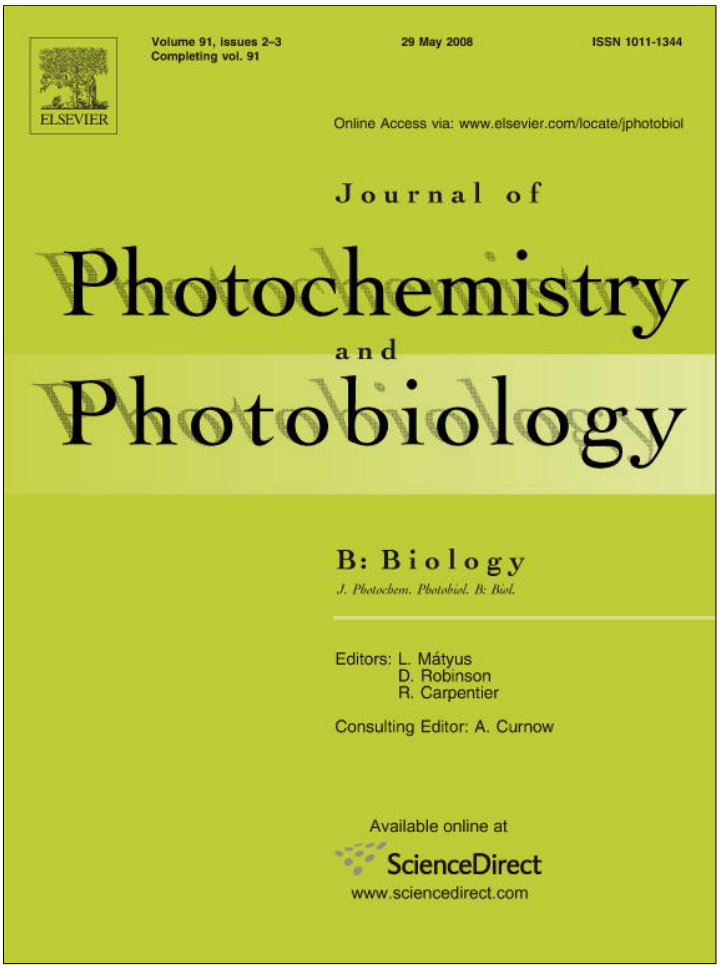

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Spectroscopic analysis of the interaction between gallium(III) and apoovotransferrin

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ABSTRACT

Ovotransferrin is a main member of transferrin family and has a dual role in both the transport of iron and antibacterial function. Gallium-67 is widely used as an imaging agent for tumors. It has been reported that Ga^{3+} can bind to apoovotransferrin at two sites, one in the N-terminal lobe and another in the C-terminal lobe. However, several details of the interaction between Ga^{3+} and apoOTf remain unclear. Here, we report detailed investigations into the interactions of Ga^{3+} with apoovotransferrin at the molecular level. First, the characteristics of Ga^{3+} binding to apoovotransferrin were analyzed using UV difference spectra. The results show that Ga^{3+} prefers to bind to the N-terminal site rather than the C-terminal site under the experimental conditions. Effective stability constants of $\log K_N = 18.88 \pm 0.24$ and $\log K_C = 17.65 \pm 0.12$ were determined. Second, conformational changes in apoovotransferrin during Ga^{3+} binding were studied using 2-p-toluidinylnaphthalene-6-sulfonate (TNS) as a fluorescence probe. Apoovotransferrin undergoes a large conformational change when Ga^{3+} binds to the N-terminal site, and a smaller conformational change when the ion binds to the C-terminal site. UV difference spectra were also used to measure the rate at which EDTA removes Ga^{3+} from ovotransferrin carrying one Ga^{3+} at the N-terminal site. Ga^{3+} removal from the N-terminal binding site follows simple saturation kinetics.

1. Introduction

Transferrins are Fe^{3+}-binding proteins such as serum transferrin (sTf), ovotransferrin (OTf), and lactoferrin (LTf) [1]. They are ~80 kDa single-chain bilobular proteins possessing one Fe^{3+}-binding site in each lobe. X-ray crystallographic data have shown that the N- and C-lobes have similar tertiary structures [2–5]. Each lobe is made up of a pair of domains (domain 1 and domain 2), with a high-affinity Fe^{3+}-binding site located in the interdomain cleft. Four of the six Fe^{3+}-coordination sites are occupied by the side-chains of amino acid residues, and the remaining two are occupied by a synergistic anion, usually carbonate under physiological conditions. X-ray solution scattering studies have shown that upon uptake and release of Fe^{3+}, all three transferrins undergo substantial conformational changes; the proteins assume open and closed conformations, respectively, in the apo- and holo-forms [6–8]. Additional studies have confirmed these conformational changes for both lobes of OTf [4,9,10]. OTf is a principal member of the transferrin family and participates in both the transport of iron and in bacterial resistance [11].

