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Aluminum Ion Removal from Monoaluminum Ovotransferrin by Pyrophosphate

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The rates at which aluminum was removed from the N- and C-terminal monoaluminum ovotransferrins by pyrophosphate were evaluated by UV difference spectra in 0.01 mol/L Hepes, pH＝7.4 and at 37 ℃. Pesudo first-order rate constants as a function of pyrophosphate concentration were measured. The results indicate that the pathways of aluminum removal are different. For the N-terminal binding site, aluminum removal follows simple saturation kinetics, while the removal of aluminum from the C-terminal binding site reverts to the combination of saturation and first-order kinetics. The saturation component is consistent with a rate-limiting conformational change in the protein as has been reported. We propose that the first-order kinetics mechanism is attributed to a pre-equilibrium process. The rate constants of saturation kinetics are accelerated from both terminals with the addition of 0.1 mol/L chloride to the monoaluminum ovotransferrin solutions, whereas the rates of the first-order kinetics are decreased for the C-terminal binding site. The effect of chloride ionic strength causes a continuing increase on k_{obs} for the N- and C-terminal binding sites. Moreover, the kinetics behavior of the N-terminal is more easily affected by chloride than that of the C-terminal. In the experiment presumably the N-terminal site is apparently kinetically more labile than the C-terminal site.

Keywords monoaluminum ovotransferrin, kinetics, chloride, pyrophosphate

Introduction

The transferrins are a family of iron-binding proteins which are the requirement of a synergistic anion for the interaction of these proteins with metals.[1,2] Although these proteins consist of a single chain of about 700 amino acid resides, they fold into two distinct, homologous lobes connected by a short polypeptide.[2-4] Each lobe contains one high-affinity iron-binding site, which is designated as the C-terminal or the N-terminal site, respectively. The ligands for each iron-binding site consist of the same set of six ligating groups: the phenolic oxygens of two tyrosines, the imidazde group of a histidine, the carboxylate group of an aspartic acid residue, and two oxygens of the bidentate carbonate synergistic anion.[2-4]

The kinetic process of the ferric release from different serum-transferrin forms *in vitro* was widely studied.[5-10] The results show that there exist different removal pathways by different ligands.[11-17] Li *et al*. studied the release of aluminum ion from the recombinant N-lobe half-molecule of serum transferrin by pyrophosphate.[18] The mechanism of iron release by ovotransferrin was also studied.[19] However, there are relatively few kinetic studies on the release of metal ions other than ferric from ovotransferrin. Ovotransferrin (OTf) is a main member of transferrin family and has a dual role in both the transport of iron and the antibacterial function.[20]

Aluminum is nonessential element and of great concern due to its large natural abundance and its possible toxic effects.[21-24] It has attracted much attention to people' health. Several different studies show that the ultimate agent for transport of aluminum in the body's plasma is believed to be the serum transferrins.[25] The binding of aluminum to transferrin inhibits the uptake of iron, and the inhibition ratio is up to 90%.[25] Therefore, it is significant to study the pathway of aluminum delivery, the state of complex, the mechanism of biomedical processes and the effect on mankind health, and more and more interest has been attracted on the subject for scientists in the fields of environment, biochemistry and chemistry.

The pyrophosphate is an important inorganic anion in biologic vivo, The interaction of pyrophosphate with many metal ions forms a soluble and stable complex compound, and it plays an important role in the progress of metal ionic metabolism.[26,27]

In the paper, the pathways of aluminum release from monoaluminum ovotransferrin were analyzed by pyrophosphate using the means of UV difference spectral technique. And we introduced the mechanism of aluminum release from the C-terminal monoaluminum

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ovotransferrin, which is attributed to a pre-equilibrium process.

Experimental

Materials

Ferrous ammonium sulfate, disodium ethylenediaminotetraacetic acid (EDTA), sodium perchlorate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), sodium chloride, aluminum nitrate and potassium pyrophosphate were all analytical grade reagents, and egg apoovotransferrin (apoOTf) was parchased from Sigma.

Stock solutions

A stock solution of aluminum nitrate in 0.10 mol/L HNO_3 was prepared and standardized by complexiметric back-titration with EDTA and zinc. The stock solution of pyrophosphate (PP_i) was prepared by dissolving weighed sample in 0.01 mol/L Hepes, pH＝7.4.

