A variant of human transferrin with abnormal properties

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Screening of human serum samples by polyacrylamide-gel electrophoresis in the presence of 6 M-urea revealed an individual who is heterozygous for a variant transferrin. The variant transferrin is able to bind two atoms of iron, but the iron in the C-terminal binding site is bound abnormally, as judged by its spectral properties, and is dissociated from the protein on electrophoresis in the presence of 6 M-urea. The iron-free C-terminal domain of the variant protein is less stable than normal to thermal and urea denaturation. Structural changes in the variant protein have not yet been characterized.

Many workers have found by using starch-gel electrophoresis and isoelectric focusing that transferrin is polymorphic in human populations (Mourant *et al.*, 1976; Thymann, 1978). Until now, however, no variant form has been found to differ from the common type of transferrin (TfC) in its iron-binding properties. Study of functionally abnormal variant molecules might reveal the role of particular amino acid residues in iron binding. In the present paper we describe an apparently rare type of transferrin variant in which the properties of one of the iron-binding sites are abnormal. This protein was discovered by screening a large number of serum samples by polyacrylamide-gel electrophoresis in a urea-containing buffer. This method, which was introduced by Makey & Seal (1976), resolves partially iron-saturated transferrin into four bands, which represent the iron-free protein (Tf), the C-terminal monoferric transferrin (TfFe), the N-terminal monoferric transferrin (FeTf) and the diferric transferrin (Fe₂Tf), in the order of increasing mobility (Evans & Williams, 1978). This resolution is the result of differences in charge and shape between the four forms of the protein. The binding of each ferric ion increases the charge on the protein by -1 , which is attributable to the ionization of a number of tyrosine residues and the concomitant binding of an anion (bicarbonate or carbonate). The presence of 6 M-urea causes the iron-free domains to unfold, which decreases the mobilities of iron-free transferrin and the two monoferric transferrins relative to that of iron-saturated protein. Evans & Williams (1980) have shown that urea decreases the mobility of the

C-terminal monoferric transferrin more than that of the N-terminal monoferric transferrin, so that although each has one iron-free and one iron-saturated domain, they have different mobilities on electrophoresis in the presence of urea. We assumed that a molecule in which one iron-binding site was markedly defective would migrate in the gel as a monoferric protein complex even after the addition of a dose of iron normally expected to saturate the protein.

Materials and methods

Preparation of human serum transferrin

Transferrin was prepared from Cohn Fraction IV of outdated human plasma and the iron removed as described previously (Evans & Williams, 1978). Transferrin from the serum of subject P.C. was isolated by immunoaffinity chromatography (March *et al.*, 1974). Further fractionation of the transferrin from the serum of subject P.C. was attempted by chromatography on DEAE-cellulose (DE-52; Whatman) and isoelectric focusing in the presence of 4 M-urea with Ampholine of pH range 5–7.

Polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis of serum samples in 6 M-urea was performed as described by Evans & Williams (1978), except that before treatment of the samples with rivanol, a saturating dose of iron was added [$10\mu\text{l}$ of 1 mM-iron(III) nitrilotriacetate]. Serum samples were obtained from the Blood Transfusion Service, Southmead Hospital, Bristol, U.K., by courtesy of the Director. Urea-gradient-gel electrophoresis was performed as described by Evans & Williams (1980). Preparative polyacrylamide-gel

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electrophoresis in 6M-urea was performed in an LKB 7900 Uniphor apparatus under the same conditions as the analytical electrophoresis, except that the stock buffer was 0.6 M-Tris/HCl/0.06 M-boric acid/10 mM-EDTA, pH 8.4.

Labelling of transferrin with [^{59}Fe]iron(III) nitrilotriacetate

[^{59}Fe]Iron(III) nitrilotriacetate was prepared as described by Woodworth (1966) by using $^{59}\text{FeCl}_3$ (from The Radiochemical Centre, Amersham, Bucks., U.K.) and used to label a sample of iron-free transferrin from subject P.C. After analytical polyacrylamide-gel electrophoresis in 6M-urea, the gel was sliced and the radioactivity measured by liquid-scintillation counting.

