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# Binding of Cu(II), Tb(III) and Fe(III) to chicken ovotransferrin

## A kinetic study

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**Abstract.** The kinetics of binding of Cu(II), Tb(III) and Fe(III) to ovotransferrin have been investigated using the stopped-flow technique. Rate constants for the second-order reaction, $k_+$, were determined by monitoring the absorbance change upon formation of the metal-transferrin complex in time range of milliseconds to seconds. The *N* and *C* sites appeared to bind a particular metal ion with the same rate; thus, average formation rate constants $k_+$ (average) were $2.4 \times 10^4\ M^{-1}\ s^{-1}$ and $8.3 \times 10^4\ M^{-1}\ s^{-1}$ for Cu(II) and Tb(III) respectively. Site preference (*N* site for Cu(II) and *C* site for Tb(III)) is then mainly due to the difference in dissociation rate constant for the metals. Fe(III) binding from Fe-nitrilotriacetate complex to apo-ovotransferrin was found to be more rapid, giving an average formation rate constant $k_+$ (average) of $5 \times 10^5\ M^{-1}\ s^{-1}$, which was followed by a slow increase in absorbance at 465 nm. This slow process has an apparent rate constant in the range $3\ s^{-1}$ to $0.5\ s^{-1}$, depending upon the degree of Fe(III) saturation. The variation in the rate of the second phase is thought to reflect the difference in the rate of a conformational change for monoferric and diferric ovotransferrins. Monoferric ovotransferrin changes its conformation more rapidly ($3.4\ s^{-1}$) than diferric ovotransferrin ($0.52\ s^{-1}$). A further absorbance decrease was observed over a period of several minutes; this could be assigned to release of NTA from the complex, as suggested by Honda et al. (1980).

**Key words:** Transferrin – Metal-binding protein

### Introduction

The transferrins are a group of homologous iron-binding glycoproteins in blood plasma, mammalian milk and avian eggs, which are called serotransferrin, lactotransferrin, and ovotransferrin, respectively (Bezkorovainy 1980; Brock 1985; Feeney and Komatsu 1966). In vivo, the transferrins bind and transfer iron and thus contribute to the effective use of iron (iron metabolism) in the physiological state. Hence it is of importance to study the binding kinetics of metal ions to transferrins and this is the subject of the present study.

Each transferrin molecule consists of a single polypeptide chain of molecular weight approximately 80 kDa, which has two major structural lobes, the *N* and *C* domains, and a short connecting part (Gorinsky et al. 1979; Anderson et al. 1987). The two domains each possess a specific site for metal ion binding, the affinity is different for the two sites (Yamamura et al. 1984; Aisen et al. 1973; Donovan and Ross 1975). The amino acid sequences of the two domains show considerably homology (Metz-Boutigue et al. 1984; Yang et al. 1984; Williams et al. 1982), and the residues in the metal-binding sites are the same (Metz-Boutigue et al. 1984; Williams et al. 1982). The domains are also capable of binding various other metal ions such as copper, terbium etc. (Aasa et al. 1963; Tan and Woodworth 1969; Luk 1971; Yamamura et al. 1988). The concomitant binding of a particular divalent anion, physiologically (bi)carbonate, is required for the formation of a stable metal-transferrin complex (Bates and Schlabach 1973; Cowart et al. 1982).

We have studied the equilibrium iron-binding properties of ovotransferrin and have found that the *N* site has a greater affinity than the *C* site for iron and that the binding is cooperative. Copper, on the other hand shows no cooperativity in binding (Yamamura et al. 1985). In the present study, we have measured the kinetics of metal binding using the stopped-flow technique. From the findings described here we propose a binding model in which the irons are bound to the two domains non-selectively and independently. The subsequent conformational change and/or stabilization of iron-binding with a divalent anion occurs cooperatively. Kinetic studies on iron binding to human serotransferrin have been performed by Bates and co-workers (Baes and Wernicke 1971; Bates

*Abbreviations*: Tf, ovotransferrin; NTA, nitrilotriacetate

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1982; Cowart et al. 1982) and by Honda et al. (1980). The model we propose here is consistent with their experimental results, but is actually established by our new findings on the bimolecular reaction and the subsequent unimolecular process.

## Materials and methods

### 1. Preparation of ovotransferrin

Ovotransferrin was purified from hen egg white by CM- and DEAE-Sepharose CL-6B column chromatography, as described previously (Yamamura et al. 1984). The concentration of apo-ovotransferrin was determined from the absorbance at 280 nm as $E^{1\%}=11.3$ (Glazer and McKenzie 1963) and a molecular weight of 77,770 (Williams et al. 1982).

### 2. Preparation of metal solutions

Fresh Fe-NTA solutions were prepared by dissolving $Fe(NO_3)_3 9H_2O$ and NTA. 3 Na in 0.05 M Tris-HCl buffer at pH 8.0 (Harris and Aisen 1973), the concentration of Fe was adjusted to 0.05 M at pH 8.0. Cu(II) and Tb(III) solutions (0.05 M) were prepared by dissolving $CuSO_4 5H_2O$ and $TbCl_3 3H_2O$, in distilled water. The solutions were diluted with buffer or distilled water to the required concentration just before each experiment. The pH was not adjusted on mixing, since the pH change due to metal binding and/or to mixing with the acidic metal solution was negligible in the buffer used.

### 3. Chemicals and buffers

All reagents used were analytical grade. Ovotransferrin was dissolved in 0.05 M Tris-HCl buffer at pH 8.0 containing 0.06 M $NaHCO_3$ in the case of reaction with Fe-NTA, or in 0.1 M Tris-HCl buffer at pH 8.0 containing 0.06 M $NaHCO_3$ in case of reaction with Cu(II) and Tb(III) ions. Final concentrations of ovotransferrin in all cases were adjusted to approximately $1.3\times10^{-4}$ M (ca. 1%) (see Results). In all cases, an amount of bicarbonate sufficient to saturate all binding sites was included.

### 4. Apparatus

Stopped-flow experiments were performed with a Union-Giken stopped-flow spectrophotometer, RA-450. The binding reactions were monitored at 465 nm for Fe-NTA (Warner and Weber 1951), at 440 nm for Cu(II) (Frankel-Conrat and Feeney 1950), and at 245 nm for Tb(III) (Yamamura et al. 1988) as these wavelengths showed the maximum absorbance change on metal binding. Data were stored in a transient memory HR-1200 (Kawasaki Electronics), and transferred to a Sord microcomputer M223. Analyses were done by the non-linear least squares Simplex method (Nelder and Mead 1964). The temperature was contolled at 30 °C ± 0.5 °C throughout.

