated iron-saturated transferrin moves as a single band on starch gel, but after treatment with trypsin it gives multiple bands that move faster than the native protein. The effect of trypsin on fully saturated human serum transferrin is different from that reported by Brock *et al.* (1976) on fully saturated bovine serum transferrin. The bovine protein is cleaved into separate iron-binding domains.

Proteolytic digestion of human serum transferrin partially iron-saturated at pH 8.5

Samples of transferrin that had been 30%-iron-saturated, at pH 8.5, with four different iron donors, iron(III)–nitrilotriacetate, $FeCl_3$, iron(II) ascorbate and $(NH_4)_2SO_4$,$FeSO_4$, were treated with trypsin

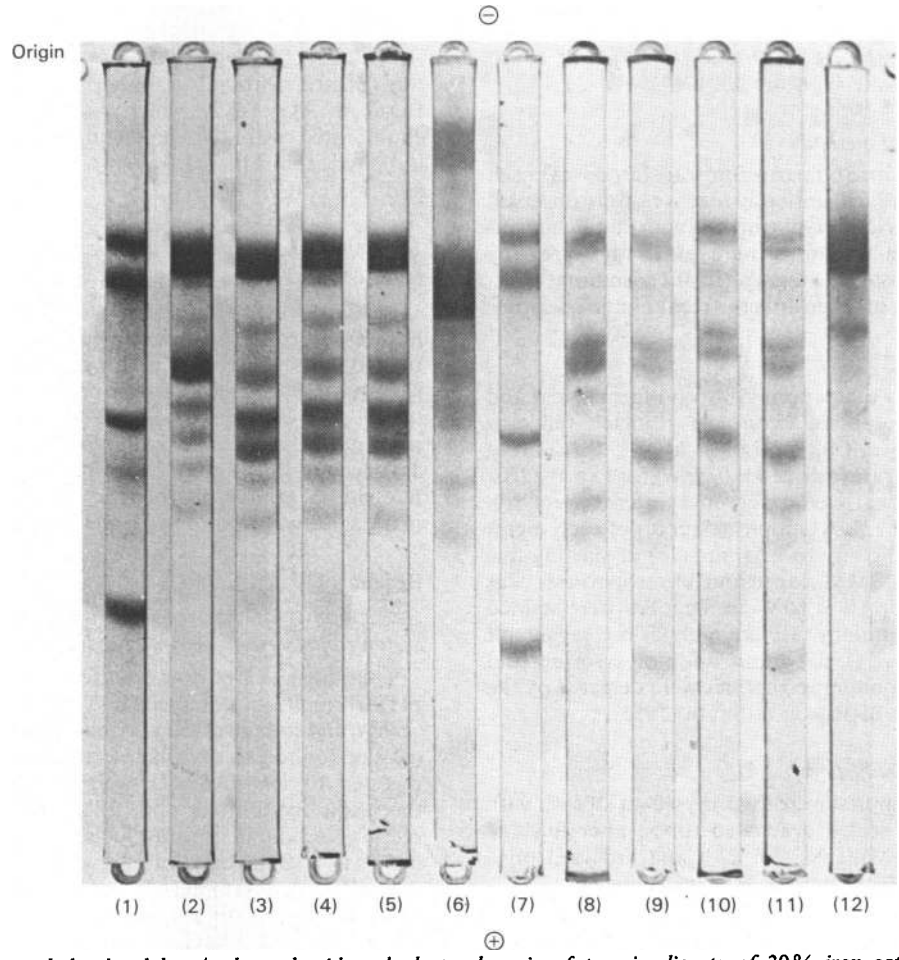

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of trypsin digests of* 30%*-iron-saturated human serum transferrin*

