Location of the Allosteric Site for 2,3-Bisphosphoglycerate on Human Oxy- and Deoxyhemoglobin as Observed by Magnetic Resonance Spectroscopy*

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2,3-Bisphosphoglycerate, the predominant phosphorylated metabolite of the human red blood cell, binds tightly to human deoxyhemoglobin and weakly, yet significantly, to human oxyhemoglobin. To locate the binding site(s) for 2,3-bisphosphoglycerate in both oxy- and deoxyhemoglobins in solution, we have measured the paramagnetic effects of hemoglobin spin-labeled with a nitroxide radical at each of the two \( \beta \)-93 cysteine sulfur atoms per tetramer on the \( ^{31}P \)-nuclei of 2,3-bisphosphoglycerate and have studied the effect of \( CaATP \), a nonlabile paramagnetic analog of \( MgATP \), bound at the allosteric site, on the EPR spectrum of the hemoglobin-bound nitroxide spin label. An appreciable paramagnetic effect of the hemoglobin-bound spin label on the transverse and longitudinal relaxation rates of the \( ^{31}P \)-nuclei of 2,3-bisphosphoglycerate is observed with both deoxy- and oxyhemoglobins, the effect being more than an order of magnitude greater on the transverse relaxation rates. These observations are consistent with the binding of 2,3-bisphosphoglycerate to both forms of hemoglobin. From the magnitude of the paramagnetic effects on the longitudinal and transverse nuclear relaxation rates, using the correlation time of \( \tau \), obtained from the ratio of the paramagnetic effects on the longitudinal and transverse relaxation rates, which is of the same order as the rotational correlation time of the hemoglobin molecule, we estimate for both forms of hemoglobin similar NMR average distances of \( \sim 15 \text{ Å} \) between the unpaired electron on the nitroxide moiety of the spin label and either of the two \( ^{31}P \)-nuclei of the hemoglobin-bound 2,3-bisphosphoglycerate molecule. Consistent with this, we obtain a \( \sim 16 \text{ Å} \) distance between the \( Cr^{3+} \) atom of \( CrATP \) bound at the allosteric site and the spin label in oxyhemoglobin from their dipolar interaction measured by EPR. These results suggest similar spatial location of the binding site of 2,3-bisphosphoglycerate in both forms of hemoglobin in solution.

The function of the hemoglobin molecule in the human red blood cell is modulated by its interaction with 2,3-bisphosphoglycerate, the predominant phosphorylated metabolite of the red cell. Glycerate-2,3-P \(_2\) binds tightly to human deoxyhemoglobin (2-9) and weakly, yet significantly, to human oxyhemoglobin (6-8, 10). For oxyhemoglobin as for deoxy, 1 molecule of glycerate-2,3-P \(_2\) is bound/tetramer of hemoglobin (8). The strong preference of glycerate-2,3-P \(_2\) for binding to the deoxy form of hemoglobin is well accepted as the mechanism for facilitated release of hemoglobin-bound oxygen to the tissues. The binding sites for glycerate-2,3-P \(_2\) and also its competitive analog, inositol hexaphosphate, to deoxyhemoglobin have been mapped out by x-ray crystallography (11, 12). However, no x-ray crystallographic studies are yet available for locating the binding site of glycerate-2,3-P \(_2\) in human oxyhemoglobin. Even the actual binding of glycerate-2,3-P \(_2\) to human oxyhemoglobin has recently been questioned (9). It has been suggested that the conformational changes in the hemoglobin molecule that accompany oxygenation may result in the loss of the binding site for glycerate-2,3-P \(_2\) (13).

In an earlier paper (14), we verified the binding of ATP to both oxy and deoxy hemoglobin and showed that ATP and MgATP bind appreciably to both forms of hemoglobin. However, whereas the affinities of phosphorylated compounds for deoxyhemoglobin are related to their net charge and differ by more than an order of magnitude, both of these compounds and several others with varying net charges have similar affinities for oxyhemoglobin (8) suggesting that the binding site in oxyhemoglobin involves fewer interactions. In this paper, we confirm the binding of glycerate-2,3-P \(_2\) to both the oxy- and deoxy forms of hemoglobin and determine the location of the binding sites in both forms in solution from a paramagnetic reference point using hemoglobin spin-labeled with a nitroxide radical at the two \( \beta \)-93 positions per tetramer. Spin-labeling of hemoglobin at positions \( \beta \)-93, a technique introduced by McConnell and co-workers (15, 16), has previously been used to study conformational properties of hemoglobin in solution (15-18). It should be emphasized that differential binding of glycerate-2,3-P \(_2\) to human oxy- and deoxyhemoglobin is sufficient to explain its role as a metabolic effector of the oxygen affinity of hemoglobin. In addition to effects on oxygen affinity, the binding by oxyhemoglobin of glycerate-2,3-P \(_2\) would affect the availability of glycerate-2,3-P \(_2\) for enzymatic reactions. Since hemoglobin provides a good model system for allosteric proteins, a study of its allosteric site and its interactions in both oxy and deoxy forms is of considerable biochemical interest.

