Iron-Binding Fragments from the Carboxyl-Terminal Region of Hen Ovotransferrin

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1. When iron-saturated hen ovotransferrin was treated with subtilisin the *N*-terminal half was digested at a faster rate than the *C*-terminal half, allowing the latter to be isolated as a single-chain fragment of mol.wt. 35000. 2. In mildly acid conditions iron-ovotransferrin loses iron preferentially from its *N*-terminal binding site. Trypsin digestion of the resulting monoferric ovotransferrin also gave rise to a *C*-terminal fragment. 3. Comparison of the *N*-terminal fragment with the *C*-terminal fragments shows differences in composition, peptide 'maps', CNBr-cleavage patterns and antigenic structures. The *C*-terminal fragments carry the carbohydrate group of ovotransferrin. 4. Both *N*-terminal and *C*-terminal fragments donate their bound iron to rabbit reticulocytes.

One of the main questions in the study of the transferrins concerns the nature of the iron-binding sites. It is generally thought that in hen ovotransferrin the two sites are very similar, although not identical, in their chemical properties (Aisen, 1973; Morgan, 1974). There is, however, little direct information on this question and the availability of protein fragments bearing the individual iron-binding sites in intact form would be useful.

Recently, two methods for isolating an iron-binding fragment that corresponds to the *N*-terminal half of hen ovotransferrin have been described. Azari and his collaborators (Tsao *et al.*, 1974*a,b*), used CNBr cleavage of the protein and obtained a fragment of mol.wt. 36000. Williams (1974), on the other hand, used limited proteolysis of a monoferric ovotransferrin in obtaining a similar fragment. The monoferric ovotransferrin was formed by incomplete saturation of ovotransferrin with iron (0.3 atom of Fe/mol of protein). The *N*-terminal fragment obtained in this way consists of a single polypeptide chain of mol.wt. about 35000, shows reversible binding of iron, and donates iron to rabbit reticulocytes at about the same rate as native ovotransferrin. It does not contain the single major carbohydrate group of this protein. From these facts it was proposed that the first atom of iron bound by ovotransferrin specifically occupies a site in the *N*-terminal region and that the second iron atom is bound by a site in the *C*-terminal region.

In the present paper it is shown that, during the removal of iron from iron-ovotransferrin by exposure to acid conditions, a monoferric ovotransferrin is formed in which the iron atom occupies the *C*-terminal binding site. Trypsin digestion of this complex gives rise to an iron-binding fragment which

represents the *C*-terminal half of the protein. A preliminary comparison of this fragment with the *N*-terminal fragment is also given. Where convenient, the new monoferric ovotransferrin will be designated OT-Fe and the monoferric ovotransferrin in which the iron atom occupies the *N*-terminal site will be designated Fe-OT.

Materials and Methods

Materials

Bovine trypsin (type XI, diphenylcarbamoyl chloride-treated) and bacterial proteinase (type VIII, from *Bacillus subtilis*) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. $^{59}\text{FeCl}_3$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hen ovotransferrin was prepared as described by Williams (1968). In this method the protein is saturated with iron before adsorption on CM-Sephadex in 0.1 M-sodium acetate, pH 5.5. After elution from the exchanger the protein was found to be only about 30% saturated with iron. The removal of iron from protein samples was carried out as described by Williams (1974).

Preparation of the *N*-terminal fragment

The *N*-terminal iron-binding fragment was prepared by trypsin digestion of ovotransferrin to which iron had been added to 30% saturation (Williams, 1974). After purification by gel filtration on Sephadex G-100 the preparation contained two iron-binding components. For some experiments this material was used without further purification and will be designated components NT. In other cases it was purified

by isoelectric focusing and the iron-binding component with pI 5.85 was isolated. This material is designated component NT5.85.

Preparation of C-terminal iron-binding fragments

Limited proteolysis was used in two ways. In the first, iron-saturated ovotransferrin was digested with subtilisin. In the second, ovotransferrin, from which iron had been partially removed by exposure to acid conditions, was digested with trypsin.