Samples of iron-free human serum transferrin in 0.1M-$NaHCO_3$, pH 8.5, were 30%-iron-saturated with iron(III)–nitrilotriacetate, $FeCl_3$, iron(II) ascorbate and $(NH_4)_2SO_4$,/$FeSO_4$ and digested with trypsin, as described in the Materials and Methods section. Gels 1–6 were run in the absence of 2-mercaptoethanol and gels 7–12 were run in the presence of 2-mercaptoethanol. Gels 1 and 7 are a marker mixture containing hen ovotransferrin (mol.wt. 80000), bovine serum albumin (mol.wt. 67000), lactate dehydrogenase (mol.wt. 36000) and cytochrome *c* (mol.wt. 12400). Gels 2 and 8 are samples of transferrin labelled with iron(III)–nitrilotriacetate. Gels 3 and 9 are samples labelled with $FeCl_3$. Gels 4 and 10 are samples labelled with iron(II) ascorbate. Gels 5 and 11 are samples labelled with $(NH_4)_2SO_4$,$FeSO_4$. Gels 6 and 12 are samples of undigested transferrin.

to digest away unoccupied iron-binding sites. The sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic pattern, in both the presence and the absence of reducing agent, is shown in Fig. 1. As can be seen from the unreduced samples (gels 2–5), the gel pattern of the sample partially saturated by using iron(III)–nitrilotriacetate shows two main species with mol.wts. 80000 and 43000, whereas the three other samples have only a trace of the second species but two components with mol.wts. 33500 and 36000. In the presence of reducing agent (Fig. 1, gels 8–11) the gel-electrophoretic pattern of the iron(III)–nitrilotriacetate sample is again different from the other three; however, the interpretation is complicated by the fact that none of the main species observed in the absence of reducing agent are single chains. As the fully saturated protein has been shown to undergo limited proteolytic cleavage, it is not surprising that the fragments do not have a single-chain structure.

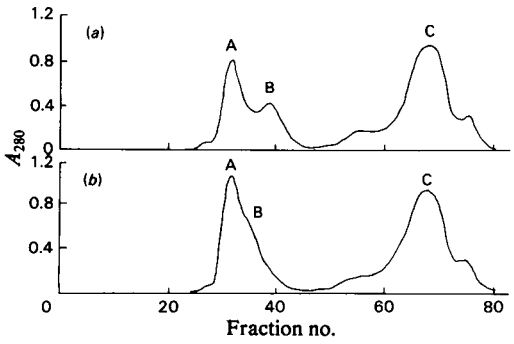

Fig. 2. *Gel-filtration patterns on Sephadex G-100 (1.2 cm × 135 cm) in 0.02 M-NH_4HCO_3 of trypsin digests of human serum transferrin partially iron saturated at pH 8.5*
(a) Protein partially iron-saturated with $FeCl_3$, iron(II) ascorbate or $(NH_4)_2SO_4$,$FeSO_4$; *(b)* protein partially iron-saturated with iron(III)–nitrilotriacetate. Fractions of volume 2.35 ml were collected.

In a separate experiment two samples of transferrin were partially saturated with iron(III) citrate and iron(III)–nitrilotriacetate and digested with trypsin, as in the above experiment. Gel electrophoresis showed that the sample labelled with iron(III) citrate gives rise to the same components as $FeCl_3$, iron(II) ascorbate and $(NH_4)_2SO_4$,$FeSO_4$.

When unreduced gels were stained for carbohydrate only the species with mol.wts. 80000 and 43000 were found to be carbohydrate-positive.

Gel filtration of each digest was carried out on Sephadex G-100. The samples that had been partially saturated with $FeCl_3$, iron(II) ascorbate, $(NH_4)_2SO_4$,$FeSO_4$ and iron(III) citrate were resolved into two protein peaks, A and B (Fig. 2*a*), both pink in colour, followed by low-molecular-weight peptides (peak C). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that peak A had a mol.wt. of 80000 and peak B had two components with mol.wts. 33500 and 36000 corresponding to those observed on gel electrophoresis of the whole digests (Fig. 1, gels 3–5). Gel filtration of the sample that had been partially saturated with iron(III)–nitrilotriacetate failed to resolve the protein components completely and the fragment with mol.wt. 43000 appeared as a shoulder on the main peak (Fig. 2*b*, peak A/B), the position of which was shifted to a slightly higher elution volume relative to peak A in the other samples. In spite of this poor resolution, a sample of the 43000-mol.wt. fragment with only a slight contamination by the other component was obtained by taking a narrow cut from the fractions on the shoulder. We will show below that the 43000-mol.wt. fragment can be resolved on Sephadex G-200.

