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Binding of Anion to Ovotransferrin: Insight into pH Dependence of Binding Ability

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The binding of anions to ovotransferrin was studied by difference UV spectra. Direct titrations of ovotransferrin with phosphate, sulfate, pyrophosphate, citrate and monovalent anion chloride produce a strong negative absorbance near 237 nm and a weak negative absorbance near 280 nm. Presuming that each anion-binding site is identical, the calculation of the binding constants was carried out by the relevant equations. The anion-binding ability is pH-dependent.

Key Words: Binding constant, Ovotransferrin, Anion, Difference UV spectra.

INTRODUCTION

Transferrins are a family of iron-binding proteins, which include serum transferrin, ovotransferrin and lactoferrin[1]. These proteins are characterized by the unique requirement that a carbonate anion is bound concomitantly with each ferric ion into a protein-carbonate-iron ternary complex[2]. It has been established that transferring binds a variety of metals. These include toxic uranyl ions, which form rather stable uranyl-transferrin derivatives[3]. Serum transferrin is iron transporter protein that mediates the delivery of iron from blood to the target cell, transferrin interacts with its receptor primarily through the C-lobe both at the cell surface and inside the endosome[4]. Ovotransferrin, a major egg white protein, possesses the cellular iron transport function *in vitro*[5].

In biological systems, the anion is (bi)carbonate, which binds to cationic protein side groups in the immediate vicinity of the metal binding site. The carbonate anion shields the metal ion from the destabilizing and also provides one oxygen as a direct legend to the metal[6]. Difference UV studies have shown that in the absence of a metal ion, a series of inorganic anions will bind to both lobes of transferrin[7] and to both the isolated N- and C-lobes of ovotransferrin[8]. Among the simple inorganic anion, there are two distinct types of anions. Synergistic anion, which bind to the protein and promote metal binding. The kinetics data suggest that not only is a synergistic anion required for tight Fe^{3+} sequestration by ferric binding protein, but also the synergistic anion plays a critical role in the process of inserting Fe^{3+} into the binding site of ferric finding protein[9]. Non-synergistic anion such as phosphate and sulfate, which interact with apoprotein but fail to promote subsequent metal binding. Harris *et al.*[10] had detected the binding of some inorganic anions to apotransferrin. Anion-binding constants were calculated from the direct titration data by nonlinear least-squares techniques. However, binding constants of anions to ovotransferrin have still not been reported so far. This paper highlights on the calculated values of some binding constants of anion binding to ovotransferrin by direct titration of ovotransferrin with some anions. The effect of pH value on binding ability of anion to ovotransferrin was experimentally examined.

EXPERIMENTAL

To avoid contamination by extraneous metal ions, all glassware was washed in acid and rinsed with deionized water that was also used to prepare all solutions. All buffer solutions were adjusted to pH value required by adding NaOH or HCl and measured at 25 ± 0.5 ºC with a Beckman acidometer. All salts were analytical grade and were used without further purification.

Iron-free ovotransferrin was purchased from sigma. A solution of ovotransferrin was prepared by dissolving about 80 mg of protein into 2 mL of 0.1 M Hepes buffer (pH 7.4) containing 0.1 M sodium perchlorate and purified by ultrafiltration as previously described. The final ovotransferrin concentration was determined from the absorbance at 278 nm using an extinction coefficient of 91, 200 $M^{-1}cm^{-1}$ [11].

Difference-UV titration of ovotransferrin with anion was carried out with a modernized cary UV-VIS spectrophotometer. The ovotransferrin was contained in Hepes buffer (pH 7.4). Defining ovotransferrin as a baseline, the difference spectra were recorded from 220 to 500 nm after titration of

anion to ovotransferrin. The equilibrium constants for the binding of anions to ovotransferrin were calculated using the method as following.

