The Interaction of Inositol Pentaphosphate with the Hemoglobins of Highland and Lowland Geese*

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We have studied the binding of inositol pentaphosphate (IPP) to the hemoglobins from two species of goose living at high and high altitudes, using the proton absorption method. Measurements were done at 25 and 37°C in a pH range between 6.0 and 8.8. The bird hemoglobins show a high affinity and a binding stoichiometry of 1 IPP molecule/hemoglobin tetramer both in the ligated and unligated state, indicating the same binding site for IPP in oxy- and deoxyhemoglobin. The results indicate that the interaction of IPP with both goose hemoglobins is very similar. For the deoxyhemoglobins of both species the IPP-binding constant shows a strong pH dependence extending over a wide pH range (i.e. ±2 × 10⁻⁶ m at pH 8.8 and ±6 × 10⁻⁶ m at pH 6.0). The binding constant of IPP for the oxyhemoglobins shows a much weaker pH dependence (i.e. ±4 × 10⁻⁶ m at pH 8.8 and ±3 × 10⁻⁶ m at pH 6.0), indicating that the interaction of IPP with the goose hemoglobin is strongly dependent on the state of ligation of the protein. The IPP binding constants for the oxy- and deoxyhemoglobins are found to be in good agreement with the IPP-induced change in oxygen affinity of both hemoglobins as estimated from oxygen binding curves.

It has been long known that the blood oxygen affinity of animals residing at high altitude is in general higher than that of lowland species of similar size (1). This pattern was observed in amphibians, mammals, and some birds (1–3). Particularly, in the latter class of animals, a large difference was found in whole blood oxygen affinity between the bar-headed goose that breeds in the highlands of Central Asia at altitudes around 4000 m and its lowland relatives, the greylag goose and the Canada goose (3). As a high oxygen affinity is of physiological significance for tolerating hypoxic hypoxia (4–7), it becomes interesting to gather information on the interaction of inositol pentaphosphate, the major phosphate compound in avian red cells, with these bird hemoglobins. We have therefore obtained estimates on the association constant of the physiological cofactor IPP with the hemoglobin of the greylag goose and the bar-headed goose at 25 and 37°C, as well as at a number of pH values.

Furthermore, avian hemoglobins are interesting with respect to their interaction with organic phosphates because they possess a relatively high number of positively charged residues at the phosphate binding site as compared to human hemoglobin (8–10). This leads to a very high affinity for organic phosphates even in the oxygenated state (11) which makes it possible to study the interaction of inositol pentaphosphate with the protein over a wide pH range. Such measurements provide information on the stoichiometry of the effecter binding to oxyhemoglobin and on the nature of the groups interacting with the phosphate.

Our results on the geese hemoglobins indicate, that, although IPP binds with 1:1 stoichiometry to both oxy- and deoxyhemoglobins, the pH dependence of the organic phosphate binding is very different for each derivative.

From the measurements presented in this paper it becomes apparent that the large difference in oxygen affinity of whole blood between the two species can be explained by an IPP-induced magnification of a small difference in intrinsic oxygen affinity of the two respiratory proteins.

MATERIALS AND METHODS

Blood was obtained from the major wing vein of unanesthetized greylag goose (Anser anser anser) and bar-headed goose (Anser indicus) and kept in ice until use. Hemoglobin solutions were prepared from the blood by standard procedures within 1 day after the blood was drawn.

The hemoglobin was freed from organic phosphates by gel filtration on Sephadex G-25 (0.1 M Tris-HCl, pH 8.5, 0.5 M NaCl). Complete removal of phosphates was checked by a total phosphate determination according to the method of Ames and Dubin (12). The major hemoglobin component, that comprised about 90% of the total, was isolated by ion exchange chromatography. A first purification was obtained on Whatman CM-52 using a linear pH gradient (starting buffer: 0.01 M phosphate, pH 7.2; limiting buffer: 0.02 M NaHPO₄). The major component was then rechromatographed on Whatman DE-52 (0.05 M Tris-HCl, pH 8.65). All chromatographic procedures were performed on carbonmonoxy derivatives at 4°C. The purity of the preparations was checked by isoelectric focusing (LKB Multiphor system).