Immunological properties of the fragments from the digests

When examined by the Ouchterlony method against an antiserum to whole human serum transferrin, each of the fragments from the samples partially saturated with $FeCl_3$, iron(III) citrate, iron(II) ascorbate and $(NH_4)_2SO_4$,$FeSO_4$ gave precipitin lines that fused with each other but showed only partial identity with that of the whole protein. The fragment of mol.wt. 43000 from the sample partially saturated with iron(III)–nitrilotriacetate also showed partial identity with transferrin, but its precipitin line crossed those from the other four samples, suggesting a complete lack of common antigenic determinants. This is illustrated in Fig. 3(*a*). The precipitin line produced by a mixture of the two types of fragment fuses with that produced by the whole protein (Fig. 3*b*), so together the fragments appear to account for all the determinants on transferrin.

Proteolytic digestion of human serum transferrin partially saturated with iron(III)–nitrilotriacetate at pH 5.5

A sample of transferrin that had been 30%-iron-saturated with iron(III)–nitrilotriacetate at pH 5.5, passed down a cation-exchange resin to remove any unbound iron and digested with trypsin after the pH of the solution had been raised to 7.45 produced the same gel-filtration pattern on Sephadex G-100 as the sample of protein that had been partially saturated with the same iron chelate at pH 8.5.

Gel filtration of digests of partially iron-saturated human serum transferrin on Sephadex G-200

The fragment derived from digestion of transferrin partially iron-saturated with iron(III)–nitrilotriacetate at either pH 5.5 or 8.5 is not fully resolved from the whole protein by gel filtration on Sephadex G-100 (Fig. 2*b*); however, gel filtration on Sephadex G-200 separated the two proteins (Fig. 4*a*). Peaks A and B both contain carbohydrate, as revealed by the orcinol/H_2SO_4 test (Winzler, 1955), and both bind iron, as judged by the A_{470}. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis confirmed that peak A was whole protein that had undergone limited proteolytic cleavage and peak B contained the fragment of mol.wt. 43000. Peaks C and D represent low-molecular-weight glycopeptides and peptides. Whereas peaks A and B have similar A_{280}/A_{470} ratios, peak B contains relatively more carbohydrate than peak A.

For comparison, gel filtration on Sephadex G-200 of a tryptic digest of transferrin partially iron-saturated with $(NH_4)_2SO_4$,$FeSO_4$ at pH 8.5 is shown in Fig. 4(*b*). Again two protein peaks, A and B,

