The preparation and partial characterization of *N*-terminal and *C*-terminal iron-binding fragments from rabbit serum transferrin

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Two iron-binding fragments of M_r 36 000 and 33 000 corresponding to the *N*-terminal domain of rabbit serum transferrin were prepared. One iron-binding fragment of M_r 39 000 corresponding to the *C*-terminal domain was prepared. The *N*-terminal amino acid sequence of rabbit serum transferrin is:

Val-Thr-Glu-Lys-Thr-Val-Asn-Trp-?-Ala-Val-Ser

One glycan unit is present in rabbit serum transferrin and it is located in the *C*-terminal domain.

Transferrins are thought to occur in all vertebrates as monomeric proteins with M_r 78 000. They transport iron in the plasma. There are two domains corresponding to the *N*-terminal and *C*-terminal halves of the protein, each of which can bind one atom of iron. A 0.6 nm (6 Å)-resolution electron-density map shows the two domains in rabbit transferrin (Gorinsky *et al.*, 1979). Iron-binding fragments corresponding to the *N*- and the *C*-terminal domains have been isolated from hen ovotransferrin, human serum transferrin and human lactoferrin by trypsin digestion of monoferric samples (Williams, 1974, 1975; Bluard-Deconinck *et al.*, 1978; Evans & Williams, 1978). In this procedure the iron-occupied domain is protected from digestion whereas the iron-free domain is not. Brock & Arzabe (1976) prepared both *N*- and *C*-terminal iron-binding fragments from diferric bovine transferrin by trypsin digestion. In this procedure a central trypsin-sensitive site is cleaved. Tsao *et al.* (1974) cleaved hen ovotransferrin with CNBr and isolated an iron-binding fragment corresponding to the *N*-terminal domain with M_r 36 000. An *N*-terminal iron-binding fragment with M_r 35 600 has also been isolated by digesting diferric human transferrin with thermolysin (Lineback-Zins & Brew, 1980). To our knowledge only one attempt has been made to isolate iron-binding fragments from rabbit transferrin (Esparza & Brock, 1980). These workers treated both iron-free and diferric rabbit transferrin with trypsin, but no iron-binding fragments were formed, presumably because of the absence of a central trypsin-sensitive site.

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Rabbit transferrin has often been used in physiological studies of iron metabolism (Martinez-Medellin & Schulman, 1972; Harris & Aisen, 1975*a,b*; van Baarlen *et al.*, 1980; Groen *et al.*, 1982), but there is little information about its structure. The amino acid content has been reported by a number of authors (Baker *et al.*, 1968; Van Eijk *et al.*, 1972; Hudson *et al.*, 1973; Strickland & Hudson, 1978). It is a glycoprotein containing glycan units of the complex biantennary type built up from a mannotrioso-di-*N*-acetylchitobiose core substituted by two *N*-acetylneuraminy- α -(2→6)-*N*-acetyl-lactosamine moieties. The number of units is one according to Leger *et al.* (1978), but two according to Strickland & Hudson (1978).

The experiments reported in the present paper show that fragments representing the iron-binding domains of rabbit transferrin can be obtained by using methods similar to those previously described for hen ovotransferrin, human transferrin and human lactoferrin. A partial structural characterization of these iron-binding fragments has been made.

Materials and methods

Preparation of rabbit serum transferrin

Transferrin was prepared from 1 litre of rabbit serum by a modification of the method of Baker *et al.* (1968). The protein was eluted from a DEAE-Sephadex A-50 column (85 cm × 3.2 cm) with a gradient from 1 litre of 5 mM-Tris/HCl buffer, pH 8.4, to 1 litre of 0.4 M-NaCl/5 mM-Tris/HCl buffer, pH 8.4. Traces of albumin were removed from the transferrin by passage down a Blue Sepharose

column (13 cm $\times$ 3 cm) in 0.1 M-KCl/50 mM-Tris/HCl buffer, pH 7.0. Iron-free transferrin was prepared by the method of Warner & Weber (1951). Iron-saturated transferrin was prepared by adding a 20% molar excess of ferric nitrilotriacetate to a solution of transferrin in 0.1 M-NaHCO₃. After 30 min at room temperature unbound iron was removed by dialysis against 10 mM-NH₄HCO₃. The protein solution was then freeze-dried.