The purified hemoglobin solutions were dialyzed against distilled water at 4°C. The carbon monoxy was removed from the hemoglobin by exposing the pigment to strong incandescent light in a rotating tonometer under a stream of pure oxygen. The solutions were kept in liquid nitrogen until use. The hemoglobin content of the samples was always less than 5%.

Before use the solutions were deionized by repeated passing through a mixed bed ion exchange column (Dowex AG, 50-X8 (D)).

Myoinositol 1,3,4,5,6-pentaphosphate (IPP) was isolated from chicken red cells. The separation between the hemoglobin and the organic phosphate was achieved by gel filtration on Sephadex G 25 (0.1 M Tris-HCl, pH 8.5, 0.5 M NaCl). Using this procedure, a good separation between IPP, ATP, and inorganic phosphate was obtained. Subsequently, the inositol pentaphosphate was precipitated as the barium salt by adding 1 M barium nitrate until no more precipitate was formed, and the precipitate was washed twice with 1 mM barium nitrate solution. Before use, the phosphate was dissolved in water by shaking with a cation exchange resin (Amberlite IR 120) and brought to the desired pH with 1 N NaOH. The purity of the sample was checked both by microanalysis for carbon and phosphorus and by 31P-NMR spectroscopy. The microanalysis gave a P/C ratio of 2.10 (theoretical value: 2.15). The 31P-NMR spectrum did not show any evidence for organic phosphate compounds other than IPP (13, 14).

The concentration of the IPP solutions was determined by measuring

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1 The abbreviations used are: IPP, myoinositol, 1,3,4,5,6-pentaphosphate; Tris, tris(hydroxymethyl)aminomethane; bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.
the total phosphate content according to the method of Ames and Dubin (12). All other chemicals used were analytical grade.

pH measurements were made with a Radiometer PHM-64 pH meter connected to a Radiometer REC-61 recorder using Radiometer C-202 C and K-401 electrodes. The titration experiments were performed in a thermostatted titration vessel which was flushed with humidified N₂ or O₂.

Hemoglobin solutions were deoxygenated in a rotating tonometer under a constant flow of pure N₂. Subsequently, the solutions were anaerobically transferred to the titration vessel. Complete deoxygenation was checked by measuring the ratio of the absorbances at 555 and 540 nm. For the measurements of the IPP binding curves an IPP solution, having the same pH as the hemoglobin solution, was added to the protein solution in steps of 0.01 to 0.05 ml. After each addition the solution was backtitrated to the original pH value by addition of a 0.007 N HCl solution. The addition of the titrants was made with specially manufactured microburettes which allowed the addition of 0.01 ml of the titrant solutions with an accuracy of about 1%.

The determination of the Bohr effect curve was made by bringing 10 ml of a deoxygenated hemoglobin solution in the titration vessel, adjusting to the desired pH by addition of 0.07 N HCl or NaOH under a nitrogen atmosphere, equilibrating the solution with pure oxygen, and finally, titrating the sample to the initial pH with 0.07 N NaOH or HCl.

Oxygen binding curves at 25°C were determined spectrophotometrically according to the method of Benesch et al. (15) using a Shimadzu UV-200 spectrophotometer. Oxygen binding curves at 37°C were measured as described in Ref. 16. All experiments were performed in the presence of 0.1 M KCl.

Sedimentation coefficients were determined with a Beckman model E ultracentrifuge at a speed of 60,000 rpm.

RESULTS

To characterize the hemoglobins of both species of goose with respect to association-dissociation behavior, ultracentrifugation experiments were performed on the CO-ligated derivatives in the concentration range 5.6 to 250 μM tetramer at pH 6, 7, and 8. The results presented in Table I indicate that no significant dissociation in dimers occurs in this concentration range. In order to obtain information on the behavior of the titratable groups in both geese hemoglobins, titration curves, i.e. pH versus the mean proton charge Z_H, of the oxygenated derivatives were measured. The differential titration curves, i.e. −ΔpH/ΔZ_H versus Z_H are shown in Fig. 1. Z_H was assumed to be zero at the isionic pH, which was 8.20 for both hemoglobins at 25°C in 0.1 M KCl. The differential titration curves show two maxima, one at pH 6.10 and the second at pH 8.6. The mean proton charge at these pH values is given by (Ref. 17, 18):

\[ Z_H = n_{\text{arg}} + n_{\text{lys}} + n_{\text{his}} + n_{\text{nh2}} - n_{\text{cooh}} \]

and

\[ Z_{Hf} = n_{\text{arg}} + n_{\text{lys}} - n_{\text{cooh}} \]  

where Z_H and Z_{Hf} are the Z_H values for the maxima at low and high pH, respectively, and n refers to the number of titratable residues of the kind designated by the index.

