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Spectral study on the binding of gadolinium ions with apoovotransferrin

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Abstract

The binding of Gd^{3+} ion to apoovotransferrin (apoOTf) was monitored by means of UV difference spectra in 0.01 M Hepes, pH 7.4 at 25 °C. Used 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) as fluorescence probe the conformational changes of protein were studied while gadolinium ions bound to apoOTf. The results show that Gd^{3+} binding produces peaks at 244 and 294 nm that is the characteristic of binding at the apoOTf specific metal-binding sites. At 244 nm the molar absorptivity of Gd–apoOTf complex is $(1.99 \pm 0.17) \times 10^4 \, \text{cm}^{-1} \, \text{M}^{-1}$. The apparent binding constants for the complexes of Gd^{3+} with apoovotransferrin are $\log K_1 = 7.61 \pm 0.14$ and $\log K_2 = 4.96 \pm 0.26$. A very large conformational change of apoovotransferrin appears when Gd^{3+} is bound to the N-terminal binding site. When Gd^{3+} is bound to C-terminal binding site there is less conformational change.
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1. Introduction

The transferrins are a superfamily of metal-binding protein, which are single-chain, 80 kDa glycoproteins, characterized by their ability to bind iron tightly (an effective binding constant is about $10^{20.7}$), but reversibly [1,2]. Since transferrin is not normally saturated with iron, it has an appreciable binding capacity for other metal ions [3]. It is known that the application of lanthanides have been widely investigated and some cells can take in lanthanide by means of phagocytosis or pinocytosis [4,5]. There is more interest in the complexes of transferrin with lanthanides [6–10].

Ovotransferrin is found in avian egg white. Ovotransferrin acts as bacteriostats by scavenging iron and keeping it from infectious organisms, that probably is derived from the fact that in vivo the protein is largely present in their apo- (iron-free) forms and is then able to bind iron so tightly that it is unavailable for bacterial growth [11]. The three-dimensional structure of ovotransferrin, as revealed by X-ray crystallography [12], is similar to those of human lactoferrin and rabbit serum transfer-

rin, being folded into two homologous lobes, each containing two dissimilar domains with one Fe^{3+} and one CO_3^{2-} bound at a specific site in each interdomain cleft. The coordination spheres of iron are identical in the two lobes. Each iron atom is linked to one aspartyl carboxylate oxygen, two tyrosyl phenolate oxygens, and one histidyl nitrogen. The remaining two coordination requirements of iron are satisfied by two oxygens of the carbonate co-anion which is secured to the protein by a network of hydrogen bonding to an arginine nitrogen, a threonine oxygen, and the N-terminus of a helix protruding into the binding cleft. Although similar, the sites are not identical, differing in their chemical, their spectroscopic, and possibly their physiological properties [13]. The fluorescence of terbium was enhanced after terbium bound to apoovotransferrin. It can be used to measure the binding constants for sequential binding two terbium ions to apoovotransferrin [14]. In the paper the binding of gadolinium to apoovotransferrin was studied by UV difference spectra.

2. Experimental

2.1. Materials

Nitrilotriacetic acid (NTA), *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (Hepes), disodium ethylenedi-

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aminetetraacetic acid (EDTA), ferrous ammonium sulfate and sodium perchlorate were all analytical grade reagents and were used as received. Chicken egg white apoovotransferrin (apoOTf) and 2-p-toluidinylnaphthalene-6-sulfonate (TNS) were purchased from Sigma.

Apoovotransferrin (100–150 mg) was dissolved into 8 mL of 0.01 M Hepes buffer, pH 7.4, and containing 0.1 M sodium perchloride. The volume of the solution was reduced at room temperature to 1 mL using an Amicon Model 8010 ultrafiltration cell fitted with an XM-50 membrane under 4.13×10^5 Pa of nitrogen gas to remove chelating agents. The dilution and concentration step was repeated four times. Then the buffer was changed into the buffer non-containing sodium perchlorate and repeated the dilution and concentration step for two times. The concentration of protein was determined from the absorbance at 278 nm using an extinction coefficient of 93,000 cm^{-1} M^{-1} [15].

