Formation of Monoferric Ovotransferrins in the Presence of Chelates

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1. When ovotransferrin is partially saturated with iron, endotherms for apo-ovotransferrin, two monoferric ovotransferrins and Fe_2 -ovotransferrin are observed by differential scanning calorimetry. The relative sizes of the endotherms are changed in the presence of the iron-chelating agents nitrilotriacetic acid and ATP. 2. When iron is added as Fe(III) -nitrilotriacetate, at Fe -nitrilotriacetate:ovotransferrin ratios less than unity, the endotherm for Fe_2 -ovotransferrin is essentially absent. At Fe -nitrilotriacetate:ovotransferrin ratios of unity the only species present in solution in appreciable concentration as evidenced by their differential-scanning-calorimetry endotherms, are two monoferric ovotransferrins in approximately equal amounts. At Fe -nitrilotriacetate:ovotransferrin ratios greater than unity, the apo-ovotransferrin endotherm is absent, and the endotherms for the two monoferric ovotransferrins decrease in size as the endotherm for Fe_2 -ovotransferrin increases. 3. In the presence of nitrilotriacetate, binding of iron to the two sites of ovotransferrin is highly anti-co-operative, but essentially indiscriminate. When monoferric ovotransferrin is formed from apo-ovotransferrin, binding at one site is slightly favoured compared with binding at the other site, but once iron has been bound at either site, the binding affinity for iron at the unoccupied site is much decreased.

Ovotransferrin (conalbumin) has two sites which bind iron(III) strongly. Measurement of the binding by equilibrium dialysis shows that the binding constant for iron is very large (of the order of 10^{30} ; Warner & Weber, 1953), but that the two stoichiometric binding constants do not differ greatly (Aisen & Leibman, 1968). There is disagreement on whether iron binding is co-operative (Woodworth, 1966) or anti-co-operative (Evans & Holbrook, 1975). However, at least one monoferric ovotransferrin has been isolated by electrophoretic methods (Wenn & Williams, 1968; Aisen *et al.*, 1973) and the isolation of two different monoferric ovotransferrins has been reported (Williams, 1975).

We have used the experimental technique of differential scanning calorimetry to investigate the binding of iron to ovotransferrin, and have shown the marked increase in heat stability produced on the binding of iron. Four different endotherms are observed on adding iron to ovotransferrin (Donovan & Ross, 1975). These endotherms have been attributed to thermal denaturation of apo-ovotransferrin, Fe_2 -ovotransferrin and two monoferric ovotransferrins.

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This paper presents further evidence for the existence of two monoferric ovotransferrin species and considers the kinetics of their formation in the presence and absence of iron-chelating agents, as well as their relative binding constants for iron.

Experimental

Materials

Apo- and Fe_2 -ovotransferrin were prepared from the white of fresh eggs from White Leghorn chickens by a modification of the method of Azari & Baugh (1967). The following absorption coefficients ($E_{1\text{cm}}^{1\%}$) were used to determine concentration: ovotransferrin in 0.2M-HCl, 11.0 at 280nm, and at pH6, 11.3 at 280nm; Fe_2 -ovotransferrin at pH6, 0.621 at 470nm (Glazer & McKenzie, 1963). A mol.wt. of 76600 was assumed (Warner & Weber, 1953). The u.v. absorption of this ovotransferrin preparation agreed with the expected absorption in the visible range when the ovotransferrin was saturated with iron, indicating absence of other protein contaminants. In addition, differential-scanning-calorimetry thermograms did not contain extraneous peaks for denaturation of the other egg-white proteins.

Tris buffer was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., nitrilotriacetic acid from Dow Chemical Co., Midland, MI, U.S.A., and ATP

from Nutritional Biochemical Corp., Cleveland, OH, U.S.A. Other chemicals were of reagent grade. Distilled water was deionized by passage through a mixed-bed ion-exchange cartridge (D0803; Barnstead Still and Sterilizer Co., Boston, MA, U.S.A.). Unchelated iron was added as a freshly prepared solution of ferrous ammonium sulphate in water. Fe-nitrilotriacetate solutions (10% molar excess of nitrilotriacetate over iron) were prepared by adding solid ferric nitrate and nitrilotriacetate to water and titrating with KOH to pH 5. All solutions were air-equilibrated.