From Fig. 1 it is clear that there is a very small, if any, difference in titration behavior between the two geese hemoglobins, both with respect to the number and the pK values of the titratable residues. From the difference between the Z_H and Z_{Hf} values it follows that in both hemoglobins about 14 histidine residues are titratable in the neutral pH region, provided all four α-NH₂ groups are titratable.

Some typical IPP binding curves are shown in Fig. 2. At all experimental conditions we observed that the binding curves show well defined equivalence points indicating a binding stoichiometry of IPP to tetrameric hemoglobin of 1:1. The continuous increase in ΔZ at IPP/Hb ratios higher than 1 is caused by the dilution of the IPP solution upon addition to the hemoglobin solution. The same effect was found when 0.01 ml of the IPP solution was added to the hemoglobin solution. The same effect was found when 0.007 N HCl solution. The addition of the titrants were made with specially manufactured microburettes which allowed the addition of 0.01 ml of the titrant solutions with an accuracy of about 1%.

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![Figure 1](http://www.jbc.org/)

**FIG. 1.** The differential titration curves (−ΔpH/ΔZ_H versus Z_H, where Z_H is the mean proton charge) of the bar-headed (●) and greylag (○) goose oxyhemoglobins. Hemoglobin concentration 37 μM tetramer, 0.1 M KCl, 25°C.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Number of protons taken up per hemoglobin tetramer (ΔZ) upon addition of IPP as a function of n, the molar ratio of IPP to hemoglobin tetramer, for the oxyhemoglobin at pH 7.2 (●) and deoxyhemoglobin at pH 8.8 (○) of the bar-headed goose. Hemoglobin concentration 25 μM tetramer, 0.1 M KCl, 25°C.

### TABLE I

Concentration dependence of the sedimentation coefficients (s_0) of the CO-ligated hemoglobin of bar-headed and greylag goose at three pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>Concentration</th>
<th>Bar-headed goose</th>
<th>Greylag goose</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>0.05</td>
<td>3.63</td>
<td>3.77</td>
</tr>
<tr>
<td>7.0</td>
<td>0.05</td>
<td>3.89</td>
<td>3.83</td>
</tr>
<tr>
<td>8.0</td>
<td>0.05</td>
<td>4.08</td>
<td>3.98</td>
</tr>
<tr>
<td>6.0</td>
<td>0.1</td>
<td>3.73</td>
<td>3.77</td>
</tr>
<tr>
<td>7.0</td>
<td>0.1</td>
<td>3.79</td>
<td>3.87</td>
</tr>
<tr>
<td>8.0</td>
<td>0.1</td>
<td>3.92</td>
<td>3.92</td>
</tr>
</tbody>
</table>
adding IPP to phosphate buffer solutions, having the same buffer capacity as the hemoglobin solution used in the experiments. The binding curves corrected for this effect were analyzed according to the equation:

$$\Delta Z' = \Delta Z_{\text{max}} \frac{K_c(n+1) + 1 - \sqrt{[K_c(n+1)]^2 - 4K_c^2c/n}}{2K_c}$$

where \( \Delta Z' \) is the number of protons taken up by the hemoglobin tetramer at an IPP/Hb ratio of \( n \), \( \Delta Z_{\text{max}} \) is the number of protons taken up per tetramer upon complete saturation with IPP, \( K_c \) is the binding constant of IPP to hemoglobin, and \( c \) is the concentration of hemoglobin tetramers. The binding curves were fitted to this equation with an iterative nonlinear least squares procedure using a Telefunken TR 440 computer.