Presuming that the numbers of anion-binding sites are n, in which binding ability is essentially independent and identical. The equilibrium equation for the system of anion-ovotransferrin is defined as:

$$nA + P \underset{K_D}{\overset{K_A}{\rightleftharpoons}} AnP \quad (1)$$

where, K_A, K_D, n, are binding constant, dissociation constant, anion-binding site, respectively. The sign A and P denote anion and protein. According to the definition of binding constant, one can calculate as:

$$[A]_t/[A]_b = 1 + 1/K_A(n[P]_t-[A]_b) \quad (2)$$

where, $[A]_t$ and $[A]_b$ are total concentration of anion, the concentration of anion binding to apoprotein for each titration point, respectively. $[P]_t$ is the total concentration of protein for each titration point. $[A]_b$ is calculated by eqn. (3).

$$[A]_b = \Delta\varepsilon/\Delta\varepsilon_M \times [P]_t \times n \quad (3)$$

where, $\Delta\varepsilon$ is difference absorptivity of anion-transferrin complex for each titration point. $\Delta\varepsilon_M$ is maximum of difference absorptivity. The plot was made by eqn. (2) on the premise that the value of n is 1,2,3,4,5…, respectively. The x-coordinate shows $1/(n[P]_t-[A]_b)$ and y-coordinate $[A]_t/[A]_b$. Thus the slope of this line should be correspond to $1/K_A$. Selecting the best fit, in which intercept should approach 1 and linear coefficient should be also about 1. Thus the number of anion-binding sites and binding constants K_A can be determined by the means.

RESULTS AND DISCUSSION

Difference UV spectra of anion binding to apoovotransferrin: The addition of anion to ovotransferrin produces a relatively strong minimum at 237nm, a weaker minimum at 280 nm. Titration curve of ovotransferrin with phosphate in Hepes buffer at pH 7.4 was shown in Fig. 1. Essentially identical spectra have been obtained from the titrations with the other anions. These anion-ovotransferrin spectra are exactly the inverse of the difference-UV spectra produced by the binding of metals to ovotransferrin. The absorbance data have been converted to absorptivities by dividing the absorbance at the 280 nm. Titration curves prepared by plotting absorptivity *versus* the accumulated concentration of the anion were shown in Fig. 2. All the final absorptivities of anion-ovotransferrin saturable sites range from 12000 to 16000 M^{-1} cm^{-1}. The large slope means the strong binding ability[8]. The preliminary conclusion was drawn as following: the binding ability of pyrophosphate-ovotransferrin is strong relatively among the anions examined.

In addition, monovalent anion chloride was also examined similarly. In contrast with divalent or trivalent anions, the binding ability of chloride-apoovotransferrin is obviously weak.

Determination of anion-binding sites and binding constants of anion to apoovotransferrin: The binding sites and binding constants were calculated by eqn. (2). The best fit was finally determined as 4 of n value, which suggests that there are four anion-binding sites in ovotransferrin. Binding constants for some anions binding to ovotransferrin were listed as shown in Table-1.

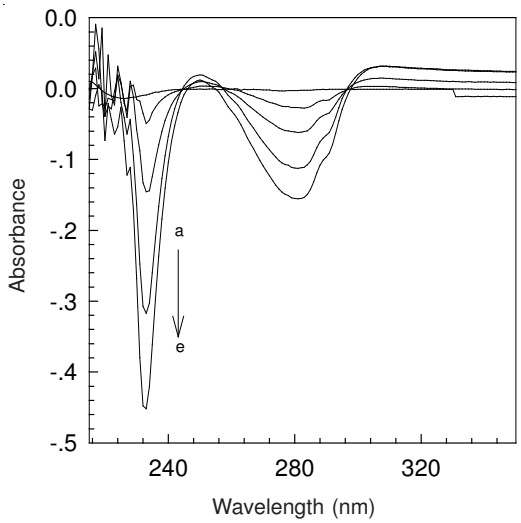

Fig. 1. Difference UV spectrum generated by the titration of apoovotransferrin with phosphate