Diferric ovotransferrin solution was prepared by adding 2 equiv. of ferrous ammonium sulfate to apoovotransferrin and was used without further purification [14].

N-terminal monoferric transferrin was prepared from apoovotransferrin by adding 1 equiv. of ferrous ammonium sulfate and was used without further purification [14].

The gadolinium stock solution was prepared by dissolving weighed sample of the oxide in some volume of dilute hydrochloride acid, and the solution was diluted to volume with distilled water. The gadolinium stock solution was standardized by complexometric titration with ethylenediaminetetraacetic acid using xylenol orange as a metal indicator in acetic acid–sodium acetate buffer at pH 5.5.

Since apoovotransferrin has a high affinity towards metal ions, precautions were taken to avoid contamination by extraneous metal ions. All glassware including cuvette were routinely soaked in 1 M HNO$_3$ and then rinsed with distilled water.

2.2. Method

Solution of apoovotransferrin in 0.01 M Hepes, pH 7.4 was added to dry, 1 cm cuvette, and a baseline of protein versus protein was recorded from 225 to 320 nm at Hewlett-Packard 8453 UV–Vis spectrophotometer. Apoovotransferrin was titrated with an acidic solution of Gd^{3+} ion at ambient bicarbonate concentration. During the titration the sample cuvette was maintained at 25 °C by a thermostated cell holder connected to an external circulating water bath (Huber). Diferric ovotransferrin and N-terminal monoferric ovotransferrin were also titrated. To correct the dilution during each titration and normalize the results from run to run, the absorbance data were converted to absorptivities ($\Delta \varepsilon$), dividing the absorbance by the analytical concentration of protein.

All fluorescence experiments were performed on a Hitachi F-2500 at room temperature, with all slit widths set to 10 nm. Excitation wavelength was 320 nm and fluorescence emission spectra were recorded with a single scan over the range 360–640 nm for TNS.

3. Results

3.1. Difference UV spectra

As apoovotransferrin is titrated by the addition of an acidic Gd^{3+} solution in 0.01 M Hepes, pH 7.4 and at 25 °C, the UV difference spectra from 225 to 320 nm are shown in Fig. 1. It can be seen that there are a larger maximum at 245 nm and a smaller maximum at 296 nm. It is the characteristic of a wide variety of metal–apotransferrin complexes [8,16]. The difference spectra
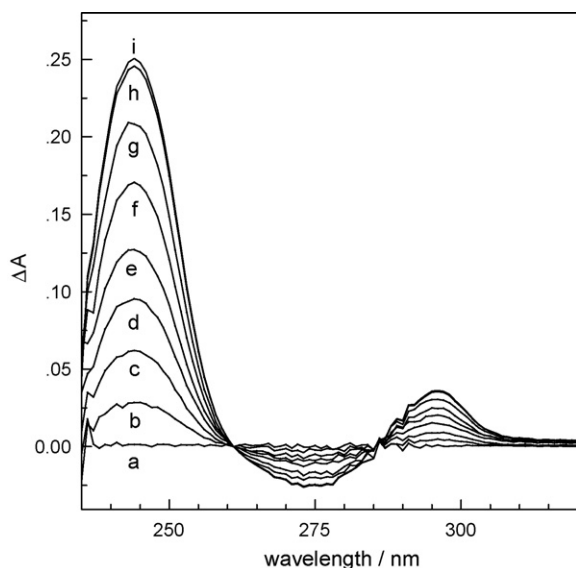