### 5. Analysis

Monophasic data were analyzed according to (2) with the assumption of a bimolecular reaction,

$$\mathrm{Tf}+\mathrm{M}\underset{k_b}{\overset{k_f}{\rightleftharpoons}}\mathrm{Tf}\cdot\mathrm{M} \tag{1}$$

Absorbance $A$ is derived as (Tachi'iri et al. in press)

$$A=A_i-(A_i-A_f)\frac{\alpha}{\beta}\left(1-\frac{\alpha-\beta}{\alpha-\beta\exp(-k_{\mathrm{app}}t)}\right) \tag{2}$$

where $A_i$ and $A_f$ are initial and final values of absorbance,

$$k_{\mathrm{app}}=k_f(\alpha-\beta) \tag{3}$$

$$\alpha=\tfrac{1}{2}\{([\mathrm{Tf}]_0+[\mathrm{M}]_0+1/K)+\gamma\} \tag{4}$$

$$\beta=\tfrac{1}{2}\{([\mathrm{Tf}]_0+[\mathrm{M}]_0+1/K)-\gamma\} \tag{5}$$

$$\gamma=\sqrt{([\mathrm{Tf}]_0+[\mathrm{M}]_0+1/K)^2-4([\mathrm{Tf}]_0[\mathrm{M}]_0-[\mathrm{Tf}\cdot\mathrm{M}]_0/K)} \tag{6}$$

and

$$K=k_f/k_b \tag{7}$$

Subscript 0 denotes concentration at $t=0$. In the present work, $[\mathrm{Tf}\cdot\mathrm{M}]_0=0$. Equation (2) with (3)–(7) was analysed by the non-linear least squares Simplex method with $A_i$, $A_f$, $k_{0n}$ and $K$ as unknown parameters.

When $K[\mathrm{M}][\mathrm{Tf}]\gg[\mathrm{Tf}\,\mathrm{M}]$, i.e. $K[\mathrm{M}]\gg1$, (2)–(6) are simplified as follows by neglecting the back reaction.

$$A=A_i-(A_i-A_f)\frac{x_0}{y_0}\cdot\left(1-\frac{1-y_0/x_0}{1-(y_0/x_0)\exp(-k_f(x_0-y_0)t)}\right) \tag{8}$$

where $x_0$ is $\max([\mathrm{Tf}]_0,[\mathrm{M}]_0)$ and $y_0$ is $\min([\mathrm{Tf}]_0,[\mathrm{M}]_0)$. In practice, (8) was used in the following analyses, as the evaluated $k_b$ were not reliable and were probably negligible. The validity of this assumption will be discussed later.

For biphasic processes the absorbance change was analysed according to the following equation,

$$A=A_i-(A_i-A_f)\frac{\alpha}{\beta}\left(1-\frac{\alpha-\beta}{\alpha-\beta\exp(-k_{\mathrm{app}}t)}\right)+\Delta C\cdot t \tag{9}$$

or

$$A=A_i-(A_i-A_f)\frac{x_0}{y_0}\cdot\left(1-\frac{1-y_0/x_0}{1-(y_0/x_0)\exp(-k_f(x_0-y_0)t)}\right)+\Delta C\cdot t \tag{10}$$

according to (8).

This was done because the two phases were very well separated and the slow process could be treated by adding a linear term ($=\Delta C\cdot t$) (see Fig. 5 b). Apparent rate constants for the slow processes were determined from other sets of experiments performed using much longer

time scales (see Fig. 5a) and assuming a single exponential decay. The initial part of the time course was omitted in this analysis.

## Results and discussion

### *1. Binding of Cu(II) and Tb(III)*

Binding kinetics of Cu(II) to apo-ovotransferrin were monitored using the absorbance change at 440 nm. Figure 1 shows a typical trace which demonstrates that the reaction is monophasic. Binding of Tb(III) to apo-ovotransferrin was monitored using the absorbance change at 245 nm, this also gave a monophasic decay (Fig. 2).

Upon increasing the metal concentration, the rates of both metal-binding reactions become faster, which suggests the reactions are (or at least include) second-order reactions. The data were analyzed according to (8) which assumes that the reaction is bimolecular. Rate constants, $k_f$, were estimated and are summarized in Figs. 3 and 4, which clearly demonstrate that the observed reactions are bimolecular since the second-order rate constants thus obtained are independent of metal concentration. The rate constants obtained for Cu(II) and Tb(III) binding are $(2.4 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$ and $(8.0 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$, respectively.

Ovotransferrin has two metal binding sites, the *N* and *C* sites. Both of these bind metals, but with different affinity as described in the Introduction. In the case of Cu(II) binding, Yamamura et al. (1985) have reported that the *N* site has a higher affinity (ratio of association constants, $K_C/K_N = 0.47 \pm 0.03$) and the sites have no cooperativity. If this difference in association constants for the two sites is ascribed to the difference in forward reaction rates, we should observe two phases in the time course of the binding process, but this is not the case. In fact, the resolving power of kinetic measurements for multiphase processes is not particularly great and one might measure an average rate constant for the two sites. Then,

$$k_f(ave) = k_{fN} f_N + k_{fC} f_C \qquad (11)$$

holds, where $k_f(ave)$ is the observed forward rate constant, $k_{fN}$ and $k_{fC}$ are forward reaction rates for the *N* and *C* sites, respectively, and $f_N$ and $f_C$ are unoccupied *N* and *C* site fractions. As the *N* site has a higher affinity, we would expect that $k_f(ave)$ shifts from $k_{fN}$ to $k_{fC}$ upon increasing the metal concentration. However, Fig. 3 demonstrates that $k_{app}$ is independent of metal concentration. Therefore, we conclude that $k_{fN} = k_{fC}$ within experimental error (ca. 10%). In fact, we could not see any evidence for the rate of binding to the *N* being different from that to the *C* site. Thus we are led to conclude that differences in association constants must be attributable to different dissociation rates, with dissociation from the *C* site being twice as fast. Yamamura et al. (1984) have reported that cupric ions are released from *N* and *C* sites with rates of $(4.08 \pm 0.22) \times 10^{-3}\ s^{-1}$ and $(8.66 \pm 2.88) \times 10^{-2}\ s^{-1}$ upon addition of 0.1 M EDTA (in the presence of 5 m*M* $NaHCO_3$). The ratio of dissociation rate constants of [*N* site]/[*C* site] (by EDTA) is 0.047.

