Lanthanide ions induce hydrolysis of hemoglobin-bound 2,3-diphosphoglycerate (2,3-DPG), conformational changes of globin and bidirectional changes of 2,3-DPG-hemoglobin’s oxygen affinity

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Abstract

The changes in structure and function of 2,3-diphosphoglycerate-hemoglobin (2,3-DPG-Hb) induced by Ln3+ binding were studied by spectroscopic methods. The binding of lanthanide cations to 2,3-DPG is prior to that to Hb. Ln3+ binding causes the hydrolysis of either one from the two phosphomonoester bonds in 2,3-DPG non-specifically. The results using the ultrafiltration method indicate that Ln3+ binding sites for Hb can be classified into three categories: i.e. positive cooperative sites ($N_I$), non-cooperative strong sites ($N_S$) and non-cooperative weak sites ($N_W$) with binding constants in decreasing order: $K_I > K_S > K_W$. The total number of binding sites amounts to about 65 per Hb tetramer. Information on reaction kinetics was obtained from the change of intrinsic fluorescence in Hb monitored by stopped-flow fluorometry. Fluctuation of fluorescence dependent on Ln3+ concentration and temperature was observed and can be attributed to the successive conformational changes induced by Ln3+ binding. The results also reveal the bidirectional changes of the oxygen affinity of Hb in the dependence on Ln3+ concentration. At the range of $[\text{Ln}^3+] / [\text{Hb}] < 2$, the marked increase of oxygen affinity ($P_{50}$ decrease) with the Ln3+ concentration can be attributed to the hydrolysis of 2,3-DPG, while the slight rebound of oxygen affinity in higher Ln3+ concentration can be interpreted by the transition to the T-state of the Hb tetramer induced by Ln3+ binding. This was indicated by the changes in secondary structure characterized by the decrease of $\alpha$-helix content. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Lanthanide ion; Hemoglobin; Conformational change; 2,3-Diphosphoglycerate hydrolysis; Oxygen affinity
1. Introduction

In recent years, lanthanides (Lns) have been used in agriculture as the additives of fertilizers to promote plant growth in China. Meanwhile, concerns about the negative effects of Ln compounds are also rising. Even though the exposure level to Lns is usually rather low, the long-term accumulation of lower doses in the body is suspected to be harmful. It has been reported that in animals i.v. injection of LnCl$_3$ solutions in lower concentrations caused an increase in erythrocyte counts in blood, and at a higher concentration hemoglobinemia and hemoglobinuria developed [1,2]. The duality is thought to be the effect on the oxygen carrying ability and the leaking of hemoglobin (Hb) from erythrocytes, respectively [1-4]. However, the events in this process are still ambiguous. To clarify the adverse effects of Lns in these aspects, we recently examined the interaction of erythrocytes with lanthanides at both the cellular and the molecular level. Lns are shown to enter erythrocytes via different routes, depending closely on the species and concentration, and then affect intra-cellular target molecules such as Hb [5-9].

Hb, as the major cytosolic protein in erythrocyte, plays a crucial role in oxygen transport from lungs to various tissues. Previous studies on the interaction of metal ions, such as Zn$^{2+}$, Cu$^{2+}$, Mg$^{2+}$ and VO$^{2+}$, with Hb showed that the metal ions affect Hb’s oxygen affinity by several different mechanisms, including 2,3-diphosphoglycerate (2,3-DPG) hydrolysis and the direct binding of metal ions to Hb [10-15]. As to the effect of Lns, by in vitro studies with Gd-DOTP and La-DOTP, two potential contrast agents for magnetic resonance imaging, Aime et al. [16] showed that Lns decrease the oxygen affinity of human HbA due to the stabilization of T-state thermodynamically. However, their results were obtained with a rather high concentration of Lns (approx. 10$^{-3}$ mol/l). More recently, it was found that orally administrated CeCl$_3$ leads to a change in oxygen affinity of Hb in rat erythrocyte [7]. The action of Ce$^{3+}$ is complicated by the shuttle between Ce(III) and Ce(IV) via a single electron transfer in physiological condition, a unique behavior among lanthanide cations. In the mechanism underlying this effect, conformational changes of Hb, oxidation of heme-Fe(II) and hydrolysis of 2,3-DPG are involved. In addition, the increase in Hb content in rat erythrocytes has been interpreted as the offset response to the change in oxygen affinity. The difference between in vivo and in vitro might be attributed to several factors, including Ln transport to erythrocytes and the coexistence of organic small molecules and proteins in blood.

The present study was aimed to investigate the effects of lanthanide cations on the structure and function of Hb, including the binding of lanthanide cations, the changes in secondary to quaternary structure and oxygen affinity. Our results reveal that the conformational change of Hb and the hydrolysis of 2,3-DPG induced by Ln$^{3+}$ binding are responsible for the bidirectional changes in oxygen affinity.

2. Materials and methods

2.1. Materials

Fresh human blood was obtained from the Beijing Red Cross Blood Bank. Sephadex G-25, pentacyclohexylammonium salt, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and Tris hydrochloride were purchased from Sigma, diphosphoglyceric acid was obtained from the pentacyclohexylammonium salt by stirring with Dowex 50-X8. The solutions were neutralized to pH 7.0 and stored at 4°C. The solutions of LnCl$_3$ were prepared by dissolving their oxides (99.99%) in concentrated HCl with subsequent heating to deplete the excess acid and dilution with purified water. The concentrations were determined by EDTA titration with xylenol orange as indicator. Thomson’s Grey solution was prepared as described previously [7]. All chemicals were of analytical grade.