Fig. 1. UV difference spectra produced by the titration of Gd^{3+} (3.3×10^{-4} M) to 2.0 mL of apoovotransferrin (2.1×10^{-5} M) in 0.01 M Hepes at pH 7.4 and 25 °C. The volume of Gd^{3+} (μL) is (a) 0, (b) 10, (c) 20, (d) 35, (e) 50, (f) 70, (g) 100, (h) 140, and (i) 180, respectively.
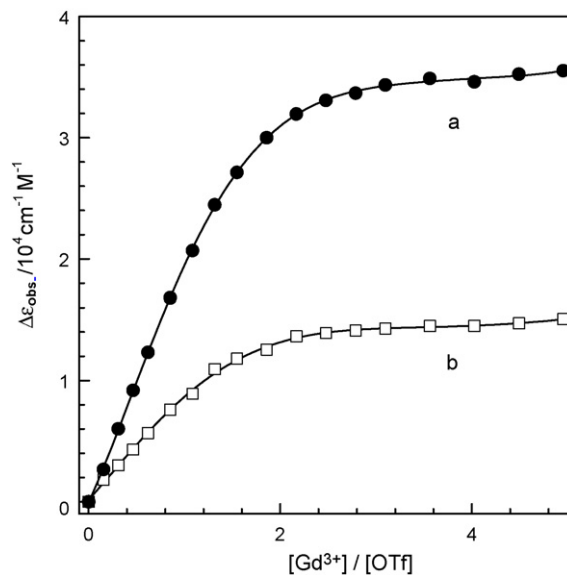

Fig. 2. The titration curves of Gd^{3+} to apoovotransferrin and Fe$_N$–ovotransferrin in 0.01 M Hepes at pH 7.4 and at 25 °C. (a) Apoovotransferrin and (b) Fe$_N$–ovotransferrin.

arise from the small perturbations in UV bands of the aromatic rings of the two tyrosine residues at each binding site that are directly coordinated to the metal ion [17]. It can be also seen that the absorbance at 245 nm is gradually increased with the addition of Gd^{3+} and approach a maximum and does not increase continuously. To correct for dilution effects and to normalize data from run to run, the absorbance data is converted to apparent absorptivities by dividing the absorbance at 245 nm by the analytical concentration of apoovotransferrin. The resulting absorptivity is plotted versus the ratio of total Gd^{3+} to the analytical apoovotransferrin concentration as Fig. 2 (curve a).

In Fig. 2 (curve a) the initial part of the titration is linear. The linear indicates that, in the early stages of the titration, apoovotransferrin is binding essentially 100% of Gd^{3+} in each aliquot of titration. Under these conditions the initial slope of the titration curve is equal to the molar absorptivity of Gd^{3+}–apoovotransferrin complex ($\Delta\varepsilon_{Gd}$). The molar absorptivity calculated by this method is $(1.99 \pm 0.17) \times 10^4 \, cm^{-1} \, M^{-1}$. The complete saturation of two binding sites of apoovotransferrin [6] should yield a final absorptivity of $4.00 \times 10^4 \, cm^{-1} \, M^{-1}$ based on the $\Delta\varepsilon_{Gd} = (1.99 \pm 0.17) \times 10^4 \, cm^{-1} \, M^{-1}$. As more Gd^{3+} is added, the increasing saturation of Gd^{3+} binding sites leads to less effective binding of addition Gd^{3+}, and the plot of absorptivity ($\Delta\varepsilon$) versus equivalent of Gd^{3+} begins to curve downward. The titration curve eventually levels off at about $3.51 \times 10^4 \, cm^{-1} \, M^{-1}$ after the addition of almost 5 equiv. of Gd^{3+}, which indicates that the two binding sites are not saturated with Gd^{3+}.

