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Isolation of the four forms of transferrin with respect to iron by high-performance liquid chromatography: comparison of three mammalian species

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Abstract

A method, based on anion exchange combined with high-performance liquid chromatography, is presented for the separation of the four different forms of transferrin with respect to iron, *i.e.* diferric, monoferric with Fe in the C-terminal lobe, monoferric with Fe in the N-terminal lobe, and apo (iron-free). Depending on the size of column applied, these forms can be obtained on a preparative scale amounting to milligrams. The procedure was also found to free transferrin from loosely bound iron and protein aggregates. Comparative studies with human, murine and rat transferrins showed that optimal resolution of these proteins depended on establishing a distinct elution programme for each species. The order in which the four forms referred to above were displaced from the exchanger differed from species to species.

1. Introduction

Transferrin (TRF), the principal iron carrier protein in mammalian blood, consists of two iron-binding lobes (N-terminal and C-terminal) interconnected by a short hinge region [1]. Since each lobe is capable of binding one atom of iron, four forms of TRF can be distinguished with respect to iron content. In 1979, the Fourth International Conference of Proteins of Iron Metabolism (Davos, Switzerland) proposed the following notation for these forms: (i) Tf (apo-transferrin, the iron-free form); (ii) Fe_N-Tf, the monoferric form with iron in the N-terminal lobe (the acid-labile binding site); (iii) Tf-Fe_C, the other monoferric form with iron in the C-terminal (the acid-stable binding site) lobe; and (iv) Fe_2-Tf, the form carrying iron in both lobes [2]. One way of detecting and quantifying these forms is by polyacrylamide gel electrophoresis in the presence of 6 *M* urea [3], followed by autoradiography of Western-blot preparations obtained with ^{125}I-labelled antibodies to TRF [4].

The affinities for iron of both lobes differ (C > N) and, depending on the prevailing pH, the difference may amount up to approximately 20-fold [5]. Moreover, several observations suggest that the lobes are functionally unequal. Thus iron absorbed from the alimentary canal is predominantly found in the N-lobe [6]; interaction of TRF with its receptor at endosomal pH

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promotes release of iron from the C-terminal site [7] in contradistinction to facilitated release from the N-terminal site when TRF is bound to heparan sulphate proteoglycan [8].

In view of the above differences, the outcome of studies with the two monoferric forms of TRF much depends on having these molecules available separated from one another and also from Tf and Fe_2-Tf. And this is not an easy task. Although it is possible to preferentially direct iron to one domain or the other by using appropriate chelators [9], usually a mixture of several molecular forms is obtained [10,11]. A preparative HPLC method utilizing cation-exchange chromatography in conjunction with stepwise elution has recently been described; however, it only yields three peaks: Fe_2-Tf, Tf, and a mixture of Fe_N-Tf and Tf-Fe_C [12].

Below we present a HPLC procedure, based on anion-exchange chromatography, for the separation of the four forms of TRF with respect to iron that also lends itself to application on a preparative scale.

2. Experimental

2.1. Isolation of transferrins

Human, mouse and rat TRF were isolated from pooled plasmas by a three-step procedure described elsewhere [13]. When chromatographed or electrophoresed under alkaline conditions in the iron-saturated state, each of these proteins can be resolved into one major (approx. 60–65% of total) and several minor components. According to structural [14,15] as well as chemical [16] analyses of TRF glycans, this behaviour is due to carbohydrate microheterogeneity. To simplify the interpretation of HPLC results, the minor subpopulations of TRF were eliminated by anion-exchange chromatography.

2.2. Preparation of transferrins for HPLC

The principal TRF component thus obtained from each species electrophoresed as a single band in alkaline polyacrylamide gel. It was converted to the apo form by dialysis against 0.1 *M* citric acid/NaOH buffer pH 4.5, followed by 0.1 *M* sodium acetate of the same pH (12 h, 500 ml each). Then the pH of the protein solution was raised to 8.0 by dialysis against 15 m*M* Tris-HCl or 15 m*M* boric acid/NaOH with several changes of the outer compartment. Portions (1–10 mg TRF) of the preparations were treated with iron using various stoichiometries (mainly 1:1). Fe^{3+} was added to TRF either as the citrate or the nitrilotriacetate (Koch-Light Lab., Colnbrook, UK) complex together with a molar excess of $NaHCO_3$. In some experiments the distribution of iron was traced by ^{59}Fe (New England Nuclear, Boston, MA, USA). After incubating for 15 min at room temperature, the sample (usually 1 ml) was injected in the HPLC column.