Method 1. Iron-saturated ovotransferrin (60 mg) in 0.2 M-NaHCO₃ (3.0 ml) was digested with subtilisin (1 mg) for 6 h at 37°C. The digest was then applied to a column (90 cm × 2 cm) of Sephadex G-100 in 0.1 M-NH₄HCO₃. Fractions (50 drops) were collected and the E_{280} was measured. Each fraction was then dried and analysed for hexose by the orcinol-H₂SO₄ method (Winzler, 1955). In another small-scale experiment the digestion was continued for 24 h, fresh lots (1 mg) of subtilisin being added every 6 h. E_{470} was monitored throughout the digestion with a Unicam SP.1800 spectrophotometer. Gel filtration was then carried out as described above.

In preparative experiments, 1 g of iron-ovotransferrin in 50 ml of 0.2 M-NaHCO₃ was digested with subtilisin (20 mg) at 37°C. Further additions of enzyme were made after 6 and 12 h. After 20 h the digest was applied in two portions to a column (90 cm × 4.5 cm) of Sephadex G-100 in 0.01 M-NH₄HCO₃. Pink-coloured fractions were pooled and, where necessary, further purified by isoelectric focusing.

Method 2. Ovotransferrin whose iron saturation had 'inadvertently' fallen to about 30% in the course of ion-exchange chromatography was dissolved in 0.2 M-NaHCO₃ at a protein concentration of 2% (w/v) and digested with trypsin (enzyme/substrate ratio 1:20, w/w) for 8 h at 37°C. A further lot of trypsin was then added and the digestion continued overnight. The digest was then dialysed against water and freeze-dried. Purification by isoelectric focusing was then carried out.

Alternatively, iron-saturated ovotransferrin was dissolved in water (protein concentration 1%, w/v). To the solution was added 0.1 vol. of 0.6 M-sodium citrate, pH 5.0. When the E_{470} of the solution had fallen to 30–35% of its initial value, anion-exchange resin was added to remove ferric citrate [8 g of Bio-Rad AG1-X2 (Cl⁻ form)/g of protein]. The resin was removed by centrifuging and solid NH₄HCO₃ was added to the solution to give 0.2 M. Trypsin digestion was then carried out as described above.

Isoelectric fractionation

Isoelectric focusing with Ampholine of pH range 5–7 was carried out as described by Williams (1974). This method was used to purify protein fragments and

also the two types of one-iron-atom-ovotransferrin complex. The monoferric ovotransferrins were prepared by isoelectric focusing of 30% iron-saturated ovotransferrin. One such complex was prepared from ovotransferrin to which iron had been added to give 30% saturation and it will be designated Fe-OT. The other complex, designated OT-Fe, was prepared from ovotransferrin whose iron content had fallen to 30% in the course of preparation. After isoelectric focusing, Ampholine and sucrose were removed from protein samples either by exhaustive dialysis against water or by passing the pooled fractions directly through a column (90 cm × 4.5 cm) of Sephadex G-100 in 0.01 M-NH₄HCO₃. Samples were then freeze-dried.

Immunological methods

Rabbits were immunized with whole ovotransferrin, fragment NT and the C-terminal fragment prepared by method 1 (see above). One rabbit was used for each immunization. The appropriate antigen (60 µg) was suspended in complete Freund's adjuvant and injected intradermally at four sites in the dorsal region. After 1 month, 1 mg of antigen (without adjuvant) was injected intramuscularly and the first bleeding was carried out 7 days later. After 4 months a further booster injection was given and the second bleeding carried out 7 days later.

Antigen-antibody reactions were observed by the Ouchterlony method in which undiluted antisera were used and the antigens were dissolved in 0.15 M-sodium phosphate buffer, pH 7.4, at a protein concentration of 0.1%.

CNBr-cleavage patterns

Protein samples (30 mg) were dissolved in 1 ml of 70% (v/v) formic acid and treated with 30 mg of CNBr for 24 h in a stoppered tube at room temperature (17°C). After dilution with water (2 ml) and de-aeration the solution was applied to a column (100 cm × 1.5 cm) of Sephadex G-100 in 5% (v/v) formic acid.

