Interaction of transferrin and its iron-binding fragments with heparin

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The interaction of heparin with transferrin (Tf; bovine and rat) and the isolated iron-binding lobes of bovine Tf were investigated. Affinity chromatography of rat Tf on heparin-agarose showed that interaction depended on both the iron content of Tf and the pH of the medium. Both the iron-free and iron-saturated forms of Tf were strongly bound by the column at pH 5.6, but only the iron-free form revealed significant affinity at pH 7.4. Desialylation of Tf moderately promoted interaction, treatment with cyclohexanedione moderately reduced interaction, and succinylation abolished it altogether. In the presence of heparin, iron release from the N-terminal lobe of native bovine Tf was

accelerated and from the C-terminal lobe it was slightly reduced. The heparin effect remained qualitatively the same on each lobe after their separation by tryptic digestion and DEAE-cellulose chromatography. The affinity of native bovine Tf for heparin was very close to that of its isolated N-terminal lobe, thus suggesting that it is this portion of the molecule that binds to the glycosaminoglycan. It is concluded that the consequences for iron-binding strength of the two transferrin lobes are diagonally opposite when Tf is bound to heparin as opposed to its natural cell-surface receptor.

INTRODUCTION

Recent work in this laboratory [1] showed that transferrin (Tf) binds to heparan sulphate proteoglycan (HSPG) purified from the rat liver. In the pH range 7.3–5.6, the strength of interaction depends on the pH ($5.6 > 7.3$). Affinity chromatography of the separated glycosaminoglycan (GAG) chains and the core protein of HSPG revealed that the binding activity is vested in the chains rather than the core protein. Thus the type of interaction under consideration here differs from the situation seen with embryonic skin fibroblasts, where Tf binds to the core protein [2].

A potentially important consequence of HSPG–Tf interaction is the accelerated release of iron from Tf at pH 5.6 [1]. In this regard, HSPG mimics the true Tf receptor, which also promotes iron release from Tf at this pH value [3]. Of the two iron-binding sites of Tf, called N- and C-terminal lobes, receptor binding at pH 5.6 destabilizes iron in the C-terminal lobe [4]. In contradistinction, our findings were consistent with the notion that HSPG binding expedites the release of iron from the N-terminal lobe of Tf [1].

Experiments presented below were aimed at the identification, by alternative means, of the iron-binding lobe of Tf from which the metal is preferentially discharged on interaction with a GAG. To this end, we first explored whether heparin could be substituted for hepatic HSPG because of its ready availability commercially. We then separated both iron-binding lobes of Tf for studying their affinities for heparin and the impact of heparin on the kinetics of their releasing iron.

MATERIALS AND METHODS

Proteins

The principal component of rat transferrin (rTf), containing a standard diantennary disialosyl glycan, was prepared as before [5]. It was used either in the diferric or the iron-free (apo) form. These forms were prepared as in an earlier study [6]. To obtain

the asialo derivative of rTf, the protein (4 mg) was treated with sialidase (EC 3.2.1.18; from *Vibrio cholerae*; Gibco Laboratories, Grand Island, NY, U.S.A.) under conditions detailed elsewhere [6]. To modify lysyl amino groups, 15 mg of rTf was incubated with 1.5 mg of succinic anhydride (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 1 M borate buffer, pH 8.0, for 30 min at 0 °C. Subsequent determination of amino groups [7] showed a reduction by 51 %. Arginyl guanidine groups in rTf were modified with 1,2-cyclohexanedione (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) as before [8]. In brief, rTf (15 mg) was allowed to react with a 550-fold molar excess of the reagent in 0.25 M borate, pH 9.0, for 2 h at 37 °C. After dialysis against 0.1 M borate/0.1 M phosphate, pH 7.0, with seven to eight changes, the extent of arginine modification was determined by treating 100–400 µg portions of the preparation with hydroxylammonium chloride (BDH Chemicals, Toronto, Ontario, Canada; a 100-fold molar excess with respect to the arginine residues present), and quantifying the resulting 1,2-cyclohexanedione dioxime as its Ni complex [9] spectrophotometrically at 550 nm. An average value of 26 mol of complex per molecule of rTf was obtained which is in the same range as the arginine content of Tfs [10], the implication being that the modification was complete or nearly complete.