2.3. HPLC procedure

A spherogel TSK DEAE-5PW (particle diameter 13 μm; 15 × 2.15 cm I.D.; Toya Soda, Tokyo, Japan) column was attached to a Gilson HPLC apparatus (Middleton, WI, USA). In the case of human and mouse TRF it was equilibrated with 15 m*M* Tris-HCl pH 8.0, specific conductivity 570–670 mho (1 mho = 1 S), and for rat TRF with 15 m*M* boric acid/NaOH pH 8.0 (100–180 mho). Both buffers contained 0.1 m*M* desferrioxamine mesylate (Sigma, St. Louis, MO, USA). These buffers were also used as the lower components of the eluting gradients. The limit solvents were 150 m*M* Tris-HCl (pH 8.0, 5400–6000 mho) for human and mouse TRF, and 15 m*M* boric acid/NaOH containing 60 m*M* NaCl (pH 8.0, 4700–5700 mho) for rat TRF, both containing 0.1 m*M* desferrioxamine mesylate. The flow-rate was 0.75 ml/min.

2.4. Analysis of the HPLC peaks

When applicable, chromatographic fractions obtained were counted in a Packard Model 5550 counter before pooling and concentrating in Centricon 30 microconcentrators (Amicon, Beverly, MA, USA). The four forms of TRF were identified as follows. The peak representing Tf

was derived from the distribution of the ^{59}Fe activity in the chromatograms taken in conjunction with the absorbance profiles at 280 nm. The three other forms were located spectrophotometrically on the basis of their characteristic iron release kinetics. The rationale behind this approach was the unequal affinities of both TRF lobes for iron, already mentioned in the Introduction, thus giving rise to pronounced differences in iron release kinetics [7,17–20]. Iron release was followed by measuring the decrease in absorbance at 295 nm caused by the protonation of TRF tyrosyl residues [17]. (The phenolic hydroxyl groups of two tyrosyls per binding site participate in coordinating the iron atom.) A Beckman Du-40 recording spectrophotometer was used for this purpose. The sample (*ca.* 1 mg protein/ml) was placed in the instrument and iron release was induced with sodium pyrophosphate at a final concentration of 10 m*M*. Changes in tyrosine ionization were then recorded at 2-min intervals over 4 h using Tf as a blank. Numerical output of the spectrophotometer was subsequently entered in Enzfitter (Elsevier-Biosoft, Cambridge, UK), a non-linear regression data analysis computer programme. The algorithm [21] underlying this programme calculates single- or double-exponential equations by minimizing the weighted sum of the squares of deviations of the calculated values from the experimental points. The type of equation ultimately selected depended on visual (overlap of experimental and theoretical values when plotted on the screen) as well as numerical (magnitude of the standard error) criteria.

3. Results and discussion

After a series of exploratory experiments, it soon became apparent that TRF from different species required different chromatographic conditions for the resolution by HPLC of their four forms with respect to iron. Consequently, more than 100 HPLC runs were performed to optimize conditions for the three transferrins under consideration here.

As already mentioned, the diferric and both monoferric forms of TRF were identified on the basis of their characteristic iron release curves. An example of spectrophotometric tracings obtained under our experimental conditions is illustrated in Fig. 1. It can be observed that the curve designated Tf-Fe_C changed slowly, in contradistinction to the Fe_N-Tf curve that showed a rapid decline. Also, the absorbance of Fe_N-Tf ceased changing after approximately 1.5 h, signifying the completion of release. Spectrophotometric readings taken after a steady value had been reached were omitted from the calculation of the results. The prominent features of Fe_2-Tf were a rapid initial change (release predominantly from the N-lobe) followed by change at a slower rate (release from the C-lobe). Single-exponential equations provided the best fit for Tf-Fe_C and Fe_N-Tf, and the sum of two exponential terms for Fe_2-Tf. The mean standard error of all fittings, expressed as a percentage of the parameters obtained, was 2.8% (median 1.4%).

Chromatographic conditions established for the resolution of the various forms of human, murine and rat TRF by HPLC are given in Figs. 2, 3 and 4, respectively. The physicochemical explanation for this kind of separation is that

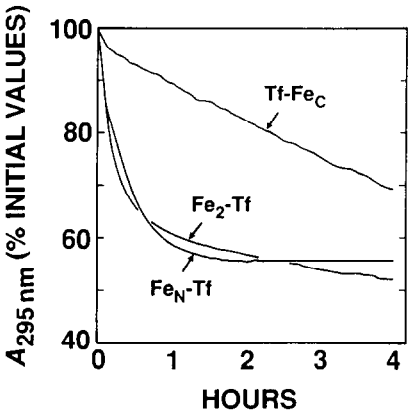

Fig. 1. Composite diagram of the spectrophotometric changes accompanying release of iron from the various forms of rat TRF. Samples were assayed individually as described in the Experimental section. They also contained 0.1 m*M* desferrioxamine mesylate from the HPLC run. Lines are reproductions of the original spectrophotometric charts. To make curves comparable, readings were normalized by assigning 100% to the values obtained before the addition of pyrophosphate. For further explanations see the text.

