A Study of the Kinetics of Iron and Copper Binding to Hen Ovotransferrin

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The kinetics of iron and copper binding to hen's-egg apo-ovotransferrin were studied by using citrate chelates of these metals at pH 9.3 in borate buffer in the presence of bicarbonate. The kinetics of the absorbance change associated with the formation of the final product show a fast process, which is pseudo-first-order, where the reagents are in excess with respect to the protein, and the citrate concentration is higher than 25 mM. At lower citrate concentration, the progress curves are clearly biphasic. There is marked dependence of the rate of the reaction on bicarbonate concentration, which may be interpreted as a displacement reaction of the ligand-metal-protein ternary complex. The kinetics have been interpreted in the framework of a reaction scheme which involves bimolecular reaction of a metal chelate to the protein and subsequent colour development by displacement of the chelator by bicarbonate. The pH-dependence of this reaction supports the belief that tyrosine residues are involved in the process of iron-binding. The overall similarity of kinetics for iron and copper binding, notwithstanding their different co-ordination preferences, suggests that the process of metal-binding or chromophore development for the two metal complexes must be similar.

Ovotransferrin is one member of a group of proteins, termed transferrins, which are found in various fluids of vertebrate animals such as serum, milk and eggs and which are characterized by their ability to form stable complexes with several different metal ions (Feeney & Komatsu, 1966).

All the proteins studied bind 2 g-atoms of metal ion per mol of protein in the pH range 6–10 in specific interaction of great complexity, where bicarbonate is somehow involved. At low pH values the metal ions are dissociated.

Little is known of the metal-binding sites on these proteins, from either structural or kinetic investigations, and the relative affinity of the two sites for the metal ions is still controversial.

In the presence of bicarbonate, iron-saturated ovotransferrin has a broad absorption band centred at about 470 nm, and the copper-saturated protein has an absorption band at 440 nm. In the present study the time-course of appearance of chromophore on mixing pure metal-free ovotransferrin with a metal chelate in the presence of bicarbonate has been followed under a variety of conditions. The kinetics of this process are complex, indicating that the final coloured product is formed through a sequence of reactions. The results appear to be consistent with a

reaction scheme involving binding of the metal chelate to the protein, followed by displacement of chelator by bicarbonate, the latter stage being associated with the largest change in absorbance.

Experimental

Materials

Hen's-egg iron-free ovotransferrin was obtained from O.T.I., Parma, Italy, in the form of a freeze-dried powder. It was dissolved in the appropriate buffer as required. From the absorbance at 280 nm and 465 nm less than 0.2% of the experimentally observable binding capacity of the apo-ovotransferrin was occupied by iron. Both apoprotein and fully iron-saturated complex migrated as single bands in starch-gel electrophoresis. In the analytical ultracentrifuge in 50 mM-sodium borate buffer, pH 9.1, at protein concentrations between 2 and 8 mg/ml a single symmetrical peak with a $s_{20,w}$ of 5.16 S was observed.

Iron-nitritotriacetate solutions (1 M) were prepared from FeCl_3 solutions and nitritotriacetate in 1 M-NaOH and diluted as required in buffer.

Buffer solutions were 50 mM-sodium borate for pH ranges 9.1–10.8 or 0.1 M-sodium phosphate buffers for pH ranges 6–8.5. FeCl_3 (1 M) and CuSO_4 solutions were made up in water, as were 1 M-citric acid and NaHCO_3 solutions. In this way a variety

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of freshly made solutions could be speedily prepared in which the iron/citrate or copper/citrate ratios could be varied, as could also the concentration of NaHCO_3 in the final mixture.

All chemicals were AnalaR where possible, and water was deionized before distillation in glass.

Methods

Spectra were measured either on a Cary model 14 or a Unicam SP.1800 recording spectrophotometer with 0.1 or 1 cm path-length cells in a thermostatically controlled cuvette holder.

The ionization of tyrosine residues was recorded spectrophotometrically by following the pH-dependence of the phenolic ionization at 293 nm by using a value of 2.3 for the millimolar absorption change (Hermans, 1962).

Stopped-flow kinetic determinations were carried out with a Gibson-Durrum apparatus (Gibson & Milnes, 1964) equipped with a 2 cm observation tube. Band widths were 1–2 nm and the dead-time of the instrument was 3–4 ms. The instrument was pre-flushed with citrate buffers before the start of the experiments and then washed extensively with the experimental buffer.