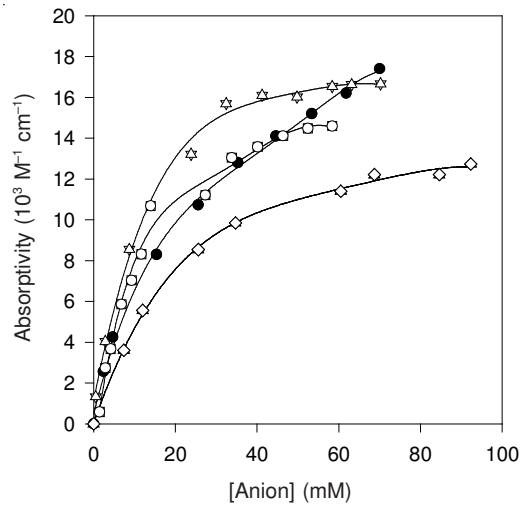

Fig. 2. Titrations of apoovotransferrin with various inorganic anions; HPO_4^{2-} (□), SO_4^{2-} (◇), cta^{3-} (●), $HP_2O_7^{3-}$ (Δ)

TABLE-1
BINDING CONSTANTS FOR SOME ANION BINDING TO APOOVOTRANSFERRIN

Anion	Dominant form	log K_A
Pyrophosphate	$HP_2O_7^{3-}$	5.15 ± 0.07
Phosphate	HPO_4^{2-}	4.78 ± 0.23
Citrate	Cta^{3-}	4.64 ± 0.09
Sulfate	SO_4^{2-}	4.17 ± 0.15
Chlorate	Cl^-	2.86 ± 0.13

Effect of pH on UV-visible spectrum of anion binding to ovotransferrin: Titrations of ovotransferrin (5.8 mM) with non-synergistic anion phosphate in 0.1 M Hepes at pH 8.1, 7.4, 6.8, 6.2 and 5.6, respectively, were shown in Fig. 3. The reaction is apt to reach saturated state at lager pH. The equilibrium reaches about 20 mM at pH 8.1 while about 40 mM at pH 7.4. When pH values are 6.8, 6.2 and 5.6, the equilibrium can't reach under the experimental condition. Particularly, phosphate can hardly bind to ovotransferrin at low concentration. However, binding ability increases sharply above 40 mM. Fig. 4 showed the UV-visible spectrum of phosphate binding to ovotransferrin at 5.8 mM. Compared with Fig. 3,

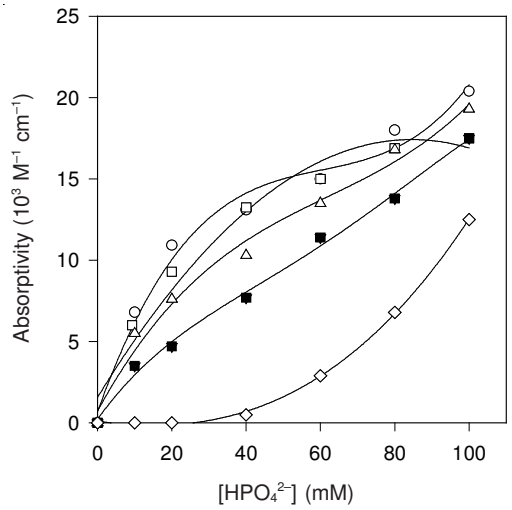

Fig. 3. Titrations for the addition of HPO_4^{2-} to apoovotransferrin at different pH value in 0.1 M Hepes. pH value: pH 8.1 (○), pH 7.4 (□), pH 6.8 (△), .pH 6.2(■), e. pH 5.6(◇)

