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The Iron-Binding Properties of Hen Ovotransferrin

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1. The distribution of iron between the two iron-binding sites in partially saturated ovotransferrin was studied by labelling with ^{55}Fe and ^{59}Fe and by gel electrophoresis in a urea-containing buffer. 2. When iron is added in the form of chelate complexes at alkaline pH, binding occurs preferentially at the *N*-terminal binding site. In acid, binding occurs preferentially at the *C*-terminal site. 3. When simple iron donors (ferric and ferrous salts) are used the metal is distributed at random between the binding sites, as judged by the gel-electrophoresis method. The double-isotope method shows a preference of ferrous salts for the *N*-terminal site. 4. Quantitative treatment of the results of double-isotope labelling suggests that in the binding of iron to ovotransferrin at alkaline pH co-operative interactions between the sites occur. These interactions are apparently absent in the displacement of copper and in the binding of iron at acid pH.

Studies on a variety of transferrins, including ovotransferrin, serum transferrin and lactoferrin, have shown that the polypeptide chain is folded into two compact regions each able to bind one atom of iron. These regions have been isolated by limited proteolysis and shown to represent the *N*-terminal and *C*-terminal halves of the molecule (Williams, 1974, 1975; Brock *et al.*, 1976; Brock & Arzabe, 1976; Line *et al.*, 1976; Bluard-Deconinck *et al.*, 1978). The two halves are different in protein structures and metal-binding properties, although amino acid-sequence studies on human serum transferrin show that their primary structures are markedly similar (MacGillivray *et al.*, 1977).

Many studies of the iron-binding properties of hen ovotransferrin have been made, but with conflicting results. Warner & Weber (1953) found a large difference between the association constants for the first and second iron atoms by equilibrium dialysis and suggested that pairs of iron atoms bind to the protein. In that work pH values of 5.6–7.0 were used and citrate was present as a competing ion. However, true equilibrium was probably not reached in that study. An approximately random distribution of iron in partially iron-saturated ovotransferrin was observed by Aisen *et al.* (1966), by using electrophoresis in free solution at pH 7.25, and by Wenn & Williams (1968), by using isoelectric focusing. In the latter study only one monoferric complex was observed, but it was later found (Williams, 1975) that both monoferric species of ovotransferrin have the same isoelectric point. It is noteworthy also that during isoelectric focusing the protein is exposed to acid conditions (pH 5.8–6.8).

Other studies could not be reconciled with a random distribution of iron. Thus Aisen & Leibman (1968*a*) found by equilibrium dialysis that the association constant for the first iron atom bound was approx. 12 times that for the second. Starch-gel electrophoresis after addition of iron–nitrilotriacetate showed that the main protein species present at 50% iron saturation was a monoferric complex and that the diferric complex was practically absent at iron content less than this (Williams *et al.*, 1970). Similarly, differential scanning calorimetry showed that when iron–nitrilotriacetate is added to an alkaline solution of ovotransferrin, the endotherm that represents the diferric protein complex is not observed at iron contents below 50% saturation (Donovan *et al.*, 1976). Evans & Holbrook (1975) found that the quenching of protein fluorescence as iron–nitrilotriacetate is added to ovotransferrin at alkaline pH is biphasic, instead of showing the smooth curve expected for random binding of iron. All these results suggested that the first iron atom is bound more strongly than the second, but they did not show which of the two sites are occupied by the first and second iron atoms.

An answer to this question was first obtained in experiments using limited proteolysis of partially iron-saturated ovotransferrin (Williams, 1974, 1975). In these experiments iron–nitrilotriacetate was added to an alkaline solution of the protein. The protein that was not protected by iron was digested away with trypsin or chymotrypsin. In this way the *N*-terminal half-molecule was obtained, suggesting that the first iron atom binds specifically to the *N*-terminal binding site. The *C*-terminal half-molecule was not detected

