The dimerization of half-molecule fragments of transferrin

John WILLIAMS and Kathleen MORETON

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

Partial proteolysis was used to prepare half-molecule fragments of hen ovotransferrin. *N*-Terminal and *C*-terminal fragments associate to form an *N*-terminal fragment–*C*-terminal fragment dimer. Variant forms of the *N*- and *C*-terminal fragments can be prepared in which a few amino acid residues are lacking from the *C*-terminal ends of the fragments. These variant fragments are partially or completely unable to associate; the suggestion that the molecular recognition sites are located in these *C*-terminal stretches of the *N*-terminal half-molecule (320–332) and of the *C*-terminal half-molecule (683–686) is in agreement with X-ray-crystallography data for human lactotransferrin [Anderson, Baker, Dodson, Norris, Rumball, Waters & Baker (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1769–1773].

INTRODUCTION

In transferrin the polypeptide chain folds up to give two compact domains formed from the *N*-terminal and *C*-terminal halves of the chain, linked by a short connecting peptide. The domains are clearly homologous, as shown by their primary structures (e.g. MacGillivray *et al.*, 1983) and three-dimensional conformations (Anderson *et al.*, 1987). It is therefore likely that the present-day transferrin molecule (M_r approx. 80000) has evolved from a half-sized ancestral form (M_r approx. 40000) that bound only one atom of iron. Despite some claims to the contrary, it has never been convincingly shown that any living species still possesses such an ancestral type of transferrin. Presumably, the ancestral transferrin served in the transport of iron and was able to interact with some kind of cell-membrane receptor, although at present nothing can be said about the evolution of the transferrin receptor.

In some respects the two domains are structurally and functionally independent. Thus they can often be isolated intact by proteolytic cleavage in or near the connecting peptide, when they retain the immunological properties and e.p.r. spectra characteristic of the corresponding regions of the whole protein (Williams, 1974; Butterworth *et al.*, 1975). Similarly, Zak *et al.* (1983) have shown that the equilibrium constant for iron-binding may be preserved in an isolated half-molecule fragment. On the other hand, the two domains are not fully independent. Co-operativity of iron-binding has been observed (Aisen *et al.*, 1978; Chasteen & Williams, 1981), and the presence or absence of iron in one domain can influence the urea-denaturation of the other (Evans & Williams, 1980). Ikeda *et al.* (1985) have shown that after trypsin cleavage of the connecting peptide of ovotransferrin the two domains remain non-covalently attached to one another and continue to show co-operative interactions. The functional significance of interactions between the domains is unknown, although Brown-Mason & Woodworth (1984) have suggested that the ability of mixtures of *N*-terminal and *C*-terminal half-molecules to bind to transferrin receptor and deliver iron to the cell depends upon their ability to associate non-covalently. In the whole protein such interactions

might serve to stabilize the orientation of the two domains necessary for binding to the receptor to take place.

Baker and his colleagues (Anderson *et al.*, 1987) have elucidated the three-dimensional structure of human lactotransferrin to a resolution of 0.32 nm. The two-domain structure is clear; each domain is composed of two non-homologous sub-domains, between which lies the iron-binding site. Contact between the domains involves relatively few residues located in two helical sections. One occurs at the *C*-terminal end of the *N*-terminal domain (residues 320–331, lactotransferrin numbering) immediately before the connecting peptide; the other is at the *C*-terminal end of the *C*-terminal domain (residues 693–703). The *C*-terminal residue itself is buried in the interface between the two domains. In the present paper we show from amino acid sequence data that the dimerization of half-molecule fragments of transferrin depends mainly upon residues found in the *C*-terminal sections of each domain. These results show that regions involved in the interactions of the two half-molecules can readily be explained, if the structure of hen ovotransferrin is essentially the same as that of human lactotransferrin.

MATERIALS AND METHODS

Preparation of transferrins

Ovotransferrins were prepared from hen, duck and quail egg-whites as described previously (Williams, 1968). Human serum transferrin was prepared as described by Chasteen & Williams (1981).