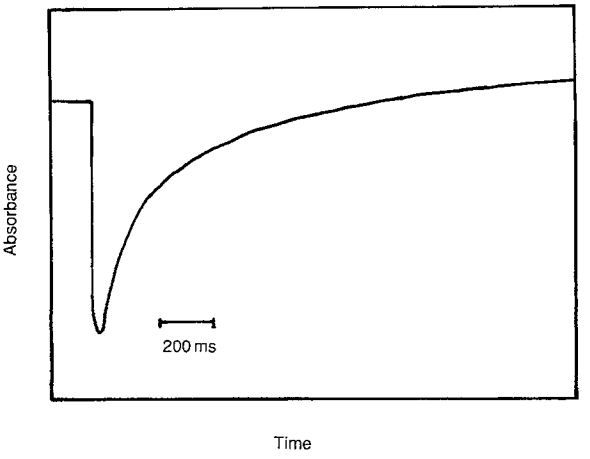



**Fig. 1.** Time course of absorbance change at 440 nm, as a probe of copper binding to ovotransferrin. Solutions (A) and (B) were mixed at a ratio of (A)/(B) = 1 vol/vol; (A) apo-ovotransferrin at $2.6 \times 10^{-4}$ M dissolved in 0.1 M Tris-HCl, pH 8.0 in the presence of 0.06 M $NaHCO_3$; (B) $5.4 \times 10^{-4}$ M $CuSO_4$ in distilled water. Ordinate represents absorbance in arbitrary units

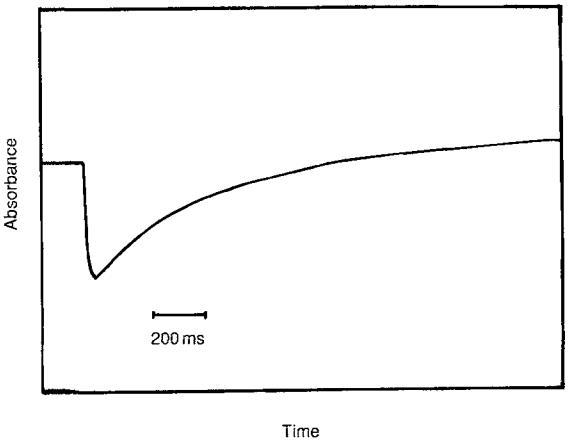



**Fig. 2.** Time course of absorbance change at 245 nm. Solutions (A) and (B) were mixed at a ratio of (A)/(B) = 1 vol/vol; (A) apo-ovotransferrin at $2.6 \times 10^{-4}$ M dissolved in 0.1 M Tris-HCl, pH 8.0 in the presence of 0.06 M $NaHCO_3$; (B) $5.4 \times 10^{-4}$ M $TbCl_3$ in distilled water. Ordinate represents absorbance in arbitrary units