Procedure

Titration with iron were made by adding small portions of solutions of iron (weighed, or measured by volume) to 0.50 ml of protein stock solution. When added separately, ATP or nitrilotriacetate was added after iron. Changes in pH produced by mixing solutions did not exceed 0.05 pH unit. Measurements of pH were made at room temperature (22°C). For calorimetric measurements, samples of 20 μ l of stock solution were weighed into differential-scanning-calorimetry hermetic pans, and the pans sealed. Differential-scanning-calorimetry thermograms (plots of heat flow as a function of temperature) were recorded on a Du Pont model 990 thermal analyser with a differential-scanning-calorimetry cell. The reference material was a sealed pan containing water. The calibration of the instrument and various tests of the validity of the thermograms have been described (Donovan & Ross, 1973; Donovan & Beardslee, 1975). The denaturation temperature (T_d) is the temperature of the peak of the endotherm (flow of heat into a sample as a protein species undergoes denaturation), observed at the usual heating rate of 10°C/min. Since the denaturation is irreversible at pH 7.5, T_d is a function of heating rate, and no characteristic T_d exists.

The area under an endotherm is proportional to the enthalpy of denaturation of the species denatured. Calculation of enthalpy of denaturation from endotherm area has been described (Donovan & Ross, 1973). Enthalpies of denaturation (10⁶ J/mol) of the ovotransferrin species are: ovotransferrin, 1.33; intermediate I, 1.6; intermediate I', 2.2; Fe₂-ovotransferrin, 2.62. Enthalpies of denaturation of the intermediates were obtained by linear interpolation between the enthalpies of denaturation of ovotransferrin and Fe₂-ovotransferrin (Donovan & Ross, 1975). Enthalpies of denaturation of a number of other proteins have a linear dependence on temperature (Privalov & Khechinashvili, 1974). For the present work, the listed enthalpies of denaturation of the four ovotransferrin species were used to calculate the relative amounts of these species present in solution from observed endotherm areas.

Results

Differential-scanning-calorimetry thermograms of ovotransferrin partially saturated with iron

Fig. 1 shows endotherms obtained for the heat denaturation of apo-ovotransferrin (curve *a*) and Fe₂-ovotransferrin (curve *d*). Binding of iron markedly increases both the temperature and enthalpy of denaturation of ovotransferrin (Donovan & Ross, 1975). When less than 2 mol of iron per mol of ovotransferrin is added in the form of ferrous (Ross *et al.*, 1968; Bates *et al.*, 1973) or ferric salts in the absence of chelator, thermograms such as curve (*b*) are obtained with four endotherms at characteristic temperatures. On the basis of material balances for both iron and protein the second and third endotherms, in the order of increasing temperature, can be attributed to the denaturation of two different monoferric ovotransferrins (Donovan & Ross, 1975). We refer to these endotherms, as well as to the monoferric species that give rise to them, as I ($T_d = 68^\circ\text{C}$) and I' ($T_d = 77^\circ\text{C}$). Additional evidence for the assignment of these endotherms has been presented (Donovan & Ross, 1975).

Thermograms such as curve (*b*) do not appear to change when the solution of iron and ovotransferrin

Fig. 1. Thermograms of ovotransferrin

(a) Apo-ovotransferrin; (b) ovotransferrin 39% saturated with iron; (c), as (b), but after 28 days at 22°C; (d) ovotransferrin fully saturated with iron. Protein concentration was approx. 100 mg/ml in 0.6 M-Tris/HCl, pH 7.5. The heating rate was 10°C/min. The bar represents a heat flow of 500 μ J/s.

is left for several days. However, after a long time at room temperature changes are observed (compare curves *b* and *c*). The size of the first and fourth endotherms (those produced by denaturation of apo- and Fe_2 -ovotransferrins respectively) decreases; there is an increase in the size of the third endotherm (I') and a smaller increase in the second (I). Accordingly, although essentially all of the iron added is bound to ovotransferrin immediately, the distribution of iron between species is not, as it previously appeared (Donovan & Ross, 1975), an equilibrium one.