Preparation of half-molecule fragments of transferrins

Two general approaches were used. In method 1 the resistance of iron-saturated transferrin to proteolytic attack is exploited. Partially iron-saturated transferrin (30–50 % saturated) is treated with proteinases to destroy the iron-free domains. Subsequent gel filtration separates intact transferrin (M_r 80000) from half-molecule fragments (M_r 40000) and small peptides. By using conditions that ensure preferential iron-binding to either the *N*-terminal or the *C*-terminal domain of the whole protein,

the corresponding half-molecules can be isolated (Williams, 1974, 1975). In method 2 iron-saturated transferrin is digested under conditions in which the connecting peptide is cleaved, simultaneously liberating both half-molecule fragments (Brock *et al.*, 1976). This method is sometimes frustrated by the absence of suitable amino acid residues in the connecting peptide, as in the case of human serum transferrin. Further purification of half-molecules prepared by either method was carried out by ion-exchange chromatography on DEAE-Sephacrose with a buffer gradient from 0.05 M-Tris/HCl, pH 7.9, to 0.4 M-Tris/HCl, pH 7.9. Protein peaks eluted from gel-filtration or ion-exchange columns were analysed by SDS/PAGE, with and without prior reduction of disulphide bridges by 1% (v/v) 2-mercaptoethanol (Evans & Williams, 1978). An *N*-terminal fragment of human serum transferrin was prepared by thermolysin digestion (Lineback-Zins & Brew, 1980).

Characterization of transferrin fragments

The complete primary structures of hen ovotransferrin and human serum transferrin are known (Jeltsch & Chambon, 1982; MacGillivray *et al.*, 1983), allowing protein fragments to be readily identified. *N*-Terminal peptides were obtained by thermolysin digestion of the maleylated protein fragment (Bruton & Hartley, 1970). After purification of the peptide by high-voltage paper electrophoresis it was identified from its amino acid composition and electrophoretic mobility (Offord, 1966). Digestion of fragments with carboxypeptidases A, B and Y (Hayashi, 1976) was used to identify *C*-terminal sequences. In some cases useful information was obtained by treatment of protein fragments with CNBr followed by isolation and identification of the peptide products.

Protein-protein interactions

Two methods were used to study the association of transferrin fragments. The first was gel filtration on columns (1.4 cm × 90 cm and 3 cm × 80 cm) of Sephadex G-100 in 0.05 M-ammonium bicarbonate. In some cases different amounts of protein were applied to the column in order to investigate possible concentration-dependence of the elution profile. For these experiments a column (1.4 cm × 90 cm) of Sephadex G-100 in 0.02 M-ammonium bicarbonate was used. The column was pumped at a constant rate of 0.1 ml/min and 45-drop fractions were collected. Different amounts of protein, from 0.5 mg to 25 mg, were applied in 0.5 ml, and A_{280} was measured. The elution profiles were normalized to a constant 'area-under-the-curve', and protein-protein interaction resulted in concentration-dependence of the elution position and of the shape of the elution profile. Ackers (1975) has pointed out that such 'small-sample-volume' experiments cannot be used to determine equilibrium binding constants. The method is, nevertheless, a convenient qualitative means of detecting interactions.

The second method was a binding assay in which a fluorescent derivative of the *C*-terminal half-molecule of ovotransferrin was added to a suspension of the *N*-terminal half-molecule immobilized on Sepharose 6B. The preparation of the fluorescent derivative of the *C*-terminal half-molecule was carried out as described by Williams *et al.* (1985). The *N*-terminal half-molecule was coupled to Sepharose 6B by the CNBr method (March

et al., 1974) at the rate of 50 mg of protein to 15 ml of settled Sepharose.