Preparation of protein samples

ApoOTf was prepared as previous description.[28] Protein concentration was determined by UV-vis spectroscopy by measuring the absorbance at 280 nm and calculating the concentration using the extinction coefficient ε_{280}＝91200 $cm^{-1}\cdot(mol\cdot L^{-1})^{-1}$.[29]

N-Terminal monoaluminum ovotransferrin (Al_N-OTf) 0.8 equivalent of freshly prepared aluminum nitrate solution was added to an apoOTf solution. The solution was stirred in the air for 10 min and then stored at 4 ℃ for an hour to reach equilibrium after aluminum was added without further purification.

C-Terminal monoaluminum ovotransferrin (Al_C-OTf-Fe_N) Adding one equivalent of ferrous ammonium sulfate to the whole apoOTf molecules in 0.01 mol/L Hepes, pH＝7.4, there are preferential oxidation and binding to the N-terminal binding site of apoOTf.[30] C-Terminal monoaluminum ovotransferrin was prepared by adding 0.8 equivalent of freshly prepared aluminum nitrate solution to the N-terminal monoferric ovotransferrin.

UV-vis spectra

To perform kinetic studies of aluminum removal from monoaluminum ovotransferrins, a solution of monoaluminum ovotransferrin (μmol/L) in 0.01 mol/L Hepes，pH＝7.4 buffer was pre-incubated at 37 ℃ to equilibrate. The PP_i solution was added to the solution, which represents a range of ligand:OTf ratios from 3∶1 to l50∶1. The spectra were monitored immediately after the addition of the ligand, at 242 nm every 6 s for 6—45 min using the UV-vis Chemstation software from an HP8453UV-vis spectrophotometer. Experiments were repeated twice at each ligand concentration. These measurements were performed in 1 cm quartz curette maintained at constant temperature (37 ℃) using an HP8453UV-vis spectrophotometer equipped with a thermostated cell holder and a circulating water bath.

Collection of kinetic data

The aluminum removal reaction was followed by using a UV-vis spectrophotometry to monitor the change of the absorbance of the monoaluminum ovotransferrin at 242 nm. The change of the absorbance at 242 nm vs. time is shown in Figure 1. Pseudo first-order rate constants were calculated from nonlinear least-square fits from Eq. (1).

$$A_t=(A_0-A_\infty)e^{-k_{obs}t}+A_\infty \quad (1)$$

where A_t, A_0 and A_∞ are the absorbance at any time t, time zero, and the infinite time, respectively.

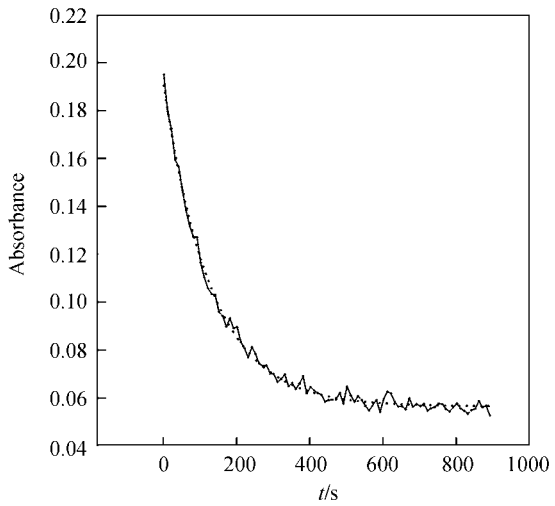

Figure 1 The variation of the $\Delta A_{242\ nm}$ vs. time. ----, fits curve of the $\Delta A_{242\ nm}$ vs. time according to Eq. (1).

Results

Aluminum removal by PP_i without added chloride

Figure 2 shows the plots of the observed constants versus the ligand concentration for aluminum removal from both N- and C-terminal monoaluminum ovotransferrins by PP_i. It can be seen from Figure 2(b) that the values of k_{obs} for the N-terminal monoaluminum ovotransferrin increase with the increasing of PP_i at low ligand concentration, but at higher concentration of the ligand the value of k_{obs} approaches a maximum value and becomes essentially independent of the concentration of PP_i. The solid line in Figure 2(b) represents calculated fits to Eq. (2). The two parameters, k_{max} and k_d are listed in Table 1. The experimental results for the N-terminal site can be fit very well to Eq. (2). It means that the pathway for aluminum removal for the N-terminal binding site is simple saturation kinetics. And such a result is usually attributed to a rate-limiting conformational change in the protein.[2]

