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The Formation of Iron-Binding Fragments of Hen Ovotransferrin by Limited Proteolysis

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1. Iron was added to hen ovotransferrin to 30% saturation and the protein was digested with trypsin or chymotrypsin. 2. Iron-binding fragments were isolated. They carried one atom of iron/mol (mol.wt. 35000) and consisted of a single polypeptide chain derived from the *N*-terminal half of the protein. Carbohydrate was not present. 3. The fragments were able to bind a variety of metals and to donate iron to reticulocytes.

All the transferrins studied so far bind two atoms of iron and two bicarbonate ions to form a coloured complex. Early work on the absorption spectrum of ovotransferrin suggested that each ferric ion is chelated to three ionized tyrosine side chains (Warner & Weber, 1953). Tan & Woodworth (1969) later found that only two tyrosine side chains are involved in the binding of one iron atom. Electron-paramagnetic-resonance (e.p.r.) studies show that two nitrogen-containing groups, thought to be the side chains of histidine residues, also interact with each iron atom (Aasa *et al.*, 1963; Aasa & Aisen, 1968). The absence of magnetic interaction between the two iron atoms shows that they are at least 1 nm (10Å) apart (Windle *et al.*, 1963; Aasa *et al.*, 1963), but from fluorescence studies on rare-earth metal–transferrin complexes a distance of at least 4.3 nm (43Å) has been proposed (Luk, 1971).

There has been much discussion about the chemical and physiological properties of the two iron-binding sites. The association constant for the binding of the second iron atom was originally thought to be much bigger than that for the first, $K_2 = 100K_1$ (Warner & Weber, 1953). Davis *et al.* (1962) later reached the opposite conclusion; $K_1 = 400K_2$. Aasa *et al.* (1963) obtained results from equilibrium dialysis indicating equivalence of the association constants. Apparent support for the equivalence of the association constants and for a random order of binding of metal ions was obtained from electrophoretic experiments on partially iron-saturated transferrin, which demonstrated the existence of a one-iron-atom–transferrin complex (Aisen *et al.*, 1966; Stratil, 1967; Wenn & Williams, 1968). Later, however, Aisen & Leibman (1968) re-estimated the association constants by equilibrium dialysis and found $K_1 = 12K_2$. A careful inspection of the starch-gel-electrophoresis patterns of partially iron-saturated ovotransferrin (Williams *et al.*, 1970) suggests a sequential binding process rather than a random one. E.p.r. spectroscopy has given further evidence that the two metal-binding sites are not chemically identical (Price & Gibson, 1972; Aisen *et al.*, 1973). Finally, Luk (1971) has claimed that transferrin binds two atoms of terbium but only one of neodymium or praseodymium. The latter rare-earth metals are chemically similar to terbium but possess larger ionic radii. Possibly, therefore, the two sites differ in their ability to bind the larger ions. Physiological studies by Fletcher & Huehns (1968) suggested that the two iron atoms in iron-saturated transferrin are not identical with respect to the transfer of iron to the reticulocyte.

Since the transferrins consist of a single polypeptide chain of mol.wt. about 77000 (Greene & Feeney, 1968; Mann *et al.*, 1970), the two metal-binding sites can only be separated by cleaving the chain. Iron binding causes a marked structural change in transferrin and it is well known that iron–transferrin is much more stable than apotransferrin towards denaturation by heat, urea and extremes of pH (Warner & Weber, 1953; Azari & Feeney, 1958). Charlwood (1971) found that iron binding causes a 1.8% increase in the sedimentation coefficient of human transferrin, the increase being divided into two approximately equal steps which accompany the binding of the two metal atoms. Immunological reactivity has also been found to change on iron binding (Tengerdy *et al.*, 1966). Of the greatest interest in the present context was the finding by Fraenkel-Conrat (1950) and by Azari & Feeney (1958) that iron-saturated ovotransferrin is very resistant to proteolysis by trypsin and chymotrypsin, whereas native apo-ovotransferrin undergoes rapid digestion.

These observations suggest the possibility that in transferrin with only one iron atom resistance to proteolysis might be found near to the metal atom, leaving the rest of the molecule susceptible to

digestion. The present paper gives an account of experiments which confirm this idea. Tsao *et al.* (1974*b*) have described the use of CNBr cleavage in isolating an iron-binding fragment from hen ovotransferrin.