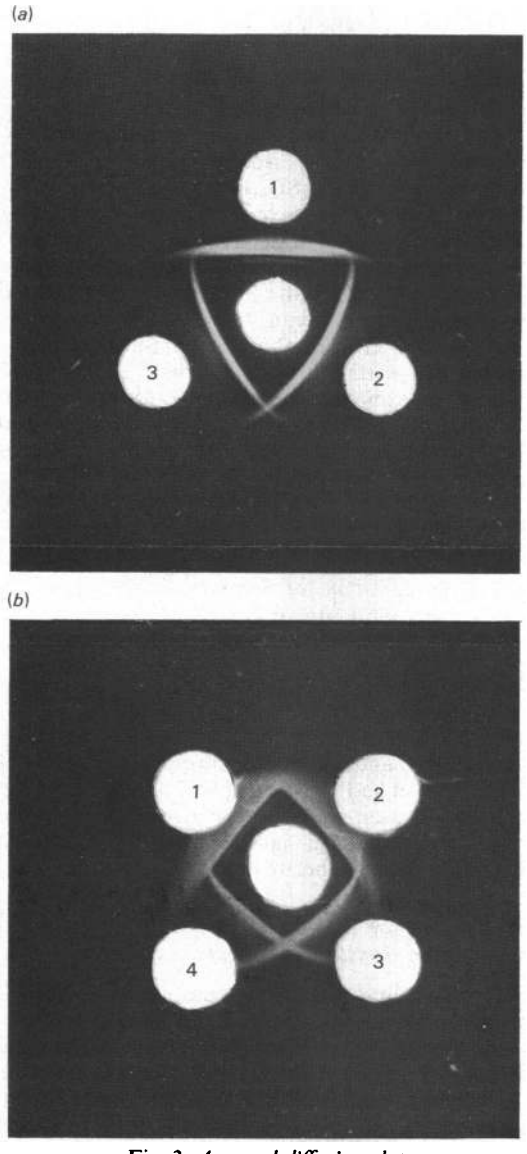

Fig. 3. *Agar-gel diffusion plate*

(*a*) Wells 1, 2 and 3 contained human serum transferrin, the fragment of mol.wt. 36000 and the fragment of mol.wt. 43000 respectively. The central well contained antiserum to human serum transferrin. (*b*) Well 1 contained human serum transferrin. Well 2 contained a mixture of equal amounts of the two fragments. Wells 3 and 4 contained the fragment of mol.wt. 36000 and the fragment of mol.wt. 43000 respectively. The central well contained antiserum to human serum transferrin.

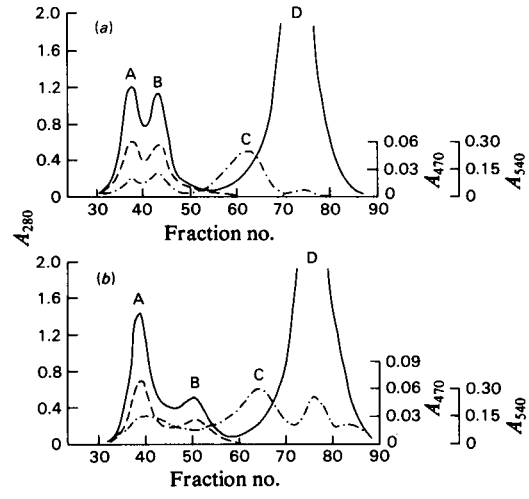

Fig. 4. *Gel-filtration patterns on Sephadex G-200 (2.4 cm × 120 cm) in 0.1 M-NH_4HCO_3 of trypsin digests of partially iron-saturated human transferrin*

(*a*) Human serum transferrin partially iron-saturated with iron(III)–nitrilotriacetate at pH8.5; (*b*) human serum transferrin partially iron-saturated with $(NH_4)_2SO_4$,$FeSO_4$ at pH8.5. ——, A_{280}; – – – –, A_{470}; –·–·, A_{540} in the orcinol assay. For details of peaks A–D see the text. Fractions of volume 6.8 ml were collected.

are found; however, although they both contain iron, peak B is essentially free of carbohydrate. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that peak B represented a fragment of mol.wt. 36000.

During the isolation procedure for human serum transferrin the protein loses some of its bound iron and the final preparation is about 40% iron-saturated. On trypsin digestion the protein yields the fragment of mol.wt. 43000.

Amino acid composition of the fragments

The amino acid compositions of human serum transferrin, the fragment of mol.wt. 36000 and the fragment of mol.wt. 43000, as isolated by gel filtration on Sephadex G-200 are given in Table 1. The fragments have similar compositions, although differences are apparent in their contents of aspartic acid, glutamic acid, threonine and phenylalanine. The sum of the compositions of the fragments agrees reasonably well with that of the whole protein.

Carbohydrate composition of the fragments

The hexose content of the 36000- and 43000-mol.wt. fragments, as determined by the orcinol/H_2SO_4 test, were found to be 1.9 and 10.1 mol of sugar/mol of protein respectively. Jamieson (1965) showed that human serum transferrin has two carbohydrate groups of identical composition, each

Table 1. *Amino acid composition of human serum transferrin and of fragments*

Results are expressed as mol of amino acid/mol of protein.