Details of the binding assay were as follows. A 0.2 ml portion of a 50% suspension of Sepharose-linked *N*-terminal half-molecule in phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.25) was placed in each of ten plastic tubes. Different volumes, from 5 μ l to 50 μ l, of a solution of the fluorescent *C*-terminal half-molecule (5 mg/ml in phosphate-buffered saline) were added, and the volume of added liquid was adjusted to 50 μ l with phosphate-buffered saline. In order to separate the free *C*-terminal fragment from that bound to the *N*-terminal fragment-Sepharose, the mixture was underlaid with 1.5 ml of 10% (w/v) sucrose, as described by Wright & Hunter (1982). The Sepharose was left to settle through the sucrose for 10 min before the supernatant solution was carefully and completely removed with a fine-tipped Pasteur pipette. Then 1 ml of 0.5 M-sodium thiocyanate solution was added to the Sepharose pellet to liberate the bound fluorescent *C*-terminal half-molecule. After centrifuging, to pack the Sepharose, the fluorescence of the thiocyanate extract was measured in arbitrary units, with a Perkin-Elmer fluorimeter (exciting wavelength 336 nm, emission wavelength 480 nm). The reciprocal of the fluorescence was plotted against the reciprocal of the volume of the *C*-terminal half-molecule solution added. The slope of a straight line, fitted by eye, through the points gave the apparent standard dissociation constant K_d^s . Experimental assays to test the effects of altered conditions of pH or salt concentration gave the apparent experimental dissociation constant K_d^{exp} , and the results are given as the relative dissociation constant K_d^* , where $K_d^* = K_d^{exp}/K_d^s$. In tests of the ability of protein fragments to inhibit competitively complex-formation between the fluorescent *C*-terminal half-molecule and the Sepharose-linked *N*-terminal half-molecule, a standard addition of 1 mg of the test material was made to each tube of the assay and the relative inhibitor constant K_i^* is given as $1/[(K_d^{exp}/K_d^s) - 1]$.

pH-dependence of K_d^*

A series of buffer solutions from pH 4.5 to pH 9.5 was prepared by adding drops of 4 M-NaOH to 0.05 M-Mes/0.05 M-Hepps, and K_d^* was determined as described above.

Effect of iron-binding upon K_d^*

This experiment was carried out by first measuring the strength of binding of the fluorescent *C*-terminal half-molecule in its iron-free and iron-saturated forms to the immobilized *N*-terminal half-molecule in its iron-saturated form. Then the experiment was repeated with the iron-free form of the immobilized *N*-terminal half-molecule, prepared by washing the protein-Sepharose with 0.2 M-KH₂PO₄/1 mM-nitilotriacetic acid/1 mM-EDTA, pH 6.0 (Brown-Mason & Woodworth, 1984).

RESULTS

Nomenclature

The half-molecule fragments studied here are referred to as, for example, H40(N), where the first letter refers to the species (H, hen; D, duck; Q, quail, M, human) followed by a number which is the apparent M_r ($\times 10^{-3}$) as judged by SDS/PAGE of an unreduced sample. The

final letter shows from which domain (N, *N*-terminal; C, *C*-terminal) the half-molecule is obtained. M_r values obtained from SDS/PAGE without reduction can depart markedly from true values. Thus, for example, the M_r values of half-molecules H44(N) and H40(C) determined from the primary structures are 36455 and 40196 respectively, and they behave as expected upon gel filtration on Sephadex G-100, with H40(C) being eluted slightly ahead of H44(N). Reduced samples of the half-molecule fragments were also analysed by SDS/PAGE in order to detect internal cleavages.