Materials and Methods

Reagents and proteins

Hen ovotransferrin was prepared as described by Williams (1968) and made metal-free by the method of Warner & Weber (1951). The anion-exchange resin used was Bio-Rad AGl (X2; 200–400 mesh; Cl^- form). The same method was used to remove metal from the fragments obtained by limited proteolysis of ovotransferrin. Bovine trypsin type XI (treated with diphenylcarbamoyl chloride) and bovine chymotrypsin type II were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Neodymium oxide and praseodymium oxide were given by Dr. G. Nickless. They were converted into the chlorides before use. Radioactive iron (as $^{59}FeCl_3$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Isolation of fragments of ovotransferrin. Metal-free ovotransferrin was dissolved in 0.1 M-$NaHCO_3$ at a concentration of 5% (w/v). Iron was added as iron nitrilotriacetate (Woodworth, 1966) to give 30% saturation as judged by E_{470}. Digestion with trypsin or chymotrypsin at an enzyme/substrate ratio of 1:50 (w/w) was carried out at 37°C for 6h. The digest was passed through a column (90cm×4.5cm) of Sephadex G-100 in 0.1 M-NH_4HCO_3. The effluent was monitored continuously at 276nm and fractions (150 drops) were collected. In one experiment (Fig. 1) samples (2ml) from each tube were dried and analysed for hexose by the orcinol–H_2SO_4 method (Winzler, 1955).

Isoelectric focusing. This method was used both to prepare ovotransferrin carrying different numbers of iron atoms (Wenn & Williams, 1968) and to purify ovotransferrin fragments. Ampholine pH5–7 was used in an apparatus of volume 440ml (LKB-Producter AB, Stockholm-Bromma 1, Sweden). Approx. 150mg of protein or fragment was focused for 3 days with 1kV. After dialysis and freeze-drying pooled fractions were redissolved and passed through a column (90cm×2cm) of Sephadex G-25 in water to remove traces of sucrose and Ampholine.

Starch-gel electrophoresis. Starch-gel electrophoresis was carried out with the discontinuous buffer system of Poulik (1957). In some experiments metals were removed from the starch powder (Williams *et al.*, 1970).

Formation of metal–protein complexes. Stock metal solutions (10mM) were prepared from iron nitrilotriacetate, ferrous ammonium sulphate, cupric sulphate, zinc acetate and the chlorides of neodymium and praseodymium. The iron-binding and copper-binding capacities of ovotransferrin and its fragments were determined by adding iron nitrilotriacetate and $CuSO_4$ respectively to 1% (w/v) solutions of the proteins in 0.1 M-$NaHCO_3$. After each addition of metal E_{470} or E_{440} was measured.

The numbers of tyrosine residues which ionize during metal binding were estimated by difference spectroscopy (Tan & Woodworth, 1969). Ovotransferrin was dissolved at 10 μM and the fragment at 20 μM in 0.01 M-Tris chloride–0.005 M-$NaHCO_3$, pH 8.5. Difference spectra were recorded with a Unicam SP. 1800 spectrophotometer and the molar extinction coefficient of ionized tyrosine in the region 239–246nm was taken as 1×10^4 litre·mol^{-1}·cm^{-1}. The iron complex was allowed to stand for 2 days before measurement.

Radioactive iron was attached to ovotransferrin and a tryptic fragment of ovotransferrin by the following method: $^{59}FeCl_3$ (0.2mCi in 0.1 M-HCl; sp. radioactivity 9.2Ci/g of Fe) was dried in a desiccator *in vacuo* over NaOH and to this was added 2.0ml of 8.1% (w/v) $FeCl_3$. The resulting solution was added to 1.07ml of 19.1% (w/v) nitrilotriacetic acid in 2M-NaOH and water was added to 10ml. Before use the solution was diluted 1:10 to give an iron concentration of 10mM. Iron-free ovotransferrin (24mg) and iron-free tryptic-fragment preparation (28mg) were each dissolved in 20ml of 0.15 M-NaCl–0.01 M-$NaHCO_3$. The concentrations of metal-binding sites in the solutions of ovotransferrin and tryptic fragments were 31 μM and 32 μM respectively. To each solution was added 0.4ml of 10mM-[^{59}Fe]iron nitrilotriacetate, this amount being sufficient to give 66% saturation of the metal-binding sites. The solutions were allowed to stand for 1h before being used in the assay of iron transfer to reticulocytes.