Amino acid	36000-mol.wt. fragment	43000-mol.wt. fragment	Sum of fragments	Human serum transferrin
Asp	27.2	34.7	61.9	62.5
Thr	10.4	16.8	27.2	24.0
Ser	19.0	17.8	36.8	33.3
Glu	26.3	32.9	58.2	54.8
Pro	19.5	16.4	35.9	32.8
Gly	28.1	31.0	59.1	56.5
Ala	36.0	33.8	69.8	63.4
½Cys	14.6	16.8	31.4	36.8
Val	18.8	17.8	36.6	38.8
Met	3.7	4.5	8.2	9.6
Ile	8.4	9.2	17.6	17.8
Leu	32.9	34.8	67.5	66.5
Tyr	13.4	14.2	27.6	31.5
Phe	18.4	13.8	32.2	31.8
His	9.8	10.1	19.9	18.6
Lys	31.5	35.5	67.0	59.6
Arg	12.4	13.5	25.9	26.9

containing 2 sialic acid residues, 8 *N*-acetylglucosamine groups, 4 mannose residues and 2 galactose residues. This was confirmed by Graham & Williams (1975) in their work on the amino acid sequence of glycopeptides from a number of transferrins, although their carbohydrate analyses indicated that each carbohydrate group contained 3 residues of both mannose and galactose. In the present work no attempt was made to determine the relative amounts of galactose and mannose in each fragment; however, the hexose contents that we obtained indicate that both carbohydrate groups lie within the fragment of mol.wt. 43000.

As the two carbohydrate groups will contribute about 5000 to the molecular weight of the fragment, the difference in molecular weight between the two fragments can be attributed mainly to the presence of carbohydrate on the fragment derived from the iron(III)–nitrilotriacetate-labelled protein.

Peptide 'maps'

Pepsin digests of the fragments as well as human serum transferrin were examined by one-dimensional electrophoresis at pH3.5 and 6.5. When the electrophoretograms were stained with ninhydrin the two fragments gave different patterns, which together appeared to account for all the bands in the transferrin digest. The ninhydrin-stained electrophoretogram, which was run at pH6.5, is shown in Fig. 5. Similarly, when the specific stains for tryptophan and tyrosine were used the fragments gave different patterns, which when combined again accounted for the bands in the digest of the whole protein.

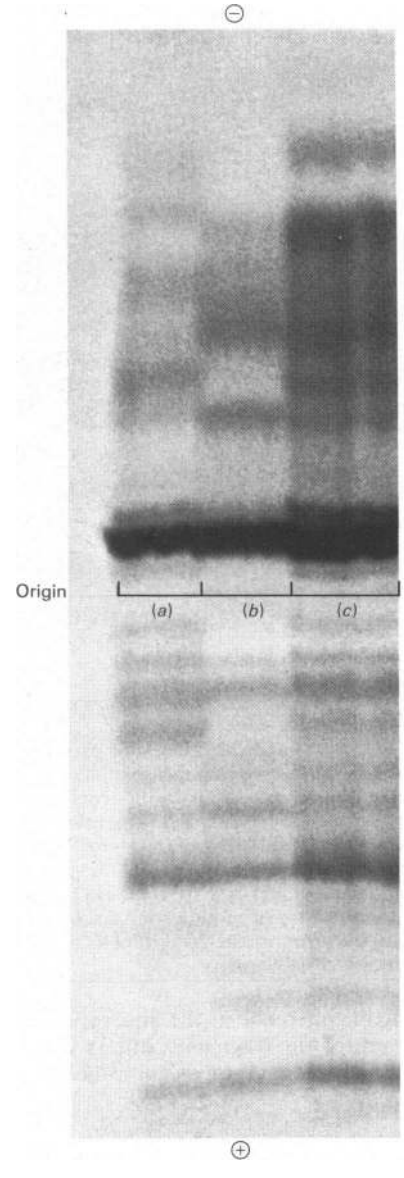

Fig. 5. *One-dimensional electrophoretogram at pH6.5 of pepsin digests of (a) fragment of mol.wt. 36000, (b) fragment of mol.wt. 43000 and (c) human serum transferrin*

The electrophoretogram was stained with the cadmium acetate/ninhydrin reagent. For details see the Materials and Methods section.

