The Distribution of Iron between the Metal-Binding Sites of Transferrin in Human Serum

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(Received 19 June 1979)

The Makey & Seal [(1976) *Biochim. Biophys. Acta* **453**, 250–256] method of polyacrylamide-gel electrophoresis in buffer containing 6 M-urea was used to determine the distribution of iron between the *N*-terminal and *C*-terminal iron-binding sites of transferrin in human serum. In fresh serum the two sites are unequally occupied; there is preferential occupation of the *N*-terminal site. On incubation of the serum at 37°C the preference of iron for the *N*-terminal site becomes more marked. On storage of serum at –15°C the iron distribution changes so that there is a marked preference for the *C*-terminal site. Dialysis of serum against buffer at pH 7.4 also causes iron to be bound much more strongly by the *C*-terminal than by the *N*-terminal site. The original preference for the *N*-terminal site can be restored to the dialysed serum by addition of the diffusible fraction.

Transferrins consist of a polypeptide chain, of mol.wt. about 80000, whose *N*-terminal and *C*-terminal halves fold up independently to give two major structural domains. Isolated domains have been obtained after limited proteolytic cleavage of ovotransferrin, serum transferrin and lactoferrin (Williams, 1974, 1975; Brock *et al.*, 1976; Evans & Williams, 1978; Bluard-Deconinck *et al.*, 1978). X-ray crystallography of transferrin at 0.6 nm resolution confirms the presence of two independently folded domains separated by a clear boundary (Gorinsky *et al.*, 1979). Amino acid-sequence studies on human transferrin show that, although they are not identical, there is considerable homology between the two halves of the protein (MacGillivray *et al.*, 1977). This would be consistent with the evolutionary origin of transferrin from a smaller ancestral protein by a process of duplication and splicing. Each structural domain carries a specific metal-binding site, and spectroscopic data indicate that they have similar but non-identical properties (Chasteen, 1977).

The structural differences between the domains result in unequal binding of metals to the two sites. Several workers have shown that *in vitro* the distribution of iron between the two binding sites is markedly dependent on pH and the nature of the anion associated with the iron (Princiotta & Zapolski, 1975; Lestas, 1976; Harris, 1977; Evans & Williams, 1978; van Eijk *et al.*, 1978; Williams *et al.*, 1978). The iron distribution also varies with the

species. For example, ferric nitrilotriacetate at pH 8 is initially bound preferentially by the *N*-terminal site of hen ovotransferrin, but by the *C*-terminal site of human transferrin. At pH 5.5 the same iron donor binds to the *C*-terminal site of both proteins. Aisen *et al.* (1978) have shown that in the presence of citrate the initial complexes formed between human transferrin and various iron donors undergo rearrangement to give an equilibrium mixture in which iron preferentially occupies the so-called 'a' site. The 'a' site is that site which retains iron at acid pH and can therefore be identified with the *C*-terminal binding site (Evans & Williams, 1978). By using isoelectric focusing in polyacrylamide gels van Eijk *et al.* (1978) also found that iron in the *N*-terminal site of monoferric human transferrin migrated to the *C*-terminal site on dialysis against phosphate buffer, pH 7.4.

At present these differences between the two metal-binding sites cannot be interpreted chemically, nor is their possible physiological significance known. One essential step is to define the distribution of iron between the sites *in vivo*, since it is known that normally human transferrin is only partially saturated with iron. In early work, with isoelectric focusing, human serum was shown to contain monoferric transferrin (Wenn & Williams, 1968), but until now there has been no information on the distribution of the iron between the two sites.

Makey & Seal (1976) introduced the use of polyacrylamide-gel electrophoresis in a buffer con-

taining 6M-urea and showed that four transferrin bands could be resolved. By combining this method with proteolytic fragmentation experiments Evans & Williams (1978) identified these bands with the following protein species in order of increasing electrophoretic mobility: (1) iron-free transferrin, (2) monoferric transferrin in which the iron atom occupies the C-terminal site, (3) monoferric transferrin in which the iron atom occupies the N-terminal site, and (4) diferric transferrin. In the present paper we describe the use of the Makey & Seal (1976) method in determining the distribution of iron in human serum. The four protein species referred to above are designated Tf, Tf-Fe, Fe-Tf and Fe₂-Tf respectively.