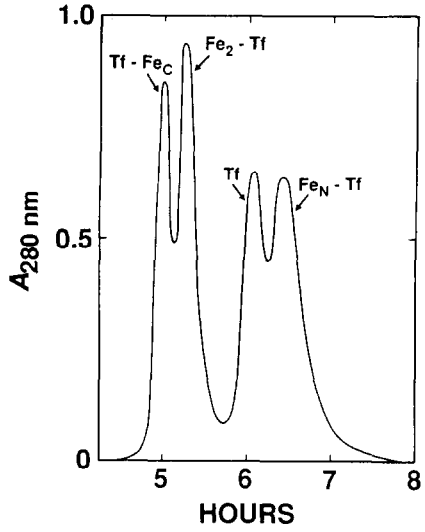

Fig. 2. Separation of the four forms of human TRF with respect to iron by HPLC. The equilibrating buffer (E) and the limit solvent (L) were Tris-HCl (see the Experimental section). A linear gradient of E and L was applied at 5 min so as to reach 80% L at 3 h. This value was then maintained for the duration of the run. Load: 10 mg TRF.

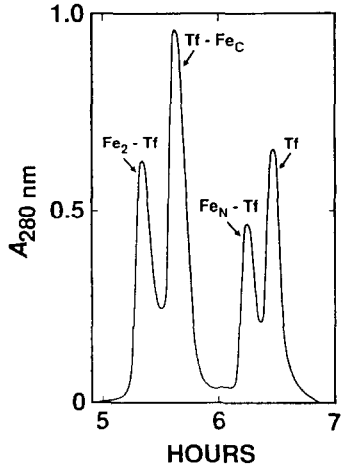

Fig. 3. Separation of the four forms of murine TRF with respect to iron by HPLC. The equilibrating buffer (E) and the limit solvent (L) were Tris-HCl (see the Experimental section). Elution commenced at 5 min with a mixture of 30% E and 70% L lasting for 3 h. Then a linear gradient was applied that reached 100% L at 5 h. Load: approximately 8 mg TRF.

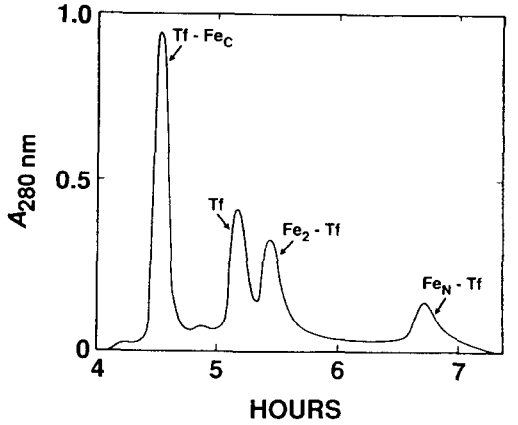

Fig. 4. Separation of the four forms of rat TRF with respect to iron by HPLC. The equilibrating buffer (E) and the limit solvent (L) were borate (see the Experimental section). Elution commenced at 5 min with a mixture of 40% E and 60% L. After 3 h, a linear gradient was applied that reached 90% L in 30 min and was maintained at this level subsequently. Load: 9 mg TRF. An explanation for the two minor peaks adjacent to Tf-Fe_C is given in the text.

metal binding alters the conformation of TRF. This has been known for some time from comparisons of the Stokes radii of Fe_2-Tf and Tf [22]. More recently, it also has been found that the magnitude of the conformational change in monoferric transferrins depends on the metal-binding site involved (Fe_N-Tf > Tf-Fe_C) [23]. Different conformations likely cause different charge distributions on the surface of the TRF molecule, thus giving rise to recognition by the HPLC column. Similar conclusions were drawn from earlier studies by conventional DEAE chromatography of TRF samples saturated to different degrees with iron [10,24].

Transferrins of various mammalian species are closely related molecules. For example, the amino acid sequence of horse, human, porcine and rabbit TRF is identical for 71–79% [25]. Therefore, the observation that the elution order of the four forms differed from species to species, is noteworthy. It seems unlikely that the type and magnitude of the conformational change on iron binding would vary greatly among mammalian transferrins. However, depending on the species, a comparable conformational change could give rise to differently

charged TRFs because the sequence is not completely identical.