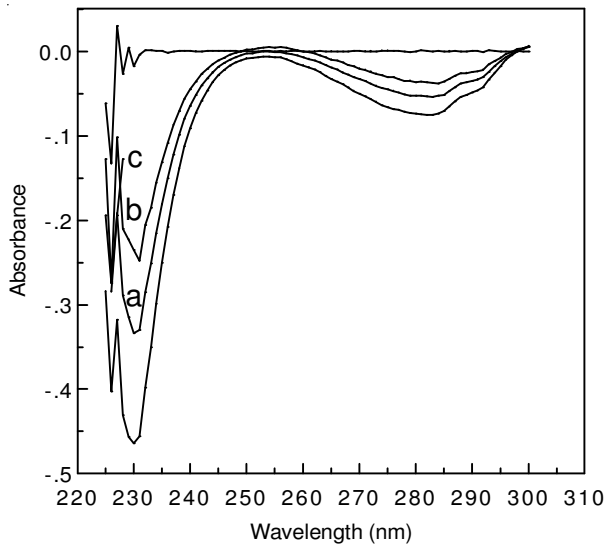

Fig. 4. Different UV spectrum produced by the addition of HPO_4^{2-} to apoovotransferrin at different pH value in 0.1 M Hepes. pH value: a. pH 8.1; b. pH 7.4; c. pH 6.8

one can draw a conclusion that specific binding of ovotransferrin to anion enhances with increased pH at low anion concentration and non-specific binding increases with decreased pH at high anion concentration.

Anion binding to ovotransferrin: The binding of anions to transferrin produces the characteristic spectrum, with a strong negative absorbance around 245 nm and a weak negative absorbance around 295 nm with a weak positive absorbance between two isobestic points[7,10,12], which arises from binding of anion to Tyr95 and Tyr188 directly or indirectly[10]. Similarly, the spectrum generated by anions binding to ovotransferrin produces a strong negative absorbance around 237 nm and a weak negative absorbance around 280 nm, which is associated with phenolic group of tyrosine and was demonstrated by measuring the UV-VIS difference spectra of model complex EHPG and HBED.

In discussing these anion-binding constants, we refer to the predominant species of phosphate, citrate and pyrophosphate as HPO_4^{2-}, cta^{3-} and $HP_2O_7^{3-}$ at pH 7.4. The binding constants of divalent or trivalent anions are about 100-times larger than that of monovalent anion chloride, which indicates that anionic charge is the over-riding factor. However, the calculated results showed that the binding constant of divalent phosphate is larger than that of trivalent citrate. There are else some factors that have essentially effect on anion to ovotransferrin. A radius of anion plays an important role in the process of anions binding to ovotransferrin. The effective radius of citrate is larger than that of phosphate and thus the resulting binding ability is the net result of anionic charge and radius. The anion-protein interaction is primarily of $NH\cdots A^-$ hydrogen bonds. For the oxaanions, large difference in electronegativity between the central atom and peripheral oxygen would lead to a higher negative charge density on the oxygen, which should results in a higher anion binding constant[12], which can also explain the reason that binding ability of phosphate is stronger than that of sulfate. As to geometrical configuration of anion, the binding sites of ovotransferrin might take advantage of the tetrahedral oxaanions over the spherical halides.

Possible anion-binding sites: There are two types of anion-binding sites. One type of anion-binding site might involve in positive charge sphere, which relates closely with tyrosine near the metal-binding site. Therefore, the binding of anions to transferrin can lead to perturbation of $\pi\rightarrow\pi$ of aromatic ring of tyrosine of metal-binding site. It is reasonable that this type of anion-binding site can be monitored using UV spectra[13]. Since the binding (bi)carbonate leads to the metal complex, it seeks likely that the anion binding site will involve in the essential arginine residue, which is hydrogen bonded to the carbonate in the Fe-CO_3-Tf ternary complex (Arg124 in transferrin)[12]. The Arg121 is consensus carbonate anchor group for holo ovotransferrin. Tyr191 of N-lobe and Tyr524 of C-lobe are directly or indirectly relevant to the binding of anion to ovotransferrin. Cyrstallographic data of the isolated N-lobe of ovotransferrin suggests that there are three SO_4^{2-}-binding sites[14]. Site 1 involves in Ser-OG and His250-NE2. Site 2 includes Ser91-OG, Arg121-NE and Arg121-NE2. Site 3 comprises Tyr191-N and Ser192-OG. The apo C-lobe was crystallized and the structure determined at 2.3 Å resolution demonstrated the existence of single bound SO_4^{2-} in the interdomain cleft[15]. The number of anion-binding sites obtained by this experiment is consistent with result obtained by crystallographic data. For transferrin, Lys206 and Lys296 might also be involved in anion binding[1]. Cheng had titrated the Lys206, Lys296 and Lys296E mutants of Tf/2N with sulfate and had confirmed that both Lys206 and Lys296 are important components of the N-lobe anion binding site[16].

