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Human melanotransferrin (p97) has only one functional iron-binding site

Edward N. Baker[a], Heather M. Baker[a], Clyde A. Smith[a], Mark R. Stebbins[b], Maria Kahn[b], Karl Erik Hellström[b] and Ingegerd Hellström[b]

[a]*Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand* and [b]*Bristol-Myers Squibb, 3005 First Avenue, Seattle, WA 98121, USA*

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The iron-binding properties of melanotransferrin, the tumour-associated antigen also known as p97, have been investigated by UV/visible and fluorescence spectroscopy, amino acid sequence comparison, and modelling. These show that, in contrast to other transferrins, melanotransferrin binds only one Fe^{3+} ion per molecule. The binding properties of its N-terminal site are similar to other transferrins, but its C-terminal site does not bind iron at all. The differences can be related to specific amino acid changes in the C-terminal site.

Iron binding; Melanotransferrin; Transferrin; Melanoma cell antigen

1. INTRODUCTION

Melanotransferrin, (MTf), also known as the tumour-associated antigen, p97, is a monomeric glycoprotein, M_r 97,000, which is expressed by human melanoma cells and certain other tissues [1–3], but is present only in trace amounts in normal adult tissues [4]. Its amino acid sequence [5] shows a high level of identity with proteins of the transferrin family (~40% identity with human transferrin or lactoferrin), it binds iron [6] and it has been proposed to play a role in iron translocation [5].

MTf does, however, differ significantly from other transferrins. It is membrane-bound, and it differs in certain key amino acid residues which in other transferrins are involved in iron binding. Examination of the MTf sequence in relation to the three-dimensional structures of human lactoferrin [7,8] and rabbit serum transferrin [9] has led to the proposal that it has an intact transferrin-type iron binding site in its N-terminal half but a possibly defective site in its C-terminal half [10]. We have tested this proposal by investigation of the iron-binding properties of MTf, and discuss the results in the light of its presumed three-dimensional structure.

2. MATERIALS AND METHODS

2.1. *Isolation of MTf*

MTf was purified from cell culture supernatants of transfected mouse melanoma clone 2a cells [11] by immunoaffinity chromatography. A typical batch purification consisted of a 20 l culture of cells contained in ten 10-shelf cell factories. The cultures were allowed to incubate at 37°C for 10 days at which time the cells had grown to confluence and were showing signs of deterioration. Cell debris was removed by filtration and the filtrate was concentrated 10- to 15-fold with a Pellicon ultrafiltration system (Millipore) using a 5 sq. ft. 30,000 NMWL casette. The retentate (approx. 2 l) was 0.2 μm filtered and applied overnight to a column (1.6 × 16 cm) of immobilized 96.5 mAb [3] that had been previously washed with 0.1 M citric acid, pH 2.2, and equilibrated in PBS, pH 7.2. The column was washed thoroughly with PBS and the bound MTf was eluted with 0.1 M citrate buffer, pH 4.0, followed by immediate neutralization with Tris. The purified MTf (approx. 10 mg) was dialysed into PBS and sterile filtered. Batch analysis consisted of gel filtration HPLC, SDS-PAGE, isoelectric focusing and double-determinant binding ELISA.

2.2. *Measurements of iron binding*

Iron was added as ferric nitrilotriacetate (FeNTA) to solutions of MTf in 0.025 M Tris–HCl, pH 7.8, containing 0.01 M $NaHCO_3$ and 0.1 M NaCl. The protein concentration was estimated using an extinction coefficient at 280 nm, $\varepsilon^{1\%}_{280} = 12.0\ cm^{-1}$, as determined by quantitative amino acid analysis. For iron titrations aliquots of FeNTA were added to iron-free MTf at a concentration of 1.2 mg/ml. Iron binding was monitored either by the increase of absorbance at 464 nm, in the visible electronic spectrum, or from the quenching of fluorescence at 338 nm. For comparison similar titrations were performed for human lactoferrin under the same conditions (using an extinction coefficient, $\varepsilon^{1\%}_{280}$, of 10.9 cm^{-1} [12]).