The packed erythrocytes were separated from fresh human blood by centrifugation (2000×g) for 10 min at 4°C to remove supernatant. They were then washed thrice with saline solution by centrifugation at 1500×g for 10 min. The solution of Hb with the 2,3-DPG not removed was prepared by the following method. The packed erythrocytes were lyed with the same volume of hypotonic buffer (20 mmol/l HEPES-HCl pH 7.4) and then centrifuged (15000×g) for 20 min to remove erythrocyte membrane. The supernatant was collected as Hb so-
Solution. To prepare 2,3-DPG-depleted Hb, 2,3-DPG was depleted along with the other low molecular weight molecules by eluting the Hb solution through a Sephadex G-25 column equilibrated with HEPES buffer (20 mmol/l, pH 7.4) by a conventional method [17]. 2,3-DPG-Hb solution was prepared by mixing 2,3-DPG with 2,3-DPG-depleted Hb at a mole ratio [2,3-DPG]/[Hb] = 2 and then removing free 2,3-DPG using ultrafiltration under purified nitrogen environment. The hemoglobin concentration was determined by Simmons’ procedure [18] with Thomson’s Grey solution as standard.

2.2. Instruments

Bio-Rad FTS-65A FT-IR spectrometer, Varian NMR spectrometer (DPX-400), dissolved oxygen meter (JPB-607, REX), pH meter (pHS-2, REX), UV-vis spectrophotometer (DMS-200), Millipore ultrafiltration equipment, Union-Giken RA401 stopped-flow spectrophotometer.

2.3. Methods

2.3.1. Determination of the oxygen saturation curve of Hb

Oxygen equilibrium experiments were conducted on the solutions of 2,3-DPG-depleted Hb and 2,3-DPG-Hb ([2,3-DPG]/[Hb] = 2) in the absence and the presence of lanthanide cations in 20 mmol/l HEPES-HCl, pH 7.4, at 25°C, respectively, according to the method described by Feng et al. [19]. The air in 30 ml Hb solution was drawn out with a vacuum pump and N₂ (containing about 5332 Pa of CO₂) was refilled, then the sample was incubated at 37°C for 3 min. This step was repeated three times. The absorbence at 560 nm and 575 nm was measured at varied oxygen partial pressures and then the degree of oxygen saturation was calculated by a conventional method [19].

2.3.2. Hydrolysis of Hb-bound 2,3-DPG as monitored by chemical determination of inorganic phosphate

After the 2,3-DPG-depleted Hb solution was deoxygenated by passing nitrogen over a rotating sample for 2 h at 4°C, the deoxyHb (1.0 × 10⁻⁴ mol/l) was incubated with 2,3-DPG (2.0 × 10⁻⁴ mol/l) at 25°C for 2 h and free 2,3-DPG in solution was removed by ultrafiltration. Then the Ln³⁺ solution was added to the recombined 2,3-DPG-deoxyHb solution in 5 × 10⁻² mol/l HEPES-HCl buffer (pH 6.7 or 7.0). By ultrafiltration (pH 6.0, after 1 h), the inorganic phosphates Pᵢ were separated. The Pᵢ concentration and total amount of phosphorus at various times and different conditions were determined by the molybdenum blue method. The degree of hydrolysis was calculated on the basis of two phosphate ester bonds in 2,3-DPG. Unless specified otherwise, all experiments were carried out with HEPES-HCl buffer (pH 6.7 or 7.0) at 25°C.

2.3.3. ³¹P NMR studies on the hydrolysis of 2,3-DPG

To study the hydrolysis of Hb-bound 2,3-DPG in the presence of lanthanide cations, a series of lanthanide cations were incubated with the cytosol containing 2.0 × 10⁻³ mol/l Hb and also 2,3-DPG and inorganic phosphates, and a solution containing 3.0 × 10⁻⁴ mol/l deoxyHb and 3.0 × 10⁻⁴ mol/l 2,3-DPG, respectively. The reactions were allowed to proceed at 37°C for 1 h and phosphoric acid (85%) was chosen as the external reference of ³¹P NMR spectra. The ³¹P NMR spectra were scanned with the operating frequency set at 162 MHz, number of transients 2000 and acquisition times of 1 s.

2.3.4. Lanthanide binding studies

Binding of lanthanide cations to 2,3-DPG-depleted Hb was examined by ultrafiltration through a 30 000 MW cutoff membrane filter (Millipore) in a stirred ultrafiltration cell. The concentration of Ln³⁺, in free or Hb-bound form, was determined by chlorophosphonazo III method with a UV-vis spectrophotometer. The ultrafiltration data were processed with similar procedures to those previously described by Schreier et al. [20], which were modified to cope with the circumstances of Hb encountered in the present study. The Scatchard parameters are expressed as follows:

\[ \frac{v}{C} = \frac{N₁K₁C^{α₁−1}}{1 + \frac{K₁}{C^{α₁}}} + \sum \frac{NᵢKᵢC}{1 + KᵢC} \]

Here v and C refer to the number of bound Ln³⁺ per Hb tetramer and the free Ln³⁺ concentration, respectively. N₁ and Nᵢ stand for the cooperative binding
sites and the independent binding sites. The latter comprise strong (S) and weak (W) independent sites, \( N_S \) and \( N_W \). \( K_I \) and \( K_S \) are the corresponding binding constants, and \( \alpha \) is the interaction exponent. The two intercepts of the Scatchard plot are

\[
(v/C)_{v=0} = N_S K_S \tag{2}
\]

\[
v(v/C)_{v=0} = N_I + N_S \tag{3}
\]

The asymptotic tangent line at \( v/C = 0 \) is given by

\[
v/C = \frac{(N_I + N_S)^2}{N_S} K_S - \frac{(N_I + N_S)}{N_S} K_S v \tag{4}
\]

The \( v/C \) intercept of this tangent line is

\[
(v/C)_{v=0} = \frac{(N_I + N_S)^2}{N_S} K_S \tag{5}
\]

The parameters \( N_I, N_S, N_W \) and corresponding binding constants are obtained from the equations described above.