in these experiments. When, on the other hand, diferric ovotransferrin was partially depleted of iron by exposure to acid conditions, limited proteolysis with trypsin yielded only the *C*-terminal half-molecule. It was later confirmed by e.p.r. spectroscopy that in the monoferric complex formed by the addition of iron–nitrilotriacetate to ovotransferrin in alkaline solution the single iron atom predominantly occupies the *N*-terminal binding site, whereas in the monoferric complex formed by partial iron depletion in acid solution the iron atom occupies the *C*-terminal site (Butterworth *et al.*, 1975). Donovan *et al.* (1976), however, have concluded from differential scanning calorimetry that when iron–nitrilotriacetate is added to ovotransferrin the first iron atom may bind indiscriminately to either binding site and that it then causes a marked decrease in the affinity for iron binding at the unoccupied site. This conclusion was based on the observation of two intermediate endotherms, which were assumed to represent monoferric protein complexes in which the iron atom was present at the two different binding sites, although no direct identification of the intermediate endotherms was made. The alternative possibility, that they represent two different conformations of the same monoferric complex, was considered unlikely, although the e.p.r. experiments of Butterworth *et al.* (1975) suggested that each bound iron atom is associated with two interchangeable environments.

The present studies were undertaken in the hope of clarifying this confused situation. The distribution of iron in partially iron-saturated ovotransferrin was studied by two methods. The first involves double-isotope iron labelling and subsequent isolation of the *C*-terminal half-molecule of the labelled protein. The second uses an electrophoretic method described by Makey & Seal (1976), who showed that human serum transferrin can be resolved into four components by polyacrylamide-gel electrophoresis in the presence of 6M-urea. The four components represent apotransferrin, two monoferric complexes and the diferric protein. It will be shown here that the distribution of iron between the two binding sites of ovotransferrin depends on the nature of the experimental conditions used.

Experimental

Nomenclature

The monoferric ovotransferrins are referred to as 'Fe-OT' or 'OT-Fe' according to whether the bound iron occupies the *N*-terminal or the *C*-terminal binding site respectively. The *C*-terminal half-molecule prepared by subtilisin digestion of iron-saturated ovotransferrin (Williams, 1975) is referred to as 'fragment CS'. The *N*-terminal and *C*-terminal half-molecules prepared by digestion of partially iron-saturated ovotransferrin with trypsin are referred to as 'fragment NT' and 'fragment CT' respectively.

Materials

Hen ovotransferrin was prepared as described by Williams (1968) and made iron-free by the method of Warner & Weber (1951). As judged by A_{470} the apo-ovotransferrin was less than 3% saturated with iron. Bovine trypsin type XI (treated with diphenylcarbamoyl chloride), bovine chymotrypsin type II and bacterial proteinase type VIII (from *Bacillus subtilis*) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Radioactive iron (as $^{59}FeCl_3$ and $^{55}FeCl_3$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Mes (4-morpholineethanesulphonic acid) was obtained from Sigma. Iron complexes were prepared as described by Woodworth (1966). Aquasol was obtained from New England Nuclear, Boston, MA, U.S.A.

Methods

Double-isotope iron labelling. In these experiments ^{59}Fe was added to ovotransferrin to give different degrees of partial saturation and ^{55}Fe was then used to give complete saturation. Fragment CS was isolated after proteolytic digestion of the labelled protein (Williams, 1975) and the ratio $^{59}Fe/^{55}Fe$ for the whole protein and for the *C*-terminal half-molecule was determined. The fractional saturation of the two metal-binding sites with the first isotope (^{59}Fe) was obtained from these ratios as described in the next section. The method assumes that no significant exchange of bound iron with free iron occurs under the conditions of the experiment. This appears to be well-founded, since Aasa *et al.* (1963) found the overall association constant to be approx. $5\times10^{23}M^{-1}$ and Aisen & Leibman (1968*b*) concluded that the rate constant for the back reaction cannot be greater than $10^{-13}s^{-1}$.

Details of the conditions used in different experiments are as follows. (i) Ovotransferrin (410mg) was dissolved in 30ml of buffer. The buffers used were 0.02M-NH_4HCO_3 and 0.1M-Tris/HCl, pH8.1. The solution was divided into 3ml portions to which were added different amounts of 0.01M-[^{59}Fe]iron–nitrilotriacetate. After 24h the A_{470} was measured and 0.01M-[^{55}Fe]iron–nitrilotriacetate was added to give complete saturation as judged by A_{470}. After a further period of 24h any unbound iron was removed by passing the sample through a column (100cm×2cm) of Sephadex G-100 in 0.02M-NH_4HCO_3. Bound iron is not removed under these conditions. A sample of the labelled protein was removed for radioactivity measurement and the rest was digested overnight with subtilisin (enzyme/substrate ratio 1:50, w/w) at 37°C. The digest was

passed through the same column of Sephadex G-100 to separate fragment CS.

