The Isoelectric Fractionation of Hen's-Egg Ovotransferrin

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1. Hen ovotransferrin was examined by isoelectric fractionation. 2. The major component observed in starch-gel electrophoresis can be isolated from the minor component. 3. When non-saturating amounts of iron are added to ovotransferrin, isoelectric fractionation demonstrates the existence of three molecular species corresponding to the metal-free protein, the one-iron-atom-protein complex and the two-iron-atoms-protein complex. 4. Isoelectric fractionation of human serum labelled with ^{59}Fe suggests that the transferrin of normal human serum also exists as metal-free protein, the one-iron-atom-protein complex and the two-iron-atoms-protein complex. 5. It is concluded that the binding constants for the first and second iron atoms are similar.

Several workers have observed that on starch-gel electrophoresis purified preparations of ovotransferrin show a major component and a minor component (Lush, 1961; Williams, 1962; Clark, Osuga & Feeney, 1963). The nature of the difference between these two varieties of ovotransferrin is not known and various attempts to prepare them in isolation have failed. We therefore decided to apply Svensson's method of isoelectric fractionation to this problem, since this method can separate proteins possessing closely similar isoelectric points (Vesterberg & Svensson, 1966). In the present paper it is shown that major and minor components of hen ovotransferrin can be separated by isoelectric fractionation.

During the work it became clear that the isoelectric patterns were relevant to the problem of iron binding by ovotransferrin and serum transferrin. At the present time there are two opposed ideas on this problem. The first, proposed by Warner & Weber (1953), is that the binding of one atom of iron modifies the structure of the proteins so that the binding of a second atom is facilitated and the two metal atoms are effectively bound as a pair. The second (Aasa, Malmström, Saltman & Vänngård, 1963) is that the metal atoms bind randomly to the two sites on the protein with approximately equal binding constants. The isolation in the present work of an ovotransferrin-iron complex containing only one atom of iron supports the latter idea.

MATERIALS AND METHODS

Reagents. Nitrilotriacetic acid was obtained from British Drug Houses Ltd., Poole, Dorset. 2-Nitroso-1-naphthol-4-sulphonic acid was obtained from Kodak Ltd., Kirkby,

Lancs. Radioactive iron ($^{59}\text{FeCl}_3$ in 0.1 N-HCl) was purchased from The Radiochemical Centre, Amersham, Bucks. Hydrolysed starch (lot no. 227-1) for gel electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada. All other reagents were of analytical grade. Solutions were made up with glass-distilled water. For experiments involving iron-free protein the apparatus was soaked in 0.1 M-citric acid and then rinsed with deionized water. Deionized water was also used in making up solutions in these experiments.

Preparation of proteins. Ovotransferrin was prepared from hen's-egg white as described by Williams (1968) and the iron-free protein was prepared by the method of Warner & Weber (1951).

Isoelectric fractionation. The isoelectric column (SC. 1, vol. 110 ml.) was obtained from LKB Produkter AB, Stockholm-Bromma 1, Sweden. Ampholyte solution (development batch no. 1, pH range 5-8, and batch no. 1, pH 5-7) was obtained from the same source. Vokam power packs (nos. 2541 and 2540, Shandon Scientific Co., London, N.W. 10) were used. To avoid heating effects the power supply was not allowed to exceed 1 W, and water at about 9° was circulated round the column. The column was silicone-treated with Repelcote (Hopkin and Williams Ltd., Chadwell Heath, Essex) before use, as recommended by Carlström & Vesterberg (1967). Current was passed through the column for about 36 hr. and during the final 24 hr. the voltage was held at 500 V. Finally, the column was drained at approx. 1 ml./min. and small fractions (0.6 ml.) were collected. The protein content of the fractions was estimated from the E_{280} value, determined with the Unicam SP.500 by using microcuvettes (1 cm. light-path, vol. 0.5 ml.). In some experiments the absorption was measured continuously with the Uvicord II (LKB 8300; LKB-Produkter AB).

Measurement of pH. Measurements were made at 20° with either a Pye model 79 pH-meter or an EIL Vibron electrometer (model 33-B) coupled to a pH-measuring unit (model C33B).

