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Characterization of Monoferric Fragments Obtained by Tryptic Cleavage of Bovine Transferrin

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1. The electrophoretically fast (F) and slow (S) fragments obtained by tryptic cleavage of bovine iron-saturated transferrin differed in carbohydrate content and peptide 'maps'. 2. A fragment capable of binding one Fe^{3+} ion per molecule was isolated after brief tryptic digestion of bovine apotransferrin and shown closely to resemble the S fragment obtained from the iron-saturated protein. 3. Fragments F and S are probably derived from the *N*- and *C*-terminal halves of the transferrin molecule respectively. 4. Bovine transferrin could donate iron to rabbit reticulocytes, but the monoferric fragments possessed little iron-donating ability.

Transferrin is a serum protein with the property of reversibly binding two Fe^{3+} ions per molecule. Its major role is in the transport of iron from storage and absorption sites to the developing erythrocyte. Originally, it was assumed that transferrin-bound iron constituted a homogeneous pool; recent evidence, however, suggests that the two iron-binding sites exhibit a functional heterogeneity, and structural data also indicate that the protein does not consist of two identical halves (for a review see Crichton, 1975). Nevertheless, the obvious difficulties of studying one iron-binding site without interference by the other have hindered progress in defining the structure and function of the two sites.

Williams (1974, 1975*a*), studying the related avian egg-white protein ovotransferrin, adopted a novel approach to the problem by degrading part of the molecule with proteolytic enzymes, leaving a half-molecule containing one iron-binding site. However, in these studies it was not possible to cleave the molecule directly into two monoferric fragments, and indeed fully saturated transferrins have generally been considered resistant to proteolysis (Azari & Feeney, 1958; Williams, 1974).

Virtually all previous studies have been carried out with transferrins from man, rabbit, rat or hen's egg-white, and little attention has been paid to transferrins of other species. However, Brock *et al.* (1976) reported that iron-saturated bovine serum transferrin, when treated with trypsin, yielded two distinct iron-binding fragments referred to as F (electrophoretically fast) and S (electrophoretically slow), and these were subsequently isolated and shown each to derive from one iron-binding site (Brock & Arzabe, 1976). In the present paper we report some of the structural and biological properties of these fragments, and also those of a monoferric fragment shown (Brock *et al.*, 1976) to be present after brief tryptic digestion of iron-free (apo) bovine transferrin. Some of this work has been reported (Brock *et al.*, 1977).

Abbreviations used: dansyl, Dns, 5-dimethylamino-naphthalene-1-sulphonyl; SDS, sodium dodecyl sulphate; Dnp, dinitrophenyl.

Materials and Methods

Reagents

Trypsin (EC 3.4.21.4) was obtained from Boehringer (Mannheim, Germany). Dithiothreitol was obtained from Calbiochem (London W1H 1AS, U.K.). Heparin, trisodium nitrilotriacetate, 2-mercaptoethanol and Rivanol (6,9-diamino-2-ethoxyacridine lactate) were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The proteinase inhibitor Trasylol was obtained from Bayer S.A. (Barcelona-15, Spain). Dansyl chloride and thin-layer polyamide sheets were obtained from BDH (Poole, Dorset, U.K.). Guanidine hydrochloride was prepared by dissolving guanidine carbonate (Hopkin and Williams, Romford, Essex, U.K.) in conc. HCl and was used without further purification after adjustment of the pH to 8.0 with solid Tris. $^{59}FeCl_3$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of the best available quality.

Transferrin

(In this paper the word transferrin used without qualification will refer to the protein of bovine origin. Transferrins of other origin will be specified as such.)

The purification of transferrin from bovine serum was carried out as described previously (Brock *et al.*, 1976). Briefly, this involved selective precipitation by Rivanol and $(NH_4)_2SO_4$ followed by purification of the transferrin-rich fraction on DEAE-Sephadex A-50 and SP-Sephadex (sulphopropyl-Sephadex) C-50 (Pharmacia, Uppsala, Sweden). The iron-free (apo) and iron-saturated (Fe_2-) forms were obtained as described previously (Brock *et al.*, 1976) except that ferric dinitrilotriacetate was used in preference to ferric citrate for saturating the transferrin.