(ii) To study the distribution of iron under acid conditions ovotransferrin was dissolved in 0.05M-Mes/0.02M-$NaHCO_3$, pH6.0, and iron was added as 0.01M-[^{59}Fe]iron–nitrilotriacetate. At all concentrations of iron used, bound and unbound iron existed in equilibrium, and the unbound iron was removed by passage of the sample through a column (100cm×2cm) of Sephadex G-100 in the pH6.0 buffer. Dissociation of iron was found to be slow relative to the gel-filtration step. To prevent dissociation of bound iron, fractions containing the protein peak were quickly made alkaline with drops of saturated NH_4HCO_3. Iron saturation was then completed as above with [^{55}Fe]iron–nitrilotriacetate.

(iii) Iron was also added to ovotransferrin in the form of iron(III)–ethylenediaminetetra-acetate or of iron(II) ascorbate. The concentration of the metal solutions was 0.01M and the buffers used were 0.04M-NH_4HCO_3 and 0.1M-$NaHCO_3$. With iron–ethylenediaminetetra-acetate, formation of the coloured complex was very slow and samples were left at 4°C for 6 days before addition of [^{55}Fe]iron–nitrilotriacetate to give complete saturation.

(iv) The displacement of bound copper by iron was also studied. Ovotransferrin dissolved in 0.1M-$NaHCO_3$ was saturated with 0.01M-$CuSO_4$, as judged by A_{440}, and then treated with [^{59}Fe]- and [^{55}Fe]-iron–nitrilotriacetate as described above. The copper–ovotransferrin complex was not subjected to gel filtration, since this caused loss of the copper. Instead, the radioactive iron solutions were added directly to the copper–ovotransferrin complex in 0.1M-$NaHCO_3$.

Radioactivity measurements. Radioactively labelled proteins were dissolved in 0.1M-$NaHCO_3$ at a concentration of 10mg/ml and 50μl samples were placed in glass vials with 5ml of Aquasol. Radioactivity was measured in a liquid-scintillation counter (Isocap 300; Nuclear Chicago). ^{55}Fe radioactivity was counted in the ^{3}H channel and ^{59}Fe in the ^{14}C and ^{3}H channels. Corrections to the observed counting rates were made by counting standard samples of ^{55}Fe- and ^{59}Fe-saturated ovotransferrin for radioactivity and the ratio ^{59}Fe/^{55}Fe was determined for each sample.

The distribution of ^{59}Fe between the *N*-terminal and *C*-terminal binding sites was obtained from these ratios, as follows. Let x be the fractional saturation of the *N*-terminal site by ^{59}Fe and y the fractional saturation of the *C*-terminal site by ^{59}Fe. Then $(1-x)$ and $(1-y)$ represent the fractional saturation of the *N*-terminal and *C*-terminal sites respectively by ^{55}Fe. If R is the ratio ^{59}Fe/^{55}Fe for the whole protein and r is the corresponding ratio for fragment CS, then

$$R=\frac{x+y}{2-(x+y)} \quad (1)$$

$$\text{and } r=\frac{y}{1-y} \quad (2)$$

$$\text{From eqn. (1) } x=\frac{2R}{1+R}-y \quad (3)$$

$$\text{From eqn. (2) } y=\frac{r}{1+r} \quad (4)$$

Substituting eqn. (4) in eqn. (3):

$$x=\frac{2R}{1+R}-\frac{r}{1+r} \quad (5)$$

The total amount of ^{59}Fe bound by the protein (C) was also obtained from the radioactivity measurements by using the equation $C=2R/(1+R)$ iron atoms/mol of protein. When these values were compared with the degree of saturation of the protein with ^{59}Fe as determined from the A_{470} the two methods agreed to within 2% for low iron contents (<20% saturation) and to within 8% for high iron contents (>80% saturation). In calculating the distribution of ^{59}Fe at different total iron contents the radioactive method for determining iron contents was used.