Trypsin digestion of hen ovotransferrin (method 2)

Diferric transferrin, digested with trypsin (enzyme/substrate ratio 1:30, by weight; 6 h at 37 °C) in 0.05 M-Tris/HCl buffer, pH 7.9, containing 0.02 M-CaCl₂ yielded two half-molecule fragments, H40(C) and H44(N). Each was a single-chain molecule. The terminal residues were determined as Ala-1 and Arg-332 for H44(N) and as Glu-342 and Lys-686 for H40(C). In some experiments the digest was heated in a boiling-water bath for 10 min. After centrifugation to remove coagulated protein, the supernatant liquid yielded one main peptide with electrophoretic mobility $m = +0.21$ at pH 6.5. Amino acid analysis identified it as the connecting peptide:

When equal amounts (w/w) of H40(C) and H44(N) were mixed and subjected to gel filtration on Sephadex G-100, they gave a peak close in position to that expected for intact transferrin (M_r 80000). If unequal amounts of the half-molecules were taken the species in excess formed a second peak at the position expected for a protein of M_r 40000. SDS/PAGE showed that the first peak contained equal amounts of the two half-molecules. When subjected to ion-exchange chromatography on DEAE-Sepharose the H40(C)-H44(N) dimer gave three peaks, identified by SDS/PAGE as the two unassociated half-molecules and the dimer. Partial dissociation of the dimer thus occurs during the chromatography.

When trypsin digestion of diferric ovotransferrin was carried out at a higher concentration of Ca²⁺ (0.05 M) two additional fragments were detected by SDS/PAGE: H32(N) and H6(N). These new fragments could also be obtained by re-digestion of H44(N) with trypsin at the higher Ca²⁺ concentration. Fragments H32(N) and H6(N) remain non-covalently associated until dissociated by SDS/PAGE, and while still associated they form a complex of M_r 80000 when mixed with fragment H40(C). Fragments H32(N) and H6(N) were separated by passage through Sephadex G-100 in 1% SDS. End-group determination and amino acid analysis showed fragment H32(N) to be a single-chain molecule from Ala-1 to Lys-279 and fragment H6(N) to be a single-chain molecule from Ser-280 to Arg-332.

Trypsin digestion of hen ovotransferrin (method 1)

Fragments H40(C) and H44(N) can be prepared from ovotransferrin by digestion of partially iron-saturated protein (method 1), as described previously (Williams, 1974, 1975). Iron presented as iron(III) nitrilotriacetate at pH 8.0 binds preferentially to the *N*-terminal domain, and after trypsin digestion gel filtration on Sephadex G-100 gives two protein peaks in the positions expected for intact transferrin and for a half-molecule. SDS/

PAGE showed, however, that the first peak contained not only intact ovotransferrin but also an equimolar H44(N)-H40(C) complex. The second peak contained only fragment H44(N).

By adding excess of fragment H40(C) to the H44(N) prepared by method 1 it was found that the latter fragment was heterogeneous. About 75% of the H44(N) was able to form a dimer with H40(C), the remainder was not. The two forms of H44(N) were denoted by H44(N)_c and H44(N)_u. They were isolated by adding sufficient H40(C) to associate with all the H44(N)_c, followed by gel filtration and ion-exchange chromatography to separate the *N*- and *C*-terminal half-molecules. The u form ran slightly ahead of the c form in SDS/PAGE. End-group determinations showed that H44(N)_c was a single-chain molecule from Ala-1 to Arg-332 and that H44(N)_u was a single-chain molecule from Ala-1 to Tyr-319. The non-dimerizing form of the *N*-terminal half-molecule has therefore arisen from a 'chymotryptic' cleavage leading to the loss of 13 residues from the *C*-terminal end.

Trypsin digestion of duck and quail ovotransferrins

When iron was presented as iron(III) nitrilotriacetate the application of method 1 to duck ovotransferrin yielded half-molecule fragments D45(N) and D39(C). Unlike the results obtained with hen ovotransferrin, the *C*-terminal fragment D39(C) was the predominant product. As with hen ovotransferrin about 25% of D45(N) was unable to dimerize with D39(C). On the other hand, duck ovotransferrin appeared not to undergo secondary cleavage at residue 279 when treated by method 2.

With quail ovotransferrin, method 1, after partial saturation with iron(III) nitrilotriacetate, gave only one fragment, Q40(C). Cross-species dimerization between any member of the group D39(C), H40(C) and Q40(C) and either D45(N) or H44(N) was readily shown by gel filtration through Sephadex G-100.