In another experiment the radioactively labelled transferrin was passed down columns (1 cm $\times$ 60 cm) of Sephadex G-100 equilibrated with the buffer solution used for polyacrylamide-gel electrophoresis and either containing or not containing 6M-urea. The amount of iron bound to the protein under these conditions was determined by measurements of A_{280} and radioactivity.

Spectroscopic methods

Absorption spectra were recorded with a Pye-Unicam SP.8-100 spectrophotometer. U.v. difference spectra were determined as described by Tan & Woodworth (1970). E.p.r. spectra were prepared by Dr. J. F. Gibson, Department of Chemistry, Imperial College London, with a Varian E-12 spectrometer. Samples were frozen in quartz tubes.

Differential scanning calorimetry

Differential-scanning-calorimetric thermograms (plots of heat flow as a function of temperature) were recorded on a Perkin-Elmer DSC-2 differential scanning calorimeter with a heating rate of 5°C/min. The protein concentration of the samples was 100 mg/ml in 0.6 M-Tris/HCl, pH 7.5.

Further analytical methods

N-Acetylneuraminic acid contents of the unfractionated transferrin of subject P.C. and normal transferrin were determined by the effect of neuraminidase on the mobility of the proteins on polyacrylamide-gel electrophoresis in 6M-urea. Cleavage patterns after CNBr, trypsin and thrombin treatment were determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Amino acid analyses and peptide mapping were performed as previously described (Evans & Williams, 1978). N-Terminal end groups were determined by the method of Gray (1967).

Results

Screening of human sera

Approx. 5000 serum samples were subjected to urea/polyacrylamide-gel electrophoresis after the addition of a dose of iron normally expected to saturate the protein so that it migrated as a single band of diferric transferrin (Fig. 1a). In one of these samples (from subject P.C.) the normal diferric transferrin band was decreased in intensity and a new slower-moving band was present (Fig. 1b). Both the fast and slow components were identified as transferrins by treatment of serum P.C. with anti-transferrin-Sepharose. Neither component was detectable in the treated serum, but both components were present in the material eluted from the Sepharose with 1 M-NH₃. When a sample of transferrin isolated from the serum of subject P.C. by immunoabsorption (Fig. 1c) was run, after addition of excess iron, on a urea/polyacrylamide gel alongside a sample of partially iron-saturated normal transferrin containing all four transferrin species (Fig. 1d), the slow component in transferrin P.C. was found to have a mobility intermediate between those of the normal N- and C-monoferric transferrins. The fast and slow

Fig. 1. Urea (6M)/polyacrylamide-gel electrophoresis of normal serum, serum from subject P.C., normal transferrin and unfractionated transferrin from subject P.C.

Samples of (a) normal serum and (b) serum from subject P.C. were treated with rivanol and subjected to 6M-urea/polyacrylamide-gel electrophoresis after addition of a dose of iron(III) nitrilotriacetate normally expected to saturate the protein. Sample (c) is unfractionated transferrin, isolated from the serum of subject P.C. by immunoabsorption, after addition of a dose of iron(III) nitrilotriacetate normally expected to saturate the protein. Sample (d) is partially iron-saturated normal transferrin unfractionated transferrin, isolated from the serum of subject P.C. by immunoabsorption, after addition of a dose of iron(III) nitrilotriacetate normally expected to saturate the protein.

transferrins observed in this way will hereafter be referred to as 'Tf_F' and 'Tf_S'. The iron content and total iron-binding capacity of serum P.C. was normal (15.9 $\mu\text{mol/litre}$ and 74.5 $\mu\text{mol/litre}$ respectively, as compared with 16.0 and 76.6 $\mu\text{mol/litre}$ for a relative whose transferrin pattern was normal). Samples of serum P.C. and a normal serum that had been stored at -15°C were subjected to urea/polyacrylamide-gel electrophoresis without prior addition of a saturating dose of iron. Both samples showed only diferric transferrin, C-terminal monoferric transferrin and apotransferrin. This agrees with the previous finding that the *N*-terminal monoferric transferrin, which predominates in fresh serum, undergoes rearrangement on storage at low temperature, thereby generating the C-terminal monoferric transferrin (Williams & Moreton, 1980). Fig. 2 shows, however, that in the absence of added iron, serum P.C. differs from the normal sample in showing a less intense C-terminal monoferric trans-