Ga^{3+} compounds are extremely useful in medical applications as radiodiagnostic and therapeutic agents [12,13]. In the body, Ga^{3+} interacts easily with transferrins. Indeed, the ion enters the cells via transferrin receptor-mediated endocytosis [14–16]. In addition, the six-coordinate ionic radii of Ga^{3+} and Fe^{3+} are almost identical (0.62 vs. 0.65 Å, respectively) [17]. As a result, human sTf and LTf bind Ga^{3+} with extremely high affinity ($K_D \approx 10^{-20}$–10^{-22} M) [18], making them an ideal diamagnetic probe for studying the metal ion binding sites of transferrins. Previously, we investigated the role of a hydrogen bond in creating differences in ion binding to the N- and C-terminal sites of apoovotransferrin (apoOTf) in solution [19]. In that study, we used Ga^{3+} as a proxy for iron binding because it could be analyzed by means of fluorescence spectra. While this approach provided new insights, it failed to clarify several details of the interaction between Ga^{3+} and apoOTf.

In the present study, the reaction of Ga^{3+} with apoOTf was analyzed using difference UV spectra. Binding constants were determined, and they revealed a stronger affinity for the N-terminal site. The conformational changes of apoOTf induced by Ga^{3+} binding were monitored using 2-p-toluidinylnaphthalene-6-sulfonate (TNS) as a fluorescence probe. The difference UV spectra were further used to measure the kinetics of Ga^{3+} removal from N-terminal monogallium ovotransferrin (Ga_N-OTf) by EDTA.

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2. Experimental

2.1. Materials

Unless otherwise noted, all reagents were analytical grade. Egg apoovotransferrin (apoOTf), N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid (Hepes), and 2-p-toluidinylnaphthalene-6-sulfonate (TNS) were purchased from Sigma. Disodium ethylenediaminetetraacetic acid (EDTA) was purchased from Beijing Chemical Plant; ferrous ammonium sulfate, from Bo Di Chemistry and Industry Ltd. (Tianjin, China); and sodium perchlorate, from Nan Shangle Chemical Plant (Beijing, China). Gallium metal (The First Reagent Factory of Shanghai, China) was high pure reagent quality.

Gallium chloride stock solutions were prepared by carefully weighing pieces of pure gallium metal into a 10-mL beaker, adding 0.5 mL of concentrated HCl, covering, and gently heating until all the metal was dissolved. More HCl was added periodically as needed to maintain vigorous hydrogen evolution at the gallium surface. This solution was then diluted to 25 mL, giving a final pH of 0.9. The gallium(III) concentration was calculated from the initial weight of the metal.

ApoOTf was prepared as previously described [20]. Protein concentration was determined from the absorbance at 280 nm using the extinction coefficient $\varepsilon_{280} = 91200\ cm^{-1}M^{-1}$ [21].

N-terminal monoferric ovotransferrin (Fe_N-OTf) was prepared by adding one molar equivalent of ferrous ammonium sulfate to apoOTf in 0.1 M Hepes (pH 7.4), and the protein-metal mixture was used without further purification [22].

Monogallium ovotransferrin (Ga-OTf) was prepared by adding 0.8 equivalent of freshly prepared gallium chloride solution to an apoOTf solution. The solution was stirred in air for 10 min and then stored for 1 h at 4 °C to reach equilibrium. The protein-metal mixture was used without further purification.

2.2. Methods

2.2.1. Difference UV spectra

Spectra of Ga^{3+} binding to apoOTf were recorded using an HP8453 UV–Vis spectrophotometer at intervals of at least 30 min, which was sufficient to allow equilibrium to be reached after each Ga^{3+} addition. To correct for dilution effects during each titration, absorbance data were converted to absorptivities ($\Delta\varepsilon$) by dividing the absorbance by the analytical concentration of apoOTf. The titration curves were then constructed by plotting $\Delta\varepsilon$ vs. r, the ratio of total metal ion to total ligand.

2.2.2. Interaction with hydrophobic probe TNS

Ga^{3+}-dependent changes in exposed hydrophobic surface of OTf were followed by monitoring the fluorescence properties of TNS as described previously [23].

2.2.3. Ga^{3+} Removal from Ga_N-OTf

The removal of Ga^{3+} from the N-terminal binding site of ovotransferrin by EDTA was monitored by difference UV spectroscopy. The Ga-OTf complex shows a positive peak near 242 nm in the difference UV spectrum. Samples for kinetics assays were prepared directly inside a 1-cm quartz cuvette. A 13.4 μM solution of apoOTf was prepared in 0.1 M Hepes (pH 7.4) in air. A baseline difference spectrum of apoprotein vs. apoprotein was recorded prior to the addition of metal ion. The addition of 0.8 equivalent of metal ion produced a solution of 10.7 μM Ga-OTf complex, as shown by the peak at 242 nm in the difference spectrum. After the Ga^{3+} had equilibrated with the apoprotein, excess EDTA in the molar ratios ranging of EDTA: OTf from 10:1 to 250:1 was added to begin the removal reaction. The rate of Ga^{3+} removal was measured from the decrease in the 242 nm peak in the difference UV spectrum. Experiments were repeated twice at each EDTA concentration. These measurements were performed in a 1-cm quartz cuvette maintained at constant temperature (37 °C) using an HP8453 UV–Vis spectrophotometer equipped with a thermostated cell holder and a circulating water bath.