Preparation of monoferric rabbit transferrins

Williams *et al.* (1982) have shown that desferrioxamine, in the presence of a high concentration of salt, removes iron preferentially from the C-terminal site of diferric human transferrin, particularly at relatively high pH. One monoferric transferrin was therefore prepared by dissolving 200 mg of diferric transferrin in 12 ml of 50 mM-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer, pH 7.4. To this was added 1.2 ml of 50 mM-sodium citrate buffer, pH 4.9, 1.1 ml of 50 mM-desferrioxamine (Desferal; CIBA-GEIGY) and 5.7 ml of 50 mM-Mops buffer, pH 7.4, containing 0.584 g of NaCl to give a final volume of 20 ml and an NaCl concentration of 0.5 M. After incubation at 37°C for 17 h, the transferrin solution was desalted on a Sephadex G-25 column (35 cm $\times$ 2 cm) and freeze-dried.

Evans & Williams (1978) found that ferric nitrilotriacetate added to human transferrin at pH 5.5 or 8.5 in quantity sufficient to give 30% saturation preferentially occupied the C-terminal site. A second monoferric rabbit transferrin was therefore prepared for the present work by adjusting iron-free transferrin to 20% iron saturation by the addition of ferric nitrilotriacetate. The transferrin sample was then desalted on a Sephadex G-25 column (32 cm $\times$ 2 cm) and freeze-dried.

Preparation of iron-binding rabbit transferrin fragments

Enzymic digestions with α -chymotrypsin and subtilisin were performed in 0.1 M-NaHCO₃, pH 8.2, or 20 mM-CaCl₂/0.1 M-Tris/HCl buffer, pH 7.8, with a transferrin concentration of 50 mg/ml and an enzyme/substrate ratio of 1:50 (w/w). Digestions were allowed to proceed for 17 h at 37°C. With α -chymotrypsin a second addition of enzyme was made 6 h after the first.

(a) Iron-binding fragments corresponding to the N-terminal domain of rabbit transferrin were prepared in one of two ways.

(i) Desferrioxamine-treated rabbit transferrin was digested with α -chymotrypsin. Portions of the digest containing 100 mg of enzyme-treated transferrin were passed down a Sephadex G-100 column (80 cm $\times$ 3.5 cm) in 10 mM-NH₄HCO₃ (flow rate 6 ml/h). Protein-containing fractions were subjected

to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and fractions containing material of M_r about 36 000 were pooled and freeze-dried.

(ii) Diferric transferrin in CaCl₂/0.1 M-Tris buffer, pH 7.8, was digested with subtilisin. A fragment of M_r about 33 000 was purified after gel filtration as described above.

(b) An iron-binding fragment corresponding to the C-terminal domain of rabbit transferrin was prepared by digesting 20%-iron-saturated transferrin with α -chymotrypsin. A fragment of M_r about 39 000 was purified after gel filtration as described above.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 6 M-urea was performed by a modification of the method of Makey & Seal (1976) as described by Chasteen & Williams (1981), except that EDTA was omitted from both the gel and the electrophoresis buffers.

Sodium dodecyl sulphate/polyacrylamide gels and samples for electrophoresis thereon were prepared as described by Evans & Williams (1978). Relative molecular masses were determined by the use of a marker mixture that contained hen ovotransferrin (M_r 78 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 44 000), bovine carbonic anhydrase (M_r 29 000) and horse heart myoglobin (M_r 18 800). Gels were stained with Coomassie Brilliant Blue R-250 as described by Evans & Williams (1978).