Effect of ATP

Since ATP acts as a chelator for iron (Neuberg & Mandl, 1949; Goucher & Taylor, 1964) and is required for transfer of iron from transferrin to hepatic ferritin (Mazur *et al.*, 1960) we added ATP after iron to determine whether it would produce a change in the differential-scanning-calorimetry endotherms. Fig. 2 suggests that the effect of ATP is to transfer iron from Fe_2 -ovotransferrin to apo-ovotransferrin, since the area of the I' endotherm has been increased at the expense of the endotherms of the apo- and Fe_2 -ovotransferrins.

Effect of nitrilotriacetate

A similar quantitative experiment was carried out with nitrilotriacetate, which increases the rate of release of iron from transferrin (Bates *et al.*, 1967b).

Fig. 2. Effect of ATP on the thermograms of ovotransferrin

(a) Ovotransferrin approximately one-third saturated with iron; (b), sample (a) plus 0.66 molecule of ATP/site (1 mol of ATP/mol of Fe); (c), sample (a) plus 5.3 mol of ATP/site (8 mol of ATP/mol of Fe). Thermograms (b) and (c) were determined 30 min after addition of ATP. Ovotransferrin concentration was 96 mg/ml in 0.6 M-Tris/HCl, pH 7.5. The heating rate was 10°C/min. The bar represents a heat flow of 200 $\mu\text{J/s}$.

Fe^{2+} was added to apo-ovotransferrin to give an iron saturation of 74%. Four endotherms were observed on differential scanning calorimetry. Nitrilotriacetate was added and thermograms were again obtained (Fig. 3). The calorimeter was operated at heating rates of 2°C and 10°C/min. In the presence of nitrilotriacetate the endotherm for Fe_2 -ovotransferrin is smaller and that for intermediate I' larger. The fractions of species, calculated from Fig. 3, are given in Table 1. These calculations, together with an auxiliary experiment, indicate that redistribution of iron is real, not just apparent.

The experiment above was carried out at two heating rates for the following reasons. Generally speaking, equilibrium conditions do not exist as a sample is heated. At slow heating rates, an approximation to the equilibrium condition can be attained (Bull & Breese, 1973; Privalov & Khechinashvili, 1974). However, at slow heating rates, kinetic processes, such as extraction of iron by nitrilotriacetate followed by denaturation of the new ovotransferrin species thus formed, would take place to a greater extent. Existence of such processes should cause the thermogram to become smeared out at slower heating rates and produce smaller calculated recoveries of iron. Although the calculated recoveries of iron are lower in the presence of nitrilotriacetate (Table 1), the four endotherms actually appear sharper at slower heating rates, as they do for a single pure protein species (Donovan & Ross, 1973; Donovan & Beardslee, 1975).

In a separate experiment, nitrilotriacetate was added to Fe_2 -ovotransferrin at ratios from 0.6 mol of nitrilotriacetate/mol of Fe to 6 mol of nitrilotriacetate/mol of Fe. A small shift of the Fe_2 -ovotransferrin

Table 1. Effect of nitrilotriacetate and heating rate on the distribution of ovotransferrin species

The fraction of species and the iron saturation are calculated from the areas under the respective endotherms of Fig. 3 and the enthalpies of denaturation of these species (Donovan & Ross, 1975). Abbreviations: T, ovotransferrin; I and I' , monoferric ovotransferrins; II, Fe_2 -ovotransferrin.

Heating rate (°C/min)	Fraction of species				Iron saturation
	T	I	I'	II	
Nitrilotriacetate absent					
10	0.12	0.13	0.11	0.62	0.74
2	0.12	0.14	0.13	0.60	0.74
Nitriloacetate present (3 mol/mol of Fe)					
10	0.08	0.14	0.30	0.48	0.70
2	0.05	0.16	0.34	0.44	0.69