Polyacrylamide-gel electrophoresis. This was carried out essentially as described by Makey & Seal (1976) by using 6.5% (w/v) polyacrylamide gels. The stock buffer contained 2.0M-Tris, 0.2M-boric acid and 0.032M-EDTA adjusted to pH8.4. This buffer was diluted 20-fold with freshly deionized 6.3M-urea for the preparation of the gel, or with deionized water for the reservoir buffer. Samples (approx. 40μg of protein) were applied in 1cm application slots and electrophoresis was carried out at 100V for 17h. A vertical gel-electrophoresis apparatus (Raven, Haverhill, Suffolk, U.K.) was used. After staining and destaining, gels were cut into 1cm-wide strips and scanned at 560nm in a Hilger and Watts spectrophotometer fitted with a Gilford scanning attachment.

Samples for electrophoresis were prepared by adding increasing amounts of iron [as iron–nitrilotriacetate, $FeCl_3$ or $(NH_4)_2SO_4$,$FeSO_4$] to ovotransferrin dissolved in 0.1M-Tris/HCl, pH8.1, or 0.1M-$NaHCO_3$. Iron distribution at low pH was studied by adding iron–nitrilotriacetate to ovotransferrin in 0.05M-Mes/0.02M-$NaHCO_3$, pH5.5, and in this experiment each sample was passed through a column (100cm×2cm) of Sephadex G-100 in pH5.5 buffer to remove unbound iron before electrophoresis.

Preparation and identification of fragments. In some experiments iron was added to ovotransferrin to 30% saturation and digestion was then carried out with trypsin or chymotrypsin at an enzyme/substrate ratio

of 1:50 (w/w). The conditions used were as follows: (*a*) iron–nitrilotriacetate was added to ovotransferrin in 0.1 M-Tris/HCl/0.01 M-$CaCl_2$, pH 8.2, and trypsin was used for the digestion; (*b*) iron(II) ascorbate was added to ovotransferrin in 0.1 M-$NaHCO_3$ and chymotrypsin was used for the digestion. Details of the isolation of the fragments and their identification with rabbit antisera directed against ovotransferrin and fragments NT and CS have been described previously (Williams, 1974, 1975). Fragment CS is immunologically identical with fragment CT, which is obtained by trypsin digestion of OT-Fe.

Results

Double-isotope iron labelling

The distribution of iron between the *N*-terminal and *C*-terminal binding sites in partially saturated ovotransferrin under different conditions is shown in Table 1. When iron, in the form of iron–nitrilotriacetate, iron–ethylenediaminetetra-acetate or iron(II) ascorbate, is added to an alkaline solution of ovotransferrin it is preferentially bound by the *N*-terminal binding site. In acid solution, however, iron–nitrilotriacetate is preferentially bound by the *C*-terminal binding site. The displacement of bound

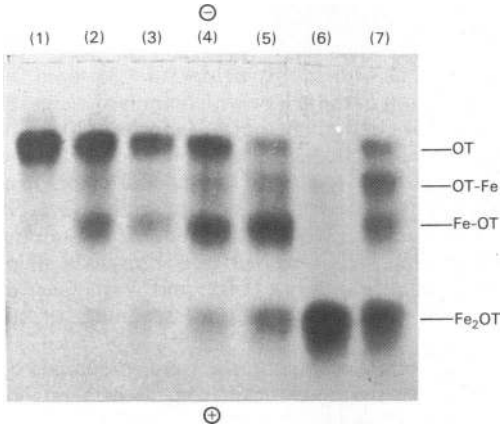

Fig. 1. *Urea/polyacrylamide-gel electrophoresis of ovotransferrin*

The first six samples represent ovotransferrin dissolved in 0.1 M-$NaHCO_3$ with different amounts of iron–nitrilotriacetate added to give 0, 20, 30, 40, 60 and 100% saturation. Sample 7 shows ovotransferrin, prepared as described in the Experimental section, with iron–nitrilotriacetate added to give 40% saturation. In the absence of added iron this material showed only bands for ovotransferrin and OT-Fe. Apo-ovotransferrin is designated OT and diferric ovotransferrin Fe_2OT.