Immunological methods

Antisera to rabbit transferrin and to the α -chymotrypsin-prepared N-terminal iron-binding fragment were raised in sheep by repeated inoculation into multiple subcutaneous sites. Protein samples were dissolved in Freund's complete adjuvant. After 8 weeks blood was collected and incubated at 37°C for 17 h. The serum was then decanted and centrifuged at 6000 g for 20 min, and the supernatant collected. Antibody-antigen reactions were demonstrated in 1% agar in 0.1 M-sodium phosphate buffer, pH 7.0 (Ouchterlony, 1958). Undiluted antisera were used. Antigens were used at a concentration of 0.2 mg/ml in 0.1 M-sodium phosphate buffer.

Carboxymethylation of rabbit transferrin

Rabbit transferrin was reduced and carboxymethylated as described by Crestfield *et al.* (1963).

Amino acid analysis

Protein in sealed evacuated tubes was hydrolysed in either 6 M-HCl/0.1% (w/v) phenol or 4 M-methanesulphonic acid at 105°C for 24, 48 and 72 h.

Amino acid sequencing

N-Terminal amino acid residues of reduced and carboxymethylated samples were determined after

reaction with dansyl chloride (Gray, 1967) and t.l.c. on polyamide sheets with the solvents described by Woods & Wang (1967). The *N*-terminal amino acid sequences of reduced and carboxymethylated rabbit transferrin samples were determined by using an Anachem APS 2400 solid-phase peptide sequencer (sequencer programme unpublished work by Dr. C. J. Brock, Department of Biochemistry, University of Bristol). A 50–100 nmol portion of protein was coupled to 300 mg of the solid support {CPG [‘3-aminopropyl-(3-aminoethyl)’ controlled-pore glass]} by using 3.5 mg of phenylene di-isothiocyanate. The protein sample was then sequenced for 10–15 Edman degradation cycles, with a double phenyl isothiocyanate coupling step on cycle 1 in order to block any remaining free amino acid groups on the solid support. The amino acid phenylthiohydantoin derivatives from each Edman degradation cycle were identified either by t.l.c. or by amino acid analysis after regeneration of the free amino acid by hydrolysis at 150°C for 4 h *in vacuo* in 6 M-HCl/0.1% SnCl₂ (Mendez & Lai, 1975).

Carbohydrate analysis

Total hexose was determined by the orcinol/H₂SO₄ method (Winzler, 1954). A standard sugar solution (0.2 mg/ml) containing galactose and mannose in a 1:2 ratio was used.

Results

Preparation of iron-binding rabbit transferrin fragments

Fig. 1 shows the elution profiles of enzyme-treated rabbit transferrin on Sephadex G-100. In each case two main peaks were seen. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the first peak was undigested rabbit transferrin and that the second peak was a fragment. The fragments obtained after α -chymotrypsin digestion of 20%-iron-saturated transferrin and desferrioxamine-treated transferrin had M_r 39 000 and 36 000 respectively. The fragment obtained after subtilisin digestion of iron-saturated transferrin had M_r 33 000. These fragments are referred to below as C39, N36 and N33 respectively. The letter indicates which domain the fragments correspond to, and the number refers to the M_r in thousands. Fig. 2 shows a sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of these fragments and of rabbit transferrin.

Immunological properties of the fragments

When examined by the Ouchterlony method against an antiserum to rabbit transferrin, each of the fragments gave precipitin lines showing only partial identity with that of rabbit transferrin. The precipitin line of fragment C39 crossed the pre-

Fig. 1. Sephadex G-100 elution profiles of rabbit transferrin samples after enzymic digestion

For experimental details see the text. (a) Desferrioxamine-treated transferrin digested with α -chymotrypsin; (b) diferric transferrin digested with subtilisin; (c) 20%-iron-saturated transferrin digested with α -chymotrypsin.

cipitin lines of both fragments N36 and N33, indicating a lack of common antigenic determinants. The precipitin lines of fragments N36 and N33 fused, indicating antigenic identity. These results are shown in Figs. 3(a) and 3(b). Fragment C39 did not react with the antiserum to fragment N36. The precipitin lines of fragments N33 and N36 fused with the precipitin line of rabbit transferrin, indicating antigenic identity when the antiserum to fragment N36 was used. These results are shown in Figs. 3(c) and 3(d).