Rates of iron removal from ovotransferrin and protein fragments

Samples of iron-saturated ovotransferrin and iron-saturated fragments (30 mg) dissolved in 2.7 ml of water were placed in a 1 cm-light-path cell, water-jacketed at 24°C. The reference cell contained water. To the sample was added 0.3 ml of 0.6 M-sodium citrate buffer, pH 5.65. The solutions were mixed rapidly and the progress of the reaction was monitored at 470 nm with a Unicam SP.1800 spectrophotometer. In some experiments NaClO₄ was added to the protein samples to give concentrations of 0.5 and 1.5 M before the addition of the citrate.

Formation of metal-protein complexes

This was carried out as described by Williams (1974) except that in preparing protein samples containing ^{59}Fe the specific radioactivity of the iron nitrilotriacetate used was 2.0 mCi/mmol of Fe instead of 0.2 mCi/mmol of Fe.

Other methods

Other methods were carried out essentially as described by Williams (1974). In some starch-gel-electrophoresis experiments, one slice of the gel was stained to reveal protein bands and side strips were removed from the rest of the gel and replaced with 1% (w/v) agar gel. Antisera were placed in troughs cut in the agar so that precipitin reactions between protein bands and the antisera could be observed.

Results

Digestion of iron-ovotransferrin with subtilisin

Subtilisin treatment of iron-saturated ovotransferrin caused a rapid initial decrease in E_{470} , which was followed by a slower rate of fall (Fig. 1). A sample of protein which had been digested for 6 h gave the gel-filtration pattern shown in Fig. 2. Four main peaks are present. Starch-gel electrophoresis showed that peak 1 represented undigested ovotransferrin, and after extensive periods of digestion this peak became very small. Peaks 3 and 4 represented low-molecular-weight glycopeptides and peptides and were not studied further. The material in peak 2 was pink in colour and contained carbohydrate. On starch-gel electrophoresis it showed a single main band and on isoelectric focusing two pink fractions were obtained with pI 5.45 and pI 5.60. Starch-gel electrophoresis of these fractions showed the same

Fig. 1. Digestion of iron-saturated ovotransferrin with subtilisin

Enzyme was added at zero time and at 6, 12 and 18 h as indicated by the arrows. E_{470} was monitored continuously. For details see the Materials and Methods section.

major band in each case, although the fraction with pI 5.60 showed an additional weak band which moved more slowly than the major band. Since further experiments showed that this major component represents the C-terminal half of ovotransferrin, it will be referred to as fragment CS.

Trypsin digestion of partially iron-depleted ovotransferrin

The starch-gel-electrophoresis pattern given by a trypsin digest of partially iron-depleted ovotransferrin is shown in Plate 1. In addition to the bands which represent the undigested protein, a faster-running component is present. The amount of undigested protein was diminished and that of the fast component increased by repeating the digestion. Isoelectric fractionation of the digest gave two main peaks with pI 5.85 and pI 5.40. The fraction with pI 5.85 was found by starch-gel electrophoresis to be the undigested protein and the pink-coloured fraction with pI 5.40 represents the fast component. Since further experiments showed that this component represents the C-terminal half of ovotransferrin it will be referred to as fragment CT. In preparing fragment CT it was immaterial whether the partial depletion of the iron content of the ovotransferrin occurred 'inadvertently' in the course of preparation of the protein or was brought about deliberately by the

Fig. 2. Gel-filtration pattern of subtilisin digest of iron-ovotransferrin

—, E_{280} ; ----, E_{540} in orcinol assay. For details of peaks 1–4 see the text.

addition of citrate buffer, pH 5.0. Therefore the former method was used as a routine.

The same protein fragment (CT), as judged by immunological properties, was also obtained by trypsin digestion of the monoferric ovotransferrin isolated by isoelectric fractionation of partially iron-depleted ovotransferrin.