Another type of anion-binding site locates in the position which is relatively far from the metal-binding site of ovotransferrin (or transferrin). Since the binding cannot lead to perturbation of $\pi\rightarrow\pi^*$ of aromatic ring of tyrosine of metal-binding site, the binding of anion to ovotransferrin (or transferrin) cannot be detected by UV-VIS spectrum. This type of anion-binding site was monitored by electro paramagnetic resonance spectrum[17].

pH Dependence of anion binding to ovotransferrin: As mentioned above, binding of anion to ovotransferrin can

lead to disturbance of phenolic group of tyrosine of the binding site, these phenolic groups are highly dependent on pH, consequently the binding of anion to ovotransferrin is pH-dependent. Hydrogen-bond between Lys296 and Lys206 in human transferrin would break with increased acidity, which might make the structure of protein become looser and so enhance the accessibility of anion[13]. In the hTf N-lobe, two critical lysine facilitate this pH-dependent conformational change allowing entry of a chelator to capture the iron. In the C-lobe, the lysine pair is replaced by a triad of residues: Lys534, Arg632 and Asp634. Mutation of any of these triad residues to alanime results in significant conformational change. The role of the three residues is probed further by conversion to the residues observed at the equivalent positions in ovotransferrin (Q-K-L)[18]. Trigger Lys209 is highly pH sensitive in the hen ovotransferrin N-lobe[19].

Present research can also draw the similar conclusion that binding of anion to ovotransferrin is pH-dependent and binding ability enhances with increased acidity (Fig. 4). One of possible reason is that a part of phenolic groups of tyrosine are protonated with increased acidity when some original hydrogen-bonded structures become looser and accessible[20]. Another possible reason is on the basis of a hypothesis that positive charges around the ligand amine acid residue grow in quantity with increased acidity and so the larger positive charges produce the much stronger anion-binding ability.

In addition, the actual form of anion in a giving buffer solution might be a predominant factor controlling the binding force at the region of low concentration of anion. For example, HPO_4^{2-} is the predominant form of phosphate at pH 7.4 and 12 % PO_4^{3-} presents in solution at pH 8.1. The higher valance of anion can exactly lead to the stronger binding ability[7]. The ratio of the higher valance of anion increases with increased pH value and so the finding from Fig. 4 is reasonable. At the region of high concentration of anion, specific binding sites are occupied by anions gradually, the degree of protonation of amino acid residues at non-specific binding sites enhances with increased acidity and consequently would result in a stronger electrostatic force. The form of anion has an effect on binding ability, however, experimental finding showed that non-specific binding stemmed from electrostatic force is predominant factor.

It is known that the conformational change caused by some trigger factor is not the change of molecule throughout but the alteration of active sites such as metal-binding sites or anion-binding sites. The two alterations have different effect on groups near the binding sites[21]. Within the neutral range, the emission peak of ovotransferrin is located in 336 nm, which mainly stem from luminescence of tryptophane[22]. Bathochromic shift occurs within the acid or basic range. The emission peak of free tryptophane is about 348 nm in aqueous solution[23]. Varying acidity from neutral to acid or basic range can primarily lead to a shift of tryptophane residue from hydrophobic region to hydrophilic region, which might result in conformational change. Therefore, one can conclude that fluorescence quenching of ovotransferrin derived from protonation of phenolic group of tyrosine within acid range and deprotonation within basic range would be caused by conformational change.

Abbreviations

Tf	serum transferrin
hTf	human serum transferrin
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
EHPG	ethylene-N,N'-bis[o-hydioxyphenylglycine]
HBED	N,N'-di[2-hydroxybenzyl] ethylenedi amine-N,N'-diaceticacid
UV spectrum	Ultraviolet- Visible spectrum
KISAB	the Kinetically Significant Anion Binding Site

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