The pH dependence of \( \Delta Z_{\text{max}} \) is shown in Fig. 3. The curves for both oxy- and deoxyhemoglobin show a rather broad pH profile. The curve for deoxyhemoglobin exhibits a maximum of about 2.2 protons/tetramer at pH 8.2, while the curve for the liganded derivative reaches a maximum value of about 1 proton at pH 7. Little difference is seen between the hemoglobins of the bar headed and greylag goose which is in accord ance with the proton titration data. The broad pH profile of \( \Delta Z_{\text{max}} \) indicates that the groups which interact with IPP show a broad pK spectrum. Especially in deoxyhemoglobin, there is a strong contribution from groups with high pK values as can be seen from the large \( \Delta Z_{\text{max}} \) values at high pH. The difference in the location of the maximum on the pH axis between oxy- and deoxyhemoglobin indicates that although IPP binds at the same binding site in both derivatives, the pK values of the groups interacting with IPP are different in oxygenated and deoxyhemoglobin.

The difference between \( \Delta Z_{\text{max}} \) for oxy- and deoxyhemoglobin should correspond to the difference in the Bohr effect in the presence and absence of organic phosphate according to:

$$\Delta Z_{\text{PP}} = \Delta Z_{\text{O}} + \Delta Z_{\text{PPmax}} - \Delta Z_{\text{Pmax}}$$

where \( \Delta Z_{\text{PP}} \) and \( \Delta Z_{\text{O}} \) refer to the Bohr effect in the presence and absence of IPP and \( \Delta Z_{\text{PPmax}} \) and \( \Delta Z_{\text{Pmax}} \) are the number of protons taken up by oxy- and deoxyhemoglobin upon binding of 1 IPP molecule. The influence of IPP on the Bohr effect of the hemoglobins of the bar-headed and greylag goose is shown in Fig. 4. Both hemoglobins show a difference in the IPP similar Bohr effect curve. The number of protons released upon oxygenation \( \Delta Z \) in the absence (\( \Delta Z_{\text{delmax}} \)) and presence (\( \Delta Z_{\text{Pmax}} \)) of IPP. Conditions as in Fig. 3; ---, calculated Bohr effect in the presence of IPP (see text) for the bar-headed goose.

**Table II**

IPP binding constants for the oxygenated (\( K_{\text{oxy}} \)) and deoxygenated (\( K_{\text{deoxy}} \)) derivatives of the bar-headed and greylag goose hemoglobins

Experimental conditions: 25 \( \mu \)M hemoglobin tetramer, 0.1 M KCl, 25°C. Values denoted by * were obtained from the binding constant at pH 8.8 and the pH profile of \( \Delta Z_{\text{max}} \) shown in Fig. 3 by the integration procedure according to Equation 5.

<table>
<thead>
<tr>
<th>pH</th>
<th>( K_{\text{oxy}} )</th>
<th>( K_{\text{deoxy}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar-headed goose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>( 2.6 \pm 1.4 \times 10^6 )</td>
<td>( 8.2 \times 10^6 )</td>
</tr>
<tr>
<td>6.7</td>
<td>( 1.3 \pm 0.2 \times 10^6 )</td>
<td>( 2.2 \times 10^6 )</td>
</tr>
<tr>
<td>7.2</td>
<td>( 5.5 \pm 0.2 \times 10^6 )</td>
<td>( 4.2 \times 10^6 )</td>
</tr>
<tr>
<td>7.7</td>
<td>( 1.7 \pm 0.4 \times 10^6 )</td>
<td>( 4.7 \times 10^6 )</td>
</tr>
<tr>
<td>8.0</td>
<td>( 9 \pm 1.6 \times 10^6 )</td>
<td>( 1.1 \times 10^6 )</td>
</tr>
<tr>
<td>8.8</td>
<td>( 5 \pm 2 \times 10^6 )</td>
<td>( 2.0 \pm 0.4 \times 10^6 )</td>
</tr>
<tr>
<td>Greylag goose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>( 3.4 \pm 0.5 \times 10^6 )</td>
<td>( 5.8 \times 10^6 )</td>
</tr>
<tr>
<td>6.7</td>
<td>( 4.7 \pm 0.6 \times 10^6 )</td>
<td>( 1.9 \times 10^6 )</td>
</tr>
<tr>
<td>7.2</td>
<td>( 3.6 \pm 0.4 \times 10^6 )</td>
<td>( 4.0 \times 10^6 )</td>
</tr>
<tr>
<td>7.7</td>
<td>( 1.5 \pm 0.1 \times 10^6 )</td>
<td>( 5.4 \times 10^6 )</td>
</tr>
<tr>
<td>8.0</td>
<td>( 1.4 \pm 0.2 \times 10^6 )</td>
<td>( 1.3 \times 10^6 )</td>
</tr>
<tr>
<td>8.8</td>
<td>( 3.2 \pm 0.9 \times 10^6 )</td>
<td>( 2.6 \pm 0.3 \times 10^6 )</td>
</tr>
</tbody>
</table>

