

CLEAVAGE OF DIFERRIC BOVINE TRANSFERRIN INTO TWO MONOFERRIC FRAGMENTS

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1. Introduction

In 1968 Fletcher and Huehns [1] suggested that the serum iron-transport protein transferrin, because of an apparent functional heterogeneity of the two iron-binding sites, might also function as a regulator of iron metabolism. Although the structure and function of these sites have subsequently been intensively investigated (see Crichton [2] for a review of recent data), such studies have been greatly hindered by the difficulty of studying the behaviour of one iron-binding site without interference by the other. It would clearly be advantageous to cleave the transferrin molecule into two monoferric fragments and thus study each iron-binding site in isolation. However, on the basis of a previous study [3] it has been generally assumed that iron-saturated transferrin (Fe_2Tf)* was resistant to proteolysis, and that the iron-free form (apoTf) was rapidly degraded to small peptides incapable of binding iron. While studying the relative susceptibilities of bovine transferrin and lactoferrin to proteolysis we observed, however, that trypsin-treatment of Fe_2Tf for 24 h caused appreciable cleavage of the molecule with release of two iron-binding fragments differing in molecular weight and electrophoretic mobility [4]. The isolation and partial characterisation of these fragments now reported provides evidence indicating that each fragment contains one of the transferrin iron-binding sites.

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*Abbreviations: Fe_2Tf , iron-saturated (diferric) bovine transferrin; apoTf, unsaturated bovine transferrin; FeNTA_2 , ferric nitrilotriacetate.

2. Methods and results

Bovine transferrin (Miles Laboratories, Slough, England) was freed from impurities by chromatography on SP-Sephadex A-50 [5], saturated with iron and incubated with trypsin for 14 h (transferrin 7.5 mg/ml, trypsin 0.15 mg/ml) in 0.1 M Tris–0.02 M CaCl_2 pH 7.8, as described in detail elsewhere [4]. Little or no loss of absorption at 470 nm occurred during digestion. Chromatography of the digest on Sephadex G-100 yielded two peaks consisting respectively of undigested transferrin and a mixture of fragments. The latter were separated on DEAE Sephadex A-50, eluted first with 0.05 M Tris–0.02 M NaCl, pH 7.6, yielding one fragment (S), then with a gradient of 0.02 M–0.1 M NaCl in Tris, yielding traces of undigested transferrin, and finally with 0.1 M NaCl in Tris, yielding the second fragment (F). The peak of fragment F was preceded by a small peak due to a minor fragment closely related to F, whose origin is not yet clear.

Cellulose acetate electrophoresis in 0.1 M Tris–0.3 M glycine buffer pH 8.7 (fig.1a) showed that fragment F moved as a single band with a faster mobility than transferrin, whereas S gave a pattern of multiple bands similar to but slower than those of transferrin. This multiple banding is probably due to genetic variants [4]. The molecular weights of fragments F and S were estimated by gel filtration on Sephadex G-100 and found to be 29 400 and 36 800 respectively. These figures agree reasonably well with the values of 32 000 and 38 500 obtained for the fragments observed in SDS-polyacrylamide electrophoresis of unfractionated digests [4].

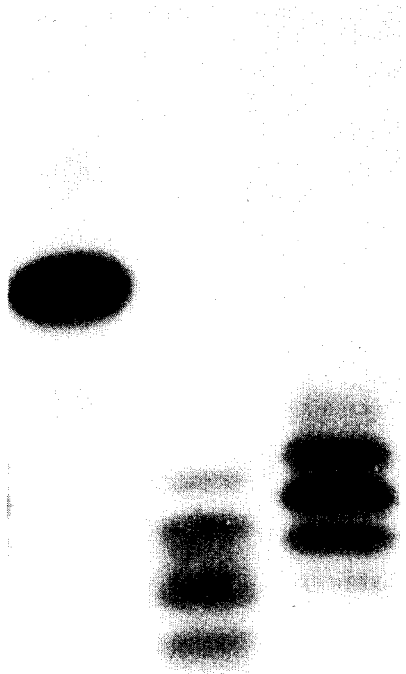


Fig.1a

The iron-binding capacity of fragments F and S was assayed at 470 nm by adding 10 μ l aliquots of 1.0 mM ferric nitrilotriacetate (FeNTA_2) to solutions of the fragments (2–2.5 mg/ml), which had first been freed of iron by dialysis against 0.02 M Na-citrate, pH 5.0, and then against 0.1 M Tris, pH 7.8, containing 0.01 % NaHCO_3 . F and S bound respectively 0.94 and 0.92 atoms of Fe per mole of fragment.

The visible spectra of S and F differed appreciably (fig.2a), the former showing greater absorption at the 460–470 nm maximum, resulting in a more pronounced 'trough' at the 400 nm minimum. An equimolar mixture of F and S gave an intermediate spectrum almost indistinguishable from that of undigested transferrin (fig.2b).