Bovine transferrin (bTf) was purified by a published method [11]. As bTf is the product of four allelic genes, and thus exhibits anionic heterogeneity, single anionic species were used with one additional component as a minor contaminant. The acid-stable C-terminal iron-binding domain of bTf and the acid-labile N-terminal one were cleaved by the method of Brock and his colleagues [12,13]. In brief, diferric bTf (25–50 mg), trace-labelled with ^{59}Fe , was incubated at 37 °C with bovine pancreatic trypsin (EC 3.4.21.4; Sigma, type I, 10000 BAEE units/mg) in the weight ratio of 50 protein to 1 enzyme. [*N* $^{\alpha}$ -Benzoyl-L-arginine ethyl ester (BAEE) is a synthetic substrate for several proteases. One BAEE unit = ΔA_{258} of 0.001 per min at pH 7.6 and 25 °C when using a reaction volume of 3.2 ml and a 1 cm path length.] The buffer was 0.1 M Tris/HCl, pH 7.8, containing 20 mM

Abbreviations used: BAEE, *N* $^{\alpha}$ -benzoyl-L-arginine ethyl ester; GAG, glycosaminoglycan; HSPG, heparan sulphate proteoglycan; Tf, transferrin; bTf and rTf, bovine and rat transferrin respectively.

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CaCl₂. After 15–17 h, the enzyme was neutralized by adding a twofold excess (w/w) of soya bean inhibitor (Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Gel filtration of the digest on Sephadex G-100 was carried out with the first preparation as described by Brock and Arzabe [13]; however, subsequently this step was found to be dispensable. Components of the digest were finally resolved by chromatography on DEAE-cellulose. The column (1.5 cm × 19 cm) and sample were equilibrated with 50 mM Tris/HCl, pH 7.6, containing 20 mM NaCl. After the sample had been loaded, 50 ml of this buffer was passed through the column, followed by an asymptotic gradient which was generated in a 250 ml chamber by mixing 50 mM Tris/HCl, pH 7.6, containing 120 mM NaCl, with the equilibrating buffer. The gradient was discontinued after 100 ml of flow, i.e. when it reached 53 mM with respect to NaCl, and was replaced by the limit solvent. The whole procedure was carried out at room temperature. Effluent fractions (2.5 ml each) were monitored for absorbance at 280 nm and ⁵⁹Fe activity.

Proteins were labelled as required with ¹²⁵I or ¹³¹I (New England Nuclear, Boston, MA, U.S.A.) by using tetrachloroglycoluril (Pierce Chemical Co., Rodeford, IL, U.S.A.) under conditions given elsewhere [14].

Heparin-agarose affinity chromatography

Sigma type-I heparin, cross-linked to 4% beaded agarose after activation by CNBr, was obtained commercially. According to the supplier, the suspension contained 750–800 µg of heparin/ml with an activity of about 140 USP units/mg of heparin. Columns (10–14 ml) were operated at room temperature at either pH 7.4 or pH 5.6. Accordingly, columns and loads were equilibrated with either 10 mM Tris/HCl, pH 7.4, or 10 mM Mes, pH 5.6. Both these buffers also contained 50 mM NaCl. Small quantities (10–30 µg) of the proteins of interest were loaded individually (¹²⁵I label alone) or in pairs (¹²⁵I and ¹³¹I). Chromatograms were developed by a linear NaCl gradient in the respective equilibrating buffer, and progress was monitored by the radioactivity content of the 1 ml fractions collected. The molarity of NaCl at the midpoint of a peak was calculated and used as a measure of affinity. In experiments with the apo form of rTf, all buffers included the iron chelator desferrioxamine mesylate (50 mg/l; Sigma).