Preparation of fragments

Fe_2-transferrin (7.5mg/ml) was digested with trypsin (0.15mg/ml) in 50mM-Tris/20mM-$CaCl_2$, pH7.8, at 37°C for 18h. Proteolysis was terminated by adding Trasylol (4 kallikrein-inactivating units per μg of trypsin: one such unit gives 50% inhibition of 1μg of trypsin), and the two monoferric fragments designated F and S were isolated from the digest by chromatography on Sephadex G-100 and DEAE-Sephadex A-50 (Brock & Arzabe, 1976).

The single fragment of apotransferrin was obtained by digesting apotransferrin with trypsin for 90min under the conditions described above, followed by chromatography of the digest on a column (90cm × 2.5cm) of Sephadex G-100 equilibrated with 75mM-NaCl. Of the three peaks eluted, the first contained undigested transferrin and the last small peptides. The middle peak contained the single fragment of apotransferrin, though it was sometimes necessary to pass this material through Sephadex G-100 a second time to remove traces of undigested transferrin.

Fragments were either stored as frozen solutions or dialysed against distilled water and freeze-dried.

Iron-binding capacity

This was determined as described previously (Brock & Arzabe, 1976).

Electrophoresis

Electrophoresis in cellulose acetate and in SDS/polyacrylamide/agarose gels was carried out as described previously (Brock *et al.*, 1976).

Gel diffusion

Immunological analyses were carried out by Ouchterlony gel diffusion with the anti-transferrin serum described previously (Brock *et al.*, 1976).

Amino acid analysis

Samples (2mg) were hydrolysed for 24h in 6M-HCl and the hydrolysates analysed in an automatic amino acid analyser (Locarte, London E.C.3, U.K.). Tryptophan was measured spectrophotometrically by the method of Goodwin & Morton (1946).

Carbohydrate analysis

Neutral sugars were determined by the method of Dubois *et al.* (1956), hexosamine by the method of Gatt & Berman (1966) and sialic acid by the *p*-dimethylaminobenzaldehyde method as described by Keleti & Lederer (1974). In this last method tubes were centrifuged for 10min in a Jouan E95 centrifuge (Jouan-Quétin, Paris-XX, France) in a model A rotor at 400*g* to sediment precipitated protein.

'Fingerprint' studies

Samples of transferrin (1mg) and fragments F and S (0.5mg each) were carboxymethylated and peptide 'mapping' was performed as described by Richardson *et al.* (1973). The α-nitroso-β-naphthol reagent was used to detect tyrosine-containing peptides.

N-Terminal analyses

The procedure described by Gray (1967) was used. The Dns-amino acids were identified as described by Milstein & Feinstein (1968), but with the use of square polyamide thin-layer sheets one-quarter the area of those used by Woods & Wang (1967).

Uptake of iron by reticulocytes

Recent reports have emphasized the effects that experimental variables may have on results obtained in experiments of this type (Workman *et al.*, 1975; Zapolski & Princiotto, 1976); consequently the method used, which is based on those of the two studies cited, will be described in detail.

Reticulocytes were obtained from a New Zealand rabbit bled on alternate days until reticulocytes constituted at least 15% of the total erythrocytes as estimated by staining with Brilliant Cresyl Blue. Coagulation was prevented by adding heparin. The cells were washed twice in 5mM-Tris/0.15M-NaCl/0.01M-glucose, pH7.45 (hereafter referred to as reticulocyte buffer). Transferrin and fragments were labelled with ^{59}Fe as follows. The proteins were rendered iron-free by dialysis against two changes of 0.02M-sodium citrate, pH5.0, followed by dialysis against two changes of reticulocyte buffer. Ferric dinitrilotriacetate, trace-labelled with ^{59}Fe, was added in sufficient quantity to give theoretical saturation of the protein. The samples were left to equilibrate at room temperature for 10min, and any excess of free iron was removed by incubation, with occasional shaking, for 10min with Dowex AG1 (X4) resin