Gel-diffusion analysis (fig.1b) showed that F and S were immunologically unrelated, but both gave a reaction of partial identity with undigested transferrin. Antiserum absorbed with S still reacted with trans-

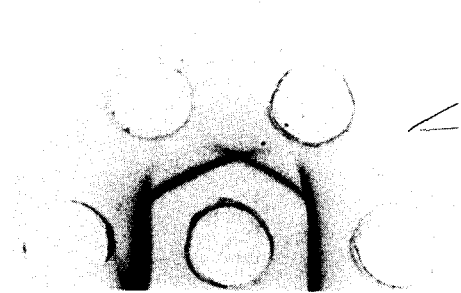


Fig.1b

Fig.1. Characterization of tryptic fragments of bovine transferrin. (a) Cellulose-acetate electrophoresis of fragment F (left), fragment S (centre) and undigested transferrin (right). Anode at top. (b) Gel diffusion pattern of fragment F (upper left), fragment S (upper right), and undigested transferrin (lower left and right) against antiserum to bovine transferrin.

ferrin and with F (which now gave a line of complete identity) but not with S, whereas antiserum absorbed with F reacted only with transferrin and S. Antiserum absorbed with both F and S no longer reacted with transferrin or with either fragment.

Thus fragments F and S, although clearly distinguishable from each other, possessed when mixed together spectroscopic and immunological properties almost identical to intact transferrin, which strongly suggested that each fragment contained one of the two transferrin iron-binding sites. To confirm this, transferrin was asymmetrically labelled with ^{59}Fe , by using the fact that one transferrin iron-binding site binds iron more readily than the other in the pH range 5.0–5.8 [6]. To one sample (A) of apoTf in 0.05 M CH_3COONa , pH 5.5, sufficient $^{59}\text{FeNTA}_2$ was added to give 30% saturation, while to a second sample (B) unlabelled FeNTA_2 was added to 70% saturation. After incubation for 30 min at 37°C the samples were dialysed against 0.1 M Tris–0.02 M

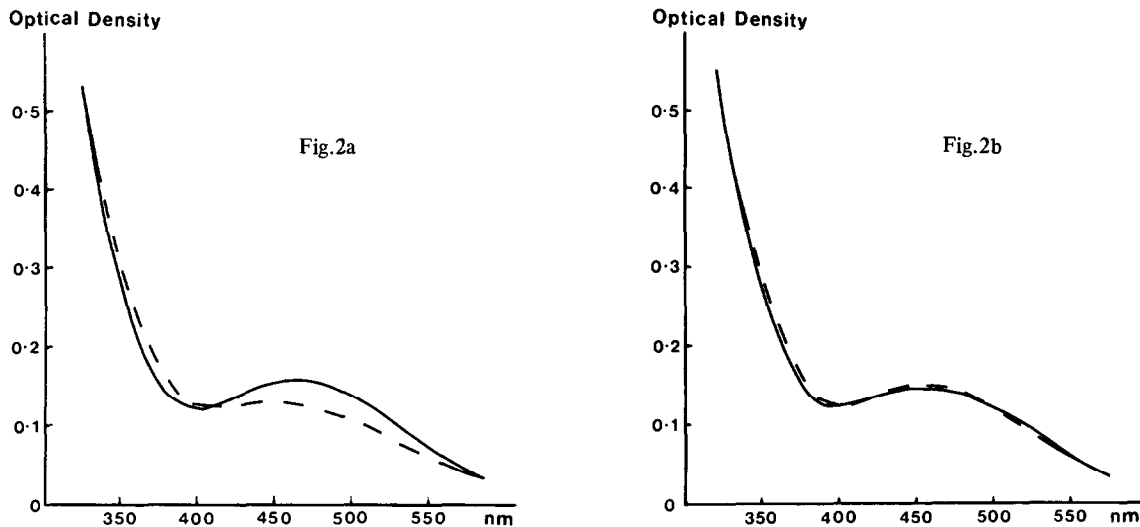


Fig.2. Visible spectra of iron-saturated bovine transferrin and tryptic fragments. (a) Fragment S (—) and fragment F (---). (b) Undigested transferrin (—) and an equimolar mixture of fragments F and S (---). Spectra were obtained in a Beckman Acta CIII spectrophotometer, with samples at 2.2 mg/ml in 0.01 M Tris-0.01% NaHCO₃, pH 7.8.

CaCl₂ pH 7.8 and then brought to 100% saturation with unlabelled FeNTA₂ (A) or ⁵⁹FeNTA₂ (B). Thus in A the ⁵⁹Fe label should be predominantly in the acid-stable site, and in B predominantly in the acid-labile site. Samples A and B were then digested with trypsin and fragments F and S isolated as described above. With A, the ratio of radioactivity in equimolar amounts of F and S was 3.34:1, whereas in B the ratio was 1:3.25. Thus Fe bound at pH 5.5 was always found predominantly in F, an observation consistent with F and S containing respectively the acid-stable and acid-labile iron-binding sites.

3. Discussion

Although monoferric proteolysis fragments have been obtained previously from ovotransferrin [7] (a related protein of avian egg-white which cannot, however, donate iron to rabbit reticulocytes [8]) there is, as far as we are aware, no previous report of monoferric fragments derived from any mammalian transferrin. There also appears to be no previous report of direct cleavage of any iron-saturated protein of the transferrin class into two monoferric fragments, the ovotransferrin fragments being obtainable only by

procedures in which one iron-binding site was digested to small peptides leaving the other iron-binding site intact [7]. Production and isolation of the two component monoferric fragments of a mammalian transferrin by the relatively simple procedure described here should help considerably in further studies of the structure and function of the two iron-binding sites, and their role in iron metabolism.

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