Amino acid analyses. Protein fractions recovered from

the isoelectric column were dialysed, freeze-dried and hydrolysed with 5.7N-HCl for 24 hr. at 105°. Analyses were carried out with a Technicon amino acid analyser. A sample of ovotransferrin with pH_I 5.78 was oxidized with performic acid for the determination of cysteic acid. Tryptophan was determined on a sample of ovotransferrin by the method of Spies & Chambers (1949). Denaturation of the protein was produced by prior hydrolysis with pepsin (Harrison & Hofmann, 1961). Amide content was determined by measuring the NH_3 content of a protein sample after heating it with 2N-HCl for 2 hr. at 100°. A suitable portion was diluted to 0.1N-HCl before application to the amino acid analyser. The NH_3 content of an unhydrolysed sample was subtracted.

Determination of iron. Since only small amounts of protein were available for iron analysis the sensitive 2-nitroso-1-naphthol-4-sulphonic acid method of Pollard, McOmie, Banister & Nickless (1957) was used.

Protein samples (5–20 mg.) were dissolved in 1.5 ml. of deionized water. After the addition of 1.5 ml. of 10% (w/v) trichloroacetic acid each sample was centrifuged. To 2.0 ml. of the supernatant was added 1.0 ml. of 5% (w/v) hydroxy-ammonium chloride and 0.025 ml. of aq. 0.005% methyl orange. Drops of 2N-NaOH were added until the methyl orange just turned yellow, and 1.0 ml. of the iron reagent [0.247% 2-nitroso-1-naphthol-4-sulphonic acid in aq. 20% (v/v) methanol] was added. The mixture was then made up to 10 ml. with 0.2M-sodium acetate buffer, pH 5.0. The E_{700} values were measured. A standard curve was prepared with $Fe(NH_4)_2(SO_4)_2$. All solutions used in the determinations were made up with deionized water. Preliminary experiments showed that protein did not interfere with the determinations.

Radioactive labelling of ovotransferrin with ^{59}Fe . A volume of 20 mm- $Fe(NO_3)_3$ that was calculated to be sufficient to give an approximate half-saturation of the iron-binding sites of iron-free ovotransferrin was mixed with 0.1 ml. of $^{59}FeCl_3$ solution. This solution was then added to the iron-free protein in the presence of an excess of $NaHCO_3$. After dialysis against water the labelled protein was subjected to isoelectric fractionation and the radioactivity present in the various components measured by drying 0.05 ml. from each fraction on an aluminium planchet and counting with a gas-flow counter.

Radioactive labelling of human serum with ^{59}Fe . Human blood was allowed to clot and the serum was removed. To 0.5 ml. of serum was added 0.005 ml. of $^{59}FeCl_3$ in 0.1N-HCl to trace-label the transferrin. In some cases, after addition of the label, non-radioactive $Fe(NO_3)_3$ was added to saturate the transferrin completely. The samples were then subjected to isoelectric fractionation. Radioactivity measurements were carried out on 0.05 ml. samples from each fraction.

Addition of non-radioactive iron to ovotransferrin. Samples of ovotransferrin saturated to different extents with iron were prepared by the addition of Fe^{3+} [as 20 mm- $Fe(NO_3)_3$] to the iron-free protein in the presence of excess of $NaHCO_3$. The degree of saturation was found by measuring the relative proportions of the three ovotransferrin species bearing 0, 1 and 2 atoms of iron/mol. of protein after isoelectric fractionation.

In some cases the iron was added in the form of an iron-nitritotriacetate complex, which was prepared essentially as described by Woodworth (1967). The iron-free protein was

dissolved in buffer (pH 8.6) containing 0.1M-tris and 1 mm- $NaHCO_3$.

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis was carried out with the buffer system of Poulik (1957). No attempt was made to remove iron from the buffers or the starch.