Assay of iron transfer to reticulocytes. Two rabbits (approx. 3kg body wt.) were given daily subcutaneous injections (1ml/kg body wt.) of phenylhydrazine (0.6%, w/v, in 0.15M-sodium phosphate buffer, pH7.4). After 5 days rabbit A had 67% reticulocytes and rabbit B 60%. In an untreated rabbit (C) the reticulocyte count was 2%. Blood was taken from each rabbit and heparinized. The cells were washed three times with cold saline–bicarbonate (0.15M-NaCl–0.01 M-$NaHCO_3$) and were finally resuspended in twice the packed-cell volume of saline–bicarbonate.

For the assay of iron transfer each tube contained 1.0ml of ^{59}Fe–protein and 0.5ml of cell suspension. Incubation was carried out at 37°C and the reaction was stopped at different times by cooling the tubes in ice and washing the cells three times with ice-cold saline–bicarbonate. Finally the cells were suspended

in a volume of 2.0ml and 100μl was dried on 3MM filter paper and the radioactivity measured by scintillation counting.

Molecular-weight determinations. Molecular weights of ovotransferrin fragments were determined by two methods. Gel filtration through a column of Sephadex G-100 was used as described by Andrews (1964). Polyacrylamide-gel electrophoresis in buffers containing 5% (w/v) sodium dodecyl sulphate was carried out as described by Fairbanks *et al.* (1971). In some experiments the sample buffer contained no reducing agent but in other cases β-mercaptoethanol was present at a concentration of 10% (v/v) and the solution was left at 18°C for 1h to allow complete reduction of disulphide bonds to occur. In one experiment performic acid oxidation (Hirs, 1967) of a fragment was carried out before the molecular weight was determined by gel electrophoresis.

Amino acid analysis. Protein samples were hydrolysed with 5.7M-HCl at 105°C in sealed evacuated tubes for periods of 24, 48 and 72h. Cystine was determined as cysteic acid after performic acid oxidation. Analyses were performed with a Technicon TSM amino acid analyser.

End-group analyses. N-Terminal residues of protein samples were identified by dansylation in the presence of urea (Gray, 1967). The isolation of *N*-terminal peptides was carried out by the method of Bruton & Hartley (1970) in which the protein is first maleylated and then digested with thermolysin. The *N*-terminal peptide was selectively purified by passage of the digest through a column of Dowex 50 (H^+ form).

Peptide 'maps'. Protein samples were dissolved in 5% (v/v) formic acid and digested with pepsin at an enzyme/substrate ratio of 1:20 (w/w) for 4h. After drying down several times the digests were dissolved in water. One-dimensional electrophoretic patterns were prepared at pH6.5 and 3.5. Volumes equivalent to 1mg of protein were applied to 2.54cm (1in) of starting line and electrophoresis was carried out for 30min with a potential gradient of 100V/cm. Peptides were stained by dipping the dried papers in the cadmium acetate–ninhydrin reagent of Heilmann *et al.* (1957). Specific stains for tyrosine and tryptophan (Jepson & Smith, 1953) were also applied.

Iron analysis. A colorimetric method with 2-nitroso-1-naphthol-4-sulphonic acid was carried out as described by Wenn & Williams (1968).

Results

Gel-filtration patterns

Enzyme digests of 30% iron-saturated ovotransferrin applied to Sephadex G-100 gave the elution pattern shown in Fig. 1. Four main peaks are present: those labelled (A) and (B) are associated with a pink colour. The orcinol–H_2SO_4 test shows that hexose

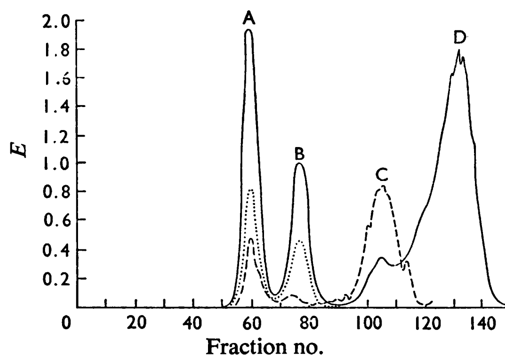


Fig. 1. *Gel filtration (on Sephadex G-100, 90cm×4.5cm) of a chymotryptic digest of 30% saturated iron–ovotransferrin*