Study of the release of iron from bTf and its tryptic fragments

The method of Morgan et al. [15] was used which measures changes in tyrosine ionization on iron release at 295 nm. (In both binding lobes, the phenolic hydroxy groups of two tyrosines form part of the co-ordination points for the metal [16].) A Beckman Du-40 spectrophotometer was set in the kinetic programme mode with recordings at 1 or 2 min intervals over 120–180 min. Test samples contained the following ingredients: buffer, 10 mM Mes, pH 6.2; desferrioxamine mesylate, 0.1 mM; disodium dihydrogen pyrophosphate (Sigma), 2.0 mM; heparin, used optionally, 0.74 mg/ml (Sigma, grade 1-A, made from pig intestinal mucosa; molecular-mass range 5–15 kDa). Bovine Tf, or its C-terminal or N-terminal fragment, was added last at a final concentration of 1 mg/ml. Then the content of the cuvette was rapidly mixed and recording started. The composition of blanks matched that of test samples except no Tf was added.

Spectrophotometer read-outs were processed by the Enzfitter computer program (Elsevier-Biosoft, Cambridge, U.K.). A two-term exponential function was fitted to the points representing diferric bTf and a single-exponential one to its monoferric fragments for calculation of rate constants (*k*).

Other techniques

Radioactivities were assayed in a Packard model 5550 γ counter with appropriate corrections for crossover. Tf concentrations were estimated spectrophotometrically at 280 nm using an absorption coefficient of 13.0 for a 1% (w/v) solution of Tf saturated with iron [17]. Alkaline (pH 8.3) PAGE and SDS/PAGE were carried out in 7.5% cross-linked slabs.

RESULTS AND DISCUSSION

Affinity of Tf for heparin

A series of experiments was designed to investigate whether heparin could bind Tf and, if so, how this binding compared with binding by hepatic HSPG. We used rTf in our earlier work on Tf-HSPG interaction [1,8] in which biosynthetic [³⁵S]HSPG was chromatographed on various forms of immobilized Tf. Therefore, studies described in this section were also carried out with rTf. Two pH values, 7.4 and 5.6, were chosen because of their relevance to the transcellular Tf cycle [4].

Capture of rTf by immobilized heparin depended on the protein's iron content and the pH of the equilibrating buffer (Figure 1). Diferric rTf had very little affinity for heparin at pH 7.4, appearing in the eluate before commencement of gradient elution. However, the iron-free form of rTf was firmly bound at the same pH and required 0.42 M NaCl in the gradient to be eluted. Apo-rTf was even more firmly bound at pH 5.6 requiring 0.54 M NaCl for elution. As shown in Figure 2, diferric rTf bound well to heparin at pH 5.6 and needed 0.306 M NaCl for displacement. Desialylation of rTf moderately enhanced interaction with heparin as is apparent from a shift of the elution peak to 0.34 M NaCl. Succinylated diferric rTf (51% loss of amino groups) did not bind to heparin at pH 5.6 (not shown). In contrast, modification of the arginyl guanidino groups did not completely abolish the affinity of rTf for the GAG but only weakened it so that the modified protein was eluted at a 10–25% lower NaCl concentration than control rTf (not shown). As the Lys/Arg ratio in transferrins is close to 2:1 [10], both types of modification affected approximately the same number of amino acids. It seems therefore that the Tf-heparin interaction is primarily mediated by lysyl amino groups.

According to our earlier data, it takes 0.161 M NaCl to disrupt diferric rTf-HSPG interaction at pH 7.4 [8]. This is in sharp contrast with the lack of affinity of rTf at the same pH for

Figure 1 Affinity of rTf for heparin-agarose as a function of its iron content and pH of the medium

Chromatographic conditions are given in the Materials and methods section. ●, Diferric rTf, pH 7.4; △, apo-rTf, pH 7.4; ▲, apo-rTf, pH 5.6; ----, concentration of NaCl in the gradient. Fraction volume was 1 ml.