Table 1. *Distribution of iron between the two binding sites of ovotransferrin at different iron contents*

The distribution is expressed as the fractional occupation of the *N*-terminal site (x) and the fractional occupation of the *C*-terminal site (y) by ^{59}Fe derived from the radioactivity measurements as described in the Experimental section. The total content of ^{59}Fe, expressed as atoms of iron/mol of protein, is given as *C*, which was also derived from the radioactivity measurements. The form of the iron and the nature of the buffer used in different experiments was: (1) iron–nitrilotriacetate, 0.1 M-Tris/HCl, pH 8.1; (2) iron–nitrilotriacetate, 0.1 M-$NaHCO_3$; (3) iron–nitrilotriacetate, 0.02 M-NH_4HCO_3; (4) iron(II) ascorbate, 0.1 M-$NaHCO_3$; (5) iron–ethylenediaminetetra-acetate, 0.04 M-NH_4HCO_3; (6) displacement of copper by iron–nitrilotriacetate, 0.1 M-$NaHCO_3$; (7) iron–nitrilotriacetate, 0.05 M-Mes/0.02 M-$NaHCO_3$, pH 6.0.

(1)		(2)		(3)		(4)		(5)		(6)		(7)	
C	*x*	*C*	*x*	*C*	*x*	*C*	*x*	*C*	*x*	*C*	*x*	*C*	*x*
0.29	0.22	0.21	0.16	0.35	0.28	0.25	0.18	0.53	0.37	0.35	0.15	0.44	0.14
0.53	0.45	0.41	0.29	0.57	0.44	0.50	0.34	0.67	0.42	0.79	0.39	0.78	0.22
0.74	0.61	0.61	0.45	0.80	0.59	0.72	0.50	0.88	0.50	0.95	0.42	1.00	0.27
0.93	0.78	0.82	0.61	0.99	0.71	0.94	0.63	1.22	0.71	1.22	0.58	1.16	0.35
1.05	0.74	1.10	0.73	1.20	0.78	1.19	0.76	1.47	0.88	1.39	0.68	1.28	0.41
1.25	0.81	1.29	0.76	1.41	0.81	1.30	0.77	1.51	0.81	1.45	0.67	1.35	0.44
1.66	0.96	1.57	0.86	1.77	0.90	1.63	0.89			1.56	0.75	1.47	0.59
		1.92	0.94	1.92	0.95	1.83	0.94					1.54	0.57

(1)		(2)		(3)		(4)		(5)		(6)		(7)	
C	*y*	*C*	*y*	*C*	*y*	*C*	*y*	*C*	*y*	*C*	*y*	*C*	*y*
0.22	0.07	0.16	0.05	0.28	0.07	0.18	0.07	0.37	0.16	0.15	0.20	0.14	0.30
0.45	0.08	0.29	0.12	0.44	0.13	0.34	0.16	0.42	0.25	0.39	0.40	0.22	0.56
0.61	0.13	0.45	0.16	0.59	0.21	0.50	0.22	0.50	0.38	0.42	0.53	0.27	0.73
0.78	0.15	0.61	0.21	0.71	0.28	0.63	0.31	0.71	0.51	0.58	0.64	0.35	0.81
0.74	0.31	0.73	0.37	0.78	0.42	0.76	0.43	0.88	0.59	0.68	0.71	0.41	0.87
0.81	0.44	0.76	0.53	0.81	0.60	0.77	0.53	0.81	0.70	0.67	0.78	0.44	0.91
0.96	0.70	0.86	0.71	0.90	0.87	0.89	0.74			0.75	0.81	0.59	0.88
		0.94	0.98	0.95	0.97	0.94	0.89					0.57	0.97

copper by iron–nitrilotriacetate occurs almost equally readily at either site.

Polyacrylamide-gel electrophoresis

It has been shown previously that ovotransferrin prepared as described here contains the monoferric complex OT-Fe (Williams, 1975) and it gave two bands in the urea/polyacrylamide-gel system. Since the slower band corresponds to ovotransferrin, the faster band must be OT-Fe. When the iron content of the protein was increased to 40% saturation by addition of iron–nitrilotriacetate two new faster bands appeared, giving a total of four bands. The fastest of these is diferric ovotransferrin, and it is proposed that the second band represents the monoferric complex Fe-OT (Fig. 1, sample 7).