### 2.3.5. The kinetics of the reaction by stopped-flow studies on fluorescence

Stopped-flow fluorescence measurements were carried out using a Union-Giken RA401 stopped-flow spectrophotometer. The 2,3-DPG-depleted Hb solution was mixed with various lanthanide cations in the mixing cell of the instrument. The intrinsic fluorescence of Hb was excited at 280 nm and its fluorescence emission was monitored within different time intervals at wavelength \( \lambda_{em} = 330 \) nm using a cut-on filter. All the reported concentrations are the final concentrations in the flow cell after mixing of the reactants. The curve fittings were carried out using the software provided with the stopped-flow instrument. The dead time of the stopped-flow apparatus was determined to be 5 ms. The data are the average values of 6–8 shots.

### 2.3.6. FT-IR studies on the secondary structure of Hb

After incubating 2,3-DPG-depleted Hb (5 \( \times \) 10^{-5} mol/l) and 2,3-DPG-deoxyHb ([2,3-DPG][deoxy-Hb] = 1) with Ln^{3+} of various concentrations at 37°C for 2 h, the solution was lyophilized and redissolved in \(^2\)H2O and stored at 4°C for 24 h for complete hydrogen-deuterium exchange. For FT-IR measurements, a suspension of 50 mg/ml Hb was used. All FT-IR spectra between 1700 cm^{-1} and 1600 cm^{-1} were collected on a Bio-Rad FTS-65A FT-IR spectrometer. The second derivative and Fourier deconvoluted spectra were fitted and analyzed by the reported procedure [21,22].

### 3. Results

#### 3.1. Effect of lanthanide cations on the saturation curve of Hb

The effect of lanthanide cations on oxygen affinity of Hb was investigated in the presence and absence of 2,3-DPG. The oxygen saturation curves given in Fig. 1 demonstrate the effect of praseodymium ions on the oxygen affinity of the recombined 2,3-DPG-Hb system. Without Pr^{3+}, the curve of 2,3-DPG-depleted Hb sits to the left of that of 2,3-DPG-bound Hb, showing a higher oxygen affinity (Fig. 1a). The 2,3-DPG-Hb curves, under the action of Pr^{3+}, shift gradually to the left depending on Pr^{3+} concentration. Conversely, for 2,3-DPG-depleted Hb, the curves shift rightward with increasing Pr^{3+} concentration from 2.5 \( \times \) 10^{-4} mol/l to 1.5 \( \times \) 10^{-3} mol/l. In relation with this, a similar bidirectional dependence on Pr^{3+} concentration was observed clearly in the oxygen partial pressure at half-saturation, \( P_{50} \), which reflects the oxygen affinity. In case of the recombined 2,3-DPG-Hb system, the value of \( P_{50} \) declines almost linearly with the increase in Pr^{3+} concentration from zero to \([Pr^{3+}]/[2,3-DPG] = 1\) (see the inset to Fig. 2a), and then it turns to increase slightly when the Pr^{3+} concentration was increased further. This means that praseodymium ions at lower concentrations can strongly promote the oxygen affinity of 2,3-DPG-bound Hb, although this tendency turns into the opposite direction at higher concentrations. But for 2,3-DPG-depleted Hb, Pr^{3+} caused a progressive, but less significant increase in \( P_{50} \) values with increasing Pr^{3+} concentration up to 2.5 \( \times \) 10^{-3} mol/l (Fig. 2b), which is similar to the results of 2,3-DPG-Hb at higher concentrations (Fig. 2a). The difference between them is distinctly dependent upon 2,3-DPG. Similar evidence was also observed in other lanthanide cations (data not shown).

The effects of different lanthanide cations (La^{3+}, Ce^{3+}, Pr^{3+}, Gd^{3+}, Tb^{3+}, Lu^{3+}) on oxygen affinity of both 2,3-DPG-depleted and bound Hb at a lower
concentration (5.0×10⁻⁵ mol/l) are displayed in Fig. 3. The results show that the \( P_{50} \) values of 2,3-DPG-Hb are affected by all lanthanide cations studied and the changes are different with various lanthanide cations. The heavier lanthanide cations with smaller radius, such as \( \text{Lu}^{3+} \), cause a more significant decrease in \( P_{50} \) value. It is noteworthy that no significant effect was observed for 2,3-DPG-depleted Hb. These results reveal that lanthanide cations enhance oxygen affinity of Hb by affecting Hb-bound 2,3-DPG. This is further supported strongly by the following results involved in \( \text{Ln}^{3+} \)-induced 2,3-DPG hydrolysis.

3.2. Lanthanide cations induce the hydrolysis of 2,3-DPG bound to Hb

The allosteric binding of 2,3-DPG to Hb results in a dramatic decrease in oxygen affinity. The binding affinity of the deoxy form (human deoxyHb) is approx. 100 times higher than that of the oxy form (oxyHb), although only one molecule of 2,3-DPG is bound per tetramer of Hb and the binding sites...
are essentially the same in both cases [23–25]. It has been reported that in the 2,3-DPG-deoxyHb system, almost 90% of 2,3-DPG is bound, but for 2,3-DPG-oxyHb only 40% [23]. In addition, since 2,3-DPG cannot bind to Hb at relatively alkaline pH [26], to study the Ln$^{3+}$-induced hydrolysis of 2,3-DPG bound in deoxyHb, the media were kept at pH 6.7 and pH 7.0 only. Ultrafiltration and chemical determination of inorganic phosphate were used to follow the reaction. The rate of hydrolysis is strongly dependent upon Ln$^{3+}$ species, their concentration and the pH of the system. A higher pH makes the reaction faster; e.g. the reaction rate at pH 7.0 is approx. 2 times that at pH 6.7. The difference among various lanthanide cations is significant, as shown in Table 1. The sequence of increasing rate of hydrolysis is: La$^{3+}$ < Ce$^{3+}$ < Pr$^{3+}$ < Lu$^{3+}$. The effect of Lu$^{3+}$ on hydrolysis of 2,3-DPG is more pronounced than that of La$^{3+}$ under the same conditions. For instance, the rate of hydrolysis of 2,3-DPG bound to deoxyHb in the presence of $8.0 \times 10^{-5}$ mol/l Lu$^{3+}$ reaches 70.3% at pH 7.0, much higher than 47.3% of La$^{3+}$. These results also indicate that lanthanide cations with smaller radius have a stronger ability to promote the hydrolysis of the phosphomonoester bond in 2,3-DPG. As shown in Fig. 4, with the Lu$^{3+}$ concentration increased up to $1.0 \times 10^{-4}$ mol/l, the hydrolysis reaches 90% rapidly. By the same time, the amount of 2,3-DPG bound to Hb decreases.