Amino acid composition

The amino acid compositions of rabbit transferrin and fragments N36 and C39 are shown in Table 1. The fragments have similar compositions, although differences are apparent in their contents of proline and phenylalanine. The sum of the compositions of the fragments is close to that of the whole protein, except in the case of half-cystine.

N-Terminal end-group determinations and sequence analysis

The *N*-terminal amino acid assignment made after reaction with dansyl chloride were: rabbit transferrin, valine; fragment N36, valine; fragment C39,

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of rabbit transferrin and fragments N33, N36 and C39 under non-reducing conditions

For experimental details see the text. Lane 1, transferrin; lane 2, fragment N33; lane 3, fragment C39; lane 4, fragment N36. Arrows indicate the approximate positions of molecular-weight markers.

Table 1. Amino acid compositions of rabbit serum transferrin and of fragments

For experimental details see the text.

Amino acid	Amino acid composition (mol of amino acid/mol of protein)			
	Fragment N36	Fragment C39	Sum of fragments N36 and C39	Transferrin
Asp	38	41	79	80
Thr	12	16	28	30
Ser	19	21	40	39
Glu	27	30	57	54
Pro	24	16	40	38
Gly	29	31	60	60
Ala	31	30	61	57
Val	26	26	52	55
Cys	18	18	36	28
Met	2	2	4	4
Ile	8	10	18	18
Leu	33	35	68	66
Tyr	7	8	15	20
Phe	16	10	26	26
Lys	30	30	60	61
His	9	8	17	20
Arg	13	14	27	29
Trp	4	3	7	7

Table 2. *N*-Terminal amino acid sequences of rabbit transferrin and fragments N36 and C39

For experimental details see the text. Two sequencer runs for each sample are shown. Residue assignments were made as described in the Materials and methods section, except where marked: *determined by dansylation; †determined only from amino acid analyses.

Cycle	Rabbit transferrin		Fragment N36		Fragment C39	
	A	B	A	B	A	B
1	Val*	Val*	Val*	Val*	Leu*	Leu*
2		Thr	Thr		Asp	Gln/Val
3	Glu	Glu	Glu		Ser	Asn
4		Lys†		Lys†	Ile	Ile
5	Thr	Thr	Thr		Ala	Ala
6	Val	Val	Val	Val	Val	Pro
7		Asx	Val/Asn	Asn	Leu	Leu
8	Trp		Trp		Leu	Leu
9					Phe	Phe
10	Ala		Ala			
11	Val		Val			
12	Ser					

Table 3. Total hexose (mannose + galactose) contents of transferrin samples determined by the orcinol/ H_2SO_4 method

For experimental details see the text. The numbers in parentheses give the numbers of hexose residues known to occur in human transferrin and hen ovotransferrin on the basis of structure determinations.

Sample	Hexose content (mol/mol of protein)
Rabbit transferrin	4.3
Human transferrin	10.9 (10)
Hen ovotransferrin	2.9 (3)
Fragment N36	0.9
Fragment C39	5.6

leucine. These results suggest that fragment N36 corresponds to the *N*-terminal domain and that fragment C39 corresponds to the *C*-terminal domain of rabbit transferrin. This is confirmed by the results shown in Table 2. Clearly the *N*-terminal sequences of both rabbit transferrin and fragment N36 are identical, i.e.:

Val-Thr-Glu-Lys-Thr-Val-Asn-Trp-?-Ala-Val-Ser

The *N*-terminal sequence of fragment C39 is:

Leu-?-?-Ile-Ala-?-Leu-Leu-Phe

Carbohydrate composition

The hexose contents of rabbit and human transferrins and hen ovotransferrin are shown in Table 3. The hexose contents of fragments N36 and C39 were also determined.