**Fig. 3.** The pH dependence of \( \Delta Z_{\text{max}} \) for the bar-headed goose oxy- (○) and deoxyhemoglobin (■) and for greylag goose oxy- (△) and deoxyhemoglobin (▲). Hemoglobin concentration 25 \( \mu \)M tetramer, 0.1 M KCl, 25°C.

**Fig. 4.** The Bohr effect of the bar-headed goose (△, ○) and greylag goose (■, ▲) hemoglobin (number of protons released per tetramer, upon oxygenation \( \Delta Z \) in the absence (△, ○) and presence (■, ▲) of IPP. Conditions as in Fig. 3; ---, calculated Bohr effect in the presence of IPP (see text) for the bar-headed goose.

Although it is not possible to calculate IPP binding constants from the IPP titration curves of deoxyhemoglobin at neutral pH, an estimate of the magnitude of this parameter can be made at pH 8.8 because of the strong pH dependence of the binding constant in deoxyhemoglobin. For both hemoglobins a value of about \( 2 \times 10^6 \) M is found at pH 8.8. Since \( \Delta Z_{\text{Pmax}} \) reflects the pH dependence of the binding constant according to (19):

$$\Delta Z_{\text{Pmax}} = -\log K_{\text{Pmax}}$$

an estimate of the binding constant at other pH values can be made by integrating the foregoing equation:
The binding constants of IPP to the deoxyhemoglobins obtained by this procedure together with the directly determined binding constants of oxyhemoglobin of both geese species at 25°C are given in Table II.

The differential binding of IPP to deoxy- and oxyhemoglobin is directly related to the influence of IPP on the hemoglobin oxygen affinity. Under the assumption that \( p_{50} \) equals the median ligand affinity, the increase in the log \( p_{50} \) value upon addition of IPP (\( \Delta \log p_{50} \)) is given by (20, 21):

\[
\Delta \log p_{50} = \frac{1}{4} \log \left( \frac{1 + K_{d_{\text{oxy}}}[\text{IPP}]}{1 + K_{d_{\text{deoxy}}}[\text{IPP}]} \right)
\]

where \( K_{d_{\text{deoxy}}} \) and \( K_{d_{\text{oxy}}} \) are the IPP binding constants for deoxy- and oxyhemoglobin, respectively, and [IPP] is the free IPP concentration. At IPP concentrations where oxy- and deoxyhemoglobin are completely saturated with IPP this equation converts to:

\[
\Delta \log p_{50} = \frac{1}{4} \log \frac{K_{d_{\text{deoxy}}}}{K_{d_{\text{oxy}}}}
\]

We measured the effect of IPP on oxygen affinity of the geese hemoglobins in order to compare these results with the \( \Delta \log p_{50} \) values calculated from the independently determined values for the IPP binding constants of deoxy- and oxyhemoglobin. Since the binding of IPP to both oxy- and deoxyhemoglobin in the pH region 6.7 to 7.7 is very strong (see Table II), Equation 7 can be used to calculate \( \Delta \log p_{50} \) values from the IPP binding constants. Provided \([\text{IPP}] > 50 \mu M\) the experimental \( \Delta \log p_{50} \) values are shown in Table III together with the calculated values. From the table it is seen that the agreement between the calculated and the observed \( \Delta \log p_{50} \) values is good.

This agreement offers proof that the method we have employed to estimate the binding constants of IPP to bird hemoglobins is reliable.

In order to get information on the interaction of IPP with the bird hemoglobins under physiological conditions we measured the IPP binding constants of oxyhemoglobin at 37°C and the oxygen binding curves in the presence and absence of IPP. From the IPP-induced shift in log \( p_{50} \) the binding constants for deoxyhemoglobin were calculated. The results are given in Table IV.