Absorption spectra of the fragments

Both fragments had an absorption maximum in the region of 460–470nm, characteristic of transferrins, and A_{280}/A_{470} ratios of 23.1 and 23.2 respectively. For comparison fully-saturated human serum transferrin has a value of 23.5 for the A_{280}/A_{470}

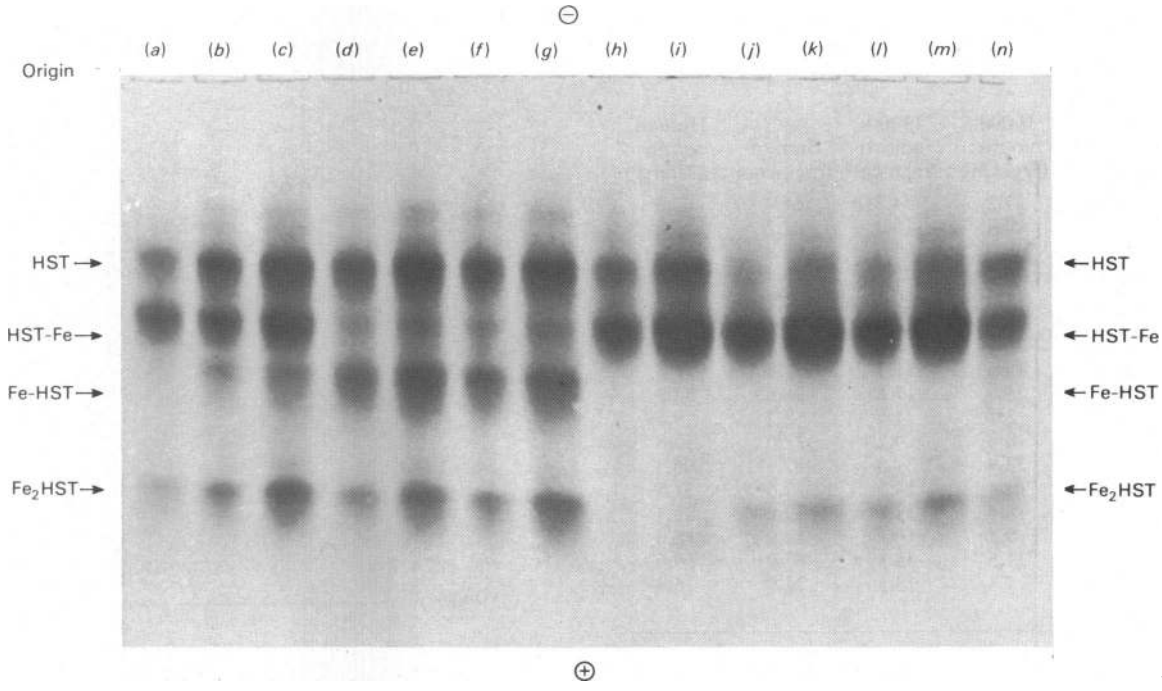

Fig. 6. *Urea/polyacrylamide-gel electrophoresis of human serum transferrin partially iron-saturated with various iron donors at pH 6.0 and 8.5*

Samples (*a*) (25 μg) and (*n*) (25 μg) are partially iron-saturated preparation of human serum transferrin. Samples (*b*) (25 μg) and (*c*) (50 μg) are proteins partially iron-saturated with iron(III)–nitrilotriacetate at pH 8.5. Samples (*d*) (25 μg) and (*e*) (50 μg) are proteins partially iron-saturated with $(NH_4)_2SO_4$,$FeSO_4$ at pH 8.5. Samples (*f*) (25 μg) and (*g*) (50 μg) are proteins partially iron-saturated with $FeCl_3$ at pH 8.5. Samples (*h*) (25 μg) and (*i*) (50 μg) are proteins partially iron-saturated with iron(III)–nitrilotriacetate at pH 6.0. Samples (*j*) (25 μg) and (*k*) (50 μg) are proteins partially iron-saturated with $(NH_4)_2SO_4$,/$FeSO_4$ at pH 6.0. Samples (*l*) (25 μg) and (*m*) (50 μg) are proteins partially iron-saturated with $FeCl_3$ at pH 6.0. HST indicates the position on the gel of iron-free human serum transferrin, HST-Fe the position of monoferric transferrin with iron in the *C*-terminal site, Fe-HST the position of monoferric transferrin with iron in the *N*-terminal site and Fe_2HST the position of fully saturated transferrin.

ratio. A slight difference in the absorption maxima was observed for the fragments, but as yet it is not known whether this is of any significance.