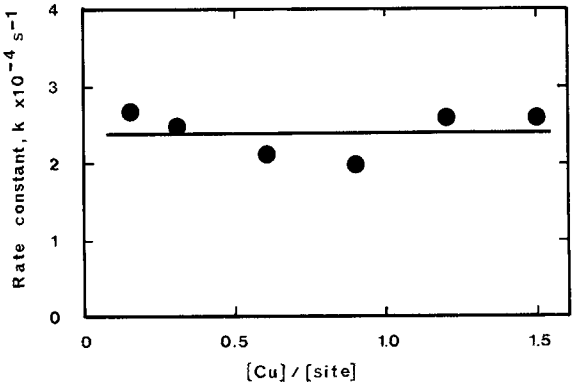



**Fig. 3.** Rate constant for copper binding to ovotransferin as a function of [Cu]//[Tf]

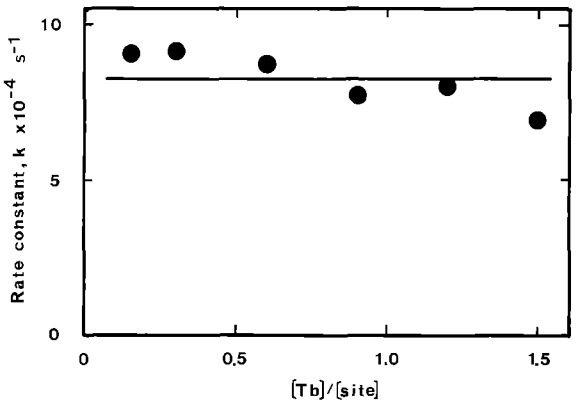



**Fig. 4.** Rate constant for terbium binding to ovoransferrin as a function of [Tb]/[Tf]

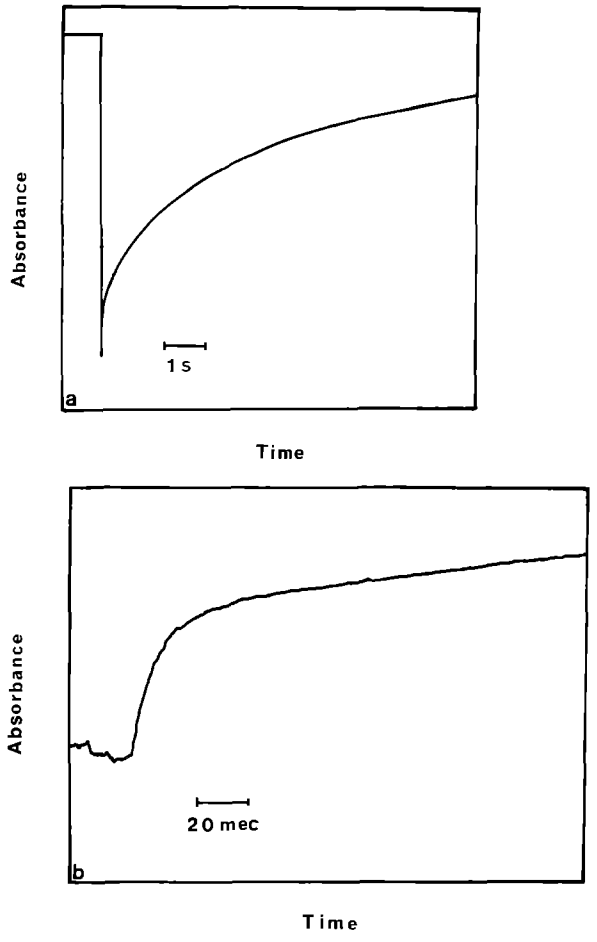



**Fig. 5a, b.** Time course of absorbance change at 465 nm, as a probe of ferric iron binding to ovotransferrin. (**a**) (top) slow phase up to 10 s. (**b**) bottom fast phase up to 200 ms. Solutions (A) and (B) were mixed at a ratio of (A)/(B)=1 vol/vol; (A) apo-ovotransferrin at $1.2 \times 10^{-4}$ M dissolved in 0.05 M Tris-HCl, pH 8.0 in the presence of 0.06 M $NaHCO_3$; (B) $2.5 \times 10^{-4}$ M Fe-NTA in 0.05 M Tris-HCl. Ordinate represents absorbance in arbitrary units

If the Cu uptake and release is a simple bimolecular reaction, this ratio (0.047) should agree with $K_C/K_N$ (=0.47), but it is one order of magnitude different. This result might indicate the existence of a different rate limiting step in Cu release induced by EDTA.

To investigate the points above in more detail, we have measured an average association constant (Experimental details are described in Appendix 1, which was $(2.6 \pm 0.1) \times 10^5$ $M^{-1}$. The average dissociation rate constant is then calculated as $(9.1 \pm 0.3) \times 10^{-3}$ $s^{-1}$. This value is of the same order as the Cu release reaction from the *C* site and suggests that Cu binding at the *C* site is a simple bimolecular reaction. On the other hand, Cu release from the *N* site is 20 times slower than that from the *C* site, which might imply the existence of an intermediate. We assumed that the backward reaction of Cu binding was negligible when analysing the forward reaction rate. This assumption was reasonable because the apparent rate constants for the uptake reactions of Cu and Tb were around 10 $s^{-1}$, whereas release reactions were in the range $10^{-2}$–$10^{-3}$.

In the case of Tb(III) binding, the situation is very similar to that for Cu(II) binding except that the *C* site has a higher affinity than the *N* site ($K_C/K_N = 2.2 \pm 0.4$ (Ichimura et al. 1989)). The average association constant $K_a$ was calculated as $(2.8 \pm 0.2) \times 10^6$ $M^{-1}$ (see Appendix 1) and the average dissociation reaction rate was estimated to be $(2.8 \pm 0.2) \times 10^{-2}$ $s^{-1}$. Since the metal release rate induced by EDTA was $(8.4 \pm 0.1) \times 10^{-2}$ $s^{-1}$ for the *N* site and $(5.8 \pm 0.1) \times 10^{-3}$ $s^{-1}$ for *C* site (Yamamura et al. 1988), it is reasonable to assume that Tb binding is a bimolecular reaction for the *N* site, whereas there might be an intermediate in Tb release from the *C* site, as appears to be the case for Cu release from the *N* site.

### 2. *Binding of Fe-NTA*

In contrast to the binding of copper or terbium, binding of Fe(III)–NTA, monitored at 465 nm, showed a multiphasic process (Figs. 5a and b). The fast phase was finished within 20 ms, there was a much slower second phase which lasted more than 10 s and one further slower process, lasting several minutes. We refer to these processes as I, II and III in what follows.

*(i) Process I.* The fastest phase was analyzed by assuming that it was a bimolecular reaction as in the case of Cu(II) and Tb(III). In the analysis, (10) instead of (8) was employed to subtract contributions of the slower phase.

Rate constants, $k_f$, were estimated using the nonlinear least squares method, and are shown in Fig. 6a. Rates thus obtained were almost independent of Fe(III) concentration with an average value of $(5.0 \pm 0.3) \times 10^5$ $M^{-1}$ $s^{-1}$, which confirms that the reaction is bimolecular. The association rate constants thus obtained are 10 times faster than the values for Cu(II) and Tb(III) binding. In all cases, association rate constants for Cu(II), Tb(III) and Fe(III) are much smaller than the diffusion-controlled limit ($\sim 10^9$ $M^{-1}$ $s^{-1}$). Differences between the rates for Fe(III), Cu(II) and Tb(III) are probably due to differences in the stereospecificity of the binding sites.

*(ii) Process II.* The second phase was analyzed according to (12).

$$F = -b \cdot \exp(-k_{2\,\mathrm{app}} t) + c \cdot t + d \qquad (12)$$

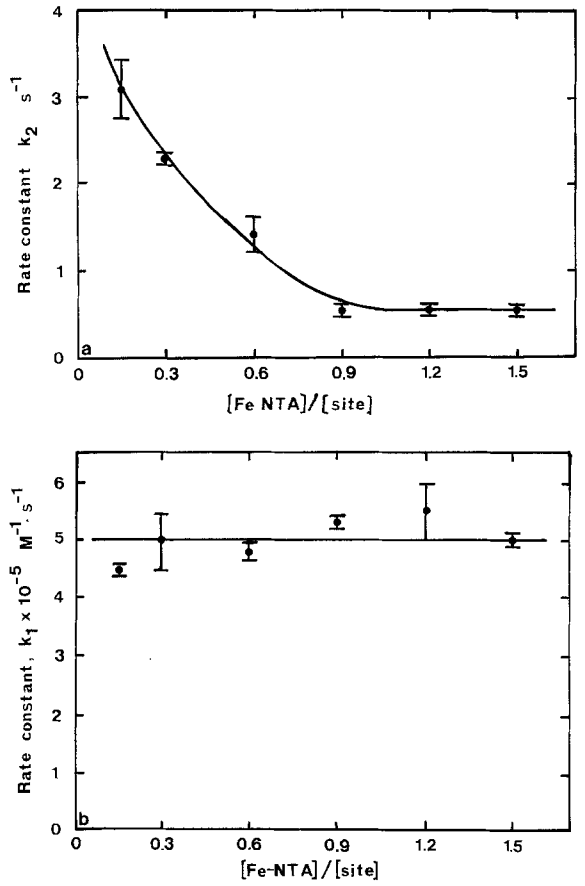



**Fig. 6a, b.** Rate constants of the slow (**a**) and fast (**b**) phases of Fe binding to ovotransferrin as a function of [Fe]/[Tf]