Figure 2 Chromatography of diferric rTf and diferric asialo-rTf on heparin-agarose at pH 5.6

Running conditions are described in the Materials and methods section. ●, Tf; ○, asialo-Tf; ----, NaCl gradient. Fraction volume was 1 ml.

heparin as found in the present study. This difference notwithstanding, heparin and HSPG compare favourably in other aspects, including marked increases in affinity for Tf on lowering the pH, and a moderate increase on removal of the sialic acids from Tf. As commercial heparin is devoid of the core protein, its capability of binding Tf must reside in the GAG chains, as found previously with HSPG [1]. Attraction between negative sulphate groups in GAGs and positively charged groups in Tf (and in some other proteins as well [8]) is a likely explanation for the behaviour of both GAGs toward Tf. Heparin is much more highly sulphated than HSPG [18], and this may explain the observation that 0.54 M NaCl was needed to disrupt apo-rTf-heparin interaction at pH 5.6 (see above) in contrast with only 0.28 M in the case of apo-rTf-HSPG [8]. Bearing these similarities and differences in mind, we concluded that heparin could be used below pH 7 to study the question of how GAGs modulate iron release from Tf.

Heparin and the monoferric fragments of bTf

In the diferric state, Tf is remarkably resistant to the action of trypsin, a feature that has been taken advantage of by Williams [19,20] and Brock et al. [12,13] to separate both iron-binding lobes of ovotransferrin and bovine serum transferrin respectively as monoferric fragments. We used this approach in order to answer two questions: (i) which of the lobes binds to heparin; and (ii) is binding paralleled by a change in iron release?

Two batches of monoferric fragments were prepared with comparable outcomes. The DEAE-cellulose chromatographic profile of one preparation is shown in Figure 3. Alkaline PAGE as well as SDS/PAGE were used to identify the peaks eluted from the DEAE-cellulose column. The first major peak seen in Figure 3 migrated under alkaline conditions more slowly than the second major peak, whereas the mobility of the two small peaks between them was close to that of bTf before exposure to trypsin (not shown). From five SDS/PAGE runs, a mass of 34–35 kDa was derived for the fragment in the first major peak and 30 kDa for the other in the second major peak. Thus the characteristics of the fragments obtained by us closely matched those produced by Brock et al. [12] as far as the results of both PAGE and SDS/PAGE were concerned. From earlier observ-

Figure 3 Separation by DEAE-cellulose chromatography of the products obtained after digesting diferric bTf with trypsin

— ^{59}Fe activity; ---- absorbance at 280 nm. Fraction volume was 2.5 ml. For further technical details see the Materials and methods section. The first major peak is the C-terminal fragment and the second the N-terminal. The minor peaks between them are insufficiently digested bTf.

ations of iron uptake by the tryptic fragments, it seems clear that the C-terminal acid-stable iron-binding site is contained in the larger fragment, whereas the N-terminal acid-labile site is in the smaller one [13]. Iron-release studies presented below support this conclusion.

When disulphide bridges were reduced in the fragments by treatment with 2-mercaptoethanol before SDS/PAGE, there was no change in the mobility and appearance of the N-terminal fragment. The C-terminal fragment, however, split into two bands, one (approx. 45%) migrating as the non-reduced fragment and the other (approx. 55%) as a protein of 30–31 kDa. The missing material of 3–4 kDa was identified in gradient SDS/PAGE (5–15% cross-linked) as three peptides (not shown). Both digests exhibited this phenomenon. The implication therefore is that the C-terminal portion of bTf is more vulnerable to trypsin action than the N-terminal one. Hydrolysis of some peptide bonds does occur, but this does not manifest itself unless the cystine residues are reduced. Thus bTf differs from ovotransferrin in which neither the N-terminal [19] nor the C-terminal fragment [20] is affected by disulphide reduction. As shown below, there was little difference between the C-terminal fragment and the C-terminal lobe of native bTf with regard to iron-release rates, suggesting that the small peptides generated by trypsin were localized to a section of the molecule that had no significant influence on the conformation of the iron-binding site.