Reversibility of iron binding to fragments

Both the iron-free fragments were titrated with iron(III)–nitrilotriacetate and $(NH_4)_2SO_4$,$FeSO_4$ at pH 8.5; however, only the fragment of mol.wt. 43000 took up its original complement of iron, as indicated by the A_{470}. The lower-molecular-weight fragment would only take up about 30% of the theoretical amount of iron, irrespective of the nature of the iron donor.

Urea/polyacrylamide-gel electrophoresis of partially iron-saturated human serum transferrin and human plasma

Human serum transferrin can be separated into four species, namely iron-free protein, two monoferric species and fully iron-saturated protein, by electrophoresis at pH 8.4 in polyacrylamide gels containing 6 M-urea (Makey & Seal, 1976). As shown in Fig. 6 addition of iron as iron(III)–nitrilotriacetate (samples *b* and *c*) to iron-free transferrin, at pH 8.5, enhances the slower-migrating of the two intermediate forms, whereas when $(NH_4)_2SO_4$,$FeSO_4$ (samples *d* and *e*) and $FeCl_3$ (samples *f* and *g*) are used as iron donors the faster-moving intermediate band is preferentially formed.

When samples of transferrin in 0.1 M-Hepes/0.01 M-$NaHCO_3$, pH 6.0, were partially saturated with the same three iron donors electrophoresis in urea/polyacrylamide gels (Fig. 6, samples *h*–*m*) revealed that in all three cases only the apoprotein and the slower of the two intermediates were present.

The isolation procedure for human serum transferrin yields protein that is about 40% iron-saturated and that on digestion with trypsin it gives rise to the fragment with mol.wt. 43000. Urea/

polyacrylamide-gel electrophoresis of this partially saturated protein (Fig. 6, samples *a* and *n*) showed that it contained mainly the slower-running monoferric species, together with small amounts of iron-free and iron-saturated transferrin.

Plasma from normal subjects was examined by this gel-electrophoresis method and found to contain only iron-free protein and the first intermediate. To check that the second intermediate can exist in plasma, iron(III)–nitrilotriacetate and $(NH_4)_2SO_4$,-$FeSO_4$ were used to saturate the transferrin partially in plasma from iron-deficient patients. It was observed that, as with the pure protein, iron(III)–nitrilotriacetate enhances the slower-migrating intermediate band, whereas the other iron donor enhances the faster-migrating intermediate.

Discussion

In the present paper we have shown that proteolytic digestion of partially iron-saturated human serum transferrin gives rise to iron-binding fragments. Under the same conditions the iron-free protein is completely degraded to low-molecular-weight peptides, whereas the iron-saturated protein undergoes some internal cleavage but retains its complement of iron, and under non-reducing conditions has the same mobility on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as the native protein. Similar observations have been made with hen ovotransferrin (Williams, 1974, 1975), except that the fully saturated protein was completely resistant to attack by trypsin. For bovine serum transferrin Brock *et al.* (1976) found that trypsin digestion of the iron-saturated protein produces two fragments that were subsequently thought to represent the two domains of the protein (Brock & Arzabe, 1976).