$$k_{obs}=\frac{k_{max}[L]}{k_d+[L]} \quad (2)$$

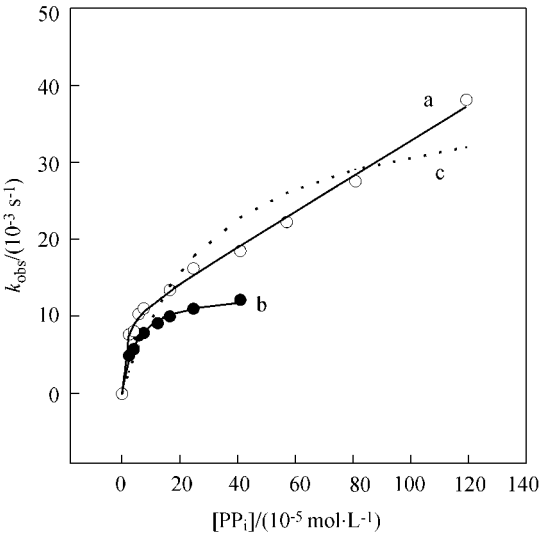

Figure 2 Apparent first-order rate constants for Al removal from C- and N-terminal monoaluminum OTf as a function of the concentration of PP_i. All solutions were buffered at pH=7.4 by 0.01 mol/L Hepes and maintained at 37 ℃. ●, Al_N-OTf; ○, Fe_N-OTf-Al_C. The curves c and b represent fitting of the data to Eq. (2) and the curve a represents fitting of the data to Eq. (3).

The plot of k_{obs} for the aluminum removal from the C-terminal binding site by PP_i vs. $[PP_i]$ is shown in Figure 2(a). Initially we have attempted to fit the data to Eq. (2), and the fitting value is shown in as curve c. However the result is so bad that we have to fit the rate constants for this system using Eq. (3), in which a first-order term is added to Eq. (3). The curve a in Figure 2 represents calculated fits to Eq. (3) using three parameters. The values of k_{max}, k_d, and k''' are listed in Table 1, too. The aluminum removal from the C-terminal binding site by PP_i can be described empirically by Eq. (3). It means that there is a complex PP_i dependence that appears to consist of saturation kinetics and first-order kinetics.

$$k_{obs}=\frac{k_{max}[L]}{k_d+[L]}+k'''[L] \quad (3)$$

Table 1 Kinetic parameters for Al(III) removal from Al_N-OTf and Fe_N-OTf-Al_C by PP_i

Sample	k_{max}/s^{-1}	$k_d/(10^{-6}\ mol\cdot L^{-1})$	$k'''/(mol^{-1}\cdot L\cdot s^{-1})$
Without added NaCl			
Al_N-OTf	0.013±0.0004	47.2±4.4	0
Al_C-OTf-Fe_N	0.010±0.0007	10.1±4.5	22.9±0.96
With 0.1 mol/L NaCl			
Al_N-OTf	0.016±0.0003	54.2±3.8	0
Al_C-OTf-Fe_N	0.012±0.0048	16.1±2.7	15.3± 0.81

Chloride effects on aluminum removal

Effect of 0.1 mol/L chloride concentration The effects of 0.1 mol/L chloride concentration on the ligand dependence of aluminum removal from both forms of monoaluminum ovotransferrins by PP_i are shown in Figure 3. The ligand dependence of aluminum removal from the N-terminal binding site follows simple saturation kinetics in Figure 3(b), namely it adapts to Eq. (2), and the values of k_{max} and k_d are also listed in Table 1.

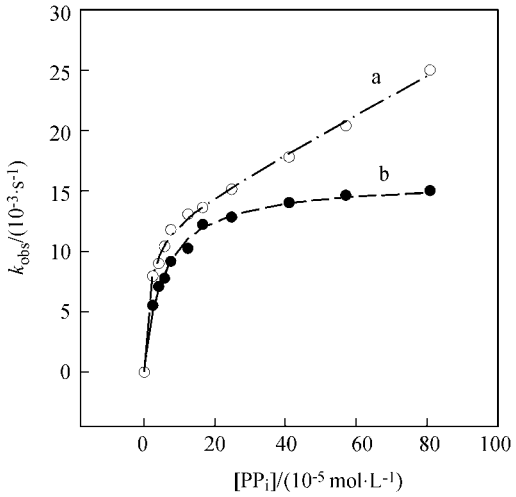

Figure 3 The variation of apparent rate constant for the removal of aluminum from monoaluminum OTf as a function of the concentration of PP_i at 37 ℃, pH=7.4, 0.01 mol/L Hepes buffer containing 0.1 mol/L chloride. ●, Al_N-OTf; the curve b represents fits of the data to Eq. (2); ○, Fe_N-OTf-Al_C, the curve a represents fits of the data to Eq. (3).