Methods

Preparation of human serum transferrin

Human serum transferrin was prepared from Cohn IV fraction as described by Evans & Williams (1978). It was made iron-free by the method of Warner & Weber (1951).

Preparation of samples

Serum samples were collected from normal adults. Some were subjected to electrophoresis within about 1h of collection, but others were first kept at different temperatures (37, 0, -15 and -70°C) for several days. When incubation at 37°C was to be used, rifampicin (20 µg/ml) was added to the samples. Some serum samples were dialysed overnight against 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, or against 0.1M-Mops [4-morpholinepropanesulphonic acid] buffer, pH 7.4.

In order to study the transferrin bands by electrophoresis it was necessary to remove most of the serum proteins by precipitation with rivanol (6,9-diamino-2-ethoxyacridine lactate) by the method introduced by Matson *et al.* (1966) to simplify the typing of serum transferrin by starch-gel electrophoresis. To 0.1 ml of serum was added 0.3 ml of reservoir buffer (see below) and 0.5 ml of 0.6% (w/v) rivanol in reservoir buffer. Two drops of glycerol were added and the mixture was centrifuged at 1000g for 5 min. A sample (50 µl) of the supernatant was applied to the gel.

Polyacrylamide-gel electrophoresis (Makey & Seal, 1976)

A stock buffer containing 2M-Tris, 0.2M-boric acid and 0.032M-EDTA adjusted to pH 8.4 with HCl was used. This buffer was diluted 20-fold with freshly deionized 6.3M-urea for the preparation of gels or with deionized water for the reservoir buffer. The gels contained 6.5% (w/v) acrylamide and 0.172% (w/v) bisacrylamide. Samples were applied

to 1 cm slots in 16 cm × 16 cm × 0.15 cm slab gels and electrophoresis was carried out at 90V for 17h. After a staining with Coomassie Blue and destaining, gels were scanned at 560nm in a Pye Unicam 8-100 spectrophotometer.

A standard protein mixture containing all four transferrin species was prepared by adding (NH₄)₂SO₄, FeSO₄ and ferric nitrilotriacetate to iron-free human transferrin (Evans & Williams, 1978). The rivanol procedure described above caused no change in the electrophoretic pattern given by this mixture. Aisen *et al.* (1978) showed that no loss or rearrangement of iron bound to transferrin occurs as a result of the electrophoresis itself.

Preparation of diffusible fraction of serum

Serum was dialysed against water for 24h in the cold and the diffusible fraction was freeze-dried. In some experiments this material was added to serum that had been dialysed against 0.2M-Mops buffer, pH 7.4, and the mixture was incubated at 37°C for 4 days. It was checked that the addition of the diffusible fraction did not cause any pH change.

Calculation of the distribution of iron

Milne (1978), in an Appendix to Williams *et al.* (1978), has derived equations relating the fractional saturation of N-terminal sites (x) and C-terminal sites (y) to the number of bound iron atoms per molecule of transferrin. [N.B. In the first of the two equations labelled (6) in Milne's (1978) Appendix there is a misprint: p/q should read q/p .] From these equations it can be inferred that the ratio of the iron-binding affinities of the N-terminal and C-terminal sites is given by $\ln(1-x)/\ln(1-y)$. The values for x and y were determined from the proportions of Tf, Tf-Fe, Fe-Tf and Fe₂-Tf found by scanning of the gels. The iron distribution is shown by plotting $-\ln(1-y)$ against $-\ln(1-x)$. A best straight line was fitted to these points by the method of least squares. In this, lines were calculated to minimize $[\delta \ln(1-x)]^2$ and $[\delta \ln(1-y)]^2$ separately and the average of these was taken to be the best line. Standard deviations of the slope and the intercept on the $\ln(1-x)$ axis are also given: the slope of this line represents the relative binding affinities of the N-terminal and C-terminal sites under the conditions of the experiments. This value does not distinguish between the binding of the first and second iron atoms to the protein, but is an overall value. Also, there is no evidence that any of the observations that we report here relate to equilibrium conditions, so that the slope of the line cannot be taken to represent the ratio of the equilibrium binding constants for the two sites. Nevertheless, if the plot of $-\ln(1-y)$ against $-\ln(1-x)$ remains linear up to high concentrations of bound metal it seems reason-

able to conclude that possible co-operative interactions between the sites must be small.