Electronic absorption spectra in the range 260–700 nm were measured with a Hewlett-Packard HP8452A diode array spectrophotometer. Fluorescence measurements were made with an Aminco SPF-500 spectrofluorimeter, with excitation at 285 nm and emission at 338 nm.

2.3. *Measurements of pH-mediated iron release*

Iron-saturated MTf (2 mg/ml) was dialysed against a series of buffer solutions in the pH range 8.0–3.0, at intervals of 0.5 pH unit. Buffer solutions used were: pH 8.0–7.0, 0.05 M Tris–HCl; pH 6.5–6.0, 0.05 M MES; pH 5.5–3.5, 0.1 M sodium acetate; pH 3.0–2.0, 0.1 M glycine-HCl. All buffer solutions contained 0.2 M NaCl. At each pH, the MTf solution was dialysed for 24 h after which its iron saturation was estimated from the ratio A_{464}/A_{280}. Parallel measurements were made on human lactoferrin and humans serum transferrin under identical conditions.

Correspondence address: E.N. Baker, Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand. Fax: (64) (6) 350 5613.

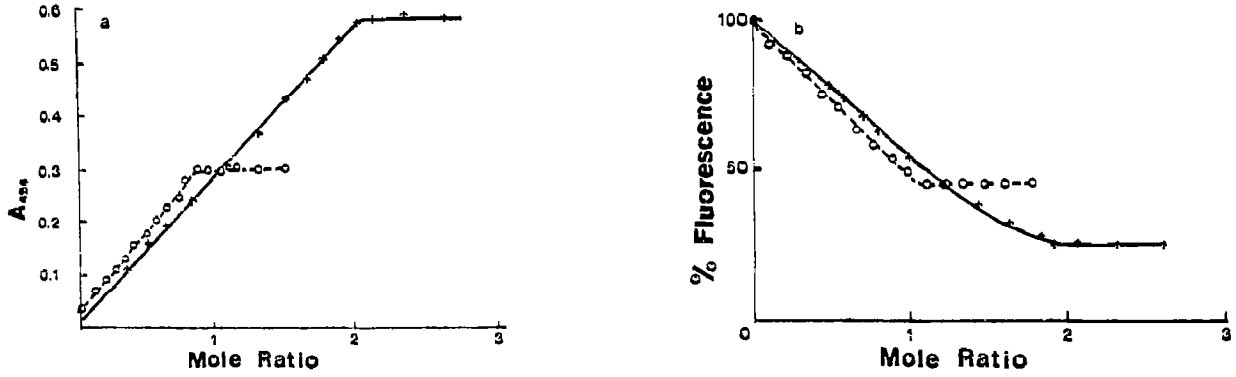

Fig. 1. Iron-binding titrations for melanotransferrin (○), compared with human lactoferrin (+). Plots of (a) the increase in absorbance at 464 nm and (b) fluorescence quenching, measured as % of initial fluorescence remaining, as a function of mole ratio (moles Fe^{3+} added/moles protein).

3. RESULTS

The electronic absorption spectrum of fully iron-saturated MTf shows a peak at 464 nm, and a minimum at 410 nm, both values essentially identical to those obtained for human lactoferrin and transferrin. The extinction coefficient for a 1% solution, however, is 0.30, which is approximately half of the corresponding values for iron saturated lactoferrin (0.58) [13] and transferrin (0.58) [13].

The iron titrations (Fig. 1a) show that the increase in absorbance at 464 nm ceases abruptly at a ratio close to 1.0 mol of Fe^{3+} per mol of MTf. This contrast with lactoferrin for which 2.0 mol of Fe^{3+} are bound per mole of protein.

A similar result is obtained from the measurements of fluorescence quenching (Fig. 1b). Although the extent of fluorescence quenching is not equal for the addition of the first and second Fe^{3+} ion to lactoferrin, at the addition of the first, accounting for approximately 70% of the observed quenching, the difference between MTf and lactoferrin is clearly evident; for MTf no further quenching is seen beyond the addition of 1.0 mol of Fe^{3+} per mol of MTf, whereas for lactoferrin quenching is not complete until a molar ratio of 2.0 is reached. The extent of quenching seen when the single Fe^{3+} ion is bound by MTf also closely matches that for the binding of the first Fe^{3+} ion to lactoferrin.