To gain more insight into the mechanism of Ln$^{3+}$-induced hydrolysis of the phosphomonoester bond in 2,3-DPG bound to Hb, the phosphorus species derived from the Hb solution containing 2,3-DPG were studied by means of $^{31}$P NMR. The $^{31}$P NMR spectrum of erythrocyte cytosol (Fig. 5a) is featured by three peaks with chemical shifts of 1.812, 2.136 and 3.012 ppm. Based on previous discussion [27], they are assigned to be inorganic phosphate (Pi), 3- and 2-phosphates of Hb-bound DPG, respectively. In the presence of $1.0 \times 10^{-3}$ mol/l Lu$^{3+}$, the signal intensities of 3- and 2-phosphates decrease with slight downfield shift, and two new peaks (3.530, 2.861 ppm) appear.
ppm) appear, which are assigned to 3'-PGA and 2'-PGA, respectively (Fig. 5b). Thus the present results reveal that the phosphomonoester bond in 3- and/or 2-phosphates of 2,3-DPG can be cleaved by Lu$^{3+}$. Moreover, the downfield shift of the $^{31}$P resonance peaks indicates the decrease in Hb-bound 2,3-DPG, as also reported by Costello [26]. As shown in Fig. 6a, the characteristic $^{31}$P signal of 3- and 2-phosphates in 2,3-DPG bound to deoxyHb was located at 3.187 and 2.203 ppm, respectively. In the presence of Lu$^{3+}$ (1.5 x 10^{-4} mol/l), the appearance of 2'-PGA and 3'-PGA signals, their decrease and downfield shift reveal the hydrolysis of 3- and/or 2-phosphate groups. However, no inorganic phosphate signal appeared in the pH 7.2 solution. An experimental observation supporting the hydrolysis and clarifying the absence of phosphate signal is the appearance of a white flocculated substance, probably Lu$^{3+}$ phosphates, when the reaction proceeds at pH 7.2. It is reasonable to suppose that the phosphate ions generated from hydrolysis were precipitated as Lu-phosphates and thus the signal of free phosphate ion disappeared. Furthermore, a wider peak at the chemical shift of inorganic phosphate actually appears at pH 6.0, suggesting that this peak might be related to inorganic phosphate (figure not shown). Similar results were observed in 2,3-DPG-oxyHb.

3.3. The binding of lanthanide cations to Hb

The equilibrium of Ln$^{3+}$ binding to 2,3-DPG-depleted Hb was studied by the ultrafiltration method.
A representative Scatchard plot at 25°C is shown in Fig. 7a. The plot acquires an asymptotic bell shape, which is similar to the curve of Mn$^{2+}$ binding to tRNA$phe$ [20]. The curve was analyzed by means of Schreier’s methods (see Section 2.3) to yield two component curves (Fig. 7b,c). However, the results indicated that the Pr$^{3+}$ binding sites fall into three categories, one group of cooperative binding sites \( N_I = 8 \pm 1 \), \( K_I = 1.05 \times 10^5 \text{ M}^{-1} \), the strong independent sites \( N_S = 8 \pm 1 \), \( K_S = 4.90 \times 10^4 \text{ M}^{-1} \) and the weak independent sites \( N_W = 48 \pm 3 \), \( K_W = 938 \text{ M}^{-1} \). Similar behavior and parameters of other lanthanide cations are also summarized in Table 2.

### Table 2

Parameters for lanthanides binding to human hemoglobin

<table>
<thead>
<tr>
<th>Lanthanide</th>
<th>( N_I )</th>
<th>( K_I (\text{M}^{-1}) )</th>
<th>( \alpha )</th>
<th>( N_S )</th>
<th>( K_S (\text{M}^{-1}) )</th>
<th>( N_W )</th>
<th>( K_W (\text{M}^{-1}) )</th>
<th>( N_{\text{total}} )</th>
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<tr>
<td>La$^{3+}$</td>
<td>11 ± 1</td>
<td>3.75 \times 10^4</td>
<td>1.90</td>
<td>15 ± 1</td>
<td>1.44 \times 10^4</td>
<td>41 ± 2</td>
<td>436</td>
<td>67 ± 4</td>
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<tr>
<td>Pr$^{3+}$</td>
<td>8 ± 1</td>
<td>1.05 \times 10^5</td>
<td>1.23</td>
<td>8 ± 1</td>
<td>4.90 \times 10^4</td>
<td>48 ± 3</td>
<td>938</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Nd$^{3+}$</td>
<td>10 ± 1</td>
<td>7.7 \times 10^4</td>
<td>1.57</td>
<td>14 ± 2</td>
<td>5.20 \times 10^4</td>
<td>44 ± 3</td>
<td>181</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>9 ± 1</td>
<td>2.64 \times 10^4</td>
<td>1.48</td>
<td>13 ± 1</td>
<td>4.41 \times 10^4</td>
<td>45 ± 3</td>
<td>426</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Eu$^{3+}$</td>
<td>8 ± 1</td>
<td>3.95 \times 10^4</td>
<td>1.97</td>
<td>12 ± 1</td>
<td>1.47 \times 10^4</td>
<td>43 ± 3</td>
<td>512</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>Lu$^{3+}$</td>
<td>9 ± 1</td>
<td>4.52 \times 10^4</td>
<td>1.68</td>
<td>11 ± 2</td>
<td>3.18 \times 10^4</td>
<td>44 ± 2</td>
<td>389</td>
<td>64 ± 5</td>
</tr>
</tbody>
</table>