(Bio-Rad, Richmond, CA, U.S.A.), equilibrated with reticulocyte buffer. The resin was removed by passage through a 0.45μm filter (Millipore Ibérica, Madrid-3, Spain) and the samples were then dialysed against two changes of reticulocyte buffer (1 litre each) and finally adjusted to a protein concentration of 0.4mg/ml. The samples (1.5ml) were mixed with an equal volume of packed reticulocytes and incubated at 37°C in a shaking water bath. Samples (0.5ml) were removed at appropriate times, diluted to 3ml with reticulocyte buffer and centrifuged at 600*g* for 2min (Jouan E95 centrifuge). A portion (1.5ml) of the supernatant was removed, made up to 3ml with reticulocyte buffer and set aside for determining transferrin-bound Fe. The remainder of the supernatant was discarded, and the cells were washed in 2×5ml of reticulocyte buffer and finally taken up in 3ml of water for determining total cell-associated Fe. The radioactivity in tubes containing supernatant and cells was measured in a Packard model 5230 gamma-counter. From the counts the proportion of iron incorporated into or associated with the cells was calculated.

Results

Preliminary characterization of the fragment obtained from apotransferrin

The single fragment obtained from briefly digested apotransferrin had a similar electrophoretic mobility and molecular weight (by SDS/polyacrylamide/agarose electrophoresis) to the fragment S isolated from digested Fe_2-transferrin (Brock & Arzabe, 1976). In Ouchterlony gel diffusion against anti-transferrin serum this fragment gave a line of complete identity with fragment S, and of non-identity with fragment F. It bound approx. 1 Fe^{3+} ion per molecule (found 0.83 ion per molecule). These preliminary observations clearly indicated that the new fragment was closely similar to, if not identical with, fragment S and it is referred to as fragment S_{apo} hereafter.

Effects of 2-mercaptoethanol on the fragments

The fragments were subjected to electrophoresis in SDS/polyacrylamide/agarose gel with and without prior reduction with 2-mercaptoethanol. Fragments F and S_{apo} gave single bands of similar mobility in both the reduced and unreduced states, but fragment S appeared to be heterogeneous, some but not all of the material running as a number of faster-moving bands after reduction (Fig. 1), indicating that some internal cleavage had occurred to a variable extent in this fragment.

Amino acid and carbohydrate composition

There were no very obvious differences in amino acid composition between the two fragments (Table 1), although fragment S was relatively

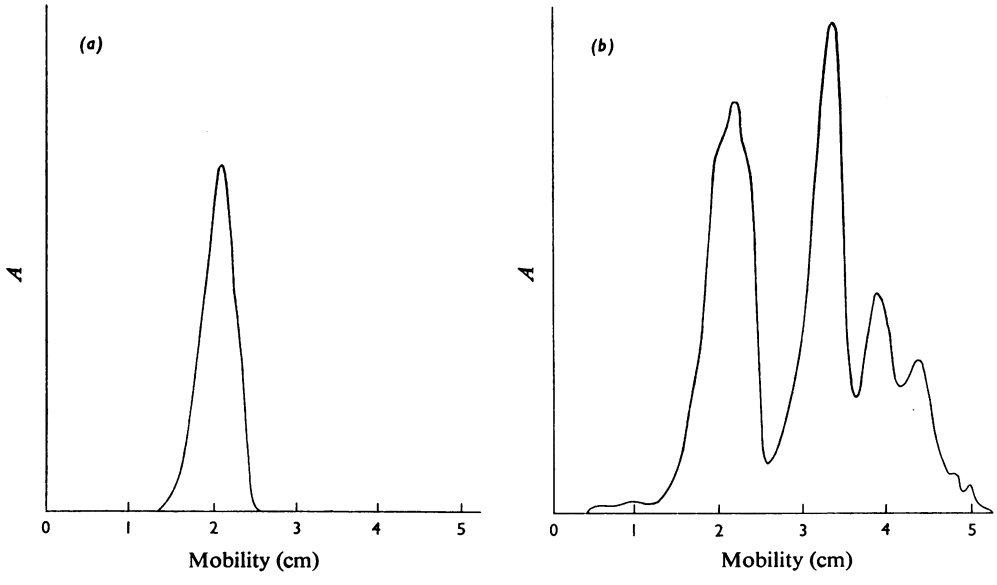


Fig. 1. *Densitometer traces of fragment S of bovine transferrin after electrophoresis in SDS/polyacrylamide/agarose gel*
Measurements were made with a TLD 100 densitometer (Vitatron, Dieren, The Netherlands) after staining of the gels with Coomassie Brilliant Blue. (*a*) Unreduced sample; (*b*) sample reduced with 1% 2-mercaptoethanol. Fragments F and S_{apo} (not shown) gave patterns essentially similar to (*a*) in both reduced and unreduced states.