Fig. 3. Effect of nitrilotriacetate and heating rate

(a) No nitriloacetate added to a ovotransferrin solution 74% saturated with iron (by addition of Fe^{2+}); (b) after addition of 3 mol of nitrilotriacetate/mol of Fe. Upper thermograms were determined at a heating rate of $10^\circ\text{C}/\text{min}$, with instrument sensitivity half that of Fig. 1; lower thermograms were determined at a heating rate of $2^\circ\text{C}/\text{min}$, with instrument sensitivity five times that for upper thermograms, to compensate for the slower heating rate. Protein concentration was 150 mg/ml in 0.6M-Tris/HCl buffer, pH 7.5.

endotherm to a lower temperature, proportional to the nitrilotriacetate added and amounting to 1°C at 6 mol of nitrilotriacetate/mol of Fe, was observed. A shoulder on the Fe_2 -ovotransferrin endotherm at the temperature characteristic of the I' intermediate was also observed. The amount of this I' endotherm was approximately linearly proportional to the amount of nitrilotriacetate added. At 3 mol of nitrilotriacetate/mol of Fe (the ratio used for the experiment of Fig. 3) 8% of the Fe_2 -ovotransferrin endotherm was converted into the I' endotherm by nitrilotriacetate. This accounts fairly accurately for the lower iron saturation calculated from the thermograms obtained in the presence of nitrilotriacetate (Table 1). Apparently nitrilotriacetate retains a small fraction of the iron (for the experiment of Fig. 3, 5% of the iron, or 0.02 mol of Fe/mol of

nitrilotriacetate) in a quasi-equilibrium manner, since the low recovery of iron is not a function of heating rate (Table 1).

Reversal of iron binding

To determine how the redistribution of iron depends on the nitrilotriacetate concentration, a solution was prepared by adding apo-ovotransferrin and Fe_2 -ovotransferrin to buffer. Unequal amounts of these two species were added to prevent domination of the thermogram by the endotherm of the diferric species. (The enthalpy of denaturation of Fe_2 -ovotransferrin is twice that of apo-ovotransferrin; see Fig. 1.) The molar ratio of iron to ovotransferrin was 0.67; the molar ratio of apo- to Fe_2 -ovotransferrin was 2. The solution was divided into three portions and nitrilotriacetate was added

to two of them. The solutions were left at room temperature. Samples were withdrawn for calorimetric measurements at various times over a period of 3 weeks. Changes in the thermograms reveal the mobilization of iron originally bound to Fe_2 -ovotransferrin at the start of the experiment. Initial thermograms and those obtained after 20 days are shown in Fig. 4. In each case the initial thermograms should show only two endotherms, for apo- and Fe_2 -ovotransferrin; we have observed such thermograms at shorter times than shown here. The major changes shown in Fig. 4 are an increase in both species I and I' in the presence of nitrilotriacetate. At the higher concentration of nitrilotriacetate, the endotherm for Fe_2 -ovotransferrin was almost eliminated from the thermogram. Our evidence indicates that the thermogram obtained after 20 days (Fig. 4c) shows an equilibrium condition, but that the other thermograms of Fig. 4 do not. It appears that in the presence of chelators, equilibrium favours the presence of species I' over species I. Calculation of the amounts of the two intermediates from both the areas and the heights of their endotherms, taking into account the difference in the enthalpies of denaturation of these species, gives $\text{I}'/\text{I} = 1.3$ at equilibrium. Keeping a sample at 22°C for 2 months did not alter this ratio.

The rate of transfer of iron to form species I'

appears to be proportional to the first power of the concentration of nitrilotriacetate (Fig. 5). Bates *et al.* (1967b) have shown that removal of iron from transferrin by EDTA is first order in EDTA. The rate of transfer of iron in the absence of a chelator is so slow as to be biochemically unimportant. The apparently significant rate of transfer in the absence of a chelator shown in Fig. 5 is greater than that observed over a longer period and may be due, in part, to difficulties in measuring the height of very small endotherms.