3. Results

3.1. Difference UV spectrophotometry of Ga^{3+} binding to ApoOTf

Gallium solutions containing various molar ratios of NTA:Ga were used to titrate apoOTf dissolved in 0.1 M Hepes (pH 7.4) buffer. A typical series of UV spectra is shown in Fig. 1. Addition of Ga^{3+} to apoOTf caused two absorbance bands to appear at 242 and 295 nm, which reflect the fact that the metal ions had deprotonated the Tyr resides at the specific iron binding sites of apoOTf and were interacting with the resulting phenolate groups. Values of $\Delta\varepsilon$ were calculated from the absorbance at 242 nm and plotted as a function of r, the ratio of total Ga^{3+} concentration to total apoOTf concentration (Fig. 2, curve 'a'). The continued increase in $\Delta\varepsilon$

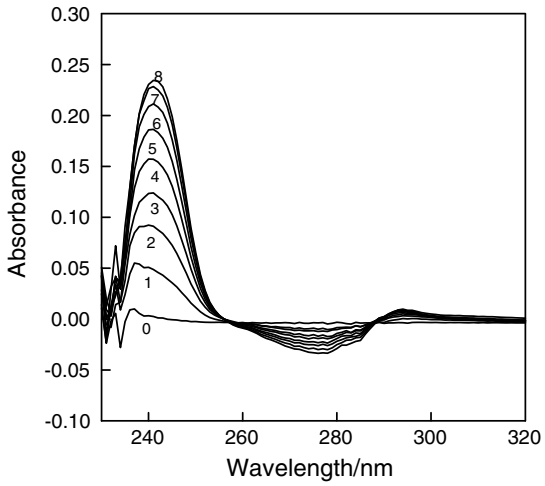


Fig. 1. Difference UV spectra generated by the titration approximately of 1.5 ml 11.6 μM apoOTf at pH 7.4 with different volumes (μL) of 0.24 mM Ga^{3+}: (0) 0; (1) 15; (2) 30; (3) 45; (4) 60;(5) 75; (6) 90; (7) 105 and (8) 120.

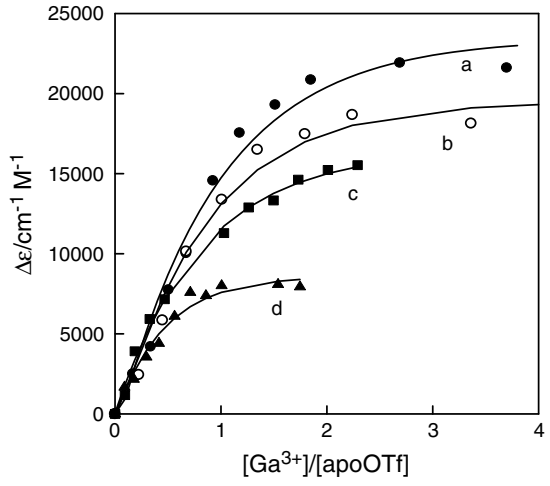


Fig. 2. Plot of $\Delta\varepsilon_{obs}$ vs. $[Ga]^{3+}/[OTf]$ for the titration of apoOTf with Ga^{3+} solution containing the indicated molar ratios of NTA:Ga^{3+}. (a) 0:1; (b) 0.3:1; (c) 5:1 and (d) 8:1.

for points beyond $r = 1$ is a strong indication that Ga^{3+} occupies both of the specific metal binding sites in OTf. The plot of $\Delta\varepsilon$ vs. r shows no obvious increase beyond $r = 1.5$. Such curvature indicates occupancy of a second site with a lower affinity, so that excess Ga^{3+} is needed to push the binding toward saturation of both binding sites of OTf. ApoOTf was titrated with $[Ga(NTA)_x]$ ($x = 0.3$, 5, or 8), and the absorbance at 242 nm was monitored. A set of titration curves for NTA:Ga ratios of 0.3:1 to 8:1 is shown in Fig. 2. At higher NTA:Ga ratios, the plots show more pronounced downward curvature at high r values due to the competition between OTf and NTA.