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The abbreviations used are: glycerate-2,3-P \(_2\), 2,3-bisphosphoglycerate; Bio-Trio, \([\text{Bio2(2-hydroxyethyl)amino}]\text{tris(hydroxymethyl)iminato}\); CrATP, Co(NH\(_3\))\(_3\)ATP, Co \(^{3+}\)(NH\(_3\))\(_3\)ATP.
For the crystalline complex of glycerate-2,3-P$_2$ with deoxyhemoglobin studied by x-ray diffraction, the organophosphate binding site lies on the 2-fold symmetry axis of the molecule in the central cavity between the globin subunits with the acidic groups of glycerate-2,3-P$_2$ forming salt bridges with a constellation of four pairs of basic groups on the $\beta$-chains, including the terminal amino groups (11). Since there is only one binding site per tetramer in the oxygenated state of hemoglobin as well, this site must also lie on the 2-fold symmetry axis of the molecule. However, whether or not binding to oxyhemoglobin occurs at the same site as to deoxyhemoglobin remains unknown. Another possible site for anion binding would be that involving the terminal amino groups of the $\alpha$-chains. CO$_2$ binds to all four amino terminal residues of the molecule. However, whether or not binding to oxyhemoglobin occurs at the same site as to deoxyhemoglobin is not known. In all cases, whether or not binding to oxyhemoglobin occurs at the same site as to deoxyhemoglobin remains unknown.

**Materials and Methods**

Oxyhemoglobin was prepared from freshly drawn blood and purified by standard procedures (8). The protein was concentrated in collagen tubes by vacuum dialysis. The concentration of hemoglobin was measured optically at 415 nm using a millimolar extinction coefficient of 134. Unless otherwise specified, all experiments were done in 0.2 M bis-Tris buffer in the presence of 0.15 M KCl to simulate physiological conditions. Deoxygenation was accomplished by passing nitrogen or argon over the hemoglobin solution in a roiling flask for 24 h. The state of deoxygenation was monitored by recording the optical spectra in the 700 to 800 nm region without any dilution using 2 mm path length cuvettes in a Cary 14 spectrophotometer and was found to be $\geq 90\%$. Methemoglobin content of samples was checked, as described in Ref. 21, before and after each experiment, and was found to be $<10\%$ in all cases.

Glycerate-2,3-P$_2$, as its cyclohexylammonium salt, was from Sigma. The cyclohexylamine was removed by passage through Dowex 50-H$^+$ and the acid was neutralized with KOH, ATP (sodium salt), inositol hexaphosphate (sodium salt), and bis-Tris were also obtained from Sigma. Chelex 100 was purchased from Bio-Rad. The concentration of glycerate-2,3-P$_2$ was determined enzymatically according to the method of Rose and Liebowitz (22) which makes use of the ability of glycerate-2-P to accelerate the hydrolysis of glycerate-2,3-P$_2$ by muscle phosphoglycerate mutase. CrATP and Co(NH$_2$)$_2$ATP were prepared according to the procedures of Cleland and co-workers (23, 24). Their concentrations were measured spectrophotometrically by adenine absorption at 260 nm using a millimolar extinction coefficient of 15.4.

The nitroxide spin label [3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinioxy] was obtained from SYVA Associates (Palo Alto, Calif.). The reaction of hemoglobin with the spin label was carried out at 4°C for 24 h in 10 mM bis-Tris buffer, pH 7.2, with a molar ratio of hemoglobin to spin label of 1:2.2 (18). The resulting solution was then passed through a Sephadex G-25 column equilibrated with 20 mM bis-Tris buffer containing 0.15 M KCl, pH 7.2. The spin-labeled hemoglobin was concentrated by vacuum dialysis and deoxygenated as described above.