Results pertaining to the affinity of the fragments for heparin are summarized in Figure 4. Both fragments possessed affinity for heparin, albeit to unequal extents. This is evident from the fact that, whereas the fragment bearing the C-terminal iron-binding lobe was displaced by 0.22 M NaCl, its counterpart was not eluted until the NaCl gradient reached 0.31 M. The affinity of undigested bTf was very close to that of the N-terminal fragment (Figure 4). The possibility is thus raised that intact bTf interacts with heparin through its N-terminal lobe, for the expectation would have been a higher molarity of eluting NaCl had both lobes been involved. It is not clear whether the C-terminal portion of intact bTf is sterically hindered in binding heparin or whether it lacks affinity for the GAG altogether. In the latter case, the chromatographic behaviour of the isolated

Figure 4 Heparin-agarose chromatography of bTf and its tryptic fragments containing the C- and N-terminal iron-binding lobes

A 14.5 ml column was used. This and the load were equilibrated with 10 mM Mes, pH 5.8, containing 50 mM NaCl and desferrioxamine mesylate (50 mg/l). All protein samples were in the apo form. For chromatography, samples were differentially labelled with radioiodine (^{125}I , ^{131}I) and run in pairs (C + N; N + bTf). Loads consisted of 10–20 μg of each protein. The straight dashed line denotes the NaCl gradient. Fraction volume was 1 ml.

Figure 5 Spectrophotometric recording of the release of iron from diferric bTf with and without the presence of heparin

Samples were in 10 mM Mes, pH 6.2, containing 0.1 mM desferrioxamine mesylate. ———, Control; —, sample containing 0.75 mg/ml of heparin. Release was prompted by 2 mM pyrophosphate. For further details see the Materials and methods section.

fragment may be an artifact of the treatment with trypsin. However this may be, heparin affinity chromatography would seem to provide a useful additional technique for studying the isolated lobes of Tf, especially in the absence of intact Tf.

Iron release from diferric and monoferric Tf has been studied extensively *in vitro* (e.g. [15,21–25]), and it is now clear that a number of factors, including pH, ionic strength and temperature, influence the value of the apparent rate constant obtained for release from either lobe [23,24]. On adding pyrophosphate [25], a reagent that destabilizes the (bi)carbonate involved in co-ordinating the Fe ions to the binding sites [26], to diferric Tf, release of iron commences in both lobes, albeit at unequal rates because of a difference in affinities for the metal. Depending on the species, this difference may be pronounced enough to give rise to two components distinguishable by exponential analysis (e.g. human and rat Tf); in other cases (e.g.

Figure 6 Release of iron from the monoferric fragments of bTf with and without heparin present

Composite illustration drawn from four spectrophotometric runs. Experimental conditions were identical with those in Figure 5. C, C-terminal fragment alone; C + h, the same with heparin; N, N-terminal fragment alone; N + h, the same with heparin.

ovine and rabbit Tf), the difference is so modest that the release curve appears to follow single-exponential kinetics [15,25].

Two preparations of bTf were used, partly to establish the type of release curves bTf would yield, and also to study the impact of heparin on release kinetics. One such experiment with and without added heparin is shown in Figure 5. Assuming a single-exponential decay model, non-linear regression analysis (see the Materials and methods section) gave a poor fit for both curves. However, reasonable results were obtained by using a two-exponential model. Thus the following apparent (see below) first-order release constants were computed for k_1 , representing release from the N-terminal lobe, and k_2 , the corresponding parameter for the C-terminal lobe: no heparin, $k_1 = 1.29 \times 10^{-2}$ ($\pm 1.7 \times 10^{-3}$ S.E.M.) min^{-1} and $k_2 = 1.44 \times 10^{-3}$ ($\pm 1.5 \times 10^{-4}$ S.E.M.) min^{-1} . With heparin present, k_1 became 2.66×10^{-2} ($\pm 1.4 \times 10^{-3}$) min^{-1} and $k_2 = 1.35 \times 10^{-3}$ ($\pm 1.6 \times 10^{-4}$) min^{-1} . The possibility that the change in k values was brought about by a pH shift on the addition of heparin to the buffer has been eliminated. Therefore these values imply that interaction of intact bTf with heparin augmented iron release from the N-terminal lobe approx. two-fold, whereas release from the C-terminal lobe was inhibited by approx. 6%. An analogous trend was noted in two sets of experiments with the monoferric fragments of bTf, one of which is illustrated in Figure 6. The release constant for the N-terminal fragment was 5.45×10^{-3} ($\pm 9.0 \times 10^{-5}$ S.E.M.) min^{-1} without heparin, and 2.27×10^{-2} ($\pm 6.2 \times 10^{-4}$ S.E.M.) min^{-1} with heparin. The corresponding values for the C-terminal fragment were 1.79×10^{-3} ($\pm 2.0 \times 10^{-5}$) min^{-1} and 1.29×10^{-3} ($\pm 1.0 \times 10^{-5}$) min^{-1} respectively. Thus heparin accelerated iron release from the N-terminal fragment over fourfold, whereas it reduced release from the C-terminal fragment to 72% of the control value.