The molecular absorption coefficient ($\Delta\varepsilon_{Ga}$) of OTf with one Ga^{3+} saturated can be calculated from the initial slope of curve 'a'. Several replicate titrations give a value of $\Delta\varepsilon_{Ga} = (17000 \pm 400)$ $cm^{-1}\,M^{-1}$. Both sites are assumed to have the same molar absorptivity. Thus, complete saturation of the two OTf binding sites would produce an observed $\Delta\varepsilon$ of ~34,000 $cm^{-1}\,M^{-1}$. It is very clear from the curves in Fig. 2 that no reasonable amount of Ga^{3+}, with or without NTA, will produce a $\Delta\varepsilon$ of 34000 $cm^{-1}\,M^{-1}$. At pH 7.4, 98.4% of the Ga^{3+} is present in solution as $[Ga(OH)_4]^-$, and the remaining 1.6% as $Ga(OH)_3$. Hence, OH^- ions appear to be the limiting factor when the $GaCl_3$ is titrated into the apoOTf solution [18]. In contrast, the accumulation of NTA is the limiting factor in systems using $Ga(NTA)_x$.

3.2. Binding constants

The absorbance data obtained at different molar ratios of NTA:Ga were used to calculate the effective Ga^{3+}-OTf binding constants as described previously [18,24]. For NTA participation in the reaction, the mass balance equations should include apoOTf, Ga^{3+}, and NTA. Therefore the system can be described using the equilibria below.

$$Ga + apoOTf \rightleftharpoons Ga - apoOTf \quad (1)$$

$$Ga + Ga - apoOTf \rightleftharpoons Ga - apoOTf - Ga \quad (2)$$

According to Eqs. (1) and (2), the effective Ga^{3+}-OTf binding constants can be defined as

$$K_1 = \frac{[Ga - apoOTf]}{[Ga][apoOTf]} \quad (3)$$

$$K_2 = \frac{[Ga - apoOTf - Ga]}{[Ga][Ga - apoOTf]} \quad (4)$$

where K_1 and K_2 are apparent binding constants dependent on the experimental conditions. [Ga] and [apoOTf] refer to the molarities of free Ga^{3+} and free apoOTf; and [Ga-apoOTf] and [Ga-apoOTf-Ga] refer to the molarities of proteins bound to one or two Ga^{3+} ions, respectively.

If it is assumed that the two binding sites have the same molar absorptivity ($\Delta\varepsilon_{Ga}$) in the Ga-OTf complex, then it is possible to estimate the [Ga] and [apoOTf] terms in the mass balance equations describing the titration of apoOTf solution with free Ga^{3+} without added NTA. This can be done using the Ga^{3+} hydrolysis constants, the effective conditional constant of Ga(NTA), and initial estimates of K_1 and K_2. Second, values of $\Delta\varepsilon_{Cal}$ at any point in the titration curve can be calculated using the above concentrations and the value of $\Delta\varepsilon_{Ga}$ calculated from

$$\Delta\varepsilon_{Cal} = \frac{\Delta\varepsilon_{Ga}K_1[Ga][apoOTf] + 2\Delta\varepsilon_{Ga}K_1K_2[Ga]^2[apoOTf]}{[apoOTf]_{tot}} \quad (5)$$

where $[apoOTf]_{tot}$ refers to the analytical concentration of apoOTf, and $\Delta\varepsilon_{Ga}$ is the molar absorptivity per bound Ga^{3+} for the Ga-OTf difference spectrum at 242 nm. Nonlinear least squares minimization is used to adjust the values of K_1 and K_2. This involves adjusting K_1 and K_2 in order to minimize the squares of the residuals between observed and calculated absorptivities, so that reliable estimates of the binding constants of Ga^{3+} to OTf can be obtained. In principle, the titration curve 'a' in Fig. 2 can be fit using the nonlinear least squares method where K_1 and K_2 are the only quantities allowed to vary. In practice, using chelating agents to complete the removal of the metal can decrease the correlation between K_1 and K_2, thereby leading to more accurate K_1 and K_2 estimates, as described previously [18,24]. Thus, the titrations with Ga^{3+} were repeated using metal ion solutions containing a range of NTA concentrations. The chelating agent competes with apoOTf for Ga^{3+} and so at any given point in the titration, there is a distribution of Ga^{3+} between apoOTf and the NTA chelating agent, and the observed absorptivity decreases as the NTA:Ga^{3+} ratio increases. A series of titration curves for Ga^{3+}-NTA solutions is shown in Fig. 2. The titration data in Fig. 2 can be fitted using Eq. (5), in which K_1 and K_2 are the only adjustable parameters.

For the 8:1 NTA:Ga titration curve, the maximum $\Delta\varepsilon$ observed (~8000 $cm^{-1}\,M^{-1}$) is much less than the calculated molar absorptivity of 17,000 $cm^{-1}\,M^{-1}$, even at $r = 2.0$. This suggests that only the stronger binding site of apoOTf is binding Ga^{3+} under these conditions, so these data were used to calculate only $\log K_1$.

The average values for the two conditional binding constants, $\log K_1 = 18.88 \pm 0.24$ and $\log K_2 = 17.65 \pm 0.12$, were determined by nonlinear least-squares fits of the titration curves at pH 7.4 in air (Fig. 2). At this pH and with a buffer concentration of 0.1 M Hepes, the ambient bicarbonate concentration is ~0.14 mM [18].