$^3$P Nuclear relaxation rates were measured at 40.5 MHz with a Varian XL-100-15 FT-NMR spectrometer in the Fourier transform mode using wide band proton noise decoupling. Typical sample volumes were ~2 ml in 12-mm outer diameter NMR tubes. The temperature of the sample was maintained at the desired value by equilibration with purified gaseous nitrogen of appropriate temperature. Special care was taken in the preparation and handling of samples for NMR studies. All reagents were passed through columns of Chelex 100, prior to use, to remove paramagnetic contamination. Longitudinal nuclear relaxation rates were measured by the standard inversion-recovery technique. Transverse relaxation rates were measured using the Carr-Purcell spin-echo technique. Absolute errors in $T_1$ measurements are estimated to be $\pm 5\%$ and those in $T_2$ measurements $\pm 10\%$.

Water proton longitudinal relaxation rates were measured by the inversion recovery null point method at 8 to 24.3 MHz on an NMR spectrometer PS 60W spin-echo spectrometer and at 100 MHz on the Varian XL-100 FT-NMR spectrometer. EPR spectral recordings were obtained on a Varian E-4 spectrometer.

**Theoretical Considerations**

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**Metal-nuclear Distance Calculations**—When outer sphere and contact hyperfine contributions are negligible and the dipolar correlation time $\tau_{D}$ is long ($\omega_{D} \tau_{D} \gg 1$), then the distance dependencies of the paramagnetic effects of a protein-bound spin label on the longitudinal and transverse nuclear relaxation rates of a fast exchanging ligand nucleus are given by (25–28):

\[
\left( \frac{f_{2}}{f_{0}} \right) = \left( \frac{f_{2} \gamma_{2}^{2} \mu^{2} 3 \tau_{2} \gamma_{2}^{2} \tau_{D}}{1 + \omega_{D}^{2} \tau_{D}^{2}} \right)
\]

\[
\left( \frac{f_{2}}{f_{0}} \right) = \left( \frac{f_{2} \gamma_{2}^{2} \mu^{2} 3 \tau_{2} \gamma_{2}^{2} \tau_{D}}{1 + \omega_{D}^{2} \tau_{D}^{2}} \right)
\]

where $T_{1}$ and $T_{2}$ are the paramagnetic contributions to the observed longitudinal and transverse relaxation rates, respectively, $q$ is the relative stoichiometry of the paramagnet and the bound ligand in the paramagnetic complex, and $f$ is the inverse ratio of the total ligand concentration to that of the paramagnetic complex. The existence of fast exchange can usually be verified by the temperature dependence of $(f_{2}/f_{0})$ which should show only a small increase with increasing temperature in the fast exchange region. In the slow exchange region, a large and opposite temperature dependence is expected. Since 1 glycerate-2,3-P$_2$ molecule interacts with spin labels at the two $\beta$-3 positions per hemoglobin tetramer, the paramagnetic effects are twice those expected from a single spin label, i.e.:
water proton relaxation rate \(1/T_{1p}\) upon binding to hemoglobin (30).