Thermolysin digestion of hen ovotransferrin

A partially iron-saturated ovotransferrin sample in which the iron predominantly occupied the *C*-terminal domain (Williams, 1975) was digested with thermolysin to yield a new half-molecule fragment H41(C), which is a single-chain molecule from Leu-336 to Phe-682. Compared with the half-molecule fragment H40(C), fragment H41(C) has six extra residues at its *N*-terminal end but lacks four residues from its *C*-terminal end. Despite the very small difference in M_r , fragment H41(C) moved perceptibly more slowly than H40(C) in SDS/PAGE. When passed through a column of Sephadex G-100, a mixture of H41(C) and H44(N)_c behaved as a dimer, although one that dissociated more readily than the H40(C)-H44(N)_c dimer.

Carboxypeptidase digestion experiments

Treatment of H40(C) with carboxypeptidase B removed only the terminal lysine residue. Gel filtration showed that the ability of the *C*-terminal half-molecule to dimerize with H44(N)_c had been diminished but not abolished. Carboxypeptidase Y removed four residues (lysine, glycine, glutamic acid and leucine) from H40(C), with complete destruction of its ability to associate with the *N*-terminal half-molecule as judged by gel filtration.

Treatment of H44(N)_c with carboxypeptidase B

Fig. 1. Effect of protein concentration upon elution profiles of (a) a mixture of equal weights of H44(N)_c and H40(C) and (b) H40(C) alone

In (a) curves 1, 2, 3 and 4 represent protein loadings of 25 mg, 10 mg, 2.6 mg and 0.5 mg respectively applied to the column in 0.5 ml. In (b) curves 1, 2 and 3 represent loadings of 23 mg, 7.3 mg and 1 mg respectively. The vertical axis represents A_{280} after normalizing each profile to the same total area. Experiments (a) and (b) were carried out on different columns of Sephadex G-100.

Table 1. Ability of different fragments of hen ovotransferrin to inhibit the interaction of fluorescent derivative of C-terminal half-molecule and immobilized N-terminal half-molecule

Results are expressed as the relative inhibitor constant K_i^* . For details of the assay see the Materials and methods section.

Fragment	K_i^*
H40(C)	0.16
H44(N) _c	0.18
H41(C)	0.45
H6(N)	0.48
M36(N)	0.68
H32(N)	0.96
H44(N) _u	1.00
Ovotransferrin	7.68

removed arginine and methionine residues, with complete loss of ability to dimerize with H40(C) as judged by gel filtration. In marked contrast with the rapid attack of carboxypeptidases upon isolated C-terminal half-molecules, attempts to remove C-terminal residues from intact ovotransferrin were unsuccessful. Only at pH 2.3

was carboxypeptidase Y able to remove five residues from the whole protein, but it then proved impossible to restore the protein to a neutral pH without irreversible precipitation.

Concentration-dependence in gel-filtration experiments

That a mixture of N-terminal and C-terminal half-molecules H44(N)_c/H40(C) can form a dissociable complex is shown by the concentration-dependence of the elution position and of the protein profile (Fig. 1a). There is no concentration-dependence in the case of the C-terminal half-molecules of hen ovotransferrin and human serum transferrin (Fig. 1b). These half-molecules thus do not show self-dimerization under the conditions of the experiment.

Competitive inhibitors of binding

Several transferrin fragments were tested for their ability to inhibit the binding of the fluorescent C-terminal half-molecule to the immobilized N-terminal half-molecule of hen ovotransferrin (Table 1).

Salt effects upon fragment interactions

Fig. 2 shows how the relative dissociation constant K_d^* is affected by different anions: Br⁻, NO₃⁻ and ClO₄⁻ weaken the association of the half-molecules, whereas Cl⁻ and F⁻ strengthen it. The promoting effect of the

Fig. 2. Effect of salts upon the relative dissociation constant K_d^* of the association of H44(C)_e and H40(C)

For details see the text.