RESULTS

Isoelectric fractionation. Isoelectric fractionation of ovotransferrin that had been approximately half-saturated with iron revealed six protein fractions (Fig. 1). Peaks 1 and 2 were colourless, but peaks 3, 4, 5 and 6 were visible as pink bands before the column was drained. Table 1 shows the pH values measured in the peak tubes, each value representing the mean from several determinations. These pH values were the isoelectric points of the protein fractions, uncorrected for the effect of sucrose. The difference in isoelectric points between peaks 1 and 3 and between peaks 3 and 5 was approx. 0.48 pH unit. The difference in isoelectric points between peaks 1 and 2, between peaks 3 and 4 and between peaks 5 and 6 was approx. 0.18 pH unit.

Starch-gel electrophoresis was carried out on fractions 1–6 recovered from the isoelectric column. Fractions 1, 3 and 5 all moved with the mobility of the slower, major, electrophoretic component of ovotransferrin. Fractions 2, 4 and 6 all moved with

Fig. 1. Distribution of E_{280} after isoelectric fractionation of partially iron-saturated ovotransferrin.

Table 1. Isoelectric points of the fractions shown in Fig. 1 measured as the pH values in the peak tubes

The value for peak 2 is a single determination.

Peak no.	Mean pH_I value $\pm$ sd.
1	6.73 $\pm$ 0.04
2	6.50
3	6.25 $\pm$ 0.05
4	6.05 $\pm$ 0.02
5	5.78 $\pm$ 0.02
6	5.62 $\pm$ 0.02

Table 2. Amino acid compositions of unfractionated ovotransferrin and of the species with $pH_{I}5.78$ and $pH_{I}5.62$

Protein fraction ...	Composition (moles/80 000 g. of protein)		
	$pH_{I}5.78$	$pH_{I}5.62$	Unfractionated
Amino acid			
Cysteic acid	29.7	29.7	29.1
Asp	83.2	81.5	82.0
Thr	38.7	38.5	36.6
Ser	51.6	46.9	45.7
Glu	80.4	75.1	77.4
Pro	31.0	30.1	28.9
Gly	58.7	58.8	56.0
Ala	59.4	57.6	54.8
Val	54.3	54.4	54.5
Met	11.5	11.6	13.1
Ile	25.5	26.9	28.0
Leu	50.2	53.8	54.7
Tyr	21.7	22.1	22.3
Phe	27.2	28.4	28.5
Lys	62.5	66.4	64.9
His	14.7	13.8	13.3
Arg	31.8	33.8	35.6
Trp	—	—	12.7
Amide	—	—	70.0

the mobility of the faster, minor, electrophoretic component.

When fully iron-saturated ovotransferrin was subjected to isoelectric fractionation only peaks 5 and 6 were observed. Iron-free ovotransferrin gave rise only to peaks 1 and 2.

Amino acid analyses. Table 2 shows amino acid compositions of peaks 5 and 6 and of unfractionated ovotransferrin. The cysteic acid value for peak 5 was obtained from a sample that had been oxidized with performic acid. There was not sufficient peak 6 material for the determination of cysteic acid, and the calculations assume that it contained the same amount as peak 5.

Iron analyses. Fully iron-saturated ovotransferrin was found to contain 1.96 atoms of iron/mol. of protein, assuming a value of 80 000 for the molecular weight of ovotransferrin. After recovery from the isoelectric column peak 5 was found to contain 1.80 atoms of iron/mol. of protein.

The iron content of 'iron-free' ovotransferrin and of peak 1 after recovery from the column was less than 0.02 atom of iron/mol. of protein.

Peak 3, from the column, was found to contain 0.70 atom of iron/mol. of protein.

Radioactive (^{59}Fe -labelled) ovotransferrin. Ovotransferrin that had been partially iron-saturated with ^{59}Fe gave the pattern shown in Fig. 2 when subjected to isoelectric fractionation. The ratio of radioactivity to E_{280} was determined for peaks 3

Fig. 2. Distribution of radioactivity ($\square$) and E_{280} ($\circ$) after isoelectric fractionation of ovotransferrin partially saturated with radioactive iron. Only that part of the graph that includes peaks 3 and 5 is shown.