Table 1. *Amino acid and carbohydrate compositions of bovine transferrin and of the transferrin fragments F and S*

Amino acid analyses (except tryptophan) were obtained from a 24h hydrolysis in 6M-HCl. Tryptophan and carbohydrates were determined as described in the Materials and Methods section. Molecular weights of 74000, 38500 and 32000 have been assumed for transferrin and fragments S and F respectively (cf. Brock *et al.*, 1976; Brock & Arzabe, 1976).

	Composition (mol/mol of protein)		
	Fragment F	Fragment S	Transferrin
Lysine	17	22	47
Histidine	7	3	12
Arginine	7	8	15
Aspartic acid	33	36	74
Threonine	11	15	26
Serine	15	14	33
Glutamic acid	17	23	50
Proline	11	6	21
Glycine	18	20	41
Alanine	21	20	45
Half-cystine	14	19	40
Valine	15	13	30
Methionine	3	4	7
Isoleucine	6	9	14
Leucine	22	16	41
Tyrosine	8	7	15
Phenylalanine	12	7	22
Tryptophan	4	3	8
Total hexose	Trace	5.4	6.6
Hexosamine	Trace	2.9	3.6
Sialic acid	Trace	3.4	3.8

somewhat richer in threonine and lysine and fragment F in phenylalanine and proline. The sum for each amino acid of the two fragments agrees reasonably well with the corresponding value for whole transferrin, whose composition, in turn, agrees reasonably well with earlier analyses (Richardson *et al.*, 1973), except for the higher half-cystine content in the present analysis.

The carbohydrate moiety of transferrin appeared to be localized entirely in fragment S, as only traces were detected in fragment F. Fragment S_{apo} also contained carbohydrate.

Peptide 'fingerprinting' analysis

The number of lysine plus arginine residues found in bovine transferrin was 62 (Table 1), so that theoretically 63 tryptic peptides should be obtained. However, since the transferrin was prepared from pooled bovine serum it is likely to contain more than one genetic variant, and differences between variants (Richardson *et al.*, 1973) may therefore increase the total number of possible peptides. The result for a typical digest of carboxymethylated transferrin is shown in Plate 1(*a*), in which some 65 peptides can be differentiated.

The results for tryptic digests of carboxymethylated fragments F and S are shown in Plates 1(*b*) and 1(*c*) respectively. The 'map' of fragment F was found reproducibly to contain 20–25 peptides and that of fragment S about 40 peptides, all of which could be identified on the transferrin 'map'. However, only four peptides were apparently common to both fragments. The genetically variable peptides TpI, TpII and TpIII (Richardson *et al.*, 1973) could all be recognized in the 'map' of transferrin, which indicates that the sample must have contained variant D_2 and A and/or D_1 and/or E. Peptides TpI and TpIII were found in the 'map' of fragment S, and peptide TpII in the 'map' of fragment F. When the 'maps' were stained with a tyrosine-specific reagent none of the tyrosine-containing peptides was found to be common to the two fragments. Both fragment S and transferrin, but not fragment F, showed two peptides remaining at the origin after chromatography. This behaviour is characteristic of glycopeptides, and would agree with the presence of carbohydrate in fragment S but not in fragment F (Table 1).