Molecular weights

Gel filtration of fragment CS on Sephadex G-100 gave one peak, and values of 31 000 and 33 000 were obtained for its molecular weight. A value of 35 500 was obtained for fragment CT. Polyacrylamide-gel electrophoresis gave single values of 36 000 for fragment CS and 44 500 for fragment CT. Since the exposure of the fragments to reducing and dissociating conditions in the latter method did not apparently lower the molecular weight, it is seen that each fragment consists of a single polypeptide chain which represents approximately half the molecular weight of ovotransferrin.

Peptide 'maps'

Peptide 'maps' of fragments NT, CS and CT were prepared to determine whether the new fragments are derived from the *N*-terminal region of the protein. After pepsin digestion, fragments NT and CS give markedly different peptide 'maps', but fragments CS and CT give very similar or identical 'maps'. This conclusion was based on the ninhydrin stain and on specific stains for tyrosine- and tryptophan-containing peptides. Fragments CS and CT are therefore apparently not derived from the *N*-terminal region of the protein.

CNBr-cleavage patterns

After CNBr treatment, ovotransferrin gave the gel-filtration pattern shown in Fig. 3(a). The patterns given by fragments NT and CS are shown in Figs. 3(b) and 3(c) respectively. Fragment CT gave the same pattern as fragment CS. Peak B in the ovotransferrin pattern was found by Kingston & Williams (1975) to represent the *N*-terminal half of the protein. Fragment NT, which was found by Williams (1974) to represent the *N*-terminal half of ovotransferrin, gave rise to peaks A and B. The nature of peak A is not known. The gel-filtration patterns suggest that fragments CS and CT represent the C-terminal half of ovotransferrin, since they give rise to peaks C, D and E. Peak F was found with both types of fragments. The position of peak E produced by the CNBr treatment of fragments CS and CT was slightly delayed as compared with the peak E produced by the whole protein, which suggests that it may be smaller.

Fig. 3. Gel-filtration patterns after CNBr treatment of (a) ovotransferrin, (b) fragment NT and (c) fragment CS

Fragment CT gave the same pattern as fragment CS. For details of peaks A-F see the text.

EXPLANATION OF PLATE I

Starch-gel-electrophoresis patterns showing effect of trypsin digestion on partially iron-depleted ovotransferrin (30% iron saturation)

The bands labelled pI5.40 and pI5.85 were isolated preparatively by isoelectric fractionation.

EXPLANATION OF PLATE 2

Agar-gel diffusion plates

(a) Wells 1, 2 and 3 contained ovotransferrin, fragment CS and fragment NT respectively. The central well contained rabbit antiserum to hen ovotransferrin. (b) Wells 1, 2 and 3 contained ovotransferrin, fragment CS and fragment NT respectively. The central well contained rabbit antiserum to fragment NT. (c) Wells 1, 2 and 3 contained ovotransferrin, fragment CS and fragment NT respectively. The central well contained rabbit antiserum to fragment CS. (d) Well 1 contained ovotransferrin. Well 2 contained a mixture of equal amounts of fragments CS and NT. Wells 3 and 4 contained fragments CS and NT respectively. The central well contained rabbit antiserum to ovotransferrin.

Table 1. *Amino acid compositions of ovotransferrin and of fragments CS, CT and NT*

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

Amino acid	Fragment CS	Fragment CT	Fragment NT	Ovotransferrin
Cysteic acid	15.6	17.3	17.5	32.1
Asp	35.4	36.1	29.9	75.4
Thr	16.9	15.0	19.7	39.3
Ser	15.4	15.5	19.9	40.2
Glu	32.0	32.1	30.7	68.9
Pro	—	14.1	12.6	28.2
Gly	26.2	27.9	23.6	54.2
Ala	21.7	21.1	30.7	55.8
Val	30.2	24.4	22.9	52.1
Met	5.4	6.7	3.5	15.9
Ile	9.6	9.0	18.8	26.0
Leu	20.4	23.8	24.5	49.0
Tyr	6.4	9.4	11.5	22.8
Phe	10.7	14.8	12.9	28.1
Lys	27.8	29.2	29.6	61.8
His	5.8	4.7	7.2	13.8
Arg	14.7	16.1	13.0	34.8