There are only modest changes in k_{max} and the presence of 0.1 mol/L chloride concentration makes k_{max} increase by about 23%. Under the same condition the ligand dependence of aluminum removal for the C-terminal site is shown in Figure 3(a), which adapts to Eq. (3), and the kinetic parameters of k_{max}, k_d and $k'''$are listed in Table 1, too. It indicates that aluminum removal from the C-terminal site follows both simple saturation and first-order kinetics. The effect of chloride concentration is much larger for the first-order component. k_{max} is increased by about 20%, while k''' is decreased by about 33%. From the experiment result, it can be derived that the first-order pathway of aluminum removal is retarded for the C-terminal site in the presence of 0.1 mol/L chloride concentration.

Effect of chloride ionic strength The effect of increasing concentration of chloride on the aluminum removal from both the terminal binding sites at a fixed PP_i/protein ratio (50/1, mol/mol) at pH=7.4 and 37 ℃ is shown in Figure 4. The addition of chloride causes a continuing increase in k_{obs} for chloride concentrations from 0.02 to 0.10 mol/L. Under the condition, the saturation kinetics pathway is greatly affected for the N-terminal binding site. The saturation kinetics and the first-order kinetics pathways are affected, but as a whole the rates of release are enhanced for the C-terminal binding sites. The slopes of these plots give a relative ordering of the release rates towards N- and C-terminal binding sites. The slope with C-terminal binding site for

the aluminum removal is a little lower than that with N-terminal binding sites. The result implies that the kinetics behavior of the N-terminal binding sites is more readily affected by chloride than that of the C-terminal binding site.

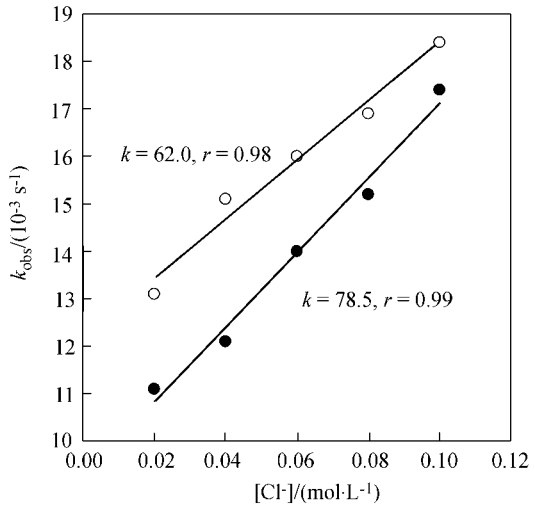

Figure 4 Variation in k_{obs} for Al removal from monoaluminum OTf as a function of the different concentration of Cl^- for 37 ℃, pH 7.4 , 0.01 mol/L Hepes buffer. ○, Al removal from Al_C-OTf; ●, Al removal from Al_N-OTf.

Discussion

Aluminum removal from the N-terminal binging site by PP_i is simple saturation kinetics in 0.01 mol/L Hepes buffer, pH=7.4 and at 37 ℃. In contrast, aluminum removal from the C-terminal binding site shows a complexity that appears to consist of saturation kinetics and first-order kinetics. Such a result is possibly attached to have extra disulphide bond at the C-lobe[12] which leads to a small conformational change from "close" to "open" when aluminum is released. Moreover, the pathway of simple saturation kinetics is primary for the C-terminal.

With the buffer described above, the effects of 0.1 mol/L chloride concentration promote aluminum removal of simple saturation kinetics from both binding sites of monoaluminum ovotransferrins. And the effect of the chloride ionic strength on k_{obs} for both terminals causes a continuing increase. Explanations of the slightly faster rate of aluminum removal by PP_i in the addition of 0.1 mol/L chloride concentration to the buffer are as follows: (1) Chloride may occupy the KISAB site,[10,31] so chloride decreases positive charge residues close to metal binding site. The behavior weakens the connection of synergistic anion (CO_3^{2-}) with OTf so that it induces a conformational change to lead to the opening of the lobes. (2) Chloride may displace the carbonate synergistic ion, which would destabilize aluminum coordination sphere in OTf. (3) Chloride may break the hydrogen bonds that maintain the protein in a "closed" conformation.