**TABLE III**

Observed and calculated changes in oxygen affinity (\( \Delta \log p_{50} \)) caused by the presence of IPP

<table>
<thead>
<tr>
<th>pH</th>
<th>log ( p_{50}^{\text{IPPP}} )</th>
<th>log ( p_{50}^{\text{IPPP}} )</th>
<th>( \Delta \log p_{50} )</th>
<th>Experimental</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.7</td>
<td>1.53</td>
<td>0.48</td>
<td>1.05</td>
<td>1.0</td>
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</tr>
<tr>
<td>7.2</td>
<td>1.31</td>
<td>0.31</td>
<td>1.00</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>7.7</td>
<td>1.00</td>
<td>0.14</td>
<td>0.86</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE IV**

The IPP binding constants for the oxyhemoglobins (\( K_{s_{\text{oxy}}} \)) of the bar-headed and greylag goose and the IPP-induced shift in oxygen affinity at 37°C

| pH   | \( K_{s_{\text{oxy}}} \) M | log \( p_{50}^{\text{IPPP}} \) | log \( p_{50}^{\text{IPPP}} \) | \( \Delta \log p_{50} \) | \( K_{s_{\text{deoxy}}} \) |
|------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 6.0  | 9.5 ± 6 \( 10^5 \) | 1.93            | 0.97            | 0.96            | 7 \( 10^5 \) |
| 7.2  | 2.2 ± 1 \( 10^6 \) | 1.57            | 0.65            | 0.92            | 1 \( 10^5 \) |
| 8.0  | 3.4 ± 1 \( 10^6 \) | 1.19            | 0.41            | 0.78            | 4.5 \( 10^5 \) |

**DISCUSSION**

As has been shown under "Results," the oxyhemoglobins of the bar-headed and greylag goose show very similar titration behavior in the pH region 5.5 to 9.0. Because the Bohr effects of both hemoglobins are practically identical, there is also no difference in titration behavior between the two hemoglobins in the deoxy form. Since the difference in primary structure between the hemoglobins of the bar-headed and the greylag goose is characterized by neutral replacements, the titration experiments indicate that these amino acid substitutions do not cause conformational differences leading to changes in pH values of the titratable residues. This implies that no major differences with respect to the geometry of the phosphate binding site are to be expected.

As can be seen from Equation 9, the shape of the IPP-binding isotherm is determined by the value of \( K_c \). Because of the very strong IPP binding of the two geese hemoglobins, we were forced to do our IPP-binding experiments at low hemoglobin concentrations (25 \( \mu M \) tetramer) in order to be able to calculate meaningful values for the binding constants. Variation of the concentration between 25 and 60 \( \mu M \) tetramer did not show any concentration dependence of the parameters deduced from the binding curves. From the \( K_{s_{\text{oxy}}} \) values presented in Table I, it is clear that in this concentration range the hemoglobins of both species of goose are predominantly tetrameric in the pH range studied.

The stoichiometry of 1:1 observed for the IPP binding to both oxygenated and deoxygenated derivatives is in good accord with results of Brygier et al. (11) on the interaction of IPP with the major component of chicken hemoglobin indicating an identical binding site in oxy- and deoxyhemoglobin. Under the assumption that IPP binds to chicken hemoglobin in a similar way as IHP does to human hemoglobin, the phosphate binding site in chicken hemoglobin would include the following residues: Val 1\( \beta \), His 2\( \beta \), Lys 82\( \beta \), Arg 135\( \beta \), Arg 143\( \beta \) and His 139\( \beta \) (8,10). The same residues are found in the geese hemoglobins at these positions (9). The higher number of positively charged residues located at the phosphate binding site in the avian hemoglobins as compared to human hemoglobin leads to a high affinity of the bird hemoglobins toward organic phosphates.