Protein compositions

The amino acid compositions of hen ovotransferrin and of its *N*-terminal and *C*-terminal fragments are given in Table 1. The composition of hen ovotransferrin is similar to that found in earlier studies (Williams, 1962; Wenn & Williams, 1968). The analyses support the suggestion (Williams, 1974) that, for many types of amino acids, the *N*-terminal fragment contains approximately half the number of residues present in the whole protein. Although the amino acid compositions of the *N*-terminal and *C*-terminal fragments are similar to one another, they are not identical, as the values for serine, alanine, methionine and isoleucine show. These data do not support the suggestion (Williams, 1974) that phenylalanine is unequally distributed between the two halves of the molecule.

Carbohydrate is absent from fragment NT but is present in the *C*-terminal fragments. Wenn (1975) has shown that the major carbohydrate group of ovotransferrin is composed of three residues of mannose and five residues of *N*-acetylglucosamine. The recovery of mannose in fragment CT (2.6 mol/mol) was 86%, but the recovery of glucosamine in fragment CS (3.1 mol/mol) was only 62%. The glucosamine content of fragment CT was not estimated. It seems probable, however, that the carbohydrate group of the protein is located in the *C*-terminal half.

Isoelectric points of the fragments

As described previously (Williams, 1974), two forms of fragment NT with pI values of 5.90 and 6.30 are found. These results were confirmed in the present experiments. Isoelectric fractionation of

fragment CS gave two bands with pI values of 5.45 and 5.61 in a single experiment. Fragment CT had pI of 5.45 (average of four determinations). The *C*-terminal half of ovotransferrin thus appears to have an isoelectric point approx. 0.5 pH unit lower than that of the *N*-terminal half.

Immunological properties of the fragments

Plate 2(a) shows that an antiserum against whole ovotransferrin reacts with fragments NT and CS, but the crossing of the precipitin lines suggests that the fragments do not share antigenic determinants. This is confirmed by the fact that each fragment reacts with its own antiserum but not with that against the other fragment (Plates 2b and 2c). Plate 2(d) shows that when equal amounts of fragments NT and CS are mixed together, the mixture reacts with anti-ovotransferrin and the precipitin line fuses smoothly with that given by ovotransferrin itself. It is likely therefore that, taken together, the *N*-terminal and *C*-terminal fragments include all or most of the antigenic determinants of the whole protein. It also seems likely that the antigenic structure of the fragments is the same as the corresponding parts of the intact molecule. Fragment CT has qualitatively the same immunological properties as fragment CS and does not cross-react with anti-(fragment NT).

Digestion of fragments CT and NT with subtilisin

The formation of fragment CS by subtilisin digestion of iron-ovotransferrin suggests that the *N*-terminal half of the protein is more rapidly digested than the *C*-terminal half. This is confirmed by the

finding that, during digestion with subtilisin, fragment NT shows a more rapid decrease in E_{470} than does fragment CT.

Metal content of fragment CS

The absorption spectrum of fragment CS in 0.1 M- NaHCO_3 showed a broad peak with λ_{max} at 460 nm. The iron content was 0.84 g-atom/35000 g. After removal of iron, fragment CS bound iron, with a

linear increase in E_{470} , up to a maximum value of 0.74 g-atom/35000 g. Copper binding occurred to a maximum value of 1.1 g-atom/35000 g to give a yellow-coloured complex with λ_{max} at 435 nm.