ferrin and a broader apotransferrin band on urea/polyacrylamide-gel electrophoresis. Electrophoresis for 40 h resolved the apotransferrin band into two components, one having the same mobility as normal apotransferrin and the other a slightly lower mobility. We have shown previously that the preferential occupation of the C-terminal binding site by iron, which occurs at -15°C , is reversed on incubation at 37°C , leading to preferential occupation of the *N*-terminal binding site (Williams & Moreton, 1980). Incubation of serum P.C. at 37°C caused diminution of the C-terminal monoferric band and the appearance of two faster-running bands. The faster of these bands corresponded to the normal *N*-monoferric transferrin and the slower band corresponded to the new band seen in Fig. 1. Gel scanning showed that in a frozen sample of serum P.C. the transferrin consisted of 64.3% apotransferrin, 25.3% monoferric transferrin (almost entirely C-terminal monoferric complex) and 10.4% diferric transferrin. After incubation at 37°C for 3 days the composition was 58.0% apotransferrin, 34.3% monoferric transferrin and 7.6% diferric transferrin.

Fig. 2. Urea (6M)/polyacrylamide-gel electrophoresis of normal serum and serum from subject P.C. after storage at -15°C

Samples of (1) serum from subject P.C. and (2) normal serum, which had been stored at -15°C , were treated with rivanol and subjected to 6M-urea/polyacrylamide-gel electrophoresis without prior addition of a saturating dose of iron.

The relative amounts of Tf_F and Tf_S

An excess of iron(III) nitrilotriacetate was added to the unfractionated transferrin from subject P.C. and the sample run on a series of polyacrylamide gels containing different concentrations of urea. Over the range 0–6M-urea the amount of Tf_S increased from 30 to 57% of the total amount of transferrin, but no further increase was observed when the urea concentration was increased to 7.6M. In the absence of iron the protein distribution was independent of urea concentration and about 57% of the protein migrated as the variant protein.

The iron content of Tf_S

The mobility of component Tf_S on 6M-urea/polyacrylamide gels was intermediate between that of the normal *N*-terminal monoferric complex and that of the normal C-terminal complex (Fig. 1). This suggested that Tf_S was itself a monoferric complex, and this was confirmed as follows: iron was removed from a sample of the mixed transferrin isolated by immunoadsorption from the serum of subject P.C. and a 4-fold excess of [^{59}Fe]iron(III) nitrilotriacetate was added. After electrophoresis, component Tf_S accounted for 57% of the transferrin protein, but only 42% of the transferrin-bound iron (Fig. 3). Therefore, component Tf_F contained 1.83 times as much iron per molecule of transferrin as did component Tf_S.

Preparative isolation of Tf_F and Tf_S

Fig. 4 shows the fractionation of ^{59}Fe -labelled transferrin by ion-exchange chromatography on

Fig. 3. Urea (6M)/polyacrylamide-gel electrophoresis of ^{59}Fe -labelled transferrin from subject P.C. A sample of iron-free unfractionated transferrin, isolated from the serum of subject P.C. by immunoadsorption, was labelled with excess [^{59}Fe]iron (III) nitrilotriacetate and applied to a 6M-urea/polyacrylamide gel. After electrophoresis, strips were cut from the gel, sliced and counted for radioactivity. The remainder of the gel was stained for protein with Coomassie Blue and scanned at 550 nm. —, A_{550} ; O, radioactivity (c.p.m.).