Results

Iron-binding studies

Comparison of different iron chelates. Preliminary experiments in which ovotransferrin (6.5 mg/ml, 78 μM in sites) in 50 mM-sodium borate buffer, pH 9.2, containing 100 mM- NaHCO_3 was titrated with standard solutions of iron-nitrilotriacetate and ferric citrate gave straight-line plots with end points of 1.91 ± 0.18 mol of iron bound per mol of protein, assuming a molecular weight of 83 000 (Fuller & Briggs, 1956). This is shown in Fig. 1.

A comparison of the two iron chelates was attempted by using rapid-mixing techniques. Two examples of the results of mixing a series of iron-nitrilotriacetate solutions, all at constant molarity of nitrilotriacetate in borate buffer, with ovotransferrin (2.6 mg/ml) in 50 mM- NaHCO_3 and 50 mM-borate buffer are shown in Fig. 2. It is noticeable that the kinetics are complex and that the half-time of the process is not predictably related to the metal-ion concentration. In another series of experiments under the same conditions but with 1:1 complex of iron-nitrilotriacetate at concentrations ranging from 5 to 0.312 mM the half-time of the measured absorbance change was about 1 s independently of the metal chelate concentration.

These results were taken to indicate that the overall observable process was rate-limited by a first-order step and discouraged further kinetic studies of the system with the iron-nitrilotriacetate complex.

Fig. 1. Titrations of ovotransferrin (6.5 mg/ml, 78 μM in binding sites) containing 100 mM- NaHCO_3 with 1 mM-iron-nitrilotriacetate

Light-path length was 1 cm and temperature was 20°C. Titration with 1 mM- Fe^{3+} in 50 mM-citrate gave identical titration curves. Extinction values were recorded minutes after mixing and were rechecked after 24 h incubation at 4°C.

Fig. 2. Tracing from experiments in which ovotransferrin (2.6 mg/ml) in 50 mM-borate buffer, pH 9.2, containing 100 mM- NaHCO_3 was rapidly mixed with 10 mM-nitrilotriacetate solutions in sodium borate buffer containing (a) 10 mM- Fe^{3+} and (b) 1 mM- Fe^{3+}

The observation wavelength was 465 nm and the temperature 20°C. The light-path was 2 cm.

Fig. 3. Progress curves of experiments in which ovotransferrin was mixed with iron-citrate, showing the plot of $\log(\Delta E_0/\Delta E_t)$ versus time

Ovotransferrin (2.6mg/ml) in 50mM-sodium borate buffer, pH9.2, containing 100mM- NaHCO_3 was rapidly mixed with 50mM-citrate in sodium borate buffer containing 1.25mM- Fe^{3+} (A, $\circ$), 0.31mM- Fe^{3+} (B, $\bullet$) or 0.08mM- Fe^{3+} (C, $\square$).

In contrast, the kinetics of the reaction using ferric citrate demonstrated a dependence on the concentration of metal ion, citrate and bicarbonate with progress curves which were clearly pseudo-first-order under most conditions. This indicated the possibility of carrying out a kinetic analysis of the system, and all further experiments were carried out with metal-citrate complexes.

When ovotransferrin was titrated with iron-citrate in the absence of bicarbonate, no colour developed. Colour did develop when bicarbonate was subsequently added. Experiments in which metal-citrate was rapidly mixed with ovotransferrin and the e.p.r. spectra were observed gave clear indication of rapid chelate binding under these conditions before colour development (E. Antonini, unpublished experiments).

Experiments were performed where ovotransferrin (2.6mg/ml in 100mM- NaHCO_3) and 50mM-sodium borate buffer, pH9.3, were rapidly mixed with solutions of iron-citrate (50mM-citrate in sodium borate buffer) containing 1.25mM-, 0.31mM- and 0.08mM- Fe^{3+} . The progress curves of $\log(\Delta E_0/\Delta E_t)$ against time for these three experiments are shown in Fig. 3. It is noteworthy that the theoretical two-site extinction change was recovered in these kinetic experiments.

Fig. 4. Effect of increasing Fe^{3+} , at constant citrate concentration, on the pseudo-first-order rate constant

The ovotransferrin was 2.6mg/ml, in borate buffer, pH9.2. Citrate was 50mM and NaHCO_3 was 100mM before mixing. The temperature was 20°C and the observation wavelength was 465nm.