Results

Iron distribution in fresh serum

Fig. 1 shows the electrophoretic pattern given by normal serum examined within 1 h of collection. In addition to the four transferrin bands there is a streak attributable to γ -globulin and a band (labelled x) of unknown identity.

In 22 samples the percentage iron saturation as calculated from the electrophoresis results ranged from 17 to 70 (mean 43.8) and the average distribution of transferrin showed Tf 39.2%, Tf-Fe 11.2%, Fe-Tf 22.9% and $\text{Fe}_2\text{-Tf}$ 26.7%. Fig. 2 shows the plot of $-\ln(1-y)$ against $-\ln(1-x)$, from which the best straight line is:

$$\ln(1-x) = 1.38(\pm 0.05)\ln(1-y) + 0.07(\pm 0.03)$$

Iron is therefore preferentially bound by the *N*-terminal site of transferrin in serum.

Fig. 1. Electrophoresis of serum samples in 6M-urea/polyacrylamide gel

Samples were prepared as described in the text. (1) Fresh serum; (2) serum which had been stored at -15°C for 6 days; (3) serum that had been stored at -15°C and then incubated at 37°C for 4 days; (M) a marker mixture containing Tf, Tf-Fe, Fe-Tf and $\text{Fe}_2\text{-Tf}$. Ig, Immunoglobulin; x, unknown.

Iron distribution in incubated serum

Eleven samples were incubated at 37°C for 4 days before electrophoretic examination. The best straight line for these samples (Fig. 3) is:

$$\ln(1-x) = 1.69(\pm 0.07)\ln(1-y) + 0.02(\pm 0.03)$$

There is an increased ratio of iron-binding affinities favouring the *N*-terminal site in these samples, which is highly significant when compared with the unincubated samples ($P < 0.001$).

Fig. 2. Plot of $-\ln(1-y)$ against $-\ln(1-x)$ for fresh serum samples

The slope of the best straight line is 1.38 ± 0.05 . In Figs. 2-7 the values for x and y were obtained from electrophoretic separation of transferrin species, as described in the text.

Fig. 3. Plot of $-\ln(1-y)$ against $-\ln(1-x)$ for the serum incubated at 37°C for 4 days

The slope of the best straight line is 1.69 ± 0.07 .

Iron distribution in serum kept at low temperature

Storage of serum at either 0 or -70°C for several days had little effect on the iron distribution. Thus in 14 fresh samples of serum the iron distribution was represented by the line:

$$\ln(1-x) = 1.32(\pm 0.04)\ln(1-y) + 0.04(\pm 0.03)$$

On storage at 0°C for 6 days the following equation was found:

$$\ln(1-x) = 1.23(\pm 0.04)\ln(1-y) + 0.03(\pm 0.03)$$

After 7 days storage at -70°C the equation was:

$$\ln(1-x) = 1.27(\pm 0.04)\ln(1-y) + 0.04(\pm 0.02)$$

On the other hand, storage of serum in a freezing compartment at -15°C for 6 days caused a marked change in iron distribution (Fig. 1); 43 samples kept under these conditions gave the equation (Fig. 4):

$$\ln(1-x) = 0.62(\pm 0.11)\ln(1-y) - 0.10(\pm 0.03)$$

The statement made earlier (Evans & Williams, 1978) to the effect that human serum contains only two transferrin species, corresponding to the apo-transferrin and the C-terminal monoferric transferrin, was based on samples stored frozen and is not valid for the iron distribution *in vivo*. A group of 24 samples that had been kept at -15°C for 2 weeks was incubated at 37°C for 4 days. This caused the migration of iron into C-terminal sites induced by low temperature to be partially reversed towards the initial state. The best straight-line equation was:

$$\ln(1-x) = 1.05(\pm 0.06)\ln(1-y) + 0.02(\pm 0.03)$$

One serum sample was examined at daily intervals while it was stored at 37°C and at -15°C . During this experiment there was no change in the percentage saturation of transferrin with iron, but the ratio $\ln(1-x)/\ln(1-y)$ showed a reversible rearrange-

Fig. 4. Plot of $-\ln(1-y)$ against $-\ln(1-x)$ for serum stored at -15°C for 6 days

The slope of the best straight line is 0.62 ± 0.11 .

ment of the iron distribution that occurred slowly over a period of several days (Fig. 5).