Fig. 3. Ouchterlony double-diffusion analysis of rabbit transferrin (Tf) and fragments N33, N36 and C39. For experimental details see the text. Plates (a) and (b) had antiserum to rabbit transferrin in the central well. Plates (c) and (d) had antiserum to fragment N36.

Fig. 4. Electrophoresis of rabbit transferrin samples on a 6M-urea/polyacrylamide gel

Urea/polyacrylamide-gel electrophoresis of rabbit transferrin (Fig. 4)

Rabbit transferrin can be separated into four species by electrophoresis in 6M-urea/polyacrylamide gels at pH8.4. These are, in order of increasing mobility on the gel, iron-free transferrin (Tf), transferrin in which the C-terminal iron-binding site is occupied (Tf-Fe), transferrin in which the N-terminal iron-binding site is occupied (Fe-Tf), and diferric transferrin (Fe₂Tf). The identity of the transferrin species was determined as follows. Tf and Fe₂Tf when electrophoresed gave the slowest- and fastest-moving bands respectively. The two intermediate bands must therefore correspond to the monoferric species. Fragment C39 was isolated from

For experimental details see the text. Lane 1, marker mixture containing all four species of transferrin; lane 2, desferrioxamine-treated transferrin; lane 3, 20%-iron-saturated transferrin.

20%-iron-saturated transferrin. Samples of 20%-iron-saturated transferrin when electrophoresed consisted mainly of Tf and the slower-moving monoferric species, which must therefore be Tf-Fe. Fragment N36 was isolated from desferrioxamine-treated transferrin. Samples of desferrioxamine-treated transferrin when electrophoresed consisted mainly of the faster-moving monoferric species, which must therefore be Fe-Tf.

Discussion

In the present paper we have described the preparation of iron-binding fragments from monoferric species of rabbit transferrin after digestion with α -chymotrypsin. All the fragments ran as single bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions. Under reducing conditions, however, more than one band was seen, indicating that some internal cleavage in the domains had occurred. With fragment N33 this internal cleavage was extensive. For this reason the fragment was not further characterized. The fact that reduced and carboxymethylated samples of fragments N36 and C39 gave a single *N*-terminal amino acid sequence suggests that the heterogeneity in these fragments must be due to partial cleavage close to the *C*-terminus, any contaminating peptides being lost during dialysis of the samples.

The *N*-terminal sequence of rabbit transferrin shows a clear homology with both human and rat transferrins (MacGillivray *et al.*, 1977; Schreiber *et al.*, 1979). Residue 9, which was not identified in the present work, is likely, by analogy with other transferrins, to be cysteine. One surprising residue assignment made on the basis of both the identification of the amino acid phenylthiohydantoin derivative and the free amino acid liberated after hydrolysis was that of threonine at position 2. In all other published *N*-terminal amino acid sequences of transferrins position 2 has been occupied by a proline residue.

The number of heteropolysaccharide units found on rabbit transferrin has been a matter of controversy. Leger *et al.* (1978) found only one heteropolysaccharide unit (of the type described in the introduction) per transferrin molecule. Strickland & Hudson (1978) found two. The present work (Table 3) indicates that rabbit transferrin possesses one heteropolysaccharide unit and that this is located in the *C*-terminal domain. The hexose contents of human transferrin (containing two heteropolysaccharide units and a total of ten hexose residues) and hen ovotransferrin (containing one heteropolysaccharide unit) and a total of three hexose residues) were correctly determined by the orcinol/H₂SO₄ method, indicating that it is sensitive enough to distinguish between transferrins con-

taining one or two heteropolysaccharide units (Jamieson, 1965; Spik *et al.*, 1975; Williams, 1975; Dorland *et al.*, 1979). The difference in the relative molecular masses of fragments N36 and C39 thus appear to be due mainly to the heteropolysaccharide unit present in fragment C39.