The results are expressed as mean values (n = 3).
3.4. Lanthanide cations trigger the fluctuation of intrinsic fluorescence of Hb

The kinetic study of Hb-Ln$^{3+}$ interactions was extended to follow the course in real time by means of stopped-flow spectroscopy. The binding of lanthanide cations to 2,3-DPG-depleted Hb was initiated in the stopped-flow mixing chamber. In the first range of 0–100 ms, as illustrated in Fig. 8, the intrin-

![Fig. 8. The kinetics of intrinsic fluorescence of human hemoglobin in the presence and absence of Tb$^{3+}$ as monitored by stopped-flow fluorometry. $\lambda_{ex} = 280$ nm, $\lambda_{em} = 330$ nm; [Hb]: $1.0 \times 10^{-3}$ mol/l; buffer: pH 7.2, 50 mmol/l HEPES, 25°C. (a) Control; (b) [Tb$^{3+}$]: $5.0 \times 10^{-3}$ mol/l; (c) computer fitted (b).](image)

![Fig. 7. (a) Scatchard plot of Pr$^{3+}$ binding to human hemoglobin in 50 mmol/l HEPES (pH 7.0) at 25°C. (b) Scatchard plot of the cooperative phase of the total Scatchard plot given in (a). (c) Scatchard plot of the independent site phase of the total Scatchard plot given in (a). The points are experimental mean values ($n = 3$) and the curve is calculated as described in the text.](image)

Fig. 8. The pseudo-first order rate constant ($k_{obs}$) depends on Tb$^{3+}$ concentration and temperature in the Hb-Tb$^{3+}$ system. Buffer: pH 7.2, 50 mmol/l HEPES; [Hb]: $1 \times 10^{-4}$ mol/l; [Tb$^{3+}$]: $\bigcirc$, $2.5 \times 10^{-3}$ mol/l; $\square$, $5.0 \times 10^{-3}$ mol/l; $\Delta$, $7.5 \times 10^{-3}$ mol/l; $\times$, $10 \times 10^{-3}$ mol/l. The results are expressed as mean values ($n = 6$).
sic fluorescence of Hb was quenched by $5 \times 10^{-3}$ mol/l Tb$^{3+}$, which is consistent with the evidence reported previously in the static fluorescence study of Hb [19]. The pseudo-first order rate constants ($k_{\text{obs}}$) for terbium ions were extracted by curve fittings and plotted against temperature and Tb$^{3+}$ concentration (Fig. 9). Since the $k_{\text{obs}}$ values depend linearly on Tb$^{3+}$ concentration, the binding of Tb$^{3+}$ to 2,3-DPG-depleted Hb can be deduced to a second order reaction with the rate equation described below:

$$r = \frac{dC_{\text{Hb}}}{dt} = k_{\text{obs}}C_{\text{Hb}} = KC_{\text{Tb}^{3+}}C_{\text{Hb}}$$

with rate constant:

$$K = k_{\text{obs}}/C_{\text{Tb}^{3+}}.$$  

According to Arrhenius' equation,

$$K = Ae^{-E_a/RT}$$  

$$\Delta H^a = E_a - RT$$  

$$A = (e \times K_b T/\hbar) \times e^{\Delta S^a/R}$$  

$$\Delta S^a = R[\ln A - \ln K_b T/\hbar - 1]$$

$$\Delta G^a = \Delta H^a - \Delta S^a T$$  

The parameters active energy ($E_a$), enthalpy ($\Delta H^a$), entropy ($\Delta S^a$) and free energy ($\Delta G^a$) for the binding of Ln$^{3+}$ to Hb are obtained by Eqs. 1a, 2a, 3a, 4a, 5a, and listed in Table 3.

The changing intrinsic fluorescence of Hb during its interaction with $5.0 \times 10^{-3}$ mol/l praseodymium ions was monitored as a function of time within a 1000 s range. The kinetic process has a feature of multiple steps, as shown in Fig. 10. The fluorescence intensity is quenched rapidly during the first 20 ms with the pseudo-first order rate constant $k_1 = 546$ s$^{-1}$ (Fig. 10a). However, within a 2 s range, the decay in fluorescence is followed by a slow increase in fluorescence, $k_2 = 3.03$ s$^{-1}$ (Fig. 10b). It is very interesting to note that, if the time was extended to 20 s, two further steps appeared, i.e. a drop and a rise in fluorescence (Fig. 10c). The $k_{\text{obs}}$ values for these four steps were estimated from curve fitting as $k_1 = 508$ s$^{-1}$, $k_2 = 2.85$ s$^{-1}$, $k_3 = 2.39$ s$^{-1}$ and $k_4 = 0.0298$ s$^{-1}$, respectively. It should also be noted that even in the longer range (1000 s), the system did not approach...
equilibrium but the fluorescence fluctuates further, e.g. starting from approx. 370 s the fluorescence decreases again with $k_5 = 5.03 \times 10^{-3}$ s$^{-1}$, and then increases within 700–750 s (Fig. 10d). However, no further change in fluorescence occurs after 1000 s. With other Ln$^{3+}$, a similar behavior of the intrinsic fluorescence in Hb was also observed. The results presented here show that after lanthanide cations initiate the conformational changes of Hb, the intrinsic fluorescence of Hb changes with the following features: (1) the decline in fluorescence intensity with fluctuations; (2) the period of each step extends for a longer time; and (3) the sequence of pseudo-first order rate constants of every step: $k_1 > k_2 > k_3 > k_4 > k_5 > k_6$.