----, E_{540} in the orcinol test for carbohydrate; ····, E_{470}; ——, E_{280}. Fractions of volume 9ml were collected. A, B, C, D are the four peaks mentioned in the text.

is associated with peaks (A) and (C). Peak A appeared to represent intact iron-saturated ovotransferrin as judged by starch-gel electrophoresis with native ovotransferrin as a marker and by the value of $E_{280}/E_{470} = 23.0$ (the ratio for iron-saturated ovotransferrin was 23.5). Peak C contained low-molecular-weight glycopeptides and peak D other low-molecular-weight peptides. Peaks C and D were not studied further. Tryptic and chymotryptic digests gave similar patterns except that peak B was relatively larger and peak D relatively smaller in tryptic digests than in chymotryptic digests.

Starch-gel electrophoresis was carried out on peak B material. Plate 1 shows that the main bands had identical mobilities in tryptic and chymotryptic digests. Isoelectric fractionation of this material produced two pink-coloured bands in each case, the more acidic band being generally the more intense. In some experiments the pink components were present in equal amounts (Fig. 2). The isoelectric points of the pink bands from chymotryptic digests were 5.90 and 6.30, and, in view of the identity of their electrophoretic mobilities and molecular weights, it is likely that the pink bands from the tryptic digests would have very similar values for their isoelectric points. The four fractions will be referred to as C5.9, C6.3, T5.9 and T6.3. They were resistant to further enzymic digestion as judged by starch-gel electrophoresis. The addition of extra iron after digestion did not alter the starch-gel-electrophoresis patterns. The presence of two pink bands in each type of digest is therefore not due to incomplete digestion or to incomplete iron saturation.

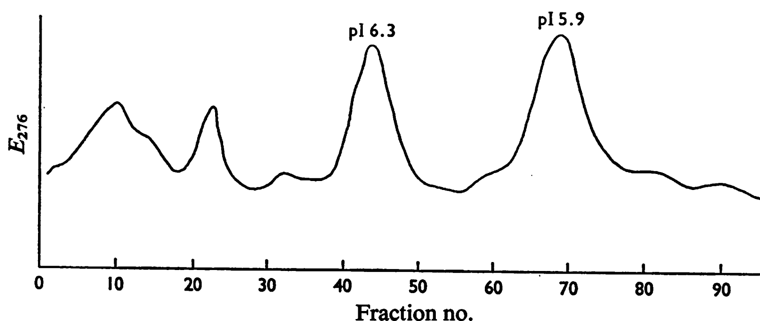


Fig. 2. *Isoelectric fractionation of peak B material (see Fig. 1) from a chymotryptic digest of 30% saturated iron–ovotransferrin*

Fraction volume was 1 ml. The peaks labelled pI6.3 and 5.9 were pink. For further details see the text.

Table 1. *Amino acid compositions of ovotransferrin and of fragments C5.9, C6.3 and T5.9*

Results are expressed as mol of amino acid/mol of protein.