The presence of the 6 basic residues at the phosphate binding site is clearly reflected in the pH dependence of the

\[ \text{W. Oberthür and G. Braunitzer, personal communication.} \]
IPP binding constant for deoxyhemoglobin (Fig. 3). At pH 9.0, there is still a considerable proton uptake due to IPP binding. The pH dependence of $K_{sxy}$ shows a quite different picture. The maximum of the $\Delta Z_{\text{max}}$ curve is positioned at pH 7.0 and $\Delta Z_{\text{max}}$ decreases with increasing pH. This observation means that the basic residues present at the phosphate binding site contribute little to the proton uptake upon IPP binding in oxyhemoglobin and furthermore implies that although IPP binds to the same binding site in oxy- and deoxyhemoglobin, it interacts with different groups in the two derivatives. With regard to this aspect, our findings support an explanation given by Bucci et al. (22) for the different pH dependence of benzenehexacarboxylate binding to deoxy and oxy human hemoglobin. However, it should be realized that the difference in the pH dependence of the phosphate binding constants of the ligated and unligated hemoglobin derivatives need not solely be caused by the fact that the phosphate interacts with different groups in oxy- and deoxyhemoglobin. It is possible that some amino acids at the phosphate binding site decrease their pK values upon ligation of the hemoglobin molecule due to weaker Cl$^-$ binding to oxyhemoglobin as compared to deoxyhemoglobin. For human hemoglobin it has been shown that a significant part of the Bohr effect is caused by oxylable chloride binding (23). It could very well be possible that some of these chloride binding sites are located in the central cavity. This would imply that the residues interacting with chloride ions have different pK values in oxy- and deoxyhemoglobin. This alternative explanation of the different pH dependence of phosphate binding to oxy- and deoxyhemoglobin is of particular relevance for the geese hemoglobins since these hemoglobins show a pronounced dependence of the Bohr effect in human hemoglobin. Either some intrinsic Bohr groups are missing or the interaction of chloride ions is different in the geese hemoglobins. Preliminary experiments on the influence of chloride ions on the Bohr effect of these bird hemoglobins$^3$ showed that at a chloride ion concentration of about 1 mm, the alkaline Bohr effect decreased to about 15% of the value found at 0.1 M KCl. This means that in the geese hemoglobins a very small intrinsic alkaline Bohr effect is present. In other words, in these hemoglobins the alkaline Bohr effect is mainly caused by a different interaction of chloride ions with deoxy- and oxyhemoglobin.

The affinity of the greylag and bar-headed goose hemoglobins for IPP is very high (see Tables II and IV). At pH 7.2 and 25°C, values for the binding constant of about $3 \times 10^6$ M$^{-1}$ for oxyhemoglobin and $4 \times 10^6$ M$^{-1}$ for deoxyhemoglobin are found. For the binding of IPP to human hemoglobin at pH 7.3, 0.1 M NaCl, and 20°C values of $2.5 \times 10^5$ M$^{-1}$ and $1 \times 10^6$ M$^{-1}$ for oxy- and deoxyhemoglobin, respectively, have been reported (24). The much higher affinity of the bird hemoglobin for IPP as compared to human hemoglobin is compatible with the presence of a higher number of positively charged residues located at the phosphate binding site. The presence of the basic residues is reflected in the high affinity of deoxyhemoglobin even at pH 8.8.

Comparing the interaction of IPP with the two geese hemoglobins it is clear that there are no significant differences, indicating that the differences in primary structure do not influence the geometry of the phosphate binding site neither in deoxy- nor oxyhemoglobin. Both hemoglobins show very similar heterotropic interactions as far as protons and IPP are involved.

From the similarity in interaction with IPP of the two geese hemoglobins we conclude that the difference in oxygen affinity observed in whole blood of these species (3) is based on a difference in the intrinsic oxygen affinity (i.e. the affinity in the absence of organic phosphates). The difference in the intrinsic oxygen affinity is very small, amounting to 1.3 mm Hg at pH 7.2 and 37°C (see Table IV). The presence of IPP magnifies this difference to 13 mm Hg. Recently, Black and Tenney extensively studied the response to hypoxic hypoxia in several species of bird (25). These authors concluded that the bar-headed goose is superior to other bird species with respect to endurance of hypoxic stress, as judged from both cardio-respiratory functions and behavioral response to hypoxia. The higher oxygen affinity of the bar-headed goose hemoglobin in the presence of IPP as compared to the greylag goose, provides this species with a better tolerance toward hypoxic hypoxia.

From an evolutionary point of view it is interesting to see that such delicate alterations in functional properties of a protein can be involved in the adaptation of a species to extreme environmental conditions to which the bar-headed goose is exposed not only during its flight over the Himalaya mountains but also during the breeding period in the highlands of Central Asia (26).

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