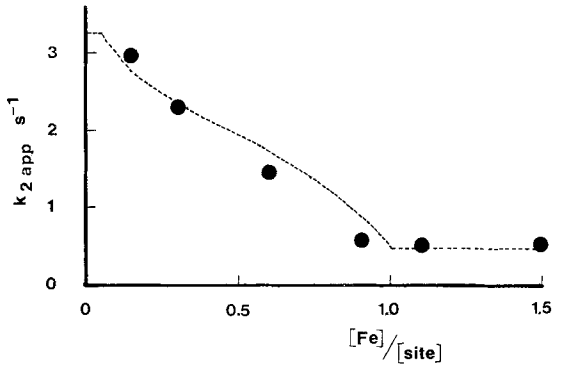



**Fig. 7.** An analysis of the formation rate for the slow phase (in Fig. 6a) according to the model (see Appendix II). Broken line was calculated according to the model shown in Appendix II. Solid circles were calculated values from the rate constants of process II (in Fig. 6a)

where $b$, $c$ and $d$ are constants. In the analysis, the contribution of the fastest process was removed by not using the initial part of the time course. As processes I and II were so well separated, this procedure was sufficient to obtain the apparent rate constant, $k_{2\,app}$. A linear term $c \cdot t$ was added for subtracting the contribution of the slowest phase. Values of $k_{2\,app}$ were thus estimated and are plotted in Fig. 6b, which shows that $k_{2\,kapp}$ decreases linearly up to [Fe]/[total $T_f$ sites] = 90%, and then becomes independent of Fe concentration for [Fe]/[total $T_f$ sites] > 120%. These results are very well explained with the following model; the rate of this process is mainly determined by whether the other site is occupied or not. We assume two rate constants, $k_1$ and $k_2$. The former corresponds to the rate when the other site is empty and the latter corresponds to the rate when the other site is occupied. With the simple analysis shown in Appendix II, we have estimated $k_1$ as 3.4 $s^{-1}$ and $k_2$ as 0.52 $s^{-1}$. Simulated results are shown in Fig. 7.

The above ideas do not exclude the possibility that the rate of process II differs for the $N$ and $C$ sites. However, the variation in rate with metal concentration is mainly determined by the state of occupancy of the other site.

*(iii) Process III.* The slowest process lasted several minutes and differed from the other processes in that the absorbance decreased (data not shown). These results are in good qualitative agreement with the data of Honda et al. (1980). They attributed this process to the release of a ligand, NTA, from an intermediate complex consisting of ovotransferrin, Fe, NTA, and $CO_3^{2-}$. A model that might explain these results is described in the next section.

*(iv) A model for Fe binding.* As far as our experiments are concerned, the metal-binding process can be summarized in a scheme shown in Fig. 8.

*(1)* Process I is a second-order reaction. The binding rate of the Fe-NTA complex for the $N$ and $C$ sites is assumed to be the same; i.e., Fe-NTA binds with a rate which does not depend on whether the other site is occupied by Fe-NTA. Thus, the binding rate constant in the scheme above is nearly the same; $k_{1N} = k_{1C} = k'_{1N} = k'_{1C}$.

*(2)* Process II shows a more complicated dependence on Fe-NTA concentration. Ovotransferrin, which binds Fe-NTA weakly, changes its conformation to form an intermediate involving Fe, NTA, ovotransferrin and $CO_3^{2-}$. In contrast to process I, the rates of formation of the intermediate complex become smaller with an increase in Fe-NTA concentration (see Fig. 6b). This result shows that the formation rate for one site is influenced by the state of occupancy of the other. Thus, the formation rate constant in the above scheme is explainable with the following relations; $k_{2N} > k'_{2N}$ and $k_{2C} > k'_{2C}$. This result implies that binding of the first Fe-NTA induces a comformational change which makes the second Fe-NTA form the intermediate complex more slowly. From our experimental results it is not possible to say anything about the relative magnitude of $k_{2N}$ and $k_{2C}$, it may be that these rate constants are in fact identical.

*(3)* From Fe(III) equilibrium binding studies (Yamamura et al. 1985), it is known that Fe(III) binds more strongly to the $N$ site than the $C$ site and that Fe(III) binding shows positive cooperativity. Our findings show that the second-order rate constants for binding of the Fe-NTA complex to the $N$ and $C$ sites are not very different. To be consistent with these results, differences in association constants would be attributed to differ-