Table 2. Effect of iron-binding upon the interaction of *N*-terminal and *C*-terminal half-molecules H44(N)_e and H40(C)

Column 2 shows the elution peak position of the dimer from a column of Sephadex G-100 in 0.02 M-ammonium bicarbonate. The column was loaded with 5 mg of each fragment in a total volume of 0.5 ml. The fraction size was 2.5 ml. In each experiment ovotransferrin was included as an internal marker, and in each case its elution position was constant at fraction 45. Column 3 shows the effect of iron-binding on the fluorescence binding assay. FeN and FeC refer to the iron complexes of the half-molecule fragments H44(N)_e and H40, and N and C to their apo forms.

Fragments	Elution position	K_d^*
FeN, FeC	50	1.00
N, FeC	50	0.90
FeN, C	47	0.42
N, C	48	0.60

F⁻ ion was large enough to be detected by gel filtration. Thus a mixture of hen ovotransferrin and the complex H44(N)_e-H40(C) was passed through a column of Sephadex G-100 in 0.02 M-ammonium bicarbonate, and 50-drop fractions were collected. The peak elution positions were 28 for the intact protein and 32 for the dimer. When 0.5 M-NaF was added to the buffer the

Table 3. Effect of pH on the strength of binding of a fluorescent derivative of the *C*-terminal half-molecule and an immobilized *N*-terminal half-molecule

Results are expressed as the relative dissociation constant K_d^* . The reference value was that given in phosphate-buffered saline at pH 7.25.

pH	K_d^*
4.5	0.52
5.0	0.73
5.5	0.90
7.0	0.92
8.0	0.93
8.5	0.97
9.0	1.30
9.5	1.52

intact protein position was unchanged at 28 but the peak position for the dimer was advanced to 30.

Iron-binding and the interactions of the half-molecules

Table 2 shows how the strength of interaction between the *N*-terminal and *C*-terminal half-molecules is affected by the presence or the absence of iron. Both the position of the protein peak eluted from a column of Sephadex G-100 and the relative dissociation constant K_d^* show that the presence or absence of iron from the *N*-terminal domain has relatively little effect but that the presence of iron in the *C*-terminal domain markedly decreased the interaction.

The data suggest that, given that the *C*-terminal domain is in the iron-free form, the presence of iron in the *N*-terminal domain increases the interaction.

Effect of pH on the interaction of the half-molecules

Table 3 shows how the relative dissociation constant K_d^* varies with pH. The interaction increases at pH values below 5.5 and diminishes at pH values above 8.5.

DISCUSSION

Location of interacting amino acid residues

The work described in this paper attempts to correlate the ability of *N*-terminal and *C*-terminal half-molecule fragments of hen ovotransferrin to form a non-covalent dimer with the presence and the absence of residues near the *C*-terminal ends of the fragments. From gel-filtration experiments the interactive residues in the *N*-terminal half-molecule appear to be in the section 320–332, as shown by the inability of fragment H44(N)_e to interact with the *C*-terminal fragment. Carboxypeptidase digestion of fragment H44(N)_e suggests that even the loss of two residues (Met-331 and Arg-332) is sufficient to diminish greatly the inter-domain interactions. In the case of the *C*-terminal domain lack of the tetrapeptide Leu-683–Lys-686 greatly [as suggested by the behaviour of fragment H41(C)], or completely [as shown by carboxypeptidase digestion of fragment H40(C)], destroys inter-domain interactions. Removal of the *C*-terminal Lys-686 residues alone, by carboxypeptidase B digestion, also weakened the interaction (see Fig. 3).

Fig. 3. Diagram of the amino acid sequence of hen ovotransferrin to show the cleavages involved in the production of half-molecule fragments

The position of the connecting peptide is shown by C.P.