The proportions of ovotransferrin, Fe-OT, OT-Fe and diferric ovotransferrin when iron is added to ovotransferrin under different conditions are shown in Fig. 2. When iron–nitrilotriacetate is added to ovotransferrin at pH 5.5 more OT-Fe than Fe-OT is formed (Fig. 2*a*), whereas at alkaline pH the formation of Fe-OT is preferred (Fig. 2*d*). When iron is added in the form of $FeCl_3$ or $(NH_4)_2SO_4$,$FeSO_4$ (Figs. 2*b* and 2*c*), there appears to be a slight preponderance of OT-Fe, although the proportions of the two species are close to those that would be expected for random distribution of iron between the two binding sites (Wenn & Williams, 1968).

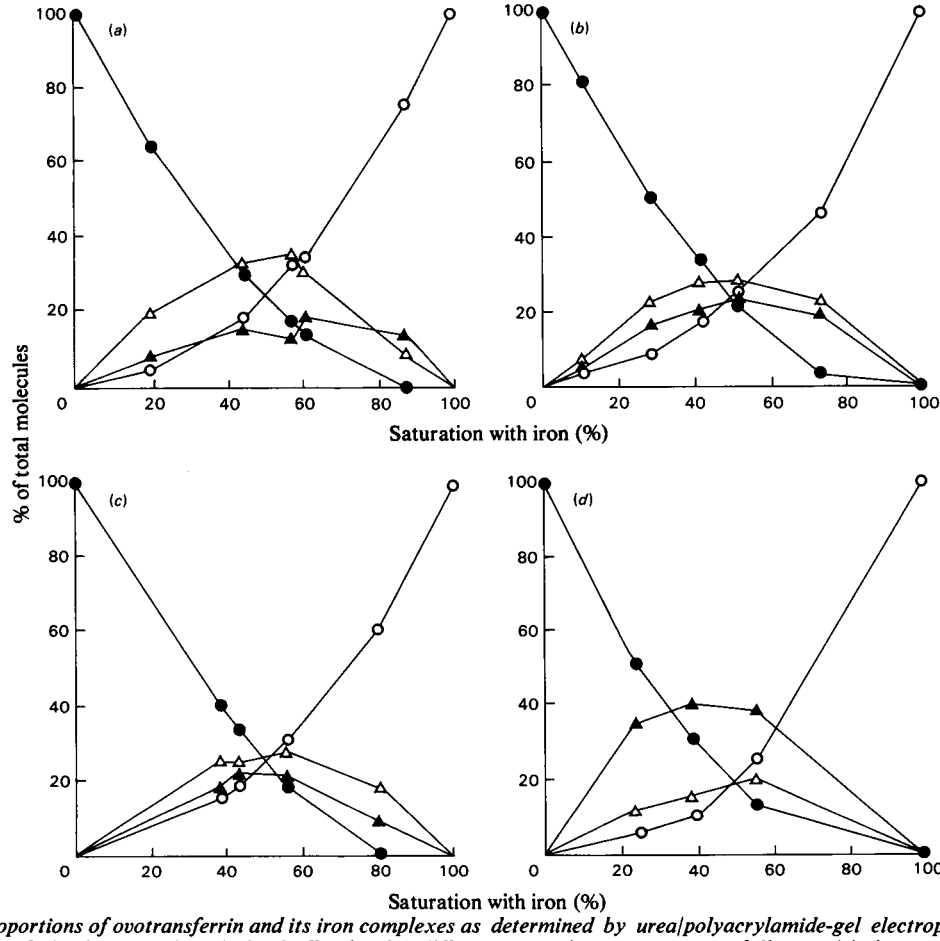

Fig. 2. *Proportions of ovotransferrin and its iron complexes as determined by urea/polyacrylamide-gel electrophoresis*
The form of the iron used and the buffer in the different experiments were as follows: (*a*) iron–nitrilotriacetate in Mes buffer, pH 5.5; (*b*) $FeCl_3$ in 0.1 M-$NaHCO_3$; (*c*) $(NH_4)_2SO_4$,$FeSO_4$ in 0.1 M-$NaHCO_3$; (*d*) iron–nitrilotriacetate in 0.1 M-$NaHCO_3$. The protein species are represented as follows: ●, ovotransferrin; △, OT-Fe; ▲, Fe-OT; ○, diferric ovotransferrin.