As is observable from the above values, rates of release of iron from the same Tf lobe were different depending on whether or not the other lobe was occupied. This phenomenon, known as site co-operativity [21–24], can exert a positive (promoting) or negative (inhibitory) effect on release from one lobe by the occupancy of the other. Positive co-operativity manifests itself in diferric/monoferric release rate ratios > 1 and negative co-operativity in ratios < 1 [22]. By comparing the release rates from diferric bTf and its monoferric fragments (i.e. monoferric N fragment and monoferric C fragment), we tentatively calculated

a co-operativity factor of 2.3 for the N-terminal lobe and 0.8 for the C-terminal lobe. These values have to be regarded as approximations only because the composition of diferric Tf changes continuously during the release reaction and, consequently, the effect exerted by one lobe on the other must be subject to changes as time progresses. For example, release of iron from the C-terminal lobe is expected to lead to a gradual erosion of the promoting effect on the N-terminal lobe. For this reason, the value of 2.3 above may represent an underestimate. Time-related changes in co-operativity are also expected to have affected the first-order release constants for diferric bTf; hence, we termed them 'apparent' (see above).

Assuming an average molecular mass of 10000 for the heparin used, the GAG-to-protein molar ratio in our release experiments was approx. 6 for whole bTf and 2.2–2.5 for its fragments. The concentration of heparin therefore cannot explain why the effect of GAG was more prominent on the tryptic fragments than on the whole protein. Spectral studies (c.d., intrinsic fluorescence) will commence shortly as part of our effort to find the answer.

As already mentioned in the Introduction, binding of Tf to its receptor or hepatic HSPG changes the metal-holding strength of the protein. The change may mean an increase or decrease, and it may affect either lobe. As a plausible explanation, it has been suggested that binding results in conformational changes [3]. Support for this hypothesis is gained from the 'flexible' nature of the overall structure of Tf [27] as well as from the fact that the iron-co-ordinating amino acids are spread out along the primary sequence [28]. Interaction of Tf with different biological structures is likely to involve different segments of the molecule, thus providing a basis for several conformations, each with its characteristic impact on the two iron-binding sites. Further studies with other Tf-binding substances should broaden our understanding of this aspect.

Heparin is not normally a constituent of blood and, consequently, its capability of binding Tf is of no real physiological importance. However, its chemical relatedness to the GAG chains of HSPG [18] renders it valuable as a model tool for studying the functional consequences of Tf interacting with sulphated proteoglycans on the cell surface, especially in the liver [1]. The picture slowly emerging is intriguing: binding to the true Tf receptor at acidic pH values promotes iron release from the C-terminal lobe and it reduces that from the N-terminal lobe [4]; in contrast, the present study indicates that binding by a GAG under acidic conditions facilitates iron release from the N-terminal lobe and moderately reduces it from the C-terminal

lobe. Finally, it should be pointed out that all the studies discussed above were carried out *in vitro*. Further work is needed to link such data to the situation prevailing *in vivo*, for our understanding of how iron is released in endosomes is limited, and a recent study suggests that additional factors are likely to be involved [29].

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