Further studies demonstrated that the kinetics of the changing intrinsic fluorescence of Hb triggered by Ln$^{3+}$ binding is closely related to Ln$^{3+}$ concentration and temperature. For instance, when [Ln$^{3+}$/[Hb] < 10, only two steps appear in the period of 2 s, but fluctuation occurs within 20 s if [Ln$^{3+}$/[Hb] > 10. The values of $k_{obs}$ for various processes and their temperature dependence are listed in Table 4.

### 3.5. Ln$^{3+}$ binding induced the change of secondary structure in Hb

The effect of praseodymium ions on the FT-IR deconvoluted and second derivative spectra of 2,3-DPG depleted-Hb is shown in Fig. 11a–d. The broad band in the amide I region (1600–1700 cm$^{-1}$) composed of six components is mainly attributed to the C = O stretching mode of the peptide backbone and to a small extent to the C-N stretching mode. Based on the well-defined criteria previously reported by FT-IR spectroscopic studies [28,29], the maximum band at 1652.5 cm$^{-1}$ is assigned to the $\alpha$-helical structure with approx. 74% content, in good agreement with that of X-ray crystallographic studies. The 1676.4 cm$^{-1}$, 1667.7 cm$^{-1}$ and 1635.5 cm$^{-1}$ bands are indicative of turns and the 1618.7 cm$^{-1}$ band is attributed to the aromatic side chain absorption, while the 1627 cm$^{-1}$ band might be related to a special $\beta$-sheet structure. However, the binding of Pr$^{3+}$ to Hb ([Pr$^{3+}$/[Hb] = 20:1) induces a significant change in secondary structure in Hb. Compared with 2,3-DPG-depleted Hb, the characteristic changes of the amide I band in the presence of Pr$^{3+}$ are the appearance of two new peaks at 1650.1 cm$^{-1}$ and 1643.9 cm$^{-1}$, which are associated with the $\alpha$-helical and random structures, respectively. Meanwhile, the dominating $\alpha$-helix content decreases markedly from 74% to 28%, but the turn structure increases from 15% to 28%. The other bands shift slightly. Fig. 12 displays the decrease in $\alpha$-helix content in Hb as a function of Pr$^{3+}$ con-

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$K$ (dm mol$^{-1}$ s$^{-1}$)</th>
<th>$\Delta H^\circ$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G^\circ$ (kJ mol$^{-1}$)</th>
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<td>44.4</td>
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</table>

The results are expressed as mean values ($n = 6$).

### Table 3

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<tr>
<th>$T$ (K)</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$K$ (dm mol$^{-1}$ s$^{-1}$)</th>
<th>$\Delta H^\circ$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G^\circ$ (kJ mol$^{-1}$)</th>
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<td>44.4</td>
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Table 4

<table>
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<tr>
<th>Ln$^{3+}$</th>
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<th>293 K</th>
<th>298 K</th>
<th>303 K</th>
<th>310 K</th>
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<td></td>
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<td>2.88</td>
<td>2.91</td>
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<td>1.01</td>
<td>2.73</td>
<td>2.75</td>
<td>2.69</td>
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<td>0.047</td>
<td>0.025</td>
<td>0.023</td>
<td>0.011</td>
</tr>
<tr>
<td>Pr$^{3+}$ 2.5 $\times 10^{-3}$ mol/l</td>
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<td>398</td>
<td>456</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>$k_2$</td>
<td>2.03</td>
<td>2.43</td>
<td>2.85</td>
<td>2.88</td>
<td>2.91</td>
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<tr>
<td></td>
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<td>2.75</td>
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<td>0.051</td>
<td>0.047</td>
<td>0.025</td>
<td>0.023</td>
<td>0.011</td>
</tr>
</tbody>
</table>

[Hb] = 1 $\times 10^{-4}$ mol/l. $k_{obs}$ is an average value ($n = 5–7$).
centration. Clearly, the α-helix content decreases with increasing Pr³⁺ concentration when [Pr³⁺]/[Hb] = 15:1, but remains nearly unchanged after [Pr³⁺]/[Hb] = 15:1.

It should be noted that 2,3-DPG binding causes a significant decrease in the α-helix content of Hb from 74% to 55%, as shown in Table 5, suggesting that the decrease in α-helical structure might be relevant with the reduction of oxygen affinity. With the addition of Pr³⁺ into the 2,3-DPG-deoxyHb system ([2,3-DPG]/[deoxyHb] = 1), a further decrease in α-helix from 54.7% to 46.3% was observed. Other lanthanide cations also induce a significant change in the secondary structure of both Hb and 2,3-DPG-deoxyHb (see Table 5). The α-helix content in the presence of various lanthanide cations decreases in the following order: Lu³⁺ > Gd³⁺ > Eu³⁺ > Pr³⁺ > La³⁺.

4. Discussion

The data presented here reveal that the bidirectional effects of lanthanide cations on the changes in oxygen affinity for 2,3-DPG-Hb depend on Ln³⁺ concentration. This is the result of the integrated effect of Ln³⁺ binding on 2,3-DPG and Hb.

4.1. Hydrolysis of 2,3-DPG attributed to the break of phosphomonoester due to Ln³⁺ binding

Our results indicate that lanthanide cations can induce the hydrolysis of Hb-bound 2,3-DPG and lead to changes in oxygen affinity. Recently, lanthanide cations have been shown to be highly effective in promoting hydrolysis of phosphate diesters, includ-
ing RNA and DNA, by monometallic or dimetallic paths [30,31]. Komiyama et al. [32] also found that lanthanide cations were of very high activity in the catalytic hydrolysis of phosphatidylinositol (PI). One of the proposed mechanisms is related to Ln$^{3+}$ binding to the phosphate group and intramolecular attack by the Ln$^{3+}$-bound hydroxide ion on the phosphate. A higher pH will facilitate the hydrolysis of 2,3-DPG binding to hemoglobin by lanthanide cations.