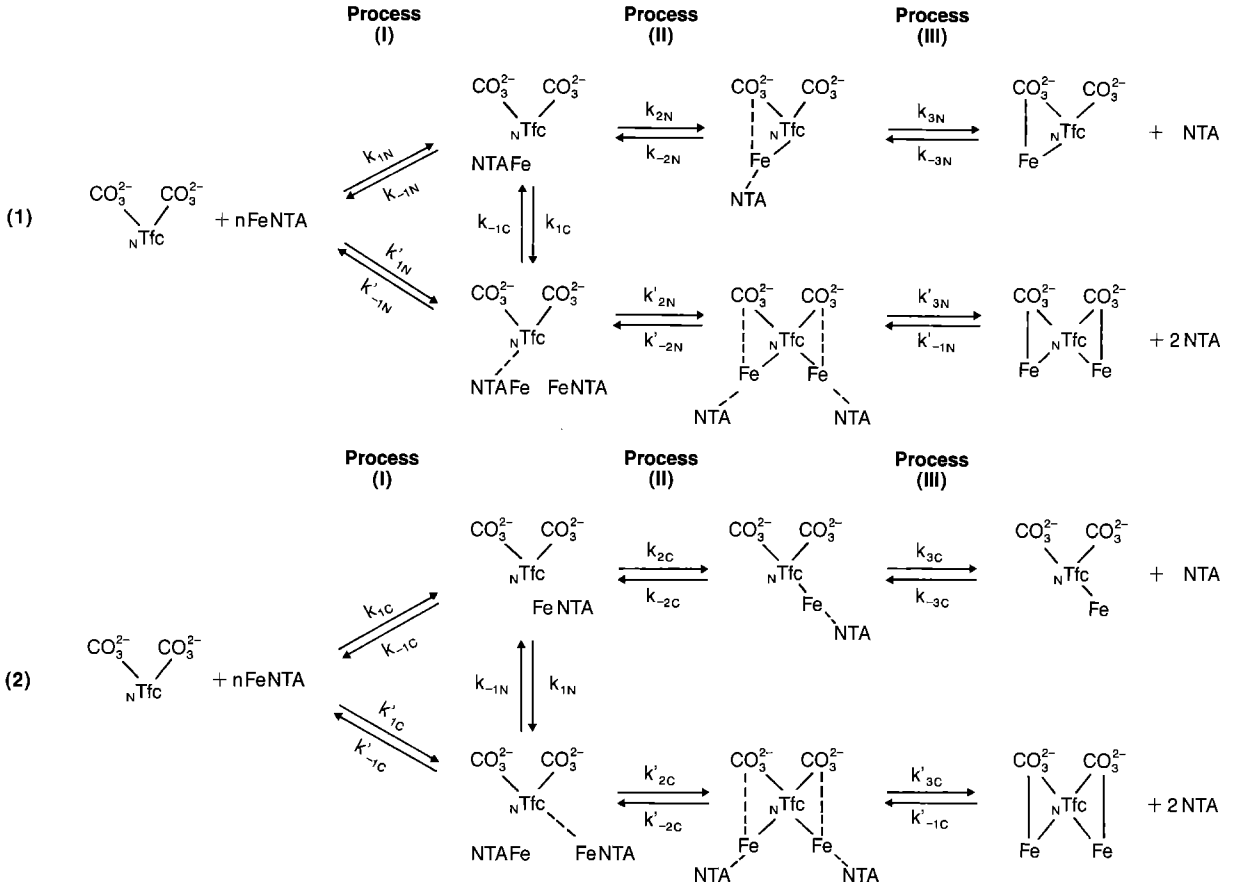
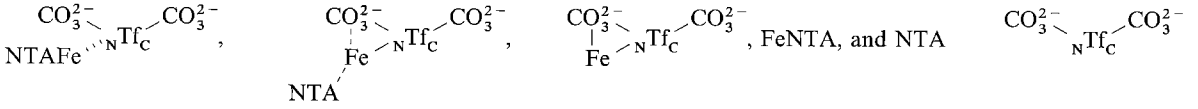



**Fig. 8.** A model scheme for the reaction of Fe-NTA with ovotransferrin. Sequences show the formation of (1) N-monoferric (upper) and diferric (lower) ovotransferrins from N-site preference bound FeNTA, and of (2) C-monoferic (upper) and diferric (lower) ovotransferrins from C-site preference bound FeNTA, respectively. The characters in sequence (i),




represent apo-ovotransferrin-carbonate, an ovotransferrin complex with weakly bound Fe-NTA, an intermediate complex involving NTA, a stable ternary complex of ovotransferrin-iron-carbonate, iron nitrilotriacetate complex, and nitrilotriacetate, respectively. $k$ is the rate constant and its subscripts indicate association ($1N$, $2N$, $3N$) or dissociation ($-1N$, $-2N$, $-3N$) rates. The symbols in the other sequence have similar meanings

ences in dissociation rate constants; $k_{-1N} < k_{-1C}$ and/or $k_{-2N} < k_{-2C}$, and $k'_{-1N} < k'_{-1C}$ and/or $k'_{-2N} < k'_{-2C}$ as in the case of Cu(II) and Tb(III).

*(v) Comparison with other reports.* Bates and coworkers (Bates and Wernicke 1971; Bates 1982; Cowart et al. 1982) have reported that the kinetics of binding of Fe-NTA to human serotransferrin are biphasic in the presence of bicarbonate ion at pH 7.45. The first phase is complete within 100 ms and it represents reaction of Fe-NTA with the metal-binding site of transferrin to form an intermediate involving NTA, iron, and transferrin in the presence of (bi)carbonate. The second phase is complete in 10 s and is thought to involve the release of NTA, so that the ternary complex of the stable iron(III)-transferrin-(bi)carbonate is formed.

Honda et al. (1980) have investigated the slower region using the stopped-flow method. They found a slow phase lasting several minutes in which the absorbance decreases. They attribute this process to the release of NTA and process III of our model probably corresponds to the process they observed. Although Bates and coworkers (Bates and Wernicke 1971; Bates 1982; Cowart et al. 1982) attribute their second phase to the release of NTA this seems unlikely when experimental results from the three groups are compared. The slower phase obtained by Bates and co-workers would appear to correspond to process II observed by us.

Thus our results partly confirm previous results but also allow us to establish a model for Fe binding which is shown in Fig. 8.

## Appendix I

An ovotransferrin molecule has two metal-binding sites. We determine an average association constant at a fixed pH of 8.3 in a buffer containing 30 m$M$ $NaHCO_3$. Cupric ion is bound to two sites without any cooperativity (Yamamura et al. 1986). An average association constant,

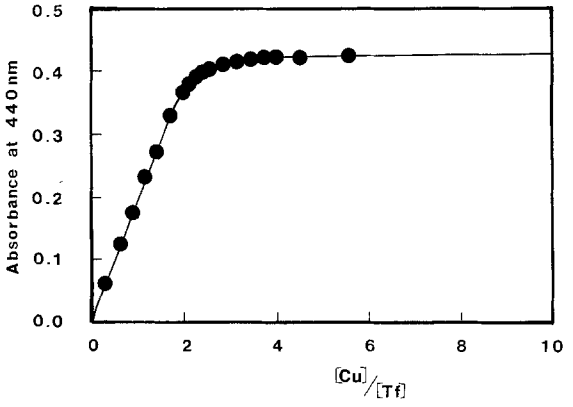



**Fig. 9.** Spectrophotometric titration of ovotransferrin with Cu(II) ion. Plots of absorbance changes at 440 nm. On the abscissa, [Tf] represents the site concentration of transferrin. Conditions: $1.2 \times 10^{-3}$ M ovotransferrin dissolved in 100 m*M* Tris-HCl buffer containing 30 m*M* $NaHCO_3$, pH 8.0. Average association constant, $K_a$ was estimated as $5.6 \times 10^3$ $M^{-1}$. The theoretical curve (solid line) was calculated with (A-7)