The calculated values of k_{max} indicate that the release rates are mildly higher for the N-site than the C-site under all conditions listed in Table 1. The phenomenon implies that the functions of both sites are different. However, the value of k''' ($k'''=0$) is not affected whether in the presence or absence of 0.1 mol/L chloride concentration for the N-terminal binding site. Based on the results above, it can be deduced that the conformational change of the N-lobe is larger than that of the C-lobe. Therefore, a conformational change is primary for the N-terminal binding site, which is the mechanism of saturation kinetics.[32] The mechanism is shown below, where the asterisk indicates on "open" conformation of monoaluminum ovotransferrins. OTf stands for the CO_3^{2-}-OTf complex and L represents PP_i.

$$\text{Al-OTf} \underset{k_{-1}\ \text{slow}}{\overset{k_1}{\rightleftharpoons}} \text{Al-OTf*} \tag{4}$$

$$\text{Al-OTf*} + \text{L} \underset{k_{-2}\ \text{fast}}{\overset{k_2}{\rightleftharpoons}} \text{Al-OTf*-L} \tag{5}$$

$$\text{Al-OTf*-L} \xrightarrow[\text{fast}]{k_3} \text{Al-L} + \text{OTf*} \tag{6}$$

Assuming steady-state conditions for both intermediates Al-OTf* and Al-OTf*-L, Eq. (7) can be derived.

$$k_{obs}=\frac{k_1k_2k_3[\text{L}]}{k_{-1}[k_{-2}+k_{-3}]+k_2k_3[\text{L}]} \tag{7}$$

The mechanism predicts that Eq. (4), the conformational change of the aluminum protein from an unreactive "closed" to a reactive "open" form, will become the rate-limiting step in the aluminum removal at high ligand concentrations. The maximum value of k_{obs}, which is designated as k_{max}, should be independent of the ligand used to remove aluminum.

The parameter of k_d which represents the ligand concentration for the C-site is a little smaller than that for the N-site in the above both cases. It means that the former release is slightly faster than the latter for the saturation kinetics.

The first-order kinetics is retarded in the presence of 0.1 mol/L chloride concentration. It appears that there is a slowly substitutional function between PP_i and Cl^- in the KISAB site.[10] This negative chloride effect can be approximately modeled by assuming competition between pyrophosphate and chloride in the KISAB[10,31] so that aluminum release can be affected by PP_i and Cl^- exchange reactions between aluminum accepting pyrophosphate and apoovotransferrin.

In view of dynamic equilibrium between "open" and "close" in the C-terminal binding site,[33] we propose that the removal mechanism of first-order kinetics from the C-terminal binding site involves a pre-equilibrium, see

the mechanism below:

$$Al_C\text{-}OTf^* + L \underset{k_a'}{\overset{k_a}{\rightleftharpoons}} Al_C\text{-}OTf^*\text{-}L \xrightarrow{k_b} Al_C\text{-}L + OTf^*$$

where Al_C-OTf*-L denotes the intermediate. A pre-equilibrium arises when the rates of formation of the intermediate and its decay back into reactants are much faster than its rate of formation of products, thus, the condition is possible when $k_a' >> k_b$ but not when $k_b >> k_a'$. Because we assume that Al_C-OTf*, L, and Al_C-OTf*-L are in equilibrium, we can write

$$\frac{[Al_C\text{-}OTf^*\text{-}L]}{[Al_C\text{-}OTf^*][L]} = K, \qquad K = \frac{k_a}{k_a'} \tag{8}$$

In writing these equations, we are presuming that the rate of reaction of Al_C-OTf*-L to Al_C-L is too slow to affect the maintenance of the pre-equilibrium. The rate of formation of Al_C-L may be written as:

$$\frac{d[Al_C\text{-}L]}{dt} = k_b[Al_C\text{-}OTf^*\text{-}L] = k_b K[Al_C\text{-}OTf^*][L] \tag{9}$$

This rate law has the form of a second-order rate law with a composite rate constant:

$$\frac{d[Al_C\text{-}L]}{dt} = k[Al_C\text{-}OTf^*][L] \tag{10}$$

$$k = k_b K = \frac{k_a k_b}{k_a'} = k''' \tag{11}$$

$$[L] >> [Al_C\text{-}OTf^*]$$

Then Eq. (12) can be derived:

$$k_{obs} = \frac{k_a k_b}{k_a'}[L] = k'''[L] \tag{12}$$

A high second-order rate constant (k''') means that PP_i has high affinity for the aluminum and is able to remove aluminum quickly from the C-terminal binding site. The two different effects of chloride affecting the two pathways for the C-terminal binding site are coincident with the two pathways being parallel.[10]

It can be arrived at the conclusion on the basis of the preceding analysis that the N-terminal site is apparently kinetically more labile than the C-terminal site.

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