Limited proteolysis

Proteolytic digestion of partially iron-saturated ovotransferrin gave half-molecules binding one atom of iron. The nature of the fragments depended on the form of the iron added. When iron–nitrilotriacetate was added to an alkaline solution of ovotransferrin the only iron-binding fragment detectable immunologically was fragment NT. On the other hand, when iron(II) ascorbate was used proteolysis yielded a mixture of the *N*-terminal and *C*-terminal fragments (NT and CT) in approximately equal amounts.

Discussion

The distribution of iron in partially saturated ovotransferrin depends on the relative affinities for iron of the *N*-terminal and *C*-terminal binding sites and on possible interactions between the sites. Such interactions would be expected to influence the relative proportions of the monoferric and diferric protein species. In the present study we have used two methods to determine the distribution of iron. The urea/polyacrylamide-gel electrophoresis method shows both the relative amounts of iron bound by the two sites and the proportions of the different protein species (Fig. 2). The isotope-ratio method, on the other hand, shows only the distribution of iron between the two sites, but it is quantitatively more precise than the electrophoresis method.

Both methods show that iron distribution depends on the form of the iron added and on the pH of the solution. Thus under some conditions there exists a preference for one or other of the binding sites, whereas under other conditions the metal atoms appear to bind equally to the two sites. To a limited extent the conclusions based on the isotope-ratio method and the electrophoresis method are supported by the results of proteolysis experiments with partially saturated ovotransferrin. When the iron–chelate complexes iron–nitrilotriacetate and iron–ethylenediaminetetra-acetate were added to ovotransferrin in alkaline solution, there was preferential occupation of the *N*-terminal site, but in acid solution iron–nitrilotriacetate prefers the *C*-terminal site (Table 1, Fig. 2). These results conflict with the conclusion of Donovan *et al.* (1976) that iron (as iron–nitrilotriacetate) binds indiscriminately to the two sites, and it therefore seems unlikely that the two intermediate endotherms observed by these authors represent the two monoferric complexes Fe-OT and OT-Fe as they suggest.

When the uncomplexed forms $(NH_4)_2SO_4$,$FeSO_4$ and $FeCl_3$ were used there appeared to be little if any preference for one site compared with the other. In the case of iron(II) ascorbate the isotope-ratio method indicated a preference for the *N*-terminal site, whereas urea/polyacrylamide-gel electrophoresis showed an essentially random distribution, the latter conclusion being supported by limited proteolysis experiments. A second apparent discrepancy between the results of different methods concerns the binding of iron–nitrilotriacetate to ovotransferrin at alkaline pH. Limited proteolysis of 30%-saturated ovotransferrin revealed only fragment NT, suggesting that the only intermediate monoferric complex is Fe-OT. Urea/polyacrylamide-gel electrophoresis, however, showed that some OT-Fe is also formed. In a previous publication (Williams & Evans, 1977) we interpreted the results of double-isotope labelling experiments and limited proteolysis experiments as indicative of sequential binding of iron–nitrilotriacetate to the two sites, since it appeared from the proteolysis experiments that the monoferric complex OT-Fe was absent. The results of urea/polyacrylamide-gel electrophoresis now necessitate a revision of this conclusion. The causes of these discrepancies are not known. In contrast with the very inefficient donation of iron as $FeCl_3$ to human transferrin (Bates & Schlabach, 1973) very rapid binding of $FeCl_3$ to ovotransferrin was observed in the present work.