When a TRF preparation contained some unbound iron (probably adsorbed on TRF as a chelate), it appeared in the eluate in less than 1 h, *i.e.* separated from the protein by several hours. Likewise, aggregated TRF appeared early as well (between 1 and 2 h). This is illustrated in Fig. 5 by the chromatogram of a preparation of mouse Tf that contained aggregated TRF and also some monosialylated and asialo derivatives of the protein, as identified by observations in an earlier study [26]. The preparation under consideration was originally homogenous, *i.e.* it electrophoresed as a single band, but this homogeneity deteriorated as a consequence of improper storage. The electrophoretic appearance of the same preparation is shown in Fig. 6.

In all mammalian species investigated thus far, TRF exhibits chromatographic as well as electrophoretic heterogeneity. As already pointed out, this is due to the intrinsic variability of TRF glycans, paralleled by a concomitant variation in the number of sialic acids present [14]. Considering that each of the glycan variants can be available in four different forms with respect to iron, the HPLC pattern becomes very complex due to additional peaks and partial overlaps (not shown), unless the protein is rendered homogenous beforehand with respect to sialic acid content. Homogeneity already achieved may suffer from subsequent microbial contamination and sialidase action. A small proportion of monosialylated TRF generated this way is the likely explanation for the presence of two minor additional peaks in Fig. 4.

We included the iron chelator, desferrioxamine mesylate, in all chromatographic solvents to prevent possible metal-ion capturing by TRF in the HPLC apparatus, although we do not know how necessary this precaution really was. The chelator was also present for technical reasons during the studies of iron release, and could have increased the rate constants (k) by shifting the equilibrium between forward and reverse reactions. In the case of diferric mouse TRF, for example, k_1, representing release from

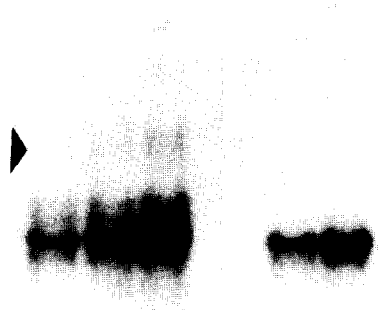

Fig. 6. Alkaline (pH 8.0) polyacrylamide gel (7.5%) electrophoresis of the mouse Tf from Fig. 5. Migration from top to bottom. The three tracks on the left are different loads (5, 10 and 20 ug) of the preparation before HPLC. The main band corresponds to the principal chromatographic peak in Fig. 5, the band behind it to peak c, and the next band to peak b. The arrowhead denotes aggregated Tf. The two tracks on the right are different loads (5 and 10 μg) of the same preparation after HPLC.

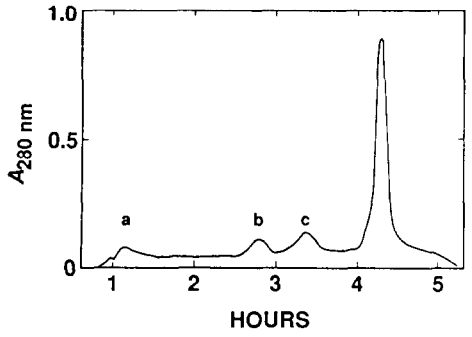

Fig. 5. Resolution of a mouse Tf preparation of inferior quality by HPLC. The equilibrating buffer (E) and the limit solvent (L), both Tris-HCl (see the Experimental section), were mixed in a ratio of 40% E and 60% L and used to develop the run during the first 3 h. Then a linear gradient was applied that reached 90% L in 30 min with no changes thereafter. The letters denote: (a) aggregated Tf; (b) asialo Tf; and (c) monosialo Tf. The large peak is unadulterated Tf. The electrophoretic appearance of the same preparation is shown in Fig. 6.

the N-terminal domain, was 0.11 ± 0.04 percent of the 'initial value' (*cf.* Fig. 1) per minute, the corresponding value for k_2 (release of the C-terminal iron atom) being 0.0032 ± 0.0007. The value of k_1 for Fe_N-Tf was 0.15 ± 0.07 as compared with a k_2 of 0.0032 ± 0.0004 for Tf-Fe_C. As for human Fe_2-Tf, the value of k_1 was 0.077 ± 0.011 and that of k_2 0.049 ± 0.002. For rat Fe_2-Tf, a k_1 of 0.092 ± 0.010 and a k_2 of 0.0010 ± 0.00008 were obtained. Rat Fe_N-Tf yielded a k_1 value of 0.104 ± 0.015 and Tf-Fe_C 0.0095 ± 0.0001. These results suggest that the diferric and monoferric forms of TRF from different species are distinct not only chromatographically but possibly also as far as their iron release kinetics is concerned. Finally, it should be pointed out that the above values do not lend themselves to a direct comparison in absolute terms with similar studies in the recent literature [7,27] because of the marked differences with respect to the composition and pH of the samples, as well as the techniques used to trace iron release.

4. Acknowledgement

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5. References

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