### RESULTS

**31P Nuclear Relaxation Studies**—To study the binding of glycerate-2,3-P₂ to hemoglobin, nuclear relaxation rates of the \(31P\) nuclei of glycerate-2,3-P₂ were determined in the presence of native and spin-labeled hemoglobin. An example of the effects of the presence of native oxy- and deoxyhemoglobins as well as of the nitroxide-spin-labeled oxy- and deoxyhemoglobins on the measured longitudinal and transverse nuclear relaxation rates of the 2-P and 3-P nuclei of glycerate-2,3-P₂ at two different temperatures is given in Table I. The presence of 2.8 mM oxy- or deoxyhemoglobin in an 11.1 mM glycerate-2,3-P₂ solution enhances the longitudinal and transverse nuclear relaxation rates of 2-P and 3-P nuclei of glycerate-2,3-P₂ at two different temperatures is given in Table I. The presence of 2.8 mM oxy- or deoxyhemoglobin in an 11.1 mM glycerate-2,3-P₂ solution enhances the longitudinal and transverse nuclear relaxation rates of 2-P and 3-P phosphorus nuclei by 2- to 3-fold at either temperature. The presence of spin-labeled hemoglobin in place of native hemoglobin produces larger enhancements of the longitudinal and transverse relaxation rates of both phosphoryl groups (Table I), presumably due to the additional paramagnetic contributions of the spin label. From the example in Table I, the presence of 4.1 mM spin-labeled oxyhemoglobin in a 9.2 mM solution of glycerate-2,3-P₂ produces a 4- to 5-fold enhancement in the longitudinal and approximately a 60-fold enhancement in transverse relaxation rate at 14°C. A somewhat larger enhancement in 1/T₁ (≈8-fold) and a somewhat smaller increase in 1/T₂ (≈55-fold) is observed at 35°C. Within the experimental error of our measurements, similar relaxation rates were obtained for the two phosphorus nuclei of the glycerate-2,3-P₂ molecule, under all conditions (Table I). The paramagnetic effects of the spin label on the longitudinal and transverse relaxation rates of the \(31P\) nuclei of glycerate-2,3-P₂ bound to hemoglobin, calculated from the respective differences between the appropriately normalized relaxation rates obtained in the presence of hemoglobin with and without the spin label, are summarized in Table II. A small correction to the magnitude of the observed effects was necessary to take into account the somewhat incomplete saturation of hemoglobin with glycerate-2,3-P₂. The presence of 3.2 mM inositol hexaphosphate in a solution containing 3.7 mM spin-labeled oxyhemoglobin and 11.4 mM glycerate-2,3-P₂ diminished the paramagnetic effect of the spin label on the transverse (1/T₂) relaxation rates of the 2-P and 3-P phosphorus nuclei of glycerate-2,3-P₂ to 5.4 and 5.6 s⁻¹ from 14 and 15 s⁻¹, respectively, due to partial displacement of glycerate-2,3-P₂ molecules from their site on hemoglobin by inositol hexaphosphate. Using the dissociation constants of glycerate-2,3-P₂ (2.0 mM (8)) and inositol hexaphosphate (0.09 mM (9)) from their respective complexes with oxyhemoglobin, 

### Table I

<table>
<thead>
<tr>
<th>Components present (mM)</th>
<th>(T_{1p}^{-1}) 14°C</th>
<th>(T_{1p}^{-1}) 35°C</th>
<th>(T_{2p}^{-1}) 14°C</th>
<th>(T_{2p}^{-1}) 35°C</th>
<th>(T_{1p}^{-1}) 14°C</th>
<th>(T_{1p}^{-1}) 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPG (11.1)</td>
<td>0.17</td>
<td>0.13</td>
<td>0.37</td>
<td>0.35</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>DPG + HbO₂ (11.1) (2.8)</td>
<td>0.29</td>
<td>0.30</td>
<td>0.92</td>
<td>0.79</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>DPG + Hb (11.1) (9.8)</td>
<td>0.30</td>
<td>0.35</td>
<td>0.97</td>
<td>0.94</td>
<td>0.31</td>
<td>0.39</td>
</tr>
<tr>
<td>DPG + SLHbO₂ (9.2) (4.1)</td>
<td>0.80</td>
<td>1.09</td>
<td>24</td>
<td>18</td>
<td>0.74</td>
<td>1.06</td>
</tr>
<tr>
<td>DPG + SLHb (9.2) (4.1)</td>
<td>0.72</td>
<td>1.04</td>
<td>24</td>
<td>19</td>
<td>0.71</td>
<td>0.94</td>
</tr>
<tr>
<td>DPG + SLHbO₂ (11.9) (3.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPG + SLHbO₂ + IHP (11.4) (3.7) (3.2)</td>
<td>5.0</td>
<td>5.0</td>
<td>8.0</td>
<td>8.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\( ^{a} T = 17°C \)