Addition of iron as Fe-nitrilotriacetate

Addition of iron to apo-ovotransferrin in the form of the Fe-nitrilotriacetate chelate (Fig. 6) gives thermograms unlike those observed when iron is added in the absence of a chelator (Fig. 1). Little or no Fe_2 -ovotransferrin is observed until almost one Fe^{3+} per ovotransferrin molecule has been added. However, both intermediates I and I' are formed, even at low degrees of saturation with iron. These solutions, on keeping for several weeks, do not appear to show changes in distribution of the species present, as evidenced by the constancy of the thermogram obtained. Although these differential-scanning-calorimetry experiments require a high concentration of protein, we have attempted to duplicate the conditions of Evans & Holbrook (1975) as far as possible.

Fig. 4. Changes in endotherms produced by nitrilotriacetate

To a solution containing apo-ovotransferrin and Fe_2 -ovotransferrin at a molar ratio of 2:1 (a), 1 mol of nitrilotriacetate/mol of Fe (b) or 2 mol of nitrilotriacetate/mol of Fe (c) was added. Initial endotherms (—) were determined at 2 h (a), 30 min (b) and 1 h (c). Final endotherms (···) were determined after 20 days at 22°C . Total protein concentration was 87 mg/ml in 0.6M-Tris/HCl, pH 7.5. The heating rate was $2^\circ\text{C}/\text{min}$. The instrument sensitivity was the same as for the lower portion of Fig. 3. Sample sizes are not identical, but average $20\ \mu\text{l}$.

Fig. 5. Mobilization of iron from Fe_2 -ovotransferrin by nitrilotriacetate

The size of the endotherm at 74°C (I') is shown as a function of time, as determined in an experiment at 22°C , similar to that of Fig. 4. Molar ratios of nitrilotriacetate to Fe^{3+} were: zero ($\circ$); 1 (Δ); 2 ($\square$).

However, the protein concentration (about 15 mg/ml, or tenfold smaller than that used for the experiment of Fig. 3) is still a factor of 30 greater than that used by Evans & Holbrook (1975). The buffer composition is the same, but its concentration is proportionately greater, and we have added HCO_3^- (as CO_2 dissolved in Tris) at 0.02 M instead of 0.01 M.

There are a number of interesting aspects of this experiment. The similarity of the second and third thermograms of Fig. 6 (Fe:ovotransferrin molar ratios of 0.5 and 0.8) to the final thermogram at 2 mol of nitrilotriacetate/mol of Fe of Fig. 4 indicates that an equilibrium distribution of iron is reached quickly when Fe-nitrilotriacetate is added to ovotransferrin. Since some of the Fe-nitrilotriacetate probably is bound initially at both sites of some molecules (binding of Fe^{2+} or Fe^{3+} is approximately random; see Fig. 1), reversal of binding of iron as the Fe-nitrilotriacetate chelate (Bates *et al.*, 1967b; Bates & Wernicke, 1971) appears to be quite rapid. However, iron bound together with the HCO_3^- is only slowly redistributed (Fig. 4a). These observations are in agreement with the binding model proposed by Bates & Schlabach (1973) namely that in the absence of chelator the anion present at the iron-binding site is normally HCO_3^- (or CO_3^{2-}); any other anion bound initially together with iron is soon

replaced by CO_3^{2-} unless CO_2 is rigorously excluded.

Thermograms of a sample prepared by addition of Fe-nitrilotriacetate to ovotransferrin (0.87 mol of Fe/mol of ovotransferrin) were obtained after 2 months at 22°C . These thermograms were not significantly different from those obtained immediately after addition of Fe-nitrilotriacetate to the ovotransferrin. The baseline used for these thermograms was the thermogram of the reheated (irreversibly denatured) sample. Gaussian curves having the peak temperatures and bandwidths previously reported (Donovan & Ross, 1975) were used to fit the thermograms. The relative amounts of species present were: ovotransferrin, 0.19; I, 0.33; I' , 0.42; Fe_2 -ovotransferrin, 0.06 (with an estimated error of ± 0.02 for all and assuming enthalpies of denaturation of these species as listed above). These fractions of species are very different from those previously obtained (Donovan & Ross, 1975) in the absence of chelators. Relative stoichiometric and site-binding constants for iron binding were calculated from these results as described below.