Since ferric ion is bound so much more tightly than the lanthanide, the presence of ferric ion at both binding sites block Gd^{3+} binding to the metal-binding sites. So diferric ovotransferrin and N-terminal monoferric ovotransferrin were also titrated with Gd^{3+}. The titration curve of N-terminal monoferric ovotransferrin is shown in Fig. 2 (curve b). There is no measurable absorbance during the titration of diferric ovotransferrin. It means that under the experimental condition the absorbance at 245 nm completely comes from the binding of Gd^{3+} on metal-binding sites of apoovotransferrin when apoovotransferrin is titrated by the addition of Gd^{3+}. Comparing the titration curve of vacant C-terminal site with the titration curve of apoovotransferrin, titration of the vacant C-terminal site begins with a much lower slope and reaches a maximum of only $1.75 \times 10^4 \, cm^{-1} \, M^{-1}$. Thus the C-terminal site appears to have a lower binding constant and Gd^{3+} binds to N-terminal binding site of apoovotransferrin preferably.

Defining the number of Gd^{3+} bound per apoovotransferrin molecule, n:

$$n = \frac{\Delta\varepsilon_{max}}{\Delta\varepsilon_{Gd}} \tag{1}$$

where $\Delta\varepsilon_{max}$ is the observed maximum absorptivity at titration curves and $\Delta\varepsilon_{Gd}$ is the molar absorptivity of Gd^{3+}–apoovotransferrin complex. It can be obtained that each apoovotransferrin can bind 1.76 of Gd^{3+}. For Fe_N–ovotransferrin n is 0.75.

3.2. Binding constants

The successive binding of Gd^{3+} to apoovotransferrin can be described by two sequential equilibria:

$$\text{apoovotransferrin} + \text{Gd} \rightleftharpoons \text{Gdapoovotransferrin} \tag{2}$$

$$\text{Gdapoovotransferrin} + \text{Gd} \rightleftharpoons \text{GdapoovotransferrinGd} \tag{3}$$
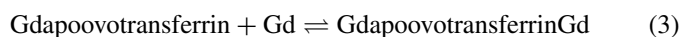
The thermodynamic binding constants for these equations are written by the following equations:

$$K_1 = \frac{[\text{Gdapoovotransferrin}]}{[\text{Gd}][\text{apoovotransferrin}]} \tag{4}$$

$$K_2 = \frac{[\text{GdapoovotransferrinGd}]}{[\text{Gdapoovotransferrin}][\text{Gd}]} \tag{5}$$

where K_1 and K_2 are apparent binding constants, which are limited to the experimental conditions. [Gd] and [apoovotransferrin] refer to the molarities of free Gd^{3+} and free apoovotransferrin, [Gd–apoovotransferrin] and [Gd–apoovotransferrin–Gd] refer to the molarities of proteins, which bind one and two Gd^{3+} ion, respectively.

It is presumed that there is no difference in the molar absorptivity for the two binding sites of apoovotransferrin [6]. Then the absorptivity at any point in titration curve can be calculated by first using initial guesses of K_1 and K_2 to solve the appropriate mass balance equation for [apoovotransferrin] and [Gd] and then using these values in Eq. (6):

$$\Delta\varepsilon_{calcd} = \frac{\begin{array}{c} \Delta\varepsilon_{Gd} K_1 [\text{Gd}][\text{apoovotransferrin}] \\ + 2\Delta\varepsilon_{Gd} K_1 K_2 [\text{Gd}]^2 [\text{apoovotransferrin}] \end{array}}{[\text{apoovotransferrin}]_t} \tag{6}$$

where [apoovotransferrin]$_t$ refers to the analytical concentration of apoovotransferrin. The molar absorptivity of Gd–apoovotransferrin complex, $\Delta\varepsilon_{Gd}$, is obtained from the initial slope of the apoovotransferrin titration curve. This leaves K_1 and K_2 as the only unknowns needed to calculate $\Delta\varepsilon_{calcd}$. In principle the titration curve in Fig. 2 can be fit by non-linear least squares using K_1 and K_2 as the only adjustable parameters to obtain K_1 and K_2. In practice using chelating agents as competition ligand can decrease the correlation of K_1 and K_2 to obtain more accurate K_1 and K_2 as descried previous [16]. So the titrations with Gd^{3+} were repeated using metal titrant solutions that contained a range of concentrations of NTA. The chelating agent competes with apoovotransferrin for Gd^{3+} and thus at any point in the titration there is a distribution of Gd^{3+} between apoovotransferrin and chelating agent, NTA, systematically reduce the observed absorptivity as the NTA:Gd^{3+} ratios increase. A series of titration curves for Gd^{3+}–NTA solutions are shown in Fig. 3. For NTA participation in the reaction the mass balance equations should include apoovotransferrin, Gd^{3+} and NTA. The titration data in Fig. 3 can be fitted using Eq. (6) yet, in which K_1 and K_2 is the only adjustable parameters.