### Table II

<table>
<thead>
<tr>
<th>State of hemoglobin</th>
<th>Atom</th>
<th>(T_{1p}^{-1}) 14°C</th>
<th>(T_{2p}^{-1}) 14°C</th>
<th>(T_{1p}^{-1}) 35°C</th>
<th>(T_{2p}^{-1}) 35°C</th>
<th>NMR average distance to the spin label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(s^{-1})</td>
<td>(s^{-1})</td>
<td>(s^{-1})</td>
<td>(s^{-1})</td>
<td>(\AA)</td>
</tr>
<tr>
<td>HbO₂</td>
<td>P-3</td>
<td>0.6 ± 0.3</td>
<td>34 ± 7</td>
<td>3.6 ± 1.9</td>
<td>14.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-2</td>
<td>0.6 ± 0.2</td>
<td>34 ± 6</td>
<td>4.0 ± 1.7</td>
<td>14.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>P-3</td>
<td>1.1 ± 0.5</td>
<td>30 ± 5</td>
<td>2.5 ± 1.2</td>
<td>13.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-2</td>
<td>1.1 ± 0.4</td>
<td>30 ± 4</td>
<td>2.5 ± 1.0</td>
<td>13.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-3</td>
<td>0.4 ± 0.2</td>
<td>26 ± 5</td>
<td>3.9 ± 2.1</td>
<td>15.1 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-2</td>
<td>0.3 ± 0.1</td>
<td>25 ± 5</td>
<td>4.4 ± 1.7</td>
<td>15.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-3</td>
<td>0.6 ± 0.3</td>
<td>20 ± 5</td>
<td>2.7 ± 1.5</td>
<td>14.9 ± 1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-2</td>
<td>0.4 ± 0.2</td>
<td>20 ± 4</td>
<td>3.4 ± 1.8</td>
<td>15.5 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) The paramagnetic effects from the spin label have been halved because of the stoichiometry of the labeling (see Equation 3).
effects may therefore be used for distance calculations. In order to calculate distances, the correlation time $\tau_c$ for the dipolar interaction of the spin label with $^{31}$P nuclei must be known. Since $1/T_{1p}$ and $1/T_{2p}$ are not exchange-limited and do not contain a contact contribution, this $\tau_c$ value may be estimated from their relative magnitudes using Equations 1 and 2. This yields for $\tau_c$ a value of $\sim 40$ ns at $T = 14^\circ$C and of $\sim 30$ ns at $T = 35^\circ$C for the interactions of the spin label with $^{31}$P nuclei for both oxy- and deoxyhemoglobins.

**Distance of Allosteric Site from the Spin Label on $\beta$-93 Cysteine Sulfur Atoms**—The existence of substantial paramagnetic effects of the spin label on $1/T_1$ and $1/T_2$ of glycerate-2,3-P$_2$ which disappear upon displacement of glycerate-2,3-P$_2$ by inositol hexaphosphate confirms binding of glycerate-2,3-P$_2$ to both forms of hemoglobin. The NMR average distance between the spin label and the individual $^{31}$P nuclei of hemoglobin-bound glycerate-2,3-P$_2$ may be calculated from $T_{1p}$ and $T_{2p}$ if these relaxation rates are not limited by chemical exchange and not dominated by outer-sphere relaxation (26). The absence of exchange limitation on $(1/T_{1p})^{-1}$ has been demonstrated in our case by the observation that $T_{1p}$ and $T_{2p}$ if these relaxation rates are not limited by chemical exchange and not dominated by outer-sphere relaxation (26). The absence of exchange limitation on $(1/T_{1p})^{-1}$ has been demonstrated in our case by the observation that $T_{1p}$ and $T_{2p}$ if these relaxation rates are not limited by chemical exchange and not dominated by outer-sphere relaxation (26). The absence of exchange limitation on $(1/T_{1p})^{-1}$ has been demonstrated in our case by the observation that $T_{1p}$ and $T_{2p}$ if these relaxation rates are not limited by chemical exchange and not dominated by outer-sphere relaxation (26).

The observed paramagnetic effects of spin-labeled oxy- and deoxyhemoglobin in itself is paramagnetic, the electron spin relaxation time of the high spin ferrous iron in this molecule is very short $\tau_e \sim 10^{-12}$ s, which is an order of magnitude shorter than the rotational correlation time of the hemoglobin molecule and than any reasonable estimate of the electron spin relaxation time of the hemoglobin bound spin label from its EPR linewidth (Fig. 1). The paramagnetic effects of the ferrous iron in deoxyhemoglobin on glycerate-2,3-P$_2$ will therefore be negligibly small and the deoxyhemoglobin-glycerate-2,3-P$_2$ complex serves as a good diamagnetic control for the spin-labeled deoxyhemoglobin complex. Accordingly, the diamagnetic effects of native oxy- and deoxyhemoglobin were subtracted out from the total effects of spin-labeled oxy- and deoxyhemoglobin to obtain the paramagnetic effects of the unpaired spin on glycerate-2,3-P$_2$ in the hemoglobin-glycerate-2,3-P$_2$ complex. Since the temperature dependence of $T_{1p}$ indicates this to be in the fast exchange region, we use the $T_{1p}/T_{2p}$ ratio to obtain $\tau_c$ via Equations 1 and 2 (Table II). The rotational correlation time of the human hemoglobin molecule is estimated to be $3 \times 10^{-8}$ s at $25^\circ$C from Stokes law. The measured correlation times of $3 \times 10^{-8}$ s therefore appear to be $\tau_r$ although the distance calculations are independent of the detailed nature of the correlation process. The magnitude of calculated $\tau_r$ also indicates that the observed effects arise from glycerate-2,3-P$_2$ rotationally bound to hemoglobin and further argue against the presence of any outer sphere effects. The increasing magnitude of $T_{1p}$ with increase in temperature is consistent with the estimated dipolar correlation times since $\omega_r \tau_r \gg 1$ at the 40.5 MHz $^{31}$P NMR frequency. It should be pointed out that $T_{2p}$ shows opposite temperature dependence because of the term proportional to $\tau_r$ in Equation 2.