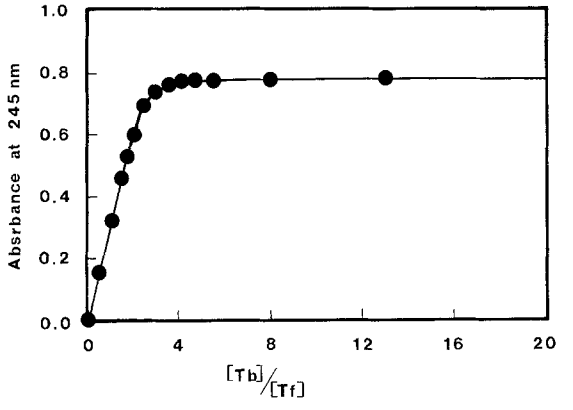



**Fig. 10.** Spectrophotometric titration of ovotransferrin with Tb(III) ion. Plots of absorbance changes at 245 nm. On the abscissa, [Tf] represents the site concentration of transferrin. Conditions: $1.2 \times 10^{-4}$ M ovotransferrin dissolved in 100 m*M* Tris-HCl buffer containing 30 m*M* $NaHCO_3$, pH 8.0. Average association constant, $K_a$ was estimated as $2.57 \times 10^2$ $M^{-1}$. The theoretical curve (solid line) was calculated with (A-7)

$K_a$, for Cu(II) binding to one site of ovotransferrin is calculated according to the following scheme and equations,

$$\mathrm{Tf} + \mathrm{Cu} \overset{K_a}{\rightleftharpoons} \mathrm{Cu\ Tf} \tag{A-1}$$

$$K_a = \frac{[\mathrm{Cu\ Tf}]}{[\mathrm{Cu}]\,[\mathrm{Tf}]} \tag{A-2}$$

$$[\mathrm{Cu}]_t = [\mathrm{Cu}] + [\mathrm{Cu\ Tf}] \tag{A-3}$$

$$[\mathrm{Tf}]_t = [\mathrm{Tf}] + [\mathrm{Cu\ Tf}] \tag{A-4}$$

where $[Cu]_t$ and $[Tf]_t$ are total concentrations. [Tf] and [Cu Tf] are concentrations of the apoprotein and the copper complex of ovotransferrin (per site). [Cu] indicates unbound copper concentration (existing as hydroxy complexes and/or Tris complexes etc.). [Cu Tf] is estimated from the specific absorption maxima of the copper complex, using $E^{1\%}_{440\,nm} = 0.54$ for the dicupric complex (Frankel-Conrat and Feeney 1950). $K_a$ is expressed as a function of [Cu Tf] using (A-2) to (A-4). Then we obtain the following equations,

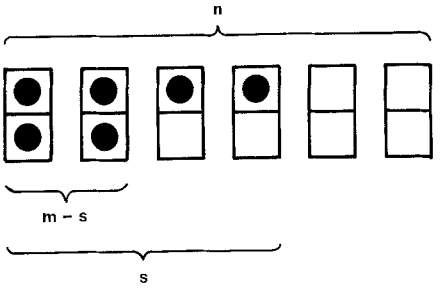



**Fig. 11.** A site-occupancy model for transferrin plus metal ions

$$[\mathrm{Cu\ Tf}] = \tfrac{1}{2}\left(x - \sqrt{x^2 - 4[\mathrm{Tf}]_t\,[\mathrm{Cu}]_t}\right) \tag{A-5}$$

where

$$x = [\mathrm{Cu}]_t + [\mathrm{Tf}]_t + 1/K_a$$

Amplitude analysis was done according to (A-7), using the non-linear Simplex method (Nelder and Mead 1964). The absorption difference due to copper binding can be written as

$$A - A_0 = \Delta\varepsilon\,[\mathrm{Cu\ Tf}] = \frac{\Delta\varepsilon}{2}[\mathrm{Tf}]_t\left(Y - \sqrt{Y^2 - 4\frac{[\mathrm{Cu}]_t}{[\mathrm{Tf}]_t}}\right), \tag{A-7}$$

where

$$Y = \frac{1}{K_a[\mathrm{Tf}]_t} + 1 + \frac{[\mathrm{Cu}]_t}{[\mathrm{Tf}]_t}$$

$A$ is absorbance, $A_0$ is the absorbance at $[Cu]_t = 0$, and $\Delta\varepsilon$ is the molecular absorption coefficient of the Cu-Tf complex. Equation (A-7) is rewritten as

$$A - A_0 = \frac{(A_f - A_0)}{2[\mathrm{Tf}]_t}\left(x - \sqrt{x^2 - 4[\mathrm{Tf}]_t[\mathrm{Cu}]_t}\right) \tag{A-8}$$

where $A_f$ represents the absorbance at saturating [Cu]. From (A-8), $K_a$, $A_0$ and $A_f$ were best-fitted by the non-linear Simplex method.

The $K_a$ thus estimated was $(2.6 \pm 0.2) \times 10^5$ $M^{-1}$. The average association constant, $K_a$ is related to the association constants for the $N$ and $C$ sites as follows:

$$K_a = \frac{K_N(1 + \lambda\, y)}{1 + y} = \frac{K_C(1/\lambda + y)}{1 + y} \tag{A-9}$$

where $y$ is the distribution ratio (=[$C$ site]/[$N$ site]) and $\lambda = K_C/K_N$ as defined by Yamamura et al. (1985). Although $K_a$ is not constant with changing Cu concentration, it does not actually change much because

$$0.75\,K_N \leq K \leq 0.83\,K_N\,.$$

In Tb(III) binding, similar equations ae used for the analysis with different molecular absorption coefficient, ovotransferrin and metal concentrations. $K_a$ for Tb(III) binding was calculated to be $(2.8 \pm 0.2) \times 10^6$ $M^{-1}$.