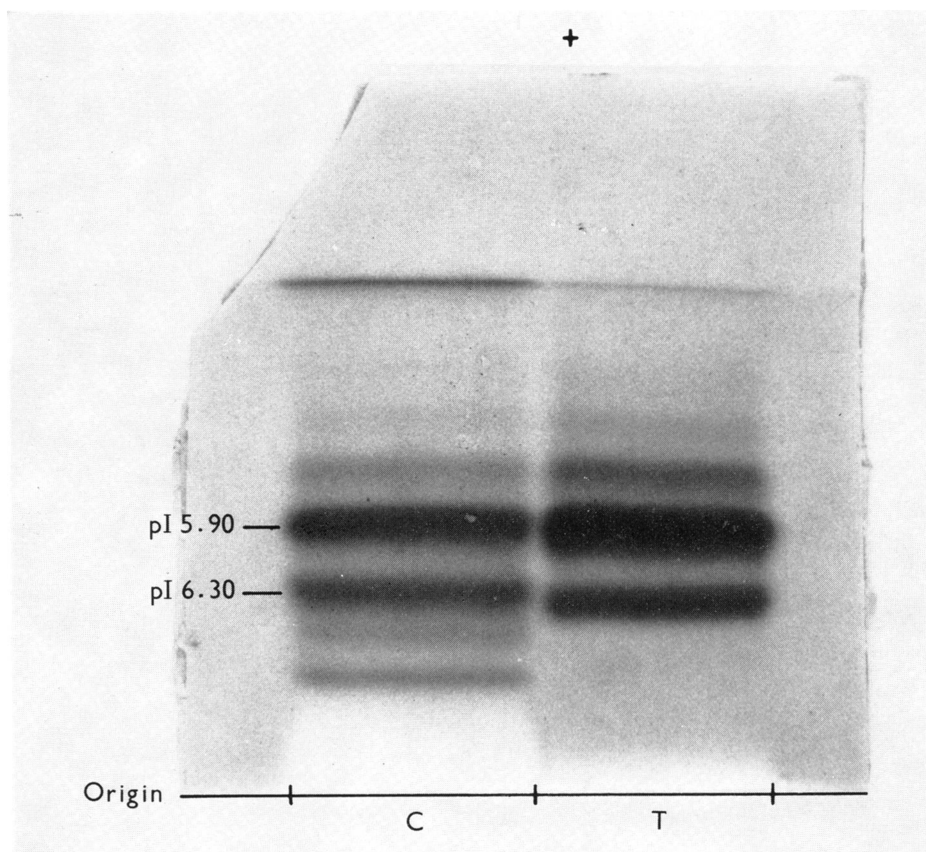
Amino acid	Fragment T5.9	Fragment C5.9	Fragment C6.3	Ovotransferrin: Present work	Ovotransferrin: Wenn & Williams (1968)	Ovotransferrin: Williams (1962)
Cysteic acid	18.5	19.0	19.5	40.6	29.1	24.4
Asp	31.6	38.6	32.2	68.4	82.0	74.7
Thr	15.2	14.9	15.3	27.5	36.6	35.2
Ser	16.9	13.9	14.8	29.8	45.7	42.7
Glu	31.8	35.5	33.4	65.3	77.4	71.2
Pro	15.4	15.3	18.5	30.3	28.9	29.4
Gly	26.1	24.8	24.3	48.5	56.0	58.9
Ala	30.0	27.7	30.9	54.9	54.8	53.0
Val	20.0	21.7	25.7	51.8	54.5	44.8
Met	0.8	2.9	1.8	10.0	13.1	11.5
Ile	16.6	14.6	16.5	30.0	28.0	24.8
Leu	23.0	24.6	23.9	50.4	54.7	49.1
Tyr	10.6	10.3	8.2	21.1	22.3	20.7
Phe	9.8	10.4	10.7	29.4	28.5	25.9
His	8.2	6.9	6.6	14.0	13.3	13.0
Lys	24.1	25.0	25.9	49.3	64.9	63.1
Arg	13.4	14.4	14.4	34.0	35.6	33.1

Molecular weights

By gel electrophoresis the three fragments T5.9, C5.9 and C6.3 had molecular weights of 35000 and reduction or performic acid oxidation of disulphide bonds caused no change in molecular weight. By gel filtration the molecular weight of fragment T5.9 was 35500 and that of fragment C6.3 was 34700. The molecular weight of fragment T6.3 was not determined. The protein fragments thus have approximately the same molecular weight and consist of single polypeptide chains.

Composition of the fragments

Table 1 shows the amino acid compositions of ovotransferrin and of fragments T5.9, C5.9 and C6.3. The three fragments appear to have similar amino acid compositions and for many types of amino acids the proteolytic fragments contain approximately one-half the number of residues found in the whole protein. Methionine and phenylalanine are notable exceptions to this generalization. The generalization is, however, only tentative because the composition of ovotransferrin obtained in this work differs markedly from earlier analyses (Williams, 1962, 1968). As Table 1 shows, the discrepancy is particularly marked in the cases of cysteic acid, threonine, serine, methionine and lysine. The cause of these differences is not clear since essentially similar methods were used in the preparation and analysis of the protein in the earlier work and in the present work. When fragment T5.9



EXPLANATION OF PLATE 1

Starch-gel electrophoresis of peak B material from a chymotryptic digest (C) and a tryptic digest (T) of 30% *saturated iron–ovotransferrin*

Electrophoresis was carried out for 4h with a voltage gradient of 13V/cm. The bands labelled pI5.90 and 6.30 were obtained pure after isoelectric focusing (Fig. 2). For further details see the text.

was analysed for hexosamine by the method of Rondle & Morgan (1955) none was found, showing that the fragment does not carry the carbohydrate prosthetic group of ovotransferrin.