3.3. Site selectivity for Ga^{3+} binding to ApoOTf

Curve 'a' in Fig. 3 denotes the titrations of apoOTf with Ga^{3+} without NTA. The initial slope of the titration curve is equal to the molar absorptivity ($\Delta\varepsilon_{Ga}$) of the Ga-OTf complex. Since the titration curve levels off at an $\Delta\varepsilon$ of ~22,500 $cm^{-1}\,M^{-1}$, which is much greater than the molar absorptivity ($17,000 \pm 400$ $cm^{-1}\,M^{-1}$), it is clear that Ga^{3+} binds to both of the OTf binding sites. The ion does not, however, appear to bind to the same extent to the two OTf binding sites (Fig. 3, curve 'a'). Thus, the equilibration of $\leqslant 1$ equiv of Ga^{3+} ion with apoOTf should result in preferential binding to the site with the better association constant.

The stronger binding site can be identified by titration of monoferric OTf [20,25]. A sample of N-terminal monoferric OTf (Fe_N-OTf) was titrated with Ga^{3+} aquo ion. The Ga^{3+} ion does not displace the more tightly bound Fe^{3+} from the Fe_N-OTf, so its binding to the vacant binding site can be monitored using difference UV spectra. The result is shown in curve 'b' of Fig. 3. The curve levels off after

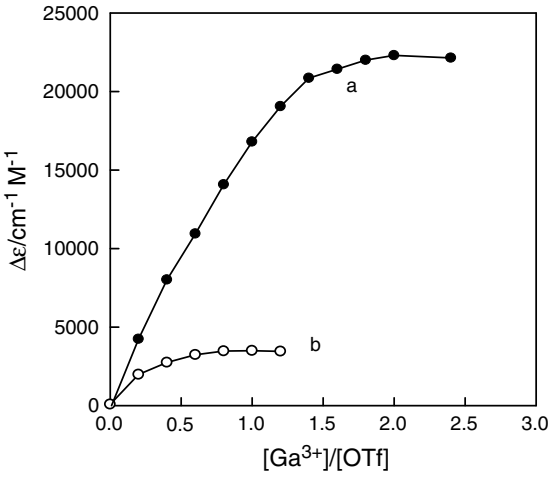


Fig. 3. Titration curves for the addition of (a) 0.24 mM Ga^{3+} to 1.5 mL 12 μM apoOTf in saturated air and 0.1 M Hepes (pH 7.4), or (b) 0.21 mM Ga^{3+} to 1.5 mL 11 μM N-terminal monoferric OTf under the same conditions.

the addition of only 0.3 equivalent of Ga^{3+} at an absorptivity of about 2300 $cm^{-1}\,M^{-1}$. Moreover, the slopes of the two titration curves in Fig. 3 are markedly different. The results indicate weaker binding to the C-terminal site. Thus it is reasonable to infer that addition of $\leqslant$1 equivalent of Ga^{3+} ion will preferentially load the N-terminal site.

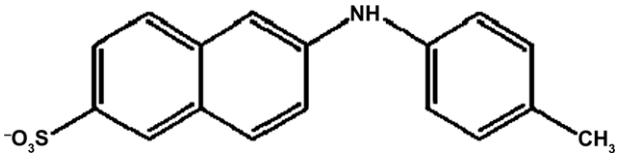
3.4. Interaction with hydrophobic probe

TNS can be used as a fluorescence probe to investigate hydrophobic regions that are exposed on the surface of proteins [23]. It interacts noncovalently with protein, and its structure is shown in Scheme 1.

Fig. 4 shows the fluorescence spectra of TNS in the absence and presence of different forms of ovotransferrin: apoOTf, Ga-OTf, and Ga_2-OTf. In all these trials, Ga_2-OTf means that the Ga: OTf molar ratio in the samples was 2.5:1. TNS emits fluorescence very weakly at 498 nm when it is excited at 320 nm in 0.1 M Hepes, pH 7.4 (Fig. 4). The addition of apoOTf, Ga-OTf, and Ga_2-OTf to TNS caused a blue shift in the fluorescence emission maximum to 441 nm. Moreover, the fluorescence intensity of TNS increased 8.85-, 6.55-, and 4.80-fold, respectively. These data strongly suggest that the different conformations of OTf expose different amounts of hydrophobic surface. The results also imply that the Ga^{3+} binding to the N-terminal site produces a large conformational change in apoOTf, whereas binding to the C-terminal site results in a more modest conformational change.