Combination of iron at constant citrate concentration. Experiments in which ovotransferrin (2.6mg/ml in 100mM- NaHCO_3) and 50mM-sodium borate buffer, pH9.3, were rapidly mixed with 50mM-sodium citrate in the same buffer containing various concentrations of Fe^{3+} are shown in Fig. 4, where the pseudo-first-order rate constant is plotted against metal ion concentration. It is clearly seen that the rate does not increase linearly with metal ion concentration, and there is some suggestion from experiments performed at high Fe^{3+} concentration that this rate will tend to a limiting value as the iron concentration increases. The initial slope of the curve corresponds to a value of a second-order rate constant of about $1.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. The effect of metal ion concentration, at fixed citrate concentration, varies with bicarbonate concentration; at low bicarbonate concentration there is no effect of metal ion concentration between 0.5 and 5mM on the rate.

Effect of varying citrate concentration at constant iron concentration. In complementary experiments using either 1mM- or 10mM- Fe^{3+} and varying the citrate concentration between 13mM- and 110mM-citrate, there was a profound effect of chelator concentration on the observed pseudo-first-order rate constant, as shown in Fig. 5. At low citrate concentrations (<5mM) the reaction time-course was no longer pseudo-first-order and showed a fast and a slow phase. When iron concentration was above 1mM, a fast phase with a rate constant of about 50 s^{-1} and a slow phase with a rate constant of 5 s^{-1} were observed, but the values of these did not change with the iron concentration when the latter was higher than 1.5mM, though the proportions of the two phases observed did change.

Fig. 5. Effect of citrate concentration at constant Fe^{3+} concentration on the pseudo-first-order rate constant

Ovotransferrin was 2.6mg/ml in borate buffer, pH9.2. The iron concentrations were: 10mM- Fe^{3+} (○) and 1mM- Fe^{3+} (●) in the same buffer, containing 100mM- NaHCO_3 , before mixing. The temperature was 20°C and the observation wavelength was 465 nm.

Effect of bicarbonate concentration on the reaction velocity. It seemed profitable to explore the concentration-dependence of the reaction velocity on bicarbonate concentration by rapidly mixing ovotransferrin in borate buffer at pH9.2 containing 100mM-, 10mM- and 1mM- NaHCO_3 respectively with buffer containing 50mM-citrate and 1mM- Fe^{3+} . Table 1 demonstrates the dependence of the observed pseudo-first-order rate constant on the bicarbonate concentration. The rate changes by tenfold across the change of two orders of magnitude in bicarbonate. The same kinetics were observed in these experiments whether bicarbonate was pre-mixed with the protein or with the iron-citrate solution.

Kinetics at constant citrate/bicarbonate ratio. In a series of experiments, the kinetics of the reaction were studied at constant ratio of citrate/bicarbonate (0.5) whereas the absolute concentration varied from 50 to 3mM in citrate.

The results are shown in Table 2 and indicate that the rate increases with decrease in citrate and bicarbonate concentration over the range 50–12.5mM

Table 1. Effect of bicarbonate concentration on the observed pseudo-first-order rate constant

Ovotransferrin (2.5mg/ml) in sodium borate buffer, pH9.3, containing various concentrations of NaHCO_3 was rapidly mixed with a buffer solution containing 1mM- Fe^{3+} and 50mM-citrate. When bicarbonate was added to the iron-citrate syringe and mixed with bicarbonate-free protein, t_4 was 250ms corresponding to $k' = 2.76\text{s}^{-1}$.

Concn. of HCO_3^- (mM) (before mixing)	k' (s^{-1})
1	0.43
10	0.95
100	3.07

Table 2. Experiments at constant citrate/bicarbonate ratio

Ovotransferrin (2.6mg/ml) in 50mM-sodium borate buffer, pH9.3, was rapidly mixed with solutions containing citrate, NaHCO_3 and Fe^{3+} in buffer. At lower citrate concentrations, the progress curves were clearly biphasic.

Concentration (mM)			k' (s^{-1})
Citrate	NaHCO_3	Fe^{3+}	
100	200	1	1.7
50	100	1	4.6
25	50	1	6.5

in citrate. At still lower citrate concentration, the progress curves were clearly biphasic.