Fig. 6. Thermograms of an ovotransferrin solution titrated stepwise with Fe-nitrilotriacetate

The molar ratio of Fe:ovotransferrin is indicated for each thermogram. Ovotransferrin concentration was 17 mg/ml at the start and 12 mg/ml at the end, in 0.6 M-Tris/HCl buffer/0.02 M- HCO_3^- , pH 7.6. Fe-nitrilotriacetate added was 1 mM, except that 0.1 M was added for the last thermogram. The heating rate was $10^\circ\text{C}/\text{min}$. Thermograms were obtained 15 min to 1 h after addition of the portion of Fe-nitrilotriacetate. The bar represents a heat flow of $100 \mu\text{J}/\text{s}$.

Discussion

Material balances for iron and protein have shown that the two intermediate endotherms produced on denaturation are monoferric ovotransferrins (Donovan & Ross, 1975). The observation of the two intermediate endotherms, but not the endotherm for Fe_2 -ovotransferrin, when Fe^{3+} -nitrilotriacetate is added at less than 1 mol of Fe/mol of ovotransferrin provides additional evidence that these two endotherms have been correctly identified as arising from the heat denaturation of two monoferric ovotransferrins.

Some arguments have been presented suggesting that both the two monoferric ovotransferrins observed calorimetrically have a single Fe^{3+} ion bound at one of the two binding sites (Donovan & Ross, 1975). These arguments do not appear to be supported by the finding that after 30% saturation of ovotransferrin with iron, by addition of Fe^{3+} -nitrilotriacetate, isoelectric focusing gives a monoferric ovotransferrin with iron bound predominantly in the *N*-terminal region (Williams, 1974). Such a result suggests that the intermediate endotherms result from denaturation of two intermediates with the single Fe^{3+} ion bound at the *N*-terminal binding site of ovotransferrin, i.e. two conformers of a single monoferric ovotransferrin. However, Williams's (1974) result depends on isolation of the monoferric ovotransferrin by isoelectric focusing and subsequent proteolytic digestion. Both of these procedures offer opportunity for exchange of iron between sites. In addition, it appears that a low recovery of the *N*-terminal fragment was obtained. This suggests that a monoferric *C*-terminal ovotransferrin, if formed, might have been preferentially digested by trypsin during the preparation procedure.

There appears to be a stronger argument than those already presented (Donovan & Ross, 1975) for interpreting the two intermediates observed calorimetrically as monoferric ovotransferrins with Fe^{3+} bound at distinct sites. Suppose the two intermediates were two different conformations of the same monoferric ovotransferrin [with Fe^{3+} at the *N*-terminal binding site, since Williams's (1974) data suggest this as the most likely possibility]. Equilibrium between these two conformations should be established quickly, since it should not be limited by slow processes such as transfer of iron from one molecule to another. But it is experimentally observed that: (a) large differences in the sizes of the intermediate endotherms are observed under identical conditions of buffer, pH and temperature (Figs. 1-4); (b) these differences in size of the intermediate endotherms persist over a period of days, unless iron-transporting molecules (chelators) are added. Accordingly, the simplest explanation for the changes observed when chelators are added is that equilibrium is established by transport of iron and

that therefore the two intermediate endotherms represent monoferric ovotransferrins with iron at different sites.

In all binding experiments observed by differential scanning calorimetry, two intermediate endotherms are found. When nitrilotriacetate or similar chelators are absent, addition of small amounts of iron always produces four endotherms, for apo-ovotransferrin, two intermediates and Fe_2 -ovotransferrin. Thus, at least qualitatively, it would appear that random binding of iron to two equivalent sites occurs in the absence of chelators. A quantitative analysis shows that formation of the intermediate I is favoured in the absence of chelator (Donovan & Ross, 1975). This is qualitatively evident in Fig. 4(a). Predominance of intermediate I over intermediate I' in the absence of chelator probably results from different rates of binding of iron to the two sites in apo-ovotransferrin and is not an equilibrium condition, as previously suggested (Donovan & Ross, 1975).