3.5. Removal of Ga^{3+} from the N-terminal binding site of ovotransferrin

Using these results on site selectivity, N-terminal monogallium ovotransferrin (Ga_N-OTf) was obtained by adding 0.8 equivalent of freshly prepared gallium chloride solution to an apoOTf solution. Therefore, samples containing 10.7 μM metal transferrin complexes with the metal bound preferentially to the N-terminal binding site were prepared as described in the Experimental Section. EDTA was added, and the rate of Ga^{3+} removal was tracked by changes in the difference UV spectrum. This reaction goes to completion only at higher EDTA concentrations. The pseudo-first-order rate constants for the removal of Ga^{3+} from ovotransferrin are plotted versus the EDTA concentration in Fig. 5. These results show that k_{obs} for the N-terminal monogallium ovotransferrin increases with EDTA concentration as long as this concentration is low, but at higher EDTA concentrations, k_{obs} approaches a maximum value and becomes essentially independent of this variable. The solid line in the plot represents calculated fits to Eq. (6) [24]. The two parameters k_{max} and k_d are listed in Table 1.



Scheme 1. The structure of TNS.

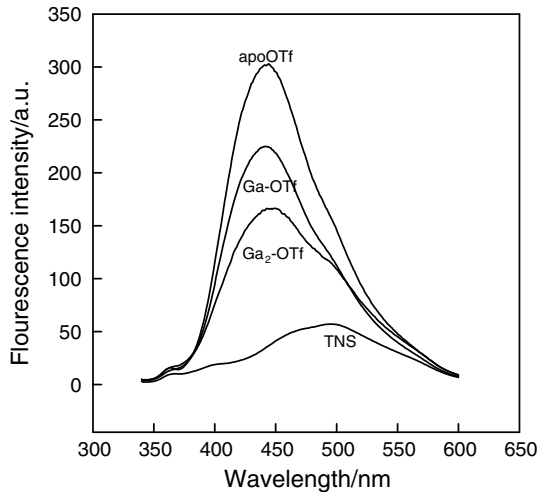


Fig. 4. Ga^{3+}-dependent exposure of hydrophobic side chains of OTf (2.78 μM). Hydrophobic exposure was monitored at room temperature in 0.1 M Hepes with hydrophobic TNS (28.3 μM). The fluorescence was measured at fixed λ_{ex} = 320 nm, slit = 10 nm.

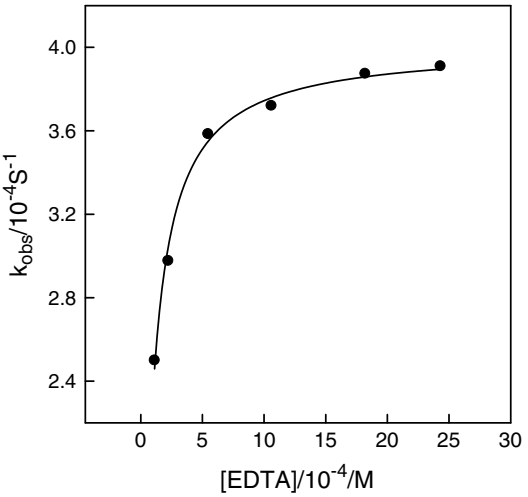


Fig. 5. Apparent pseudo-first-order rate constants for Ga removal from Ga_N-OTf as a function of the concentration of EDTA. All solutions were buffered at pH 7.4 by 0.1 M Hepes and maintained at 37 °C. The curves represents fitting of the data to Eq. (6).

Table 1
Kinetic parameters for Ga^{3+} removal from Ga_N-OTf by EDTA

Sample	$k_{max}/(10^{-4}\,s^{-1})$	$k_d/(10^{-5}\,M)$
Ga_N-OTf	4.0 ± 0.032	7.1 ± 0.037

$$K_{obs} = \frac{k_{max}[L]}{k_d + [L]} \quad (6)$$

The experimental results for the N-terminal site can be fit very well to Eq. (6). This implies that simple saturation kinetics describes the pathway for Ga^{3+} removal from the N-terminal binding site. This usually indicates a rate-limiting conformational change in the protein [25].

4. Discussion

Complexation of metal ions to the phenolic group of the tyrosine residues of ovotransferrin in the specific iron binding site perturbs the Π–Π* transitions of the aromatic group and leads to two absorption bands at ~240 and ~295 nm in the difference UV spectrum. The changes in the UV spectrum of OTf upon binding of Ga^{3+} are similar to those observed previously for the binding of other metal ions to the specific Fe^{3+} binding sites. The new bands at 242 and 295 nm are attributable to binding to tyrosine ligands (Π–Π* transitions). The difference UV spectra suggest that Ga^{3+}

can bind to both of the iron binding sites of apoOTf. The titration curve is linear through the addition of ~1.3 equivalent of Ga^{3+} with a slope of 17,000 cm^{-1} M^{-1}. The curve levels off at a $\Delta\varepsilon$ value of about 22,500 cm^{-1} M^{-1}. The partial saturation of the weaker binding site is probably due to competition from hydrolysis of Ga^{3+}.