Table 3. Ratio of the specific radioactivity (area under radioactivity curve/area under E_{280} curve) of peak 3 to the specific radioactivity of peak 5

Expt. no.	Ratio of specific radioactivities.
1	1.113:2.000
2	1.023:2.000
3	0.998:2.000

and 5, and the values obtained in three experiments (Table 3) show that peak 5 contains twice as much iron per protein molecule as does peak 3.

Distribution of the ovotransferrin varieties. The data presented above suggest that peaks 1 and 2 represent the major and minor components of ovotransferrin in the iron-free state, that peaks 3 and 4 represent these molecules bearing one atom of iron and that peaks 5 and 6 are the fully iron-saturated forms of the ovotransferrin components. Preparations of ovotransferrin containing different amounts of iron were examined by isoelectric fractionation and the relative proportions of molecules carrying 0, 1 and 2 atoms of iron were estimated from the areas under the E_{280} curve. The relative proportions of these three species that

Fig. 3. Relative proportions of iron-free ovotransferrin (C_0), half iron-saturated ovotransferrin (C_1) and fully iron-saturated ovotransferrin (C_2) at different degrees of iron-saturation. The curves represent the theoretical distributions, assuming random binding of iron atoms at two sites on each protein molecule. The relative amounts of the three types of protein are given by the equations $C_0 = C - X + \frac{X^2}{4C}$, $C_1 = X - \frac{X^2}{2C}$ and $C_2 = \frac{X^2}{4C}$, where C , the total amount of protein, is equal to $C_0 + C_1 + C_2$, and X , the amount of iron, is expressed in terms of C and has values from 0 to $2C$. The experimentally obtained values are plotted thus: ●, C_0 ; ■, C_1 ; ▲, C_2 .

Fig. 4. (a) Distribution of radioactivity in human serum, trace-labelled with ^{59}Fe and subjected to isoelectric focusing for 24 hr. (b) Similar experiment in which the serum was trace-labelled with ^{59}Fe and then saturated with non-radioactive iron.

would be expected if the two atoms of iron were to bind randomly to the two binding sites on the protein, i.e. with equal binding constants, were determined theoretically. Fig. 3 shows that the experimental values agree reasonably well with the theoretical curves.

Iron-binding components in human serum. Isoelectric fractionation of human serum after the addition of a trace amount of ^{59}Fe gave the pattern of radioactivity shown in Fig. 4. Two major peaks of radioactivity were present at $\text{pH } 5.2$ and $\text{pH } 5.5$. After the addition of a saturating amount of non-radioactive iron, radioactivity at $\text{pH } 5.5$ was markedly diminished and that at $\text{pH } 5.2$ increased. In these experiments the duration of the run was reduced to 24 hr., since it was found that when the transferrin was allowed to remain for 3 days at its isoelectric point ($\text{pH } 5.2$) the more acid component gave rise to the component with $\text{pH } 5.5$, probably because of loss of iron.

DISCUSSION

These experiments show that the minor component of ovotransferrin observed in starch-gel electrophoresis possesses an isoelectric point about 0.18 pH unit lower than that of the major electrophoretic component. Williams (1962) suggested that the two species are immunologically related to one another and the amino acid compositions obtained in the present study show a close similarity between them. Although there was insufficient material for iron analysis on the minor component, the isoelectric patterns suggest that it is similar to the major component in being able to bind either one or two atoms of iron.

Warner & Weber (1951) were the first to show, by means of moving-boundary electrophoresis, that the isoelectric point of iron-saturated ovotransferrin ($\text{pH } 5.8$) is lower than that of the iron-free protein ($\text{pH } 6.8$). They also observed a third boundary of intermediate mobility and suggested that it might represent ovotransferrin molecules with only one atom of iron/molecule. The isoelectric points obtained in the present work agree closely with those of Warner & Weber (1951): 6.73 for the iron-free protein, 6.25 for the intermediate species carrying one atom of iron and 5.78 for the iron-saturated protein. Aisen, Leibman & Reich (1966) also examined human serum transferrin and hen ovotransferrin by free electrophoresis and in each case observed three species with differing mobilities. The relative proportions of the species varied with the degree of saturation with iron. Equilibrium-dialysis experiments on the binding of iron by serum transferrin were carried out by Aasa *et al.* (1963), who concluded that the metal binds randomly to two independent and equivalent sites on the

protein. The distribution of the iron-free, half-saturated and fully iron-saturated ovotransferrin species observed by isoelectric fractionation (Fig. 3) clearly agrees with this conclusion.