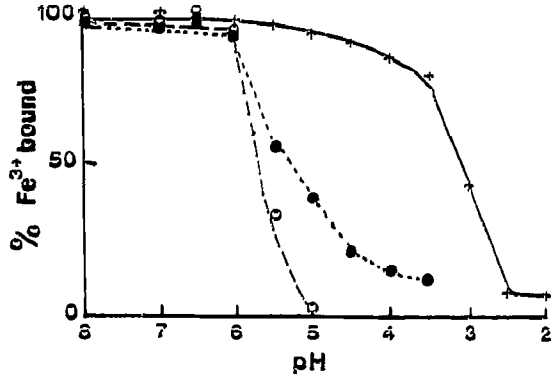

Fig. 2. Effect of pH on iron release by melanotransferrin (○), compared with human lactoferrin (+) and human serum transferrin (●).

The iron release experiments show that loss of iron from MTf begins at a pH of 6.0 and is complete at pH 5.0 (Fig. 2). This compares with transferrin, for which release occurs over the pH range 6.0–4.0, and lactoferrin, for which iron release takes place at considerably lower pH, beginning at pH 3.5 and complete at pH 2.5.

4. DISCUSSION

A characteristic property of other members of the transferrin family (serum transferrin, ovotransferrin, lactoferrin) is the ability to bind reversibly two Fe^{3+} ions, together with two CO_3^{2-} ions, per molecule. These molecules have a strong 2-fold internal amino acid sequence homology which is reflected in their being folded into two globular lobes, representing the N-terminal and C-terminal halves of the polypeptide chain [7–9]. Each lobe has a similar 3D structure and a similar binding site, with the same groups involved in binding the Fe^{3+} and CO_3^{2-} ions.

The present results show clearly that MTf binds only one Fe^{3+} ion per molecule. It is unlikely that this results from changes to the overall 3D structure. The high sequence identity (~40%) with other transferrins implies a very similar structure, as in other homologous proteins [14]. The 46% internal sequence identity, higher even than in other transferrins, also implies similar folding of the N and C-terminal halves. The failure to bind a second Fe^{3+} ion must then result from specific changes within the C-terminal binding site as proposed previously [10].

In the N-terminal half of MTf all four of the iron ligands found in other transferrins, Asp-60, Tyr-92 and His-253 (lactoferrin numbering) are present, as are the groups involved in binding the CO_3^{2-}, Thr-117 and Arg-121 [5]. This must be the site of iron binding in MTf. The similarity of the absorption spectrum implies that the binding site is very like those of other transferrins. The pH dependence of iron release from MTf is also