Using difference UV titrations Harris [6] demonstrates that with both binding sites apotransferrin binds two lanthanide ions, either smaller or larger lanthanide ions and the amount of
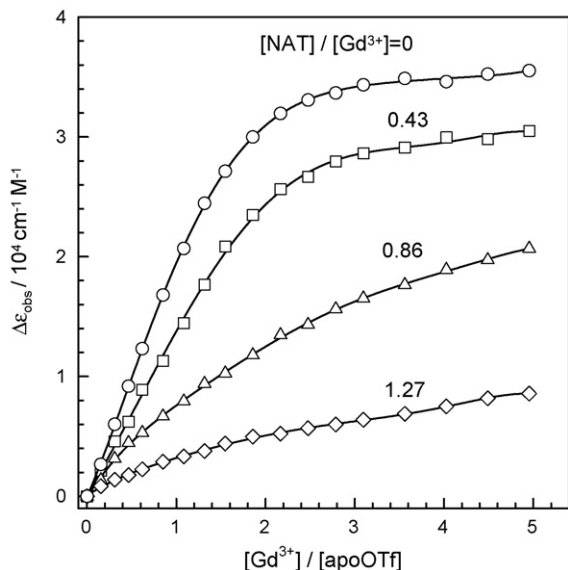
Fig. 3. The titrations of approximately 2.1×10^{-5} M apoovotransferrin in 0.01 M Hepes at pH 7.4 and 25 °C with 3.3×10^{-4} M solutions of Gd^{3+} that contained varying NTA:Gd ratios. NTA:Gd is 0, 0.43, 0.86, and 1.27, respectively.

saturation is affected by the concentration of bicarbonate. For an ambient bicarbonate concentration is about 0.14 mM at pH 7.4, it will interfere with Gd^{3+} binding to apoovotransferrin. The solubility product of $RE_2(CO_3)_3$ follows in the range of 10^{-31} to $10^{-33.4}$ [18]. This gives an upper limit on the concentration of free Gd^{3+} in the range of 10^{-6} to 10^{-7} M. Since the Gd^{3+}-carbonate species not included in mass balance equations the non-linear least squares fitting are restricted to points for which the calculated concentration of free Gd^{3+} is not allowed to exceed 10^{-6} M. From Fig. 3 the apparent binding
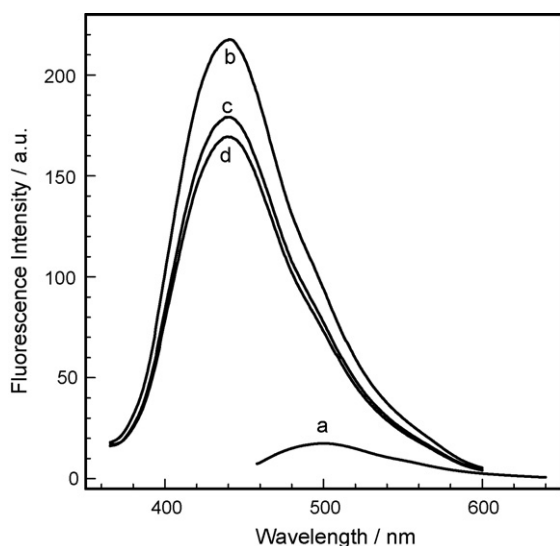

Fig. 4. The fluorescence spectra of TNS in the absent (a) or in the present of apoovotransferrin (b), Gd_N–ovotransferrin (c), and Gd_N–ovotransferrin–Gd_C (d), respectively, in 0.01 M Hepes, pH 7.4 at room temperature. The concentration of TNS is 2.6×10^{-6} M, and the concentrations of proteins are 1.3×10^{-6} M.

constants for the complexes of Gd^{3+} with apoovotransferrin are $\log K_1 = 7.61 \pm 0.14$ and $\log K_2 = 4.96 \pm 0.26$.