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References

- Baker, E., Shaw, D. C. & Morgan, E. H. (1968) *Biochemistry* **7**, 1371–1378
- Bluard-Deconinck, J. M., Williams, J., Evans, R. W., Van Snick, J., Osinski, P. A. & Masson, P. L. (1978) *Biochem. J.* **171**, 321–327
- Brock, J. H. & Arzabe, F. R. (1976) *FEBS Lett.* **69**, 63–66
- Chasteen, N. D. & Williams, J. (1981) *Biochem. J.* **193**, 717–727
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Dorland, L., Haverkamp, J., Vliegthart, J. F. G., Spik, G., Fournet, B. & Montreuil, J. (1979) *Eur. J. Biochem.* **100**, 569–574
- Esparza, I. & Brock, J. H. (1980) *Biochim. Biophys. Acta* **622**, 297–307
- Evans, R. W. & Williams, J. (1978) *Biochem. J.* **173**, 543–552
- Gorinsky, B., Horsbaugh, C., Lindley, P. F., Moss, D. S., Parkar, M. & Watson, J. L. (1979) *Nature (London)* **281**, 157–158
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151
- Groen, R., Hendricksen, P., Young, S. P., Leibman, A. & Aisen, P. (1982) *Br. J. Haematol.* **50**, 43–53
- Harris, D. C. & Aisen, P. (1975a) *Nature (London)* **257**, 821–823
- Harris, D. C. & Aisen, P. (1975b) *Biochemistry* **14**, 262–268
- Hudson, B. G., Ohno, M., Brockway, W. J. & Castellino, F. J. (1973) *Biochemistry* **12**, 1047–1053
- Jamieson, G. A. (1965) *J. Biol. Chem.* **240**, 2914–2920
- Leger, D., Tordera, V., Spik, G., Dorland, L., Haverkamp, J. & Vliegthart, J. F. G. (1978) *FEBS Lett.* **93**, 255–260
- Lineback-Zins, J. & Brew, K. (1980) *J. Biol. Chem.* **255**, 708–713
- MacGillivray, R. T. A., Mendez, E. & Brew, K. (1977) *Proc. Int. Conf. Proteins of Iron Storage and Transport*, pp. 133–141. Grune and Stratton, New York
- Makey, D. G. & Seal, U. S. (1976) *Biochim. Biophys. Acta* **453**, 250–256
- Martinez-Medellin, J. & Schulman, H. M. (1972) *Biochim. Biophys. Acta* **264**, 272–284
- Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **58**, 47–53
- Ouchterlony, Ö. (1958) *Prog. Allergy* **5**, 1–78
- Schreiber, G., Dryburgh, H., Mullership, A., Matsuda, Y., Inglis, A., Phillips, J., Edwards, K. & Maggs, J. (1979) *J. Biol. Chem.* **334**, 12013–12019
- Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquet, S. & Montreuil, J. (1975) *FEBS Lett.* **50**, 296–299

- Strickland D. K. & Hudson, B. G. (1978) *Biochemistry* **18**, 2549–2554
- Tsao, D., Morris, D. H., Azari, P. R., Tengerdy, R. P. & Phillips, J. L. (1974) *Biochemistry* **13**, 403–407
- van Baarlen, J. V., Brouwer, T., Leibman, A. & Aisen, P. (1980) *Br. J. Haematol.* **46**, 412–426
- Van Eijk, H. G., Van Dijk, J. P., Van Noort, W. L., Leijnse, B. & Monfoort, C. H. (1972) *Scand. J. Haematol.* **9**, 267–270
- Warner, R. C. & Weber, I. (1951) *J. Biol. Chem.* **191**, 173–180
- Williams, J. (1974) *Biochem. J.* **141**, 745–752
- Williams, J. (1975) *Biochem. J.* **149**, 237–244
- Williams, J., Chasteen, N. D. C. & Moreton, K. (1982) *Biochem. J.* **201**, 527–532
- Winzler, R. J. (1954) *Methods Biochem. Anal.* **2**, 279–311
- Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370