Effect of pH on the reaction. With a series of buffers of 100mM-sodium phosphate or borate covering the range pH7.25–9.9, and with 50mM-citrate with 1mM- FeCl_3 , the pseudo-first-order rate constant was measured at 20°C at four pH values. The results are shown in Fig. 6 and show a 15-fold variation across this pH range. Attempts to measure rates at pH10.5 were unsuccessful because of the difficulty of preserving the ferric-citrate chelate.

Copper-binding studies

The binding of copper by ovotransferrin was studied by using cupric-citrate as donor, by methods similar to those used for iron binding.

Effect of citrate concentration and copper concentration respectively. The results of experiments in which ovotransferrin (2.6mg/ml) in sodium borate buffer at pH9.2 and in the presence of 50mM- NaHCO_3 was rapidly mixed with cupric-citrate in borate buffer are shown in Figs. 7 and 8. As in previous experiments, it was necessary to keep the concentration of either citrate or copper constant while the other component was varied. The form of these two curves as shown in Figs. 7 and 8 are very similar

Fig. 6. Effect of pH on pseudo-first-order rate constant (O) for iron binding to ovotransferrin (4mg/ml) in 50mM buffers containing 100mM-NaHCO₃

The iron was 2mM and citrate 50mM before mixing. The temperature was 20°C and the observation wavelength 465nm. ΔE_{293} (apoprotein minus holoprotein) (●), indicating the number of tyrosine residues titrated at each pH value, is also shown.

Fig. 7. Effect of Cu²⁺ at constant citrate concentration on the pseudo-first-order rate constant for copper binding to ovotransferrin

Borate buffer was 50mM, pH9.3, containing 100mM-NaHCO₃. The temperature was 20°C and the observation wavelength was 440nm. Protein was 2.6mg/ml and citrate was 50mM (before mixing).

Fig. 8. Effect of citrate concentration at constant Cu²⁺ concentration of the pseudo-first-order rate constant

Ovotransferrin was 2.6mg/ml in borate buffer, pH9.3. The Cu²⁺ concentration was 1mM in the same buffer with 100mM-NaHCO₃. Temperature was 20°C and observation wavelength was 440nm.

Table 3. Effect of bicarbonate concentration on the observed pseudo-first-order rate constant for colour development

Ovotransferrin was 2.6mg/ml in 50mM-sodium borate buffer containing 100mM-NaHCO₃. CuSO₄ was 1mM and citrate was 1mM in borate buffer.

Bicarbonate concentration (mm) (before mixing)	k' (s ⁻¹)
1	2.75
10	3.7
100	8.6

shown in Table 3 and replicate exactly the behaviour seen in the iron-binding systems.

Discussion

In all attempts to understand the physiological mechanism operating in iron binding to the transferrins a large initial problem is the complex chemistry of ferric salts. At neutral pH, these form large polymeric complexes from which the release of low-molecular-weight ferric salts is very slow. Attempts to titrate transferrin with FeCl₃ showed that between 5 and 25% of the iron was bound to human transferrin when 1 molecular equivalent of FeCl₃ was added (Bates & Schlabach, 1973). Thus iron chelates have been used as ferric donors.

The form of iron-citrate chelates has been experimentally determined by Spiro *et al.* (1967a,b), who showed that at low pH the equimolar ferric-citrate molecule exists which polymerizes at higher pH to form a hydroxy ion polymer surrounded on the surface by citrate molecules. However, in the presence

to those shown in Figs. 4 and 5, except that the rates observed are lower for copper binding under all conditions.

No attempt was made to measure the pH-dependence of the copper-binding reaction.

Effect of bicarbonate concentration on copper binding. There is no clear evidence implicating bicarbonate in the copper complex of ovotransferrin, and a series of experiments similar to those reported for iron binding were undertaken. The results are

of excess of citrate, an anionic chelate is formed at high pH values which contains 2 citrate molecules per iron atom, each citrate having its hydroxyl proton ionized. From proton-magnetic-relaxation rates (Spiro *et al.*, 1967*a,b*) it appears that, at ratios of citrate/ Fe^{3+} of 50 and above, the dicitrate chelate is the predominant species and there are no polymeric molecules present.