The site selectivity of metal ion binding to transferrins has been extensively investigated. The selectivity varies with the metal ion, the protein, and the synergistic anion. A preference for the C-terminal site of transferrin has been observed in difference UV titrations of some trivalent ions such as the lanthanides [26], Al^{3+} [24], Ga^{3+} [25], and Bi^{3+} [27], and there is clear NMR evidence that Ti^{3+} binds preferentially to the C-terminal lobe with carbonate as the synergistic anion [28]. On the other hand, Al^{3+} binds preferentially to the N-terminal site of transferrin in the presence of oxalate [29]. The addition of $Al(NO_3)_3$ to apoOTf leads to preferential loading of the N-terminal site [20], as does the addition of $GaCl_3$ in the presence of bicarbonate [30]. Similar to our results, titrations of monoferric OTf with La^{3+} showed stronger binding to the N-terminal site at pH 7.4 using difference UV spectra [31].

The data on Ga^{3+} binding to N-terminal monoferric OTf clearly indicate that Ga^{3+} binds more tightly to the N-terminal site. It is likely that the titration curves of apoOTf reflect saturation of the stronger N-terminal site during the initial phase, followed by partial occupancy of the weaker C-terminal site. This result is consistent with a previous report [30].

Two linear free energy relationships for the complexation of Ga^{3+} and Fe^{3+} are shown in Fig. 6. Each data point represents a ligand. The *x* coordinate is the log of the stability constant of the ligand with Fe^{3+}, while the *y* coordinate is the log of the stability constant of the same ligand with Ga^{3+}. The data in Fig. 6 represent the ligands that bind through a combination of aliphatic amines and oxygen donors [32]. It has been shown that the serum transferrin binding constants for several metal ions conform to this type of LFER [24]. Thus the LFER provides a basis for using the Fe^{3+}-OTf binding constants to estimate the values for the Ga^{3+}-OTf binding constants. The data points for the $\log K_1$ and $\log K_2$ values for ovotransferrin are shown as the open circle and triangle in Fig. 6. It can be seen that there is a good correlation, which is described by Eq. (7), with a correlation coefficient of $r = 0.993$.

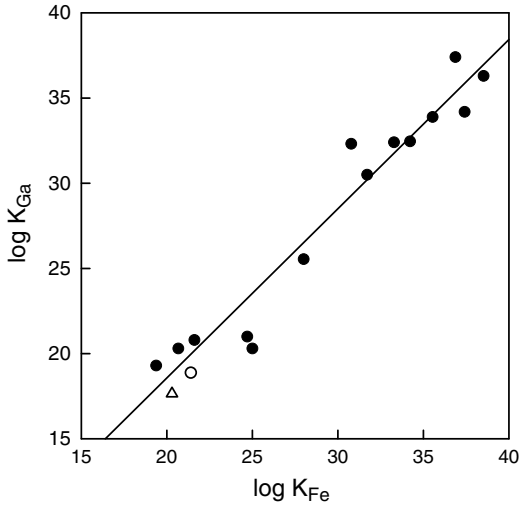


Fig. 6. Linear free energy relationships (LFER) for the complexation of Ga^{3+} and Fe^{3+} with oxygen and nitrogen donors. Each data point consists of the log of the stability constant of a given ligand with Fe^{3+} as the x coordinate and the log of the stability constant of the same ligand with Ga^{3+} as the y coordinate. The filled circles represent ligands that coordinate through a combination of oxygen and nitrogen donor atoms. The points for ovotransferrin are shown as the open circle and triangle which represent two binding constants for Ga-ovotransferrin.

$$\log K_{Ga} = 0.99.3 \log K_{Fe} - 1.30 \quad (7)$$

The data for $\log K_1$ and $\log K_2$ values are shown, respectively, as the open circle and triangle in Fig. 6. The two binding constants of $\log K_1 = 18.88 \pm 0.24$ and $\log K_2 = 17.65 \pm 0.12$ are within the LFER region, which implies that the size of the metal ion is suited to the interdomain cleft of the protein. It is reasonable to assign K_1 to the stronger N-terminal site and K_2 to the weaker C-terminal site.