N-Terminal sequences

The *N*-terminal amino acid sequence of hen ovotransferrin is Ala-Pro-Pro-Lys-Ser (Graham & Williams, 1974). Fragments T5.9, C5.9 and C6.3 all had *N*-terminal alanine and the *N*-terminal peptide obtained by the maleylation–thermolysin method had mobility $(m) = +0.45$ at pH 6.5 and stained pink with the cadmium acetate–ninhydrin reagent. The sequence was determined by the dansyl-Edman method and was the same as that of the *N*-terminal peptide of hen ovotransferrin. The three fragments therefore carry the *N*-terminal part of ovotransferrin.

Peptide 'maps'

One-dimensional electrophoretograms of pepsin digests of fragments C5.9 and C6.3 at pH 6.5 and 3.5 gave identical patterns when stained with ninhydrin and the specific stains for tyrosine and tryptophan. The two fragments thus appear to possess markedly similar amino acid sequences.

Iron contents of fragment C5.9

The pink colour of the fragments suggested that they contained iron complexed in a manner similar to that in iron–transferrin. For fragments C5.9 and C6.3 the ratio E_{280}/E_{470} was 23.5 and the iron content of fragment C5.9 by colorimetric analysis was 0.152% (w/w), equivalent to 0.98 g-atom of iron/mol. The absorption spectrum of fragment C5.9 showed a broad peak with its maximum at 465 nm.

Metal binding by tryptic fragments

Metal binding was studied in a sample of peak B material from a tryptic digest which had not been subjected to isoelectric focusing and therefore contained both fragments T5.9 and T6.3. After removal of iron this material was able to bind a variety of metals. Fig. 3 shows the linear uptake of iron and copper to maximum extinctions at 0.84 g-atom of Fe/35000 g and 0.82 g-atom of Cu/35000 g. The decrease in slope shown by the curve for copper as it approaches saturation suggests that this metal is less tightly bound than iron. The iron complex was salmon-pink in colour with $\lambda_{max.}$ at 468 nm and the copper complex was yellow with $\lambda_{max.}$ at 450 nm. When 1 equiv. of iron (as iron nitrilotriacetate) was added to the copper complex there was a slow shift of the absorption maximum from 450 to 468 nm, complete in about 30 min.

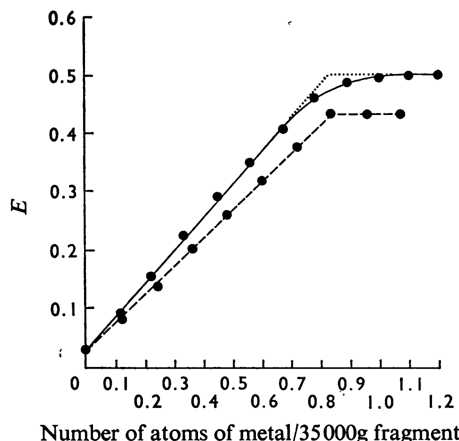


Fig. 3. *Titration of tryptic fragment (1%, w/v, in 0.1 M-NaHCO$_3$) with 10 mM iron and copper*

For copper E_{440} (——) was measured and for iron E_{470} (----). For further details see the text.

Table 2. *Numbers of ionized tyrosine residues, estimated by difference spectroscopy in tryptic fragments and in ovotransferrin, on metal saturation*

The results for the tryptic fragments are expressed as mol of ionized tyrosine/metal-binding site, and those for ovotransferrin as mol of ionized tyrosine/two metal-binding sites.

	Tryptic fragments	Ovotransferrin
Fe^{3+}	1.76	3.70
Zn^{2+}	1.14	2.30
Nd^{3+}	1.27	2.00
Pr^{3+}	0.94	1.97

The same protein sample was used in estimating the numbers of tyrosine residues ionizing during metal binding by difference spectroscopy. Table 2 shows the results for the binding of iron, zinc, neodymium and praseodymium by the protein fragments and by native iron-free ovotransferrin. The difference spectra given by the native protein and the tryptic fragments were very similar (Fig. 4).