N-Terminal analyses

Transferrin and fragment F both showed an

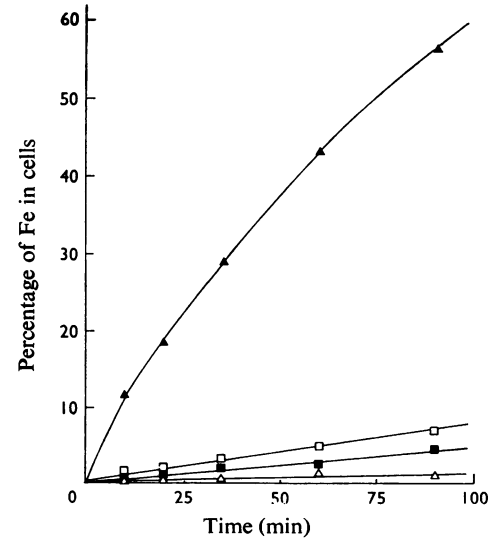
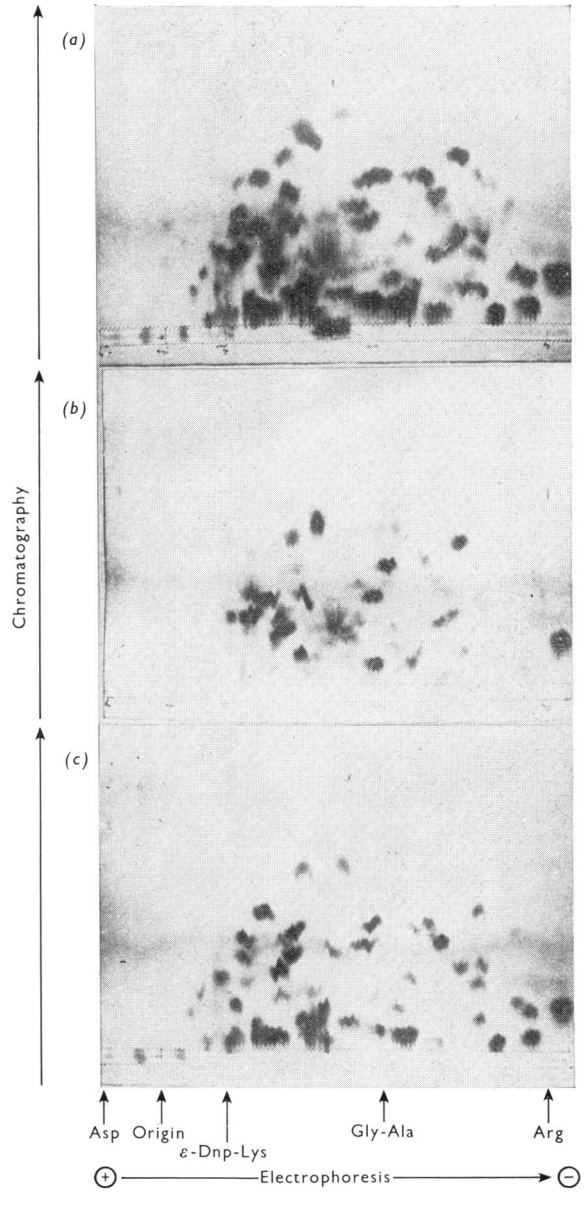


Fig. 2. *Incorporation of ^{59}Fe into rabbit reticulocytes from bovine transferrin* (▲) *and its monoferric fragments F* (△), *S* (■) *and* S_{apo} (□)
For details see the text.



EXPLANATION OF PLATE 1

'Fingerprints' of tryptic peptides of carboxymethylated transferrin and transferrin fragments

These were obtained by electrophoresis at pH3.5 in the first dimension and by ascending chromatography in butan-1-ol/water/pyridine/acetic acid in the second dimension, and development with ninhydrin (Richardson *et al.*, 1973). The samples run were (*a*) transferrin, (*b*) fragment F and (*c*) fragment S.

N-terminal amino acid residue of aspartic acid. Analysis of fragment S was hindered by the fact that the polypeptide chain had been cleaved to a variable extent by the initial trypsin treatment. This fragment showed aspartic acid, serine and glycine as *N*-terminal amino acids. Further purification of Dns-fragment S was attempted by SDS/polyacrylamide-gel electrophoresis followed by elution and *N*-terminal analysis of the fluorescent bands, but the method proved unsatisfactory as insufficient fluorescent material could be eluted from the gel. *N*-Terminal analysis of the single-chain fragment S_{apo} showed serine and glutamic acid.