Rates of iron removal from fragments

Figs. 4(a) and 4(b) show the removal of iron from ovotransferrin and its *N*-terminal and *C*-terminal fragments, as judged by the fall in E_{470} . Fragment

Fig. 4. Rates of fall of E_{470} by ovotransferrin and fragments CS and NT in 0.1 M-sodium citrate, pH 5.65

Additions of sodium perchlorate were made as shown. (a) Loss of iron by fragments CS and NT. (b) Loss of iron by iron-saturated ovotransferrin. The theoretical curve was obtained by summing the curves for fragments CS and NT [see (a)]. (c) Loss of iron by monoferric ovotransferrin complexes. —, Fe-OT; ····, OT-Fe; ----, partially iron-saturated ovotransferrin prepared as described in the Materials and Methods section.

NT loses its iron much more rapidly than does fragment CS, and intact ovotransferrin shows an intermediate rate (Fig. 4b). However, summation of the curves for the two fragments does not reproduce the curve given by the whole protein. In the presence of perchlorate, iron is lost less rapidly both by the fragments and by the intact protein.

Transfer of iron to reticulocytes

Fragments CS and NT and ovotransferrin were all able to donate bound iron to rabbit reticulocytes at the same rate. There was no uptake of iron by control cells that contained only 1.6% reticulocytes.

One-iron-atom-ovotransferrin complexes

Two types of one-iron-atom-ovotransferrin complex were isolated by isoelectric focusing as described in the Materials and Methods section. They were both obtained as pink-coloured bands with pI 6.4. Fig. 4(c) shows that after treatment with sodium citrate, pH 5.65, the rate of loss of iron from OT-Fe is lower than that from Fe-OT. The curve (E_{470} against time) given by OT-Fe is very similar to that given by fragment CS (Fig. 4a). The rate of loss of iron from Fe-OT, on the other hand, is not as high as that from fragment NT or from iron-saturated ovotransferrin (Figs. 4a and 4b).

After digestion of the one-iron-atom-ovotransferrin complexes with trypsin, starch-gel electrophoresis showed the presence, in each case, of two main bands. The slower-moving band represents undigested ovotransferrin. The faster band, which has the same electrophoretic mobility in both cases, represents protein fragments, since its mobility is the same as that of fragments CT and NT. In both cases the faster band gave a precipitin reaction with antiserum to fragment NT and also with antiserum to fragment CS. Thus both complexes appear to yield a mixture of fragments NT and CT on trypsin digestion. It is difficult to estimate the relative amounts of the two types of fragment in each case, although Fe-OT appears to have given rise predominantly to fragment NT, since only a weak reaction was obtained with antiserum to fragment CS. On the other hand, when Fe-OT is formed by the addition of iron to apo-ovotransferrin at alkaline pH values and exposure to acid conditions is avoided, trypsin digestion yields only fragment NT and no reaction with antiserum to fragment CS is obtained.

Starch-gel electrophoresis of the fragments

In starch-gel electrophoresis at pH 8.6 the mobilities of fragments CT and NT are almost identical whereas fragment CS moves much faster.

Discussion

In the present paper two iron-binding fragments (designated CS and CT) from hen ovotransferrin are described. Since the new fragments differ from the *N*-terminal fragment described by Williams (1974) in many respects, it is suggested that they represent the *C*-terminal region of the protein. The *N*-terminal and *C*-terminal fragments differ in peptide 'maps', CNBr-cleavage patterns, amino acid and carbohydrate compositions, and in immunological properties. The immunological data appear to be particularly significant, since they show that together fragments CS and NT account for all the antigenic determinants possessed by ovotransferrin. The fragments differ also in their susceptibilities to digestion by subtilisin and in the rates at which they lose iron when exposed to mildly acid conditions. Moreover, it has been shown that the *N*-terminal and *C*-terminal fragments give characteristic e.p.r. (electron-paramagnetic-resonance) spectra (R. M. Butterworth, J. F. Gibson & J. Williams, unpublished work). Nevertheless, direct structural evidence for the view that the new fragments represent the *C*-terminal half of ovotransferrin is still lacking. For example, it has not yet been shown that fragments CS and CT carry the *C*-terminal residue of ovotransferrin, nor have the *N*-terminal amino acid sequences of the fragments been determined.