The data on the fractional saturation of the two sites given in Table 1 can also be examined quantitatively, as follows: assuming that iron atoms are bound independently by the two sites with probabilities p for the *N*-terminal site and q $(= 1-p)$ for the *C*-terminal site the fractional saturations, x and y, of the *N*-terminal and *C*-terminal sites respectively are given by

$$q\ln(1-x)/p = \ln(1+x-C)$$

and

$$p\ln(1-y)/q = \ln(1+y-C)$$

where C is the total amount of iron bound (see the Appendix). A graph of $\ln(1+x-C)$ plotted against $\ln(1-x)$ should therefore give a straight line of slope q/p, the relative affinities of the *C*-terminal and *N*-terminal binding sites. Non-linearity, indicating that the ratio q/p was not constant as the amount of iron was increased, could be due to co-operative interactions between the sites.

Fig. 3 shows the graphs obtained for some of the experimental conditions used here. For iron added as iron(II) ascorbate or iron–nitrilotriacetate at alkaline pH the graph is curved and the slope q/p changes from an initial value of approximately 0.25 to a final value of approximately 2.3 (data of Expt. 3, Table 1). This suggests that the preferred binding of iron at the *N*-terminal site leads to an enhanced binding affinity at the *C*-terminal site through an interaction between the sites. When iron merely displaces copper from the binding sites the graph appears to be linear with slope $q/p = 1.2$. Finally, when iron–nitrilotriacetate binds to ovotransferrin at pH 6.0 the graph is linear with slope $q/p = 4.0$, showing that at acid pH the *C*-terminal site binds

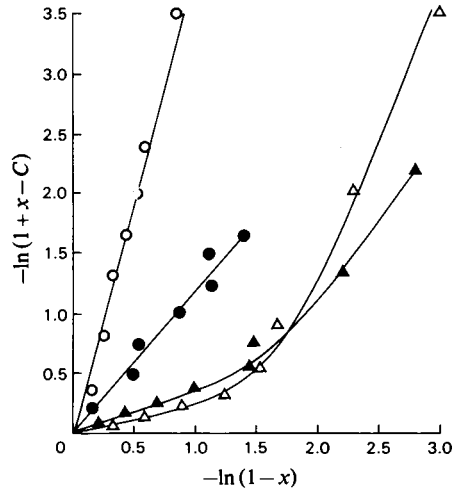

Fig. 3. *Fractional saturation of the N-terminal site (x) as a function of the total iron content (C) expressed as a graph of $-ln(1+x-C)$ against $-ln(1-x)$ (see the text and the Appendix)*

The form of iron and buffer used are as follows: ○, iron–nitrilotriacetate added to ovotransferrin in Mes buffer, pH 6.0 (Expt. 7, Table 1); ●, the displacement of bound copper by iron–nitrilotriacetate in 0.1 M-$NaHCO_3$ (Expt. 6, Table 1); △, iron–nitrilotriacetate added to ovotransferrin in 0.02 M-NH_4HCO_3 (Expt. 3, Table 1); ▲, iron(II) ascorbate added to ovotransferrin in 0.1 M-HCO_3 (Expt. 4, Table 1).

iron with more affinity than the *N*-terminal site. Co-operative interactions appear to be absent at this pH, but the degree of saturation with ^{59}Fe reached in this experiment ($C = 1.54$), and also in the copper-displacement experiment, is lower than in the other cases shown, so that no direct comparison can be made.

The difference between the relative binding affinities of the *N*-terminal and *C*-terminal sites appears to be present in the isolated 'half-molecule' fragments since Evans *et al.* (1977) found by differential-scanning calorimetry that iron–nitrilotriacetate added to an alkaline solution containing fragments NT and CT was preferentially bound by the *N*-terminal fragment. It would be of interest to know whether the weak co-operative effects observed with the intact protein also persist in a mixture of fragments, suggesting non-covalent association of the separate domains.

As pointed out in the Methods section the overall association constant for the binding of iron by human transferrin is approx. $5 \times 10^{23}\,M^{-1}$ (Aasa *et al.*, 1963) and the rate constant for the dissociation of the complex cannot exceed $10^{-13}\,s^{-1}$ (Aisen & Liebman, 1968*b*). Since differential binding affinities of the two sites on transferrin have now been established it must be concluded that the initial metal–protein complex formed when an iron chelate is added to the protein has an appreciable rate of dissociation and that a process of 'locking in' subsequently occurs, possibly as the chelate moiety is displaced by the carbonate ion (Aisen *et al.*, 1967).

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