3.3. Gd^{3+} binding induced changes in the conformation of apoovotransferrin

A useful probe of proteins' conformational changes is the hydrophobic fluorophore TNS [19], because its fluorescence is altered when it binds to hydrophobic patches on the accessible surface of proteins. Fig. 4 shows the fluorescence spectra of TNS in the absence and presence of ovotransferrin (apoovotransferrin, Gd–ovotransferrin and Gd–ovotransferrin–Gd). As seen from Fig. 4, the addition of apoovotransferrin, Gd–ovotransferrin and Gd–ovotransferrin–Gd leads to the fluorescence intensity of TNS increase by 13, 10.5 and 10 times, respectively, and concomitant blue shifts from 496 to 446 nm in the maximum wavelength of emission, indicating the exposure extent of hydrophobic patches on the molecular surface of proteins is different.

4. Discussions

The natural substrate of apoovotransferrin is Fe^{3+}. For the metal UV difference spectral titrations show a sharp break at the point of saturation of the two metal-binding sites. However, a distinct end point is seldom observed with other metal ions [7]. It is such for Gd^{3+} UV difference spectra titration curve, in which there is no a distinct break at 2 equiv. of Gd^{3+}. In fact the final $\Delta\varepsilon_{obs}$ value is consistently less than twice the $\Delta\varepsilon_{Gd}$ value that would be expected for fully formed Gd^{3+}–apoovotransferrin–Gd^{3+}, indicating that the two binding sites of apoovotransferrin are not saturated even in the presence of a relatively large excess of Gd^{3+}.

In pH 7.4 Fe^{3+} binds specifically to the N-terminal binding site of apoovotransferrin [20]. It can be seen that Gd^{3+} binds to N-terminal binding site of apoovotransferrin preferably from Fig. 2. The sequential macroscopic binding constants for the binding of two Gd^{3+} to apoovotransferrin are $\log K_1 = 7.61 \pm 0.14$ and $\log K_2 = 4.96 \pm 0.26$. The difference between $\log K_1$ and $\log K_2$ is 2.65. Since Gd^{3+} binds to N-terminal binding site of apoovotransferrin preferably (see Fig. 2), it is reasonable to assign K_1 to the stronger N-terminal site and K_2 to the weaker C-terminal site.

Crystal structure of diferric ovotransferrin shows that the overall conformation is similar to those of human lactoferrin and rabbit serum transferrin [12] and apoovotransferrin had identical properties of iron binding and release. The N-lobe was shown to have undergone a very large conformational change, whereas the C-lobe remained essentially unchanged even though no metal was bound [11]. TNS is one of a class of compounds which do not fluoresce in water but fluoresce both in organic solvents or hydrophobic environment [21]. After adding apoovotransferrin to the solution of TNS the increase of the fluorescence intensity and concomitant blue shifts from 496 to 446 nm in the maximum wavelength of emission are owing to change of TNS in hydrophobic environment of apoovotransfer-

rin. The fluorescence intensity of TNS is weaker in the presence of Gd–apoovotransferrin or Gd–apoovotransferrin–Gd than of apoovotransferrin. It means that most conformational change of apoovotransferrin is attributed to the binding of first equivalent Gd^{3+} the N-terminal binding site of protein preferably, and second equivalent Gd^{3+} is bound to C-terminal binding site, concomitant essentially unchanged.

Acknowledgements

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