Iron distribution in dialysed serum

In serum that had been dialysed against Hepes buffer, pH 7.4, and then incubated there was migration of iron into C-terminal sites of transferrin, and this was accelerated by the addition of citrate to 1 mM (Fig. 6). In a series of five dialysed samples the equation of the best line was:

$$\ln(1-x) = 0.15(\pm 0.05)\ln(1-y) + 0.06(\pm 0.14)$$

In these samples the N-terminal monoferric complex Fe-Tf was undetectable in the electrophoretograms, and the N-terminal site was occupied by iron only in the form of $\text{Fe}_2\text{-Tf}$. The addition of citrate to a sample of undialysed serum had little effect on the previously noted migration of iron into N-terminal sites of transferrin on incubation.

Fig. 7 shows the effect of adding the diffusible fraction of serum on the iron distribution in serum previously dialysed against 0.2M-Mops buffer, pH 7.4. The diffusate promoted the migration of bound iron into N-terminal binding sites at 37°C and this effect was reversed by subsequent storage at -15°C .

The diffusate also caused rearrangement of iron distribution when added to a solution of transferrin in buffer. Iron-free transferrin was dissolved in 0.1M-Mops buffer, pH 7.4, at a concentration of 10 mg/ml. After partial saturation with ferric nitrilotriacetate,

Fig. 5. Ratio $\ln(1-x)/\ln(1-y)$ in a serum sample stored at 37°C and at -15°C

A serum sample was divided into two parts. One was kept at 37°C for 5 days and then at -15°C for 5 days (●). The other (○) was kept first at -15°C and then at 37°C .

Fig. 6. Effect of 1 mM-citrate at 37°C on dialysed and undialysed serum as shown by the ratio $\ln(1-x)/\ln(1-y)$

The dialysed and undialysed samples were divided into two portions. To one portion (●) 1 mM-citrate was added. No citrate was added to the other portion (○).

Fig. 7. Effect of incubating the diffusible fraction of serum with dialysed serum for 4 days at 37°C as shown by the ratio $\ln(1-x)/\ln(1-y)$

Different amounts of the freeze-dried diffusible fraction were added to the dialysed serum.

(8 mg/ml) for 2 days caused nearly complete replacement of Tf-Fe by Fe-Tf.

Discussion

Many studies, referred to in the introduction, have shown that the two metal-binding sites of human transferrin are non-identical under a variety of conditions *in vitro*. The present demonstration that in serum the transferrin-bound iron preferentially occupies the N-terminal site shows that the sites are also non-identical under physiological conditions, although the physiological significance of the difference is not known.

On dialysis, the normal preferential binding of iron to the N-terminal site is replaced by a strong preference for the C-terminal site. This agrees with the findings reported by Aisen *et al.* (1978). Our observations suggest that the physiological distribution of iron is determined by one or more low-molecular-weight substances present in serum. The identity and mode of action of this substance is unknown, but it is suggested that it may be an iron-chelating substance that can donate iron to transferrin with a specific preference for the N-terminal binding site. Alternatively, this substance may act as an allosteric regulator that binds to one or both structural domains of the protein, thereby changing their relative iron-binding affinities. The rearrangement of iron distribution that occurs at -15°C suggests that a relatively large entropy factor is involved in the interaction.

We are grateful to Dr. J. C. G. Milne who gave invaluable help with numerical methods, and to Dr. R. W. Evans for the preparation of transferrin from human serum.

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urea/polyacrylamide-gel electrophoresis of the solution showed two bands representing Tf and Tf-Fe. Incubation of this solution with the diffusate

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