Transfer of iron to reticulocytes

Fig. 5 shows that after 66% saturation with iron the tryptic fragments and ovotransferrin were able to transfer iron to reticulocytes at approximately equal rates. The uptake by cell sample A (67% reticulocytes) was slightly greater than by cell sample B (60% reticulocytes). There was very little uptake by cell sample C (2% reticulocytes). For sample A ovotransferrin appeared to donate its iron more

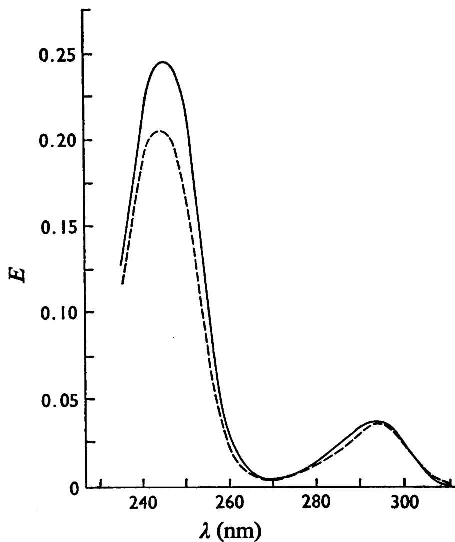


Fig. 4. *U.v. difference spectra for the praseodymium complexes of ovotransferrin (----) and of tryptic fragment (——)*

For details see the text.

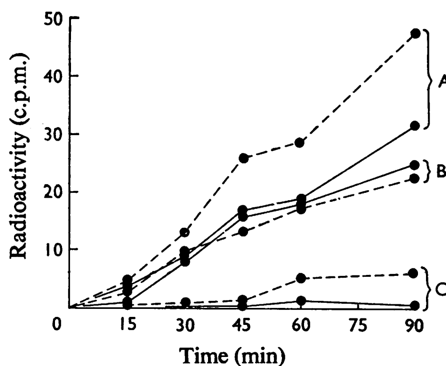


Fig. 5. *Uptake of ^{59}Fe from tryptic fragment (——) and from ovotransferrin (----) by rabbit blood cell samples A, B and C, which contained 67, 60 and 2% reticulocytes respectively*

For details see the text.

rapidly than the tryptic fragments but for cell sample B the rates were essentially the same.

Origin of iron–ovotransferrin in enzyme digests

The presence of iron-saturated ovotransferrin in digests of 30% iron-saturated ovotransferrin is unexpected. Raising the iron amount to 50%, which

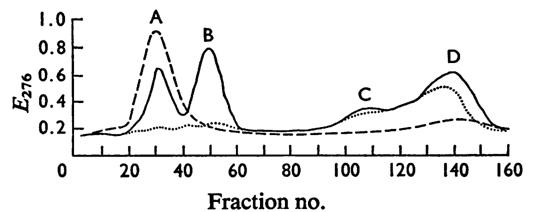


Fig. 6. *Gel filtration on Sephadex G-100 of tryptic digests of apo-ovotransferrin (····), one-iron-atom–ovotransferrin (——) and iron-saturated ovotransferrin (----)*

A column of 90cm×2cm was used and fractions (3ml) were collected. Peaks A, B, C and D are indicated (see Fig. 1). For further details see the text.

would be expected to give the maximum proportion of the one-iron-atom–ovotransferrin complex, gave a greatly increased amount of iron-saturated ovotransferrin and a very small amount of iron-binding fragment (peak B). Partially iron-saturated ovotransferrin was fractionated by isoelectric focusing into iron-free protein, one-iron-atom–protein complex and iron-saturated protein, and each fraction was digested with trypsin. Gel filtration (Fig. 6) showed that the iron-binding fragments arise only from the one-iron-atom–protein complex, which also gives rise to iron-saturated ovotransferrin in the course of digestion. Starch-gel electrophoresis confirmed that before digestion the one-iron-atom–protein complex was essentially free from contamination by iron-saturated ovotransferrin.