The observed paramagnetic effects of spin-labeled oxy- and deoxyhemoglobin on the $^{31}$P nuclear relaxation rates are comparable, suggesting significant occupancy of the allosteric site in both cases, at the glycerate-2,3-P$_2$ levels used in our experiments. The neglect of hyperfine contact interactions between the paramagnetic spin label and the phosphorus nuclei of glycerate-2,3-P$_2$ assumed in the derivation of Equations 1 and 2 is justified by the smallness of the observed paramagnetic effects and by the slowness of electronic relaxations ($\tau_e \geq 10^{-8}$ s; $\tau_{wP} \gg 1$) (26, 31). From the observed paramagnetic effects of the spin label on the $^{31}$P nuclei (Table II), using the correlation time $\tau_e = 4 \times 10^{-8}$ s for spin-labeled oxy- and deoxyhemoglobins at $14^\circ$C, we obtained the NMR average distances between either of the two phosphoryl groups and the paramagnetic spin label at the $\beta$-93 positions to be $15.3 \pm 1.5$ Å in the deoxygenated state and $14.1 \pm 1.3$ Å in the oxygenated state of hemoglobin. Similar distances are obtained from the nuclear relaxation data at $35^\circ$C. The two phosphoryl groups of glycerate-2,3-P$_2$ appear to be equidistant from the spin-labeled positions within the error level of our NMR measurements. The distances of either phosphoryl group from the two $\beta$-93 sulfur atoms in the deoxyhemoglobin-glycerate-2,3-P$_2$ complex are known from x-ray crystallographic study to be 15.5 and 23.5 Å (11), the inverse sixth root of the mean of the inverse sixth power of the two distances being $17.2 \pm 1$ Å. According to the crystallographic model, the two phosphoryl groups are located at the center of the tetramer and are symmetrically arranged with respect to the 2-fold symmetry axis of the molecule which exists because of the presence of two identical $\beta$- and two identical $\alpha$-chains in the tetramer.
CrATP causes an enhancement in its paramagnetic effects on 1/T_1 of water protons. A titration measuring the observed enhancement of the 1/T_1 of water protons, in a solution containing 2.3 mM CrATP and oxyhemoglobin varying in the range 0 to 2.2 mM, was theoretically fitted to yield a dissociation constant of 0.6 mM for CrATP from its complex with oxyhemoglobin at pH 6.4 (Fig. 2). The fit also yielded a value of 2.7 ± 0.3 for the bound state enhancement (ε_b) of the paramagnetic effect of Cr³⁺ on water protons in the CrATP- oxyhemoglobin complex. Since the rotational correlation time of the CrATP complex of oxyhemoglobin is long (~3 × 10⁻⁸ s) compared to the electron spin relaxation time τ, of CrATP, the latter must be the correlation time for the dipolar interaction of Cr³⁺ with water protons. The observed enhancement in the bound state yields a value of ~4 × 10⁻¹⁰ s for this correlation time since the dipolar correlation time for free CrATP has previously been determined to be ~1.4 × 10⁻¹⁰ s (30). This value of correlation time is consistent with the observed temperature independence (±10%) of ε_b over the range 20 to 35°C and with the absence of any significant frequency dependence (±10%) of the observed paramagnetic effects of CrATP-HbO₂ complex on the longitudinal relaxation rate of water protons at 8, 15, 24.3, and 100 MHz. The latter observations set an upper limit of 5 × 10⁻¹⁰ s on the value of τ. Since the number of fast exchanging protons in the inner sphere of Cr³⁺ cannot increase by more than a factor of 2 (the factor of 2 allowing the possible protonation of metal-bound hydroxyl groups in free CrATP upon binding to hemoglobin), a lower limit of 2 × 10⁻¹⁰ s on τ, is provided by the observed ε_b of 2.7.