Fragments CS and CT are prepared with subtilisin and trypsin respectively, and this would be expected to cleave the polypeptide chain at different positions. The two fragments have different electrophoretic mobilities despite the similarities in their pI values and molecular weights. However, as judged by peptide 'maps', CNBr-cleavage patterns and immunological properties they must have very similar structures.

The *N*-terminal and *C*-terminal fragments have been obtained here by trypsin digestion of the appropriate one-iron-atom-ovotransferrin complex, i.e. Fe-OT and OT-Fe respectively. At pH 8.0, iron is bound specifically by the *N*-terminal site, to give Fe-OT, but at pH 5.0, iron is lost preferentially from the *N*-terminal site, to give predominantly OT-Fe. A possible explanation of these findings is that both the absolute and relative values of the association constants for the binding of iron to the two sites are pH-dependent. In this work the monoferric ovotransferrin complexes were isolated by isoelectric focusing at pH 6.4 and, as judged by the nature of the fragments formed on trypsin digestion, it appears that some exchange of iron atoms between the binding sites had occurred. Nevertheless, this exchange is unlikely to have been extensive, because the rates of loss of iron from the two complexes at pH 5.65 were markedly different and because the isolated complexes give e.p.r. spectra that are characteristic of the separate *N*-terminal and *C*-terminal fragments

(R. M. Butterworth, J. F. Gibson & J. Williams, unpublished work).

Several authors have proposed that the two iron-binding sites of serum transferrin have different physiological functions (Fletcher & Huehns, 1968; Hahn, 1973; Zapolski *et al.*, 1974). For example, it has been suggested that reticulocytes accept iron preferentially from one site. Isolated iron-binding fragments and the two types of monoferric transferrin complex may prove useful in testing these ideas. The present experiments show that the *N*-terminal and *C*-terminal fragments of hen ovotransferrin donate iron equally rapidly to rabbit reticulocytes. This gives no support to the theory mentioned above, but it must be stressed that the transferrin and the reticulocytes did not originate from the same species and that specific cellular discrimination between the binding sites of the protein might conceivably not operate between different groups of animals. It would also be desirable to study the ability of other types of iron-accepting cells to take up iron from transferrin fragments.

Clearly it is important that the fragments should be structurally intact in order to make valid comparisons with the whole protein. The immunological evidence suggests that the antigenic properties of the fragments are the same as those of the corresponding regions in the protein. The susceptibility of the fragments to digestion by subtilisin and the rates with which they lose iron under mildly acid conditions also appear to be characteristics that belong to the corresponding regions of the protein, since otherwise the preparation of fragments CS and CT by the methods described here would not have been possible. Nevertheless, it has been noted that the rate of iron loss by the whole protein at pH 5.65 does not appear to be a simple sum of the rates for the two fragments.

The use of NaClO₄ in these experiments was prompted by the observations of Price & Gibson (1972), which showed that NaClO₄ causes the prominent broad e.p.r. signals originating from the *N*-terminal site to be greatly diminished. The relatively much smaller broad signals associated with iron bound at the *C*-terminal site also disappear, and the resultant sharp spectra from the two sites are very similar. In the present experiments NaClO₄ caused a decrease in the rates of loss of iron at pH 5.65 from both the *N*-terminal and *C*-terminal sites. Thus the abolition of the broad signals and the accentuation of the sharp signals in the e.p.r. spectra appear to be associated with an increase in the tightness of binding

of iron. A considerable difference in the rates of iron loss was still present in 1.5M-NaClO₄ and was not diminished by raising the concentration of NaClO₄ to 3M.

This work and that reported earlier (Williams, 1974) is consistent with a structural model of ovotransferrin in which most of the protein is accounted for as two structurally independent regions which represent approximately the *N*-terminal and *C*-terminal halves. Such a structure might conceivably have arisen by duplication of a protein of molecular weight about 40000, as many have suggested. It is clear, however, that numerous changes in the two halves must have occurred in the course of evolution, and investigations into the primary structures of the fragments may be expected to throw some light on this problem.

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