Proteolytic digestion of human serum transferrin saturated to 30% with iron(III)–nitrilotriacetate at either pH5.5 or pH8.5 produces a carbohydrate-containing fragment of mol.wt. 43000. However, when four other iron donors, iron(III) citrate, $FeCl_3$, iron(II) ascorbate and $(NH_4)_2SO_4$,$FeSO_4$, are used partially to saturate the protein at pH8.5 a different fragment is obtained after digestion with trypsin. This second fragment lacks carbohydrate and has mol.wt. 36000. In some experiments this fragment was accompanied by a minor fragment of slightly lower molecular weight, possibly owing to further digestion. Neither the 36000-mol.wt. fragment nor the 43000-mol.wt. fragment possesses a single chain; however, this is not surprising in view of the fact that the fully saturated protein itself undergoes limited cleavage with trypsin.

Although the smaller fragment is readily separated from undigested protein by gel filtration on Sephadex G-100, the other fragment could only be completely resolved from transferrin by gel filtration on Sephadex G-200. As well as differing in molecular weight and carbohydrate composition the fragments have different peptide 'maps' and amino acid compositions and are immunologically distinct. Their only similarity is in their absorption spectra, which are almost identical and characteristic of all transferrins. Studies on human serum transferrin (MacGillivray *et al.*, 1977) have shown that the two carbohydrate groups on the protein lie within the *C*-terminal half of the protein, so we conclude that the fragment with mol.wt. 43000 must represent the *C*-terminal region and the other fragment the *N*-terminal region. The availability of the isolated iron-binding domains will simplify further investigations into the structural properties of the iron-binding sites.

The observation that iron(III)–nitrilotriacetate, a non-physiological iron donor, has a preference for the *C*-terminal site at both pH5.5 and pH8.5 agrees with the work of Harris (1977*a*), who also found that one particular site, designated A, exhibits a tendency to bind iron first at both acidic and neutral pH. We now know that the A site lies in the *C*-terminal region of the protein. In hen ovotransferrin the *N*-terminal site preferentially binds iron, when added as iron(III)–nitrilotriacetate, at pH8.5, but at acidic pH the *C*-terminal site takes up iron preferentially (Williams *et al.*, 1978).

Although the other iron donors that we examined, which showed a preference for the *N*-terminal site, might be expected to resemble more closely the form in which iron is present in the body, the distribution of iron on transferrin in normal plasma suggests that this is not so. By using a urea/polyacrylamide gel, which resolves iron-free human serum transferrin, the two monoferric transferrins and fully saturated transferrin (Makey & Seal, 1976), we have compared the pattern obtained when iron in different forms is added to the pure protein with the pattern in normal blood. Human plasma, where the transferrin is about 30% iron-saturated (Giovaniello & Peters, 1963), contains two species, iron-free transferrin and a monoferric form that corresponds to that obtained when iron(III)–nitrilotriacetate is added to the isolated protein at pH6.0 or 8.5 and therefore has iron in the *C*-terminal site. Transferrin in the plasma of iron-deficient patients is essentially iron-free, so it was possible to confirm that, as with the pure protein, addition of iron(III)–nitrilotriacetate to plasma enhances the monoferric form with iron in the *C*-terminal site, whereas the other donors enhance the other monoferric species.

It is noteworthy that, as for hen ovotransferrin (Williams *et al.*, 1978), the monoferric human serum transferrin species with iron in the *C*-terminal site has a lower mobility in urea/polyacrylamide gels than the form with iron in the *N*-terminal site. In the original

report on this gel method (Makey & Seal, 1976) the exact basis for the separation of the monoferric transferrins was uncertain. However, now that we know that in both hen ovotransferrin (Williams, 1975) and human serum transferrin there is an asymmetric distribution of the carbohydrate groups between the two halves of each protein it is tempting to postulate that it is this difference that results in the monoferric species having different mobilities.

For several years there have been reports for and against the hypothesis of Fletcher & Huehns (1967, 1968) that the two sites of transferrin have different physiological roles. Most recently Harris (1977*b*) showed conclusively that iron is taken up in equal proportions from both sites of fully saturated human serum transferrin by human reticulocytes. This result was taken as definitive evidence that there is no functional difference between the sites. However, as our results show that normal plasma does not contain appreciable amounts of diferric transferrin, a more correct demonstration of the role of the two sites requires a comparative study with the specifically labelled forms. Now that we can prepare these by using different iron donors a more valid test is possible.

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