Fig. 4. Chromatography of unfractionated transferrin from subject P.C. on DEAE-cellulose DE-52 $^{59}\text{FeCl}_3$ (100 μl) was mixed with 1 μl of 0.5 M- FeCl_3 and 1 μl of 0.5 M-disodium nitrilotriacetate and added to 10 mg of iron-free unfractionated transferrin in 1.0 ml of 1 M- NaHCO_3 . After incubation for 12 h at 4°C the protein was desalted on a column of Sephadex G-25 (1 cm $\times$ 60 cm) equilibrated with 0.01 M-Tris/HCl, pH 8.0, and applied to a column of DEAE-cellulose DE-52 (1 cm $\times$ 12 cm) in the same buffer. Protein was eluted with a linear gradient (total volume 500 ml) consisting of equal volumes of 50 mM- and 200 mM-Tris/HCl, pH 8.0. Fractions (50 drops) were collected and were assayed for protein (●) and radioactivity (O).

DEAE-cellulose DE-52. Two components were obtained and they had equal iron contents, which presumably corresponded to the diferric state. The two components were immunologically identical with each other when tested by the Ouchterlony method with sheep antisera against either component. On urea/polyacrylamide-gel electrophoresis the first peak gave component Tf_F and the second peak gave two bands; the stronger band was Tf_S , but a weak band running ahead of Tf_F was also present. This result suggested that Tf_S was a monoferric transferrin that arose from a diferric transferrin (peak II, Fig. 4) during electrophoresis. Other isolation methods were tried because of the failure to obtain peak II in a pure state by chromatography.

Isoelectric focusing failed to resolve the two components when ampholine of pH range 5–7 was used. The addition of 4 M-urea greatly improved the resolution, probably because of the ability of urea to raise the isoelectric point of some proteins (Ui, 1967). Wenn & Williams (1968) found that, at its isoelectric point of 5.2, diferric transferrin undergoes partial loss of iron, resulting in a diffuse pattern.

Preparative urea/polyacrylamide-gel electrophoresis gave the best separation of Tf_F and Tf_S (Fig. 5), and determination of the ratio A_{470}/A_{280} confirmed that Tf_S contained only half as much iron per molecule as Tf_F .

Urea-gradient/polyacrylamide-gel electrophoresis

Unfractionated iron-free transferrin isolated from the serum of subject P.C. by immunoadsorption gave the denaturation pattern shown in Fig. 6(a).

Fig. 5. Preparative polyacrylamide-gel electrophoresis of unfractionated transferrin from subject P.C. in the presence of 6 M-urea

A 70 mg portion of iron-saturated unfractionated transferrin, isolated from the serum of subject P.C. by immunoadsorption, was applied to a 6 M-urea/polyacrylamide gel in the LKB Uniphor 7900 apparatus. Protein eluted from the gel was collected in 3 ml fractions.

Two protein species are present, each of which denatures in two steps, the second step occurring at the same urea concentration for both components. The first step, which Evans & Williams (1980) have shown to be attributable to the unfolding of the C-terminal domain of the protein, occurs at markedly different urea concentrations for the two components. Comparison with the pattern given by normal human serum transferrin (Evans & Williams, 1980) shows that the C-terminal domain of the variant transferrin unfolds at a lower urea concentration than normal.

When the iron-saturated transferrin was subjected to urea-gradient electrophoresis it gave the pattern shown in Fig. 6(b). Components Tf_F and Tf_S

Fig. 6. Electrophoresis of unfractionated transferrin from subject P.C. on urea-gradient/polyacrylamide gels (a) Electrophoresis of a sample of iron-free unfractionated transferrin. (b) Electrophoresis of a sample of unfractionated transferrin after addition of excess iron(III) nitrilotriacetate. (c) Electrophoresis of a mixture of iron-free and iron-saturated unfractionated transferrin.

are present throughout the urea concentration range used. Tf_F shows no denaturation, but Tf_S shows a single denaturation step, in agreement with its proposed monoferric nature. By mixing iron-free and iron-saturated samples it was shown that the single denaturation step of Tf_S coincides with the first denaturation step of the iron-free variant protein (Fig. 6c). Therefore we conclude that Tf_S is an *N*-terminal monoferric complex of a variant transferrin that arises from the diferric form of the protein during electrophoresis.