Attempts to determine the *C*-terminal amino acid residues of transferrin and the fragments by carboxypeptidase digestion by the method of Ambler (1967) were unsuccessful, with either reduced amidomethylated or undenatured proteins, as no significant release of amino acids occurred.

Uptake of iron by reticulocytes

Bovine transferrin readily donated iron to rabbit reticulocytes (Fig. 2). Uptake from fragment F was virtually nil, amounting to only about 1% after 90min, and this minimal uptake could probably be attributed to traces of intact transferrin in the fragment preparation. With fragments S and S_{apo} the uptake, although also low (up to 7% at 90min), was reproducibly found to be higher than the uptake from fragment F and too high to be due solely to contaminating transferrin.

Discussion

The properties reported in this paper of the two fragments F and S obtained by tryptic cleavage of bovine Fe_2-transferrin clearly support the previous conclusion that each fragment contains one of the iron-binding sites of transferrin (Brock & Arzabe, 1976). Thus only fragment S contains carbohydrate, and 'fingerprints' of tryptic digests of the two fragments are almost totally different. The latter result argues against any large-scale duplication of amino acid sequence within the transferrin molecule as suggested by Mann *et al.* (1970), but is not incompatible with a limited internal homology, as reported for human transferrin (MacGillivray & Brew, 1975).

The fact that bovine Fe_2-transferrin can be cleaved directly into two monoferric fragments shows that the two iron-binding regions are independent and that no disulphide bridges exist between the two regions. Such a proposal has been made for human transferrin (McGillivray & Brew, 1975) and for ovotransferrin (Williams, 1975*b*), although conclusive proof has been lacking.

N-Terminal analyses revealed aspartic acid as the *N*-terminal amino acid of bovine transferrin, which is at variance with the findings of Graham & Williams (1975), who reported alanine to be *N*-terminal, but consistent with those of Bezkorovainy & Grohlich (1974), who found aspartic acid, but not alanine, to be present as an *N*-terminus in CNBr-digested transferrin. Since fragment F also showed aspartic acid as the unique *N*-terminal amino acid, it seems likely that this fragment corresponds to the *N*-terminal half of the transferrin molecule. *N*-Terminal analyses of fragment S were not meaningful owing to internal cleavages in the polypeptide chain, but no *N*-terminal aspartic acid was observed in the apparently single-chain fragment S_{apo} derived from apotransferrin, which suggests that this fragment is not *N*-terminal. The fact that this fragment showed two *N*-terminal amino acids (serine and glutamic acid) is unexplained, although it might indicate an internal cleavage so close to one end of the molecule that it could not be detected by electrophoresis of reduced S_{apo} on SDS-containing gels. The designation of fragment F as the *N*-terminal half of the transferrin molecule must therefore be considered as tentative, especially in view of the fact that confirmation could not be obtained by carboxypeptidase *C*-terminal analyses. This designation would, however, be in accord with the studies of ovotransferrin by Williams (1974, 1975*a*), who found the carbohydrate moiety to be present in the *C*-terminal fragment, which would thus be analogous to fragment S.

When apotransferrin is digested with trypsin, degradation to small peptides occurs much more rapidly than with Fe_2-transferrin (Brock *et al.*, 1976). However, if digestion is carried out for only a short time it is possible to isolate a fragment closely resembling fragment S (S_{apo}), but the part of the molecule giving rise to fragment F is immediately degraded to small peptides. It may therefore be inferred that the degree to which the loss of bound iron destabilizes the structure is greater for the site corresponding to fragment F than for that corresponding to fragment S.

Williams (1975*a*) reported that monoferric fragments of ovotransferrin donated iron to rabbit reticulocytes, but Zapolski & Princiotto (1976) subsequently suggested that only minimal uptake had in fact occurred in the experiments of Williams (1975*a*), and this would thus accord with the inability of fragments F and S to donate iron to reticulocytes. It therefore seems reasonable to conclude that those structural features responsible for iron binding by transferrin are not in themselves sufficient to permit interaction with reticulocytes.

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