### Table 5

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>La$^{3+}$</th>
<th>Pr$^{3+}$</th>
<th>Eu$^{3+}$</th>
<th>Gd$^{3+}$</th>
<th>Lu$^{3+}$</th>
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</thead>
<tbody>
<tr>
<td>Hb</td>
<td>73.67%</td>
<td>49.47%</td>
<td>44.36%</td>
<td>42.97%</td>
<td>39.68%</td>
<td>35.12%</td>
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<tr>
<td>2,3-DPG-deoxyHb</td>
<td>54.74%</td>
<td>48.93%</td>
<td>46.25%</td>
<td>39.13%</td>
<td>36.42%</td>
<td>31.56%</td>
</tr>
</tbody>
</table>

$[\text{Ln}^{3+}]/[\text{Hb}] = 10:1; [\text{Ln}^{3+}]/[2,3\text{-DPG-deoxyHb}] = 10:1; [2.3\text{-DPG}]/[\text{deoxyHb}] = 1:1.$ The data are the average values of two experiments.

Scheme 1. Proposed mechanism for the hydrolysis of 2,3-DPG binding to hemoglobin by lanthanide cations.
phosphate diesters, because high pH favors the binding of hydroxide ions to Ln\(^{3+}\), as shown in the following:

\[
\text{Ln(H}_2\text{O)}_{6}^{3+} + n\text{H}_2\text{O} \rightarrow [\text{Ln(H}_2\text{O)}_{6-n}(\text{OH})_{n}]^{3-n} + n\text{H}_3\text{O}^+ 
\]

Our data reveal that the 2,3-DPG hydrolysis induced by Ln\(^{3+}\) is enhanced by the increase in pH from 6.7 to 7.0 (Table 1). For the hydrolysis of 2,3-DPG induced by metal ions, Stankiewicz [33] suggested that \(\text{VO}^{2+}\) causes 2,3-DPG hydrolysis with site specificity by cleaving the 2'-phosphomoester bond. However, \(^3\text{P}\) NMR spectra show that Ln\(^{3+}\) can cleave either the 2'- or the 3'-phosphomoester bond of 2,3-DPG in Hb solution without site specificity (Figs. 5 and 6). According to the conformation of 2,3-DPG bound to Hb, this might be explained by the following cleaving mechanism. Lanthanide cations non-selectively chelate 2'-phosphate or 3'-phosphate and one carboxylate group to form an unstable seven/eight-membered ring structure, and then the Ln\(^{3+}\)-bound hydroxide ions attack intramolecularly the phosphomoester bond by the nucleophilic mode (Scheme 1).

4.2. Ln\(^{3+}\) binding results in the conformational change of globin

Our previous studies suggested that the lanthanide cations cannot replace the iron in the heme or react directly with the heme ring, since the Soret bands of Hb are not disturbed [19]. However, in Hb there should be a large number of amino acid residues, which can potentially be involved in Ln\(^{3+}\) binding. This possibility is supported by our results of ultrafiltration studies. As mentioned above, three kinds of binding sites were deduced, i.e. positive cooperative sites, non-cooperative strong sites and non-cooperative weak sites. Fushitani et al. [34] reported that calcium ions bind to Hb by two ionic bonds with \(-\text{NH}_2^+\) and \(-\text{OOC}^-\) groups. We can deduce that the lanthanide cation, as a Ca\(^{2+}\) analogue, is possibly bound to Hb by a similar way, as shown in Scheme 2.

The intrinsic fluorescence generated from tryptophan residues, two \(\alpha\text{Trp14, two } \beta\text{Trp15 and two } \beta\text{Trp37, is generally much more sensitive to ligand-induced changes in the quaternary structure of Hb. Among them, } \beta\text{Trp37, located at the } \alpha_1\beta_2 \text{ interface, has been assigned as the primary source of fluorescence emission and sensitive to the oxyHb (R-state) to deoxyHb (T-state) transition [35]. In general, the ligand-induced R- to T-state transition is accompanied by an increase in intrinsic fluorescence [36]. The kinetics of the reaction between lanthanide cations and Hb reveals that Ln\(^{3+}\) binding to Hb causes the fluctuation of intrinsic fluorescence in Hb. In the initial 100 ms, fluorescence quenching is closely related to the binding of Ln\(^{3+}\) to Hb. This can be interpreted by the evidence that energy transfer might occur from the tryptophan side chain to the bound Ln\(^{3+}\). Based on the Ln\(^{3+}\) concentration-dependent fluorescence fluctuation (Fig. 10), it seems reasonable to assume that as the first step, lanthanide cations bind to the highly affinity and perhaps the positive cooperative sites, and induce the conformation of globin in Hb to change to a more open structure. As the second step, more lanthanide cations bind to non-cooperative strong sites and cause the conformation to change further so that several buried sites become exposed to the environment, and then further binding and a further conformational change follow. When Ln\(^{3+}\) bind to amino acid residues located adjacent to \(\beta\text{Trp37} \text{ at the } \alpha_1\beta_2 \text{ interface, the dipole-dipole non-radiative energy transfer between } \beta\text{Trp37-Trp and Ln}\(^{3+}\) results in fluorescence quenching [37]. The fluctuation in fluorescence intensity, dominated by \(\beta\text{Trp37}, \text{ can be attributed to the successive conformational changes. Moreover, data obtained from FT-IR spectra of Hb in the presence of higher Ln}\(^{3+}\) concentrations revealed that Ln\(^{3+}\) binding altered the secondary structure of Hb significantly, as characterized by the lower \(\alpha\)-helix content, which is consistent with our previous in vivo and in vitro results [7,19]. In view of the marked change in \(\alpha\)-helix content that occurs in the case of [Ln\(^{3+}\)]/[Hb] < 20 (Fig. 12), the change in secondary structure involving \(\alpha\)-helix content might result mainly from the binding of Ln\(^{3+}\) to both the positive

\[\text{Scheme 2.}\]

\[\text{–NH}_2\text{ \hspace{0.5cm} OOC–} \]

\[\text{–NH}_2\text{ \hspace{0.5cm} OOC–} \]

\[\text{–NH}_2\text{ \hspace{0.5cm} OOC–} \]
cooperative sites and the non-cooperative strong sites.