Spectra

The unfractionated transferrin prepared by immunoadsorption from the serum of the subject P.C. gave an absorption spectrum with absorption maximum at 448 nm after being saturated with iron. Normal diferric transferrin has a broad absorption maximum at 465–470 nm. After fractionation by preparative urea/polyacrylamide-gel electrophoresis, different absorption maxima were found

Fig. 7. Absorption spectra of iron-saturated samples of unfractionated transferrin, Tf_F and Tf_S .

Absorption spectra of (a) unfractionated transferrin (—), isolated from the serum of subject P.C., (b) Tf_F (---) and (c) Tf_S (— · —) were recorded after saturation of the binding sites with iron(III) nitrilotriacetate.

for the two components in their diferric forms: 465 nm for Tf_F and 435 for Tf_S (Fig. 7). Differences were also found between the e.p.r. spectra (Fig. 8). On the other hand, although the u.v. difference spectra of the iron, copper, aluminium and zinc complexes of Tf_F and Tf_S showed marked differences from one metal to another, the two proteins gave indistinguishable spectra.

Differential scanning calorimetry

Pooled iron-free normal transferrin showed two denaturation peaks at 64.4 and 74.4°C when a heating rate of 5°C/min was used. For iron-free component Tf_F , denaturation steps occurred at 65.2 and 75.2°C and for iron-free Tf_S they occurred at

Fig. 8. E.p.r. spectra of iron-saturated samples of fractionated transferrin, Tf_F and Tf_S .

E.p.r. spectra of (a) unfractionated transferrin, from the serum of subject P.C., (b) Tf_F and (c) Tf_S were recorded after saturation of the binding sites with iron(III) nitrilotriacetate.

63.4 and 75.3°C. Thus for normal transferrin the denaturation steps are separated by 10°C, but for the variant transferrin the separation is increased to 11.9°C.

Trypsin digestion of Tf_s

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the principal fragment produced by partial proteolysis of Tf_s (isolated by isoelectric focusing in 4M-urea) with trypsin had mol.wt. of 36 000, which corresponds to the value expected for the *N*-terminal fragment (Evans & Williams, 1978). The *C*-terminal fragment (mol.wt. 42 000) was not seen. This result supports the suggestion that Tf_s is an *N*-terminal monoferric complex.

Gel filtration in urea-containing solutions

Tf_s appears to arise, in our experiments, by the loss of the iron atom bound to the *C*-terminal site of a variant diferric transferrin on electrophoresis in

urea/polyacrylamide gels. ^{59}Fe labelled diferric transferrin from subject P.C., which had not been fractionated, was passed down two identical columns (1 cm $\times$ 30 cm) of Sephadex G-25 equilibrated in one case with the electrophoresis running buffer minus urea and in the other case with buffer plus 6 M-urea. The protein-bound iron determined by counting the radioactivity associated with transferrin was the same in both cases. Thus 6 M-urea alone does not seem to remove iron from the variant diferric transferrin.

Amino acid composition, end-groups, peptide maps and *N*-acetylneuraminic acid content

The amino acid compositions of Tf_F and Tf_s show no significant differences. Dansylation of the unfractionated mixture gave only valine as the *N*-terminal amino acid. After treatment with CNBr the unfractionated mixture and a sample of pooled normal transferrin gave identical patterns on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The fragments generated by thrombin digestion of the reduced and alkylated proteins were identical, as also were one-dimensional peptide maps of pepsin digests, which were stained with ninhydrin and with specific amino acid stains. The four-step decrease in mobility of Tf_s on treatment with neuraminidase confirmed that, in common with normal transferrin, it has four *N*-acetylneuraminic acid residues on its carbohydrate chains and its abnormal mobility is not due to lack of *N*-acetylneuraminic acid (Fig. 9).

Discussion

These experiments were undertaken in the hope of discovering transferrin variants capable of binding only one atom of iron. In this way we hoped to be able to identify particular residues playing critical roles in iron binding. In the event we have discovered in subject P.C. an individual who is heterozygous for a variant transferrin which binds two atoms of iron but which displays a number of novel abnormalities.