Thus with 25 mM-citrate and 0.5 mM- Fe^{3+} , at pH 9.2, the only species of iron chelate present should be the ferric-dicitrate molecule.

However, the metal chelate species which reacts with the protein is probably the monocitrate-iron complex. If this is so, under conditions where the prevailing species is the dicitrate complex, there will be a competition for the monocitrate species as between the protein and the excess of free citrate. The experiments reported in Table 2 seem to agree with this suggestion.

The rate of iron binding by ovotransferrin is thus dependent on the nature of the metal chelates used. This is shown in the present experiments by the widely different behaviour of the nitrilotriacetate and citrate complexes.

Schade *et al.* (1949) first demonstrated that CO_2 was implicated in the formation of iron complexes of human serum transferrin. Warner & Weber (1953) showed that the species required was either bicarbonate or carbonate. Uncertainty still surrounds the role of bicarbonate, and two suggestions have been made. In the first, bicarbonate is required for the initial binding of iron to the ovotransferrin, and the second suggestion is that it is involved in the formation of the coloured complex.

Aisen *et al.* (1967) have shown, using electron-paramagnetic-resonance spectroscopy, that ternary complex-formation between metal, chelate and transferrin takes place without the involvement of the HCO_3^- anion, and they suggested that HCO_3^- was involved in the rate-limiting step of chromophore development by displacement of the chelator by bicarbonate.

The kinetics reported in this paper support such a ligand-exchange mechanism for colour development. On the basis of the considerations outlined above we propose the following minimum scheme for the reaction of apo-ovotransferrin with ferric-citrate in the presence of bicarbonate:

It is assumed that the absorbance change occurs predominantly at stage (3). It is also assumed that the equilibrium in reaction (1) is attained very rapidly and that reaction (3) proceeds via dis-

sociation of the citrate bound to the metal-protein complex. Thus under conditions where reaction (2) is slow in comparison with reaction (3), such as at very low metal ion concentrations, the measured rate represents the second-order combination reaction of the metal ion with the protein modified by the pre-equilibrium step (1).

On the other hand, when the formation of protein-metal-ligand is fast in comparison with the rate of reaction (3) the observed rate would reflect essentially the rate of displacement of citrate by bicarbonate. It would be expected that under these conditions the observed rate would depend markedly on the relative citrate-bicarbonate concentrations.

The experimental data reported in this paper do conform with good approximation to the reaction scheme shown above. From the initial slope of the plot of the values of the rate versus the metal ion concentration at high citrate and low iron concentrations the rate constant for the second-order process is estimated to be higher than $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for iron, the rate for copper being somewhat lower. As the metal ion concentration is increased and the [citrate]/[bicarbonate] ratio is decreased the rate of the process tends toward limiting values for both iron and copper, reflecting the fact that the displacement reaction becomes rate-limiting.

At low citrate concentrations (<5 mM) where the fast phase was observed, the limiting second-order rate constant is higher, $2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Under these conditions, the fast phase may be taken to represent the combination of the reactive species, possibly iron monocitrate, with the protein. The behaviour of the slower phase is similar to the overall response at high citrate concentration. The values for the fast phase seem to approach a limit of 50 s^{-1} . In view of the very low [citrate]/[bicarbonate] ratio under these conditions, in the framework of the scheme shown above, this may be taken to approximate the rate of dissociation of citrate from the iron-protein complex.

Quite apart from the validity of the scheme proposed, the results emphasize the large number of variables which must be taken into account in this work. These include the nature and reactivity of the metal chelate species and their interconvertibility as well as the role of bicarbonate.

The effect of pH on reaction velocity indicates that a basic group with an apparent pK above 10 is involved in iron binding. Wishnia *et al.* (1961) first showed that the proton equilibria of apo-ovotransferrin and the iron-saturated material showed a difference of six titratable phenol groups between the apo-protein and the iron-ovotransferrin molecules. The pH-dependence of the rate (Fig. 6) is consistent with a direct role for tyrosine in the binding of the iron. Further studies involving protein fluorescence quenching and proton release are being currently

conducted to shed more light on the detailed mechanism of iron binding.

Finally the question of the equivalence or non-equivalence of the two iron-binding sites remains unresolved and it remains for future experiments using specifically modified ovotransferrins to clarify this point as well as to attempt to delineate the chemical nature of the groups on the protein that bind the ferric ion.

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