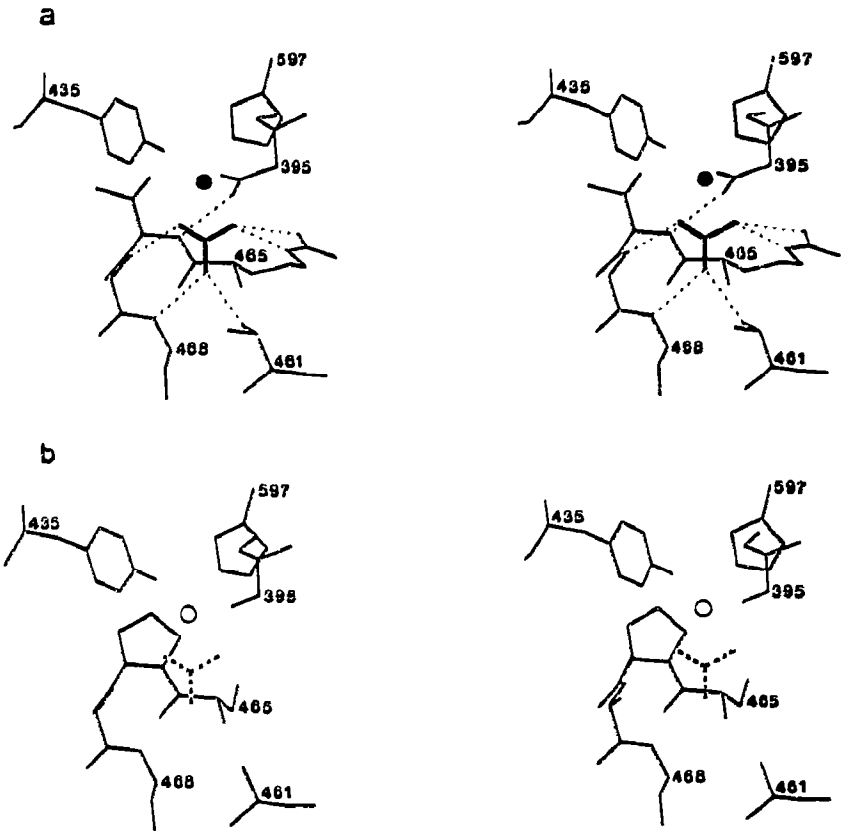

Fig. 3. Stereo views of (a) the C-lobe iron binding site of human lactoferrin, showing the iron atom (●), the CO_3^{2-} ion, three of the four iron binding residues (Asp-395, Tyr-435 and His-597) and residues of the anion binding site, together with important hydrogen bonds (dashed lines), and (b) the equivalent site in melanotransferrin (with the positions of the Fe^{3+} and CO_3^{2-} ions in lactoferrin shown for reference).

similar to that of the 'acid-labile' N-terminal site of serum transferrin, which loses iron over the pH range 6.0–5.2 [13] (the tailing-off to lower pH for transferrin corresponds to release from its 'acid-stable' C-terminal site). In all these respects, therefore, the iron-binding site in MTf resembles a typical serum transferrin-type N-terminal site.

In the C-terminal half of MTf, however, at least four specific amino acid changes are found, of residues which are totally conserved in other transferrins:

(i) Asp-395, which binds to iron through one carboxylate oxygen and hydrogen-bonds across the binding cleft to Thr-466 through its other carboxylate oxygen, is changed to Ser. Ligation through the Ser hydroxyl would be weaker and modelling (Fig. 3) suggests that the bond would be longer unless there is some structural readjustment. Moreover it could not simultaneously hydrogen-bond across the binding cleft as Asp does;

(ii) Arg-465, which hydrogen-bonds to the CO_3^{2-} ion, is changed to Ser. Although the Ser hydroxyl could still hydrogen-bond to the anion, the charge interaction would be lost;

(iii) Thr-461, which hydrogen-bonds to the CO_3^{2-} ion, is changed to Ala, so that this hydrogen bond is lost; (iv) Thr-466, which hydrogen-bonds across the binding cleft to Asp-395, either through its peptide NH (in lactoferrin) or its OH (in transferrin) is changed to Pro, so this hydrogen bond is also lost.

None of these changes, shown in Fig. 3, seems sufficient on its own to abolish binding of Fe^{3+} and CO_3^{2-}, but taken together they clearly are. Whether the binding site is also disrupted by other nearby changes is difficult to say, and only an X-ray structure analysis will finally answer this question.

Why does MTf not bind iron in both lobes as other transferrins do? The C-terminus is probably involved in membrane anchoring [5], although the exact mechanism of attachment has not been described. It is also known that the potential glycosylation sites and the antigenic sites of melanotransferrin are all on the N-terminal half [3]. This suggests that the close association of the C-lobe with the cell membrane either limits access of iron or prevents the opening of the binding cleft which is necessary for iron binding [15]. Alternatively, it is possible that only the outer N-lobe is required for whatever iron translocation function MTf possesses. Either way, evolutionary mutations have been able to occur in the C-lobe binding cleft in the absence of the selective pressures of maintenance of an iron-binding function, even though the overall structure is conserved.

Even in 'normal' transferrins the C-terminal site has somewhat different properties from the N-terminal site,

in that it releases iron less readily, with less facile opening of the binding clef [15,16]. There have been suggestions that the two lobes could have different functional roles [17], and recent studies of the transferrin receptor suggest that receptor binding is required to 'pull' iron from the C-terminal site [18]. When taken with our results, and the recent report of a more distantly related transferrin from the hornworm, *Manduca sexta*, which apparently also has a defective C-terminal site, with a different pattern of substitutions from MTf [19], it appears that the N-lobe has remained fairly constant in its properties while the C-lobe has become differentiated during evolution since the original gene duplication event.

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