The abnormality which led to the discovery of the variant is that when the diferric form of the protein is subjected to urea/polyacrylamide-gel electrophoresis under the conditions described, one atom of iron is lost from the *C*-terminal binding site, resulting in the formation of an *N*-terminal monoferric transferrin which runs a little slower than the normal *N*-terminal monoferric complex on electrophoresis. It is difficult to explain the loss of the iron atom. The presence of 6 M-urea is not enough by itself to cause loss of the metal. It appears that even in the absence of urea some iron is lost from the variant protein on electrophoresis and, as the urea concentration is increased, more of the variant protein migrates as the monoferric complex until, in

Fig. 9. Electrophoresis of normal and unfractionated transferrin on a 6M-urea/polyacrylamide-gel after incubation with neuraminidase

Neuraminidase (25 units) was added to 0.5 ml of normal transferrin and unfractionated transferrin (10 mg/ml) in 0.1 M-sodium acetate/0.01 M- $Ca(NO_3)_2$, pH 5.0, and the solutions incubated at 37°C. Samples (40 μ l) were removed over a period of 36 h, diluted to 200 μ l with 0.2 M- $NaHCO_3$ /1.25 mM-iron(III) nitrilotriacetate containing 10% (v/v) glycerol and examined by analytical polyacrylamide-gel electrophoresis in the presence of 6 M-urea. Samples 1 to 9 are normal transferrin, samples 10 to 18 are unfractionated transferrin. Samples 9 and 10 were taken before addition of neuraminidase, samples 8 and 11 after 12 min, samples 7 and 12 after 24 min, samples 6 and 13 after 48 min, samples 5 and 14 after 1 h 36 min, samples 4 and 15 after 3 h 12 min, samples 3 and 16 after 4 h 48 min, samples 2 and 17 after 6 h 24 min and samples 1 and 18 after 36 h.

6 M-urea, it is all in that form and amounts to 57% of the total transferrin. Once the variant protein has been isolated by preparative polyacrylamide-gel electrophoresis in 6 M-urea, it subsequently migrates as a monoferric complex irrespective of the concentration of urea in the gel. In the absence of iron the apparent relative amounts of the normal and variant transferrins are independent of the concentration of urea in the gel.

The iron-free form of the variant also runs a little slower than the normal apoprotein on 6 M-urea/polyacrylamide-gel electrophoresis. In urea-gradient/polyacrylamide-gel electrophoresis the C-terminal domain of the iron-free variant protein is denatured at a lower urea concentration than is normal. Similarly, thermal denaturation of the C-terminal domain appears to occur at a slightly lower temperature than normal. Donovan & Ross (1976) suggested that the two domains of iron-free human serum transferrin denatured separately, on the basis of calorimetric studies, but could not determine which domain was the less stable. From our results on the variant protein we can state that the C-terminal domain is less stable than the N-terminal domain.

At least one of the iron atoms in the diferric form of the variant is liganded in an abnormal way to the protein, as judged by the pronounced yellow colour of the complex (λ_{max} 435 nm) and by the e.p.r. spectrum. In many respects the protein resembles normal transferrin, and it may be noted that the subject has no clinically significant haematological changes. Although a structural change is evidently present in the C-terminal domain, it could not be detected by immunological tests or by fragmentation with thrombin, CNBr, trypsin or pepsin. The change is therefore likely to be a single amino acid substitution or a small deletion. We have shown previously that, in normal serum incubated at 37°C, iron is preferentially bound to the N-terminal binding site and that on storage at -15°C a reversible rearrangement takes place, leading to a preferential binding of iron to the C-terminal site (Williams & Moreton, 1980). Electrophoresis of serum from subject P.C. suggests that the variant transferrin behaves in a similar way. In samples incubated at 37°C the N-terminal sites of the normal and variant

transferrins were occupied, but storage at -15°C caused the monoferric transferrin zone to be depleted and the apotransferrin zone to be increased. This suggests that, at low temperature, rearrangement to give the C-terminal monoferric variant protein has taken place. This protein species, however, cannot be observed by polyacrylamide-gel electrophoresis, since the iron atom is lost and the protein migrates as apotransferrin.

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