The crystal structure of diferric ovotransferrin shows that the overall conformation is similar to that of human LTf and rabbit sTf [4]. Like these proteins, apoOTf binds and releases iron. The structure of apoOTf has been determined, and both lobes are in the open conformation [6,9]. The opening of the C lobe is equivalent to 75% of the opening of the N lobe [6]. The two lobes of OTf undergo very similar conformational changes during iron binding and release. Here conformational changes of the protein were studied with TNS, since it belongs to a class of compounds that do not fluoresce in water but that fluoresce in organic solvents and other hydrophobic environments [33]. Namely, fluorescent quantum yield is increased in hydrophobic environments, while fluorescent quantum yield is smaller in hydrophilic environments. So the conformational change of OTf induced by Ga^{3+} binding to one or two binding sites can be inspected by TNS. In our experiments, adding apoOTf to the solution of TNS increased the fluorescence intensity and caused a blue shift in the emission peak from 498 to 441 nm. These effects are due to changes in the hydrophobic microenvironment of TNS interacting with apoOTf, since TNS monitors the accessibility of a hydrophobic patch on the OTf. The fluorescence intensity of TNS decreases at 441 nm for Ga-OTf and Ga_2-OTf. This indicates decreased accessibility to the hydrophobic patch on the OTf. It can be seen from the Fig. 4 that only the fluorescence intensity is decreased and the emission peak does not changed. So we infer that the hydrophobic microenvironment of TNS interacting with apoOTf, Ga-OTf or Ga_2-OTf is the same, but the extent of exposed hydrophobic patch on the protein is different. The fluorescence intensity of TNS increased 8.85-, 6.55-, and 4.80-fold, respectively. The fluorescence intensity of TNS is the strongest in the presence of apoOTf, it decreases substantially in the presence of Ga-OTf, and then it decreases slightly more in the presence of Ga_2-OTf. According to the observed site selectivity, the Ga_N-OTf complex is formed when one molar equivalent of Ga^{3+} is added to apoOTf. Therefore, here Ga-OTf is in fact Ga_N-OTf. Ga_2-OTf means that N-terminal site of apoOTf is saturated by Ga^{3+}, while C-terminal site is partly saturated. Hence, the conformational change of Ga_2-OTf mainly results from occupancy of the N-terminal site, while binding at the C-terminal site induces a smaller conformational change [6]. So we infer that the exposed hydrophobic patch seems to be a specific property of the N-terminal site of apoOTf. Therefore, we presume that Ga^{3+} binding at the C-terminal site induces a small conformational change in the protein, whereas most conformational change in ovotransferrin occurs when Ga^{3+} binds at the N-terminal site. This interpretation is supported by the fact that the C-lobe contains an interdomain disulfide bridge (Cys478-Cys671), which may restrict domain opening, though it does not completely prevent it [9].

The kinetics of release of different metal ions from serum transferrin has been widely studied in vitro [25]. This work has shown that different metal ligands use different removal pathways. The mechanism of iron release from ovotransferrin has also been studied [10]. However, there have been few studies on the kinetics of release of other metal ions from ovotransferrin.

The saturation kinetics of Ga^{3+} release observed in the present study is consistent with the mechanism described initially by Bates [34] and Harris [35]. The mechanism is shown below, where the asterisk indicates on "open" conformation of mono gallium

ovotransferrins. OTf stands for the CO_3^{2-}-OTf complex and L represents EDTA.

$$\text{Ga-OTf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Ga-OTf}^* \quad \text{Slow} \tag{8}$$

$$\text{Ga-OTf}^* + \text{L} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Ga-OTf}^* - \text{L} \quad \text{fast} \tag{9}$$

$$\text{Ga-OTf}^* - \text{L} \overset{k_3}{\rightarrow} \text{Ga-L}^* + \text{OTf}^* \quad \text{fast} \tag{10}$$

The mechanism predicts that Eq. (8), the conformational change of the gallium protein from "closed" native form to "open" active form, will become the rate-limiting step in the Ga^{3+} 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 Ga^{3+}. According to this mechanism, k_{max} corresponds to k_1, the forward rate constant for the conformational change in the protein. The kinetic parameter k_d corresponds to the ligand concentration required to reach half-saturation. In this study the EDTA concentration required to reach half-saturation is about 7.1×10^{-5} M. The mechanism of simple saturation kinetics for Ga^{3+} ion removal by EDTA is similar to that previously reported for the release of Al^{3+} ion by PP_i [36].

At least three explanations for the mechanism of Ga^{3+} removal by EDTA in our experiments are possible. (1) EDTA may occupy the kinetically significant anion binding (KISAB) site [6,37], leading to a decrease in the positive charge density close to the metal binding site. This weakens the interaction between synergistic anion (CO_3^{2-}) and OTf, inducing a conformational change that leads to opening of the lobes. (2) EDTA may displace the carbonate synergistic ion, which would destabilize the Ga^{3+} coordination sphere in OTf. (3) EDTA may break the hydrogen bonds that maintain the protein in a "closed" conformation.

Based on the results above, it can be deduced that the mechanism of saturation kinetics for Ga^{3+} removal from the N-terminal monogallium ovotransferrin by EDTA depends on a primary conformational change.

In summary, there is a strong binding site for Ga^{3+} on the N-terminal site of ovotransferrin under the experimental conditions tested. The fluorescence intensity of TNS is weaker in the presence of Ga-OTf or Ga_2-OTf than in the presence of apoOTf. Most of the conformational change in apoOTf occurs when the first Ga^{3+} binds preferentially to the N-terminal site, and a small amount of conformational change occurs upon binding of the second Ga^{3+} to the C-terminal site. Lastly, our results indicate that Ga^{3+} removal from N-terminal monogallium ovotransferrin by EDTA shows simple saturation kinetics.

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