When a chelator such as nitrilotriacetate or ATP is added either with the iron or after addition of iron, the endotherms observed with differential scanning calorimetry appear in proportions different from those in the absence of a chelator. In particular, in experiments in which iron is added as Fe-nitrilotriacetate, carried out under conditions similar to those of Evans & Holbrook (1975), the size of the endotherm for apo-ovotransferrin gradually decreases to zero as the Fe:ovotransferrin molar ratio approaches unity. The endotherm of Fe_2 -ovotransferrin only appears to a significant extent at Fe:ovotransferrin molar ratios greater than unity. Thus the stoichiometric binding constant for iron to apo-ovotransferrin is much greater than that for iron to monoferric ovotransferrin. Negative co-operativity exists between sites. These observations and conclusions are in agreement with the results and interpretations of Evans & Holbrook (1975), who observed a linear decrease in fluorescence for the binding of up to one Fe^{3+} per ovotransferrin molecule, followed by another, but different, linear fluorescence decrease as a second Fe^{3+} is bound. At Fe:ovotransferrin molar ratios less than unity, random binding, in which some Fe_2 -ovotransferrin would be produced, is excluded by the absence of a non-linear decrease in fluorescence quenching in their experiments (Evans & Holbrook, 1975), and the absence of an endotherm characteristic of Fe_2 -ovotransferrin in ours. When Fe^{2+} is added to ovotransferrin in the absence of chelators, both the fluorescence changes observed by Evans & Holbrook (1975) and differential-scanning-calorimetry experiments (Donovan & Ross, 1975) indicate positive co-operativity between sites, when it is assumed (incorrectly) that the distribution of iron between sites is an equilibrium distribution (see above).

When Fe-nitrilotriacetate and ovotransferrin

solutions are mixed, some Fe_2 -ovotransferrin may be formed, since it is observed when iron is added without chelator. It is well known that nitrilotriacetate facilitates transfer of Fe^{3+} (Bates *et al.*, 1967a; Aisen *et al.*, 1967; Bates & Wernicke, 1971) and it is probable that Fe-nitrilotriacetate is initially bound as Fe^{3+} -nitrilotriacetate, the nitrilotriacetate later being displaced by CO_3^{2-} or HCO_3^- (Aisen *et al.*, 1967; Bates *et al.*, 1967a,b; Bates & Schlabach, 1973). Accordingly at low iron saturation, any Fe_2 -ovotransferrin formed must immediately lose one Fe-nitrilotriacetate molecule, which would then become bound by an apo-ovotransferrin molecule.

It appears that (a) the first Fe^{3+} ion is bound about equally strongly to either site, and (b) binding of a second Fe^{3+} ion is much less favoured. This marked anti-co-operativity may be attributable to a conformational change produced by binding of the first Fe^{3+} ion, where 'first' is to be interpreted as the first Fe^{3+} ion to bind to either site on the apo-ovotransferrin. Experimental evidence for a conformational change produced by iron binding has been presented by Emery (1969) and Ulmer (1969). This anti-co-operativity is similar to the half-of-the-sites reactivity observed for multiple-subunit enzymes (Stallcup & Koshland, 1973; Malhotra *et al.*, 1974).

Binding of iron to the two sites is actually non-equivalent. When the estimated enthalpies of denaturation of the I and I' species are taken into account (Donovan & Ross, 1975), calculations show that 1.3 times as much intermediate I' is formed as intermediate I (see Figs. 4 and 6). The non-equivalence of the two sites is compatible with the two-stage linear decrease in fluorescence with iron binding observed by Evans & Holbrook (1975). Since this ratio (I'/I = 1.3) is observed both on direct addition of Fe-nitrilotriacetate (Fig. 6) and when an excess of nitrilotriacetate is added to a mixture of ovotransferrin and Fe_2 -ovotransferrin (Fig. 4), it appears that formation of the intermediate which gives the endotherm I' is favoured by the equilibrium binding of Fe^{3+} in the presence of chelators such as nitrilotriacetate or ATP.