## Appendix II

Consider $n$ transferrins with $m$ iron atoms. Each iron atom is bound to free sites independently. If the other site is empty the association rate is $k_1$, and if the other site is occupied, the association rate is $k_2 (k_1 > k_2)$. Then,

$$k_{app} = k_1 f_1 + k_2 f_2 \qquad \text{[B-1]}$$

where $f_1$ and $f_2$ are the fractions of protein molecules with one and two sites occupied. Assume that $s$ molecules bind at least one iron and then $(m-s)$ molecules bind two iron atoms. Denote zero, one and two sites occupied combinations as $C_0$, $C_1$ and $C_2$. Then

Number of molecules with no sites occupied: $n-s$
Number of molecules with one site occupied: $2s-m$
Number of molecules with two sites occupied: $m-s$

Then

$$C_0 = \sum_{s=m/2}^{m} \frac{n!}{(n-s)!\,(2s-m)!\,(m-s)!} \qquad \text{[B-2]}$$

$$C_1 = \sum_{s=m/2}^{m} \frac{n!}{(n-s)!\,(2s-m-1)!\,(m-s)!} \qquad \text{[B-3]}$$

$$C_2 = \sum_{s=m/2}^{m} \frac{n!}{(n-s)!\,(2s-m)!\,(m-s-1)!} \qquad \text{[B-4]}$$

$$f_1 = \frac{C_1}{C_1 + C_2}, \quad f_2 = \frac{C_2}{C_1 + C_2} \qquad \text{[B-5]}$$

In Fig. 7, [B1] is illustrated.

Thus, concentration dependency for the apparent rate constants is explainable with the assumption that the rate of binding is much faster when the other site is empty.

## References


Aasa R, Malmsrom RB, Saltman P, Vangerd T (1963) The specific binding of iron(III) to transferrin and conalbumin. Biochim Biophys Acta 75:203–222

Aisen P, Lang G, Woodworth RC (1973) Spectroscopic evidence between the iron-binding sites of conalbumin. J Biol Chem 248:647–653

Anderson BF, Baker HM, Dodson EJ, Norris GE, Rumball SV, Wates JM, Baker EN (1987) Structure of human lactoferrin at 3.9 A resolution. Proc Natl Acad Sci USA 84:1769–1772

Bates GW (1982) Metal ion and anion exchange reactions of serum transferrin; the role of quaternary complexes and conformational transitions: In: Saltman P, Hegenaiuer J (eds) The biochemistry and physiology of iron. Elsevier, North Holland, pp 3–18

Bates GW, Schlabach MR (1973) A study of the anion binding sites of transferrin. FEBS Lett 33:289–292

Bates GW, Wernicke J (1971) The kinetics and mechanism of Iron(III) exchange between chelates and transferrin IV. The reaction of transferrin with iron (II) NTA. J Biol Chem 246:3679–3685

Bezkorovainy A (1980) Chemistry and metabolism of the transferrin In: Biochemistry of nonheme iron. Plenum Press, New York, pp 127–206

Brock JH (1985) Transferrins. In: Harrison P (ed) Topics in molecular and structural biology, vol 7. Verlag Chemie, Weinheim Basel, pp 178–261

Cowart RE, Kojima N, Bate GW (1982) The exchange of $Fe^{3+}$ between acetohydroxamic acid and transferrin. Spectrophotometric evidence for a mixed ligand complex. J Biol Chem 257:7560–7565

Donovan JW, Ross KD (1975) Non equivalence of the metal binding sites of conalbumin. J Biol Chem 250:6022–6025

Feeney RE, Komatsu StK (1966) The transferrins. Struct Bond 1:149–206

Frankel-Conrat H, Feeney RE (1950) The metal-binding activity of conalbumin. Arch Biochem 29:101–113

Glazer AN, McKenzie HA (1963) The denaturation of proteins IV. Conalbumin and iron(III)-conalbumin in urea solution. Biochim Biophys Acta 71:109–123

Gorinsky B, Horsburgh C, Lindley PC, Moss DS, Parker M, Watoson JL (1979) Evidence for the bilobal nature of diferric rabbit plasma transferrin. Nature 281:157–158

Harris DC, Aisen P (1973) Facilitation of Fe(II) autoxidation by Fe(III) complexing agents. Biochim Biophys Acta 329: 156–158

Honda K, Nishikata I, Sasakawa S (1980) Kinetics of specific binding of iron(III)-NTA to human apotransferrin and of the ligand exchange of the resulting complex using the stopped-flow technique. Chem Lett (Jpn) 1980:21–24

Ichimura K, Kihara H, Yamamura T, Satake K (1989) Negative cooperativity of chicken ovotransferrin on Al(III)-binding. J Biochem 106:50–54

Luk CK (1971) Study of the nature of the metal-binding sites and estimate of the distance between the metal-binding sites in transferrin using trivalent lanthanide ions as fluorescent probes. Biochemistry 10:2838–2843

Metz-Boutigue M-H, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P (1984) Human lactotransferrin: Amino acid sequence and structural comparisons with other transferrins. Eur J Biochem 145:659–676

Nelder JA, Mead R (1964) A simplex method for function minimization. Comput J 5:308–313

Tan AT, Woodworth RC (1969) Ultraviolet difference spectral studies of conalbumin complexes with transition metal ions. Biochemistry 8:3711–3716

Warner RC, Weber I (1951) The preparation of crystalline conalbumin. J Biol Chem 191:173–180

Williams J, Elleman TC, Kingston IB, Wilkina AG, Kuhn KA (1982) The primary structure of hen ovotransferrin. Eur J Biochem 122:297–303

Yamamura T, Hagiwara S, Nakazato K, Satake K (1984) Copper complexes at *N*- and *C*-site of ovotransferrin: quantitative determination and visible absorption spectrum of each complex. Biochem Biophys Res Commun 119:298–304

Yamamura T, Ikeda H, Nakazato K, Takimura K, Satake K (1985) "Cooperativity between the *N* and *C* domains of ovotransferrin observed on iron binding and thermal denaturation". In: Spik G, Montreuil J, Crichton RR, Mazurier (eds) Proteins of iron storage and transport. Elsevier, Amsterdam, pp 53–56

Yamamura T, Ichimura K, Hayashi A, Tsuda T, Taniguchi T, Toi R, Kawashima S, Maeda Y, Kihara H, Satake K (1988) Lanthanoid complex of iron-transport protein, transferrin. Kinetic study on release of the metal from *N* and *C* binding sites. Nippon kagaku kaishi (in Japanese) 1988:452–458

Yang F, Lum JB, McGill JR, Moore CM, Naylor SL, van Bragt PH, Baldwin WD, Bowman BH (1984) Human transferrin: cDNA characterization and chromosomal localization. Proc Natl Acad Sci USA 81:2752–2756