Titration of spin-labeled oxyhemoglobin with CrATP significantly decreases the amplitude of the EPR signal from the free radical (Fig. 1). In the presence of a saturating level of CrATP (12.5 mM) in a 1.5 mM solution of spin-labeled oxyhemoglobin in 0.15 M KCl (pH 6.4), ~20 ± 4% reduction in the amplitude of the major peak of the EPR spectrum, but no large change in linewidth, is observed in comparison with an equivalent level of Co(NH₃)₅ATP, a diamagnetic analog of CrATP, in an otherwise identical solution. The width of this derivative peak as measured by the separation between the positive and negative extrema (29) for the solution of spin-labeled hemoglobin containing Co(NH₃)₅ATP was ~3.4 G. From these data, the interaction coefficient C in Equation 4 was estimated to be 0.6 ± 0.1 according to the procedure of Leigh (29). Substitution of the various magnetic parameters and τ, in Equation 4 yields a value of 16 ± 2 Å for the distance between the Cr³⁺ atom and the paramagnetic nitroxide moiety of the spin label on oxyhemoglobin. This Cr³⁺ to spin label distance of 16 ± 2 Å measured by EPR methods is in good agreement with the Cr³⁺ to glycerate-2,3-P₂ distance measured by the nuclear relaxation technique, providing further support to our determination of the location of the allosteric site on oxyhemoglobin.

![EPR spectra of the nitroxide spin label bound to oxyhemoglobin (1.5 mM in tetramer) at pH 6.4 (μ = 0.15 M) in the presence of (upper) 12.5 mM CrATP and (lower) 12.5 mM Co(NH₃)₅ATP.](http://www.jbc.org/content/280/12/8254/F1)

**Fig. 1.**

**Discussion**

From the present study, it is concluded that the binding site for glycerate-2,3-P₂ to oxyhemoglobin is in the same part of the hemoglobin molecule as the deoxyhemoglobin binding site, that is at the interface of the two β-chains. Earlier, we showed that the stoichiometry of binding of glycerate-2,3-P₂ to oxyhemoglobin is the same as for deoxyhemoglobin, i.e. 1 molecule bound/hemoglobin tetramer (8). Unlike binding to deoxyhemoglobin, the binding constants to oxyhemoglobin for a number of compounds with different net charge, i.e. glycerate-2,3-P₂, ATP, MgATP, ADP, and glycerate-1,3-P₂ measured under simulated physiological conditions, are the same within the error of the measurements. Crystallographic data indicate that the binding of glycerate-2,3-P₂ to deoxyhemoglobin involves salt bridges to residues in the two β-chains: valines-1, histidines-2, lysines-82, and histidines-143 (11). In the deoxy structure, the NH₂-terminal valine residues are too far apart for both to interact with glycerate-2,3-P₂ and the binding involves some strain which is reflected in a motion that brings the entire A helix closer to the E and F helices.
The crystallographic data indicate that these valine residues move farther apart on oxygenation. Perrella et al. (19) have shown that glycerate-2,3-P₂ interferes with the binding of CO₂ to the NH₂ terminal valines of the β-chains of deoxyhemoglobin but not of oxyhemoglobin. Therefore, these residues (and also histidines-2) probably do not participate in the binding of glycerate-2,3-P₂ to oxyhemoglobin. It is possible that lysines-82 and histidines-143 may be involved in binding to oxyhemoglobin but without more knowledge of the geometry of human oxyhemoglobin in this region of the molecule, it is not possible to say how many simultaneous interactions are feasible. The decrease in the number of groups associated with binding can explain the weakened binding of phosphorylated compounds to oxyhemoglobin. A more definitive analysis of the residues involved in the interaction requires either crystallographic studies of the glycerate-2,3-P₂ complex with oxyhemoglobin or binding studies with appropriate human hemoglobin variants or nonhuman hemoglobins, e.g., horse hemoglobin has a glutamine in place of histidine-2 on the β-chains and human fetal hemoglobin has a serine in place of histidine-143 on the β-chains.

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