The three-dimensional structure of human diferric lactotransferrin at 0.32 nm resolution (Anderson *et al.*, 1987) shows that the interacting residues occur mainly in two helical sections, at the C-terminal ends of the two domains (residues 321–332 and 675–686, in ovotransferrin numbering). Thus the peptide sequences give results that are in general agreement with the X-ray crystallography. The resistance to the removal of C-terminal residues from the intact protein is explained by the X-ray data, showing the C-terminus to be buried between the domains. It is proposed to call the recognition site at 321–332 the N binding site and its corresponding or homologous sequence in the C-terminal domain at 658–669 the N' site. The binding site in the C-terminal domain at 675–686 is the C binding site.

The structure–function correlations described here are made possible by the fortunate coincidence that both the majority of the interacting residues and the variations in the sites of proteolytic cleavage occur near the C-terminal ends of the half-molecule fragments. The correlation of the presence or absence of an amino acid residue with changes in the strength of inter-domain interactions does not prove that amino acid to be directly involved in the structure of the recognition site. Only structural data can supply this proof.

Some qualitative aspects of the interaction

Gel filtration by the 'small-application-volume' method used here allows only semi-quantitative estimations of binding constants for the various proteolytic fragments to be made. The fluorescence binding assay permits more detailed analysis. The inability of intact ovotransferrin to inhibit the interaction between the N-terminal and the C-terminal half-molecules [K_i^* 7.68 as compared with 0.16 for H40(C) and 0.18 for H44(N)_c; Table 1] is important in showing that the half-molecules associate by means of recognition sites that are not exposed in the intact protein. It has not, however, been

shown that the orientation of the domains in the dimer is the same as that present in the intact protein. The K_i^* values in Table 1 show that fragments that appear from gel-filtration experiments to be totally unable to undergo dimerization [such as fragment H44(N)_u] may nevertheless retain some ability to interact, but the location of the amino acid residues responsible for this cannot be determined from the variant fragments available at present.

Conservation of recognition sites

Cross-species dimerization occurs between half-molecule fragments from hen, duck and quail ovotransferrins, as judged by gel-filtration experiments. An N-terminal fragment of human serum transferrin M36(N) showed some ability to inhibit the interaction of the hen half-molecules (K_i^* 0.68), although no dimer formation could be detected by gel filtration. It is likely that inter-domain interactions occur in all soluble transferrins, though probably not in the membrane-bound melanotransferrin (Rose *et al.*, 1986). This protein possesses an N site at 345–356 (melanotransferrin numbering) and the homologue of this site, N', at 694–705. The C site would be expected to occur at 713–723, but from position 710 to position 738 the polypeptide chain comprises a tail sequence of alanine, proline and leucine residues that is thought to anchor the protein in the cell membrane.

The three sites (N, N' and C) have a generally hydrophobic nature, but the factors that can influence the interactions are evidently complex. They include conformational effects due to iron-binding, and electrostatic effects as shown by pH-dependent and salt-dependent changes.

Weeds & MacLachlan (1974) suggested that internally duplicated proteins with inter-domain interactions might be preceded in evolution by a self-dimerizing monomer stage. This idea appears particularly useful in the case of

the transferrins, where the problem of retaining the hypothetical ancestral half-molecule in the bloodstream has been noted (Williams *et al.*, 1982*b*). No sign of self-dimerization was detected in gel-filtration experiments with fragments M43(C) and H40(C).

Concluding comments

Brown-Mason & Woodworth (1984) found that isolated half-molecule fragments of hen ovotransferrin do not bind or donate iron to chick-embryo red blood cells. Addition of the contralateral half-molecule rapidly caused binding and iron-donation to occur at a rate close to that for intact hen ovotransferrin. They suggested that prior dimerization of the half-molecules is essential to bring about binding to the cell-membrane receptor. The availability of half-molecules whose ability to dimerize has been lost by removal of a few C-terminal residues could be useful in testing this theory.