Discussion

These experiments show that proteolytic digestion of the one-iron-atom–ovotransferrin complex yields fragments which contain the bound iron atom. They consist of a single polypeptide chain and are approximately one-half the size of the original protein. The fragments carry the *N*-terminal alanine residue of hen ovotransferrin. The fact that disulphide-bond cleavage causes no apparent change in molecular weight suggests that the *N*-terminal half of ovotransferrin constitutes a region possessing only internal disulphide bonds.

Both trypsin and chymotrypsin give two iron-binding fragments from ovotransferrin and these differ in charge but not apparently in molecular weight. Although the two enzymes would be expected to cleave the chain at different points, the results of starch-gel electrophoresis suggest that the products from the two types of digest are closely similar. The cause of the charge difference between the two fragments is not yet known but the close similarity of their peptide 'maps' and the fact that both

carry the *N*-terminus of the original protein suggest that their primary structures must be very similar. The absence of carbohydrate from these fragments shows that the carbohydrate group of ovotransferrin is not present in the *N*-terminal half of the protein.

Metal atoms are bound by the fragments to give complexes with similar colours, visible absorption spectra and u.v. difference spectra to those given by the native protein. Therefore although no direct study of the question has been made, it appears likely that a bicarbonate ion takes part in the metal–protein fragment complex, as it does in the intact protein. Since the fragments can donate their iron to reticulocytes, they evidently possess the structural features necessary to interact with the transferrin–receptor site of the reticulocyte membrane. Kornfeld's (1968) conclusion that the carbohydrate groups of human transferrin play no part in iron binding or in the transfer of iron from transferrin to the reticulocyte is supported by the present work.

The metal-binding site present in the proteolytic fragments is probably the one which binds the first of the two iron atoms. There was no evidence in the present experiments for the existence of an iron-binding fragment other than the *N*-terminal fragment. This supports the theory that the order of iron binding by ovotransferrin is sequential and not random. The isolated fragments bind iron, copper, zinc, neodymium and praseodymium. It was suggested by Luk (1971) that human serum transferrin is able to bind only one atom of neodymium or praseodymium. The numbers of tyrosine residues ionizing in ovotransferrin during the binding of iron and zinc in the present experiments are close to those found by Tan & Woodworth (1969), but the values for neodymium and praseodymium are somewhat higher than those obtained by Luk (1971) for human transferrin. The numbers of ionized tyrosine residues in the fragments are approximately equal to one-half of those found in the original protein. This suggests that similar numbers of tyrosine residues are involved in each of the two metal-binding sites. It appears likely that two atoms of neodymium and praseodymium rather than one are bound by native ovotransferrin, although further work on this is required.

In order for iron-saturated ovotransferrin to arise during the digestion of one-iron-atom–ovotransferrin it is necessary for free iron atoms to be bound by one-iron-atom–ovotransferrin itself. It may be suggested therefore that the first iron atom does not confer complete resistance to proteolysis upon the *N*-terminal half of the molecule. Some of the molecules may undergo digestion and release their bound iron. Nevertheless, the isolated fragments appear to be fully resistant to further enzymic attack.

The information available on the primary structure of transferrin (Elleman & Williams, 1970; Tsao *et al.*, 1974*a*; I. B. Kingston & J. Williams, unpublished work; Graham & Williams, 1974) does not at present support the view that the molecule consists of two similar or identical halves. The observations reported here, however, are consistent with the view that the binding sites for the first and second iron atoms are formed from the *N*-terminal and *C*-terminal halves of the molecule respectively and that these halves are structurally and functionally to a large extent independent of one another. Tsao *et al.* (1974*b*) have found that the *N*-terminal fragment, obtained by CNBr cleavage of hen ovotransferrin, binds one atom of iron. The molecular weight of this fragment was 36000. This fragment evidently corresponds closely to the fragments obtained by limited proteolysis. The fragment described by Tsao *et al.* (1974*b*) apparently consists of two polypeptide chains linked by disulphide bonds. The single-chain fragments described in the present paper contain one or more residues of methionine which would be expected to provide cleavage points for CNBr. The structure of the metal-binding site in the CNBr-derived fragment may therefore not be the same as in the native protein or in the proteolytic fragments. The fact that the absorption maximum of the iron complex of the CNBr fragment was at 430nm suggests that the metal-binding site has undergone some structural change, as compared with the native structure.

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