4.3. 2,3-DPG hydrolysis and the conformational change of Hb are both responsible for Ln$^{3+}$-induced changes in oxygen affinity

2,3-DPG, the physiological allosteric effector of the Hb function, has been taken as indicative of the switch of the quaternary conformation of the Hb tetramer from the high to the low oxygen affinity form. According to the crystal structure of 2,3-DPG-Hb, the 2,3-DPG molecule forms salt bridges with N-terminal amino groups, His2 and His143 of both β-chains, and with Lys82 of one β-chain [38]. It seems that a nearly linear relation between 2,3-DPG hydrolysis and the conformational cooperative sites and the non-cooperative strong sites.

It should be noted that the in vitro saturation curves from Hb will produce a large increase in the oxygen affinity and a left shift of the saturation curve. It has been reported that Gd-DOTP and La-DOPT might bind to the tetramer at the 2,3-DPG binding site and the apparent affinity of Gd-DOTP for HbA-NO decreased with increasing 2,3-DPG concentration [16]. Thus, Ln$^{3+}$ is likely to be a potential competitor for 2,3-DPG binding to Hb. Moreover, Perutz et al. [40,41] suggested that introducing other positive charges into the central cavity of the 2,3-DPG binding region should destabilize the deoxyHb (T-state) structure and therefore enhance the oxygen affinity. Our data show that the saturation curve shifts rapidly to the left with the increase in Ln$^{3+}$ concentration at the range of [Ln$^{3+}$]/[Hb] < 2. The present results are consistent with their postulation. Based on the results obtained from ultrafiltration and $^{31}$P NMR spectra, it is conceivable that the hydrolysis of 2,3-DPG induced by Ln$^{3+}$ binding can cause the break of salt bridges βHis2-2,3-DPG and βHis143-2,3-DPG and the subsequent separation of 2,3-DPG from Hb. This should account for the increase in oxygen affinity (Fig. 2). However, the data also show that the effects cannot be explained merely in terms of the hydrolysis of 2,3-DPG because the saturation curve gradually shifted to the right again with a further increase in Ln$^{3+}$ concentration. The trend of changing $P_{50}$ for 2,3-DPG-Hb at higher Ln$^{3+}$ concentrations is qualitatively in agreement with the results obtained from 2,3-DPG-depleted Hb in the same range of Ln$^{3+}$ concentrations (Fig. 2). The conformational changes of Hb due to Ln$^{3+}$ binding, as shown in FT-IR spectra and stopped-flow experiments, are involved in the effect of Ln$^{3+}$ at higher concentrations on the oxygenation of Hb. Perutz et al. [42,43] have shown that during the transition between deoxyHb (T-state) and oxyHb (R-state), the α1β2 subunit interface undergoes a sliding movement, whereas the α1β1 subunit interface remains nearly unchanged. The intrinsic fluorescence is mainly originated from βTrp37, located at the α1β2 interface. The fluctuation indicates that the α1β2 interface is involved in the conformational change at higher Ln$^{3+}$ concentration. Thus, at [Ln$^{3+}$]/[Hb] ≈ 2, Ln$^{3+}$ might bind directly to globin and its effect on oxygen affinity would be attributed to the conformational fluctuation at the α1β2 interface and the shift of the conformational state toward the T-state (i.e. the state with lower oxygen affinity). As shown in stopped-flow and FT-IR experiments, the conformation change and the fluorescence fluctuation occurred in the same range of Ln$^{3+}$ concentrations. It would be reasonable to suggest that the lanthanide cations bind first to Hb-bound 2,3-DPG by the nucleophilic mode and then cause its hydrolysis. This stage is closely related to the increase in oxygen affinity at lower Ln$^{3+}$ concentration. However, more lanthanide cations will bind to Hb when the Ln$^{3+}$ concentration becomes higher. Then the effect of Ln$^{3+}$ on oxygen affinity will be related to the conformational change induced by Ln$^{3+}$ binding. It should be noted that the in vitro saturation curves of Hb after reaction with CeCl$_3$ could not acquire a double sigmoid shape, as in animal tests with CeCl$_3$ fed rats [7]. This might be explained partly by suggesting that there are several cerium species coexisting in vivo.

In summary, the binding of Ln$^{3+}$ to the 2,3-DPG-Hb system was shown to cause a non-linear change in oxygen affinity depending on Ln$^{3+}$ concentration. This effect is a result of both the hydrolysis of 2,3-DPG and the conformational change of Hb due to Ln$^{3+}$ binding. If the Ln$^{3+}$ concentration is rather low, Hb will be switched from the T-state with lower oxygen affinity to the R-state with higher oxygen affinity, due to the hydrolysis of 2,3-DPG induced by Ln$^{3+}$ binding. However, further Ln$^{3+}$ binding to Hb can also reverse this transition to a certain extent.
through the conformational fluctuation. Through these studies we also gain further insight into the understanding of erythrocyte response to the uptake of lanthanide cations.

Acknowledgements

This project was supported by the National Natural Science Foundation of China.

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