Finally, the existence of dimerizing half-molecule fragments of transferrin resolves a long-standing inconsistency in work carried out in this laboratory. Trypsin digestion of ovotransferrin partially saturated with iron(III) nitrilotriacetate gave rise on subsequent gel filtration to a 'fragment peak' composed solely of the N-terminal half-molecule. It was therefore proposed that iron(III) nitrilotriacetate binds exclusively to the N-terminal domain of hen ovotransferrin and only enters C-terminal sites when all N-terminal sites are occupied (Williams, 1974). On the other hand, double isotope labelling of ovotransferrin with radioactive iron showed preferential, rather than exclusive, binding of the metal to the N-terminal domain (Williams *et al.*, 1978). It is now clear that the 'fragment peak' in gel-filtration experiments (method 1, as described here) contains only the excess N-terminal half-molecule fragments, and that the preference of iron(III) nitrilotriacetate for the N-terminal iron-binding site is not absolute.

REFERENCES

- Ackers, G. K. (1975) *Proteins* 3rd Ed. **2**, 1–94
- Aisen, P., Leibman, A. & Zweier, J. (1978) *J. Biol. Chem.* **253**, 1930–1937
- Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M. & Baker, E. N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1769–1773
- Brock, J. H., Arzabe, F., Lampreaeve, F. & Piniero, A. (1976) *Biochim. Biophys. Acta* **446**, 214–225
- Brown-Mason, A. & Woodworth, R. C. (1984) *J. Biol. Chem.* **259**, 1866–1873
- Bruton, C. J. & Hartley, B. S. (1970) *J. Mol. Biol.* **52**, 165–178
- Butterworth, R. M., Gibson, J. F. & Williams, J. (1975) *Biochem. J.* **169**, 559–563
- Chasteen, N. D. & Williams, J. (1981) *Biochem. J.* **193**, 717–727
- Evans, R. W. & Williams, J. (1978) *Biochem. J.* **173**, 545–552
- Evans, R. W. & Williams, J. (1980) *Biochem. J.* **189**, 541–546
- Hayashi, R. (1976) *Methods Enzymol.* **45**, 568–587
- Ikedu, H., Banuchi, Y., Nakazato, K., Tanaka, Y. & Satake, K. (1985) *FEBS Lett.* **182**, 305–309
- Jeltsch, J. M. & Chambon, P. (1982) *Eur. J. Biochem.* **122**, 291–295
- Lineback-Zins, J. & Brew, K. (1980) *J. Biol. Chem.* **255**, 708–713
- MacGillivray, R. T. A., Mendez, E., Shewale, J. G., Sinha, S. K., Lineback-Zins, J. & Brew, K. (1983) *J. Biol. Chem.* **258**, 3543–3553
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Arch. Biochem. Biophys.* **60**, 149–152
- Offord, R. E. (1966) *Nature (London)* **211**, 591–593
- Rose, T. M., Plowman, G. D., Teplow, D. B., Dreyer, W. J., Hellstrom, K. E. & Brown, J. P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1261–1265
- Weeds, A. G. & MacLachlin, A. D. (1974) *Nature (London)* **252**, 646–649
- Williams, J. (1968) *Biochem. J.* **108**, 57–67
- Williams, J. (1974) *Biochem. J.* **141**, 745–752
- Williams, J. (1975) *Biochem. J.* **149**, 237–244
- Williams, J., Evans, R. W. & Moreton, K. (1978) *Biochem. J.* **173**, 535–542
- Williams, J., Elleman, T. C., Kingston, I. B., Wilkins, A. G. & Kuhn, K. A. (1982*a*) *Eur. J. Biochem.* **122**, 297–303
- Williams, J., Grace, S. A. & Williams, J. M. (1982*b*) *Biochem. J.* **201**, 417–419
- Williams, J., Moreton, K. & Goodearl, A. D. J. (1985) *Biochem. J.* **228**, 661–665
- Wright, J. S. & Hunter, W. M. (1982) *J. Immunol. Methods* **48**, 311–325
- Zak, O., Leibman, A. & Aisen, P. (1983) *Biochim. Biophys. Acta* **743**, 490–495

Received 11 September 1987/3 November 1987; accepted 5 January 1988