A more quantitative statement of the anti-co-operativity can be made. The stoichiometric binding constants K_1 and K_2 can be written as:

$$K_1 = \frac{[\text{I} + \text{I}']}{[\text{T}][\text{F}]} \quad K_2 = \frac{[\text{II}]}{[\text{I} + \text{I}'][\text{F}]} \quad (1)$$

Where T denotes apo-ovotransferrin, I and I', the intermediates, II, Fe_2 -ovotransferrin and F, Fe^{3+} . The participation of nitrilotriacetate, HCO_3^- and H^+ is ignored. Their concentrations will effectively disappear when ratios of constants are calculated at a fixed pH. The site binding constants are specified by the equilibrium:

In the previous paper (Donovan & Ross, 1975) these constants were represented by capital letters. Here they are written in the lower case, the more common usage (Klotz, 1974). This prevents their confusion with the stoichiometric constants, which are always written in upper-case letters. The site binding constants are given by, for example:

$$k_1 = \frac{[\text{I}]}{[\text{T}][\text{F}]} \quad k_3 = \frac{[\text{II}]}{[\text{I}][\text{F}]} \quad (3)$$

Although the concentration of free Fe^{3+} (or, equivalently, Fe-nitrilotriacetate) is unknown, knowledge of the ratios of the four protein species suffices to determine the ratios of the binding constants. As stated in the Results section, one set of molar fractions of species present at equilibrium is given by: T, 0.19; I, 0.33; I', 0.42; II, 0.06. From eqn. (1), $K_1/K_2 = 40$, which corresponds to a difference in free energy of binding of 9.2 kJ/mol for the two Fe^{3+} ions. Assuming $k_4 = 1$, the relative site binding constants, from eqn. (3) are: k_1 , 10; k_2 , 13; k_3 , 1.3; k_4 , 1. These constants show the relatively large difference between the strength of binding of the first and second Fe^{3+} ions and the relatively small difference between the two sites in their affinity for Fe^{3+} ions.

Evans & Holbrook (1975) estimated that K_1/K_2 must be greater than 30. Our value of 40 for this ratio is consistent with their estimate. Evans & Holbrook (1975) have termed the binding 'sequential'. In a limited sense any binding to two sites is sequential, because on a particular molecule one site is filled before the other. If our conclusion that the two monoferric ovotransferrins have iron bound at different sites is correct, then only in this limited sense can the term 'sequential' be applied to the binding of iron. Therefore the more general term 'anti-co-operative' is more appropriate to describe the binding.

This equilibrium distribution of iron between sites, observed for experiments *in vitro* in the presence of chelators, may, in a sense, be irrelevant. Transfer of iron from the binding sites of ovotransferrin must occur by means of a chelator or other iron receptor in order for the rate of transfer to have metabolic importance *in vivo*. When HCO_3^- (or CO_3^{2-}) is the anion bound at the metal-binding site, removal of iron is much slower than when the anion is nitrilotriacetate or ATP. Accordingly it is possible that iron-transfer agents transfer iron to specific sites on transferrins and 'lock' the iron in those sites by placing a HCO_3^-

(or CO_3^{2-}) anion with it *in vivo*. Receptors may retrieve the iron from a specific site by a similar process, operated in reverse. Alternatively some other anion, which is only slowly replaced by CO_3^{2-} , may be metabolically important. Thus the distribution of iron between sites *in vivo* may bear no relationship whatever to that observed *in vitro*.

Some time ago, it was suggested that a first Fe^{3+} ion is bound to ovotransferrin at an 'inner' site before a second can be bound at an 'outer' site (Woodworth, 1966). Later, this terminology was modified, so that 'inner' refers to the first site to which Fe^{3+} is bound and 'outer' to the second site (Aisen *et al.*, 1973; Williams & Woodworth, 1973), with the disclaimer that these designations may not refer to unique locations in the metal-free protein (Aisen *et al.*, 1973). Whereas the spectroscopic experiments of Aisen *et al.* (1973) and Butterworth *et al.* (1975) appear to be consistent with differences between the first and second binding sites, release of iron by ovotransferrin to chick embryo erythrocytes shows no difference between the two sites (Williams & Woodworth, 1973). In contrast with ovotransferrin there are marked differences in release of the first and second Fe^{3+} ions from human transferrin (Fletcher & Huehns, 1967; Harris & Aisen, 1975; Princiotto & Zapolski, 1975). Our initial differential-scanning-calorimetric investigation of the heat denaturation of human transferrin shows striking differences from ovotransferrin, even in the absence of iron.

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