Other workers have suggested that the binding constant for the second atom of iron is much greater than that for the first, so that iron atoms effectively bind to the protein in pairs. This conclusion was reached by Warner & Weber (1953) from a study of the dissociation of iron-saturated ovotransferrin in the presence of citrate and by Woodworth (1967) from a study of the rate of formation of the coloured complex of transferrin and iron-nitrilotriacetic acid. A study of iron exchange between transferrin and EDTA by means of equilibrium dialysis (Davis, Saltman & Benson, 1962) also suggested that the binding constants of the first and second atoms of iron were widely different ($pK_1 = 27.7$, $pK_2 = 30.3$). The isolation of the ovotransferrin molecule bearing one atom of iron appears to be incompatible with these data and conclusions, but it is difficult to understand the origin of this contradiction.

Schade (1963) studied the uptake of ^{59}Fe by liver cells from iron-transferrin (or siderophilin) and concluded that 'a one-iron-atom-siderophilin complex would not be expected to exist as a stable and observable molecular species in serum'. Since this conclusion conflicts with the data obtained with ovotransferrin it was decided to test it in the following manner.

It is generally believed that transferrin is the principal iron-binding component of serum, where it exists in a partially unsaturated state. If a one-iron-atom-transferrin complex exists in serum it would be expected that the addition of a trace of radioactive iron would give rise to two radioactive protein components: one would represent the addition of ^{59}Fe to the iron-free protein and the other would represent the binding of ^{59}Fe by the one-iron-atom-transferrin complex. By analogy with the ovotransferrin results the former radioactive product should have a higher pH_1 than the latter, and saturation of the trace-labelled serum with non-radioactive iron should convert the radioactive one-iron-atom-protein complex into the fully saturated form. If, alternatively, the serum transferrin exists only as a mixture of metal-free and fully saturated molecules, and iron binding is an 'all-or-none' phenomenon, then the addition of a trace of ^{59}Fe should lead to only one radioactive product corresponding to the fully saturated form.

The data shown in Fig. 4 demonstrate that two main radioactive products are present after trace-labelling of serum with ^{59}Fe , and that saturation with non-radioactive iron largely abolishes the more alkaline component ($pH_1 5.5$) and increases the acid component ($pH_1 5.2$). This suggests strongly that the former represents the one-iron-atom-trans-

ferrin complex and the latter the fully saturated transferrin.

As mentioned in the Results section, it was observed that when the fully saturated transferrin was allowed to focus at its isoelectric point for long periods a partial reversion to the one-iron-atom-transferrin complex occurred. It is likely that the low pH at the isoelectric point of the fully saturated transferrin facilitates loss of iron whereas the resultant one-iron-atom-transferrin complex is relatively more stable because of its higher isoelectric point. This probably also explains the presence of a small amount of one-iron-atom-transferrin even when focusing was carried out for 24 hr.

The failure of starch-gel electrophoresis to separate the forms of ovotransferrin carrying different amounts of iron has also been observed for human serum transferrin by Roop & Putnam (1967). By avoiding contamination from iron present in the starch, however, Sass-Kortsak & Vamosi (1964) were able to separate human serum transferrin in the iron-saturated state from the iron-free protein. Similarly, the separation of transferrin from iron-transferrin by polyacrylamide-gel electrophoresis in tris-EDTA-borate buffer at pH 9.2 was reported by Yashioka, Fujii & Ito (1966). In this case, however, the iron-transferrin had a lower mobility than the iron-free protein, and this is the reverse of what would be expected from their isoelectric points. Possibly the resolution depended more on a conformational difference than on a charge difference, since Bezkorovainy (1966) has shown that the axial ratio of iron-saturated transferrin is higher than that of the iron-free protein. Stratil (1967) has recently reported that starch-gel electrophoresis can be used to separate ovotransferrin molecules bearing different numbers of iron atoms.

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