The Effects of Temperature and pH on the Binding of ATP to Carp (Cyprinus carpio) Deoxyhemoglobin*

(Received for publication, June 11, 1979)

George S. Greaney,† Mitchell K. Hobish,‡ and Dennis A. Powers§
From the Mergenthaler Laboratory for Biology, The Johns Hopkins University, Baltimore, Maryland 21218

The linkage relationships between the binding of protons, organic phosphate, and oxygen to hemoglobin are well documented phenomena. These interactions provide a model for the investigation of linkage equilibria. Since ATP is the major organic phosphate in the erythrocytes of many fish, the binding of ATP to a representative fish hemoglobin (Carp I) was investigated. In these studies, both temperature and pH were varied so that their effects on ATP binding could be elucidated. The experimental binding constants were fitted to an equation that included the two major ionizable forms of ATP present within the experimental pH range. The resultant fitted parameters indicate three protons are taken up as deoxyhemoglobin binds the fully ionized form of ATP while only two protons are bound for the partially ionized form. The enthalpy of binding (ΔH) provides insight concerning the possible physiological and ecological significance of this phenomenon.

DEFINITIONS AND ASSUMPTIONS

When a hemoglobin tetramer (Hb) binds 4 molecules of a ligand (e.g. O₂) and 1 molecule of a second ligand (e.g. ATP), the equilibria can be depicted by the following scheme:

\[ Hb \rightleftharpoons HbO \rightleftharpoons Hb(O₂) \rightleftharpoons Hb(O₂)ATP \]

One assumes that the temperature, pH, etc. are held constant and that the hemoglobin does not dissociate or polymerize. This system can be described by 9 of the 13 thermodynamic equilibrium constants, if other conditions (e.g. pH) are held constant. However, when another ligand, such as proton concentration, is allowed to vary, the simultaneous equilibria involve many additional constants.

The simultaneous equilibria for deoxyhemoglobin (Hb), protons (H), and ATP can be written as:

\[ Hb \rightleftharpoons HbH \rightleftharpoons HbH₂ \rightleftharpoons HbH₃ \rightleftharpoons ... \]

\[ HbATP \rightleftharpoons HbHATP \rightleftharpoons HbH₂ATP \rightleftharpoons HbH₃ATP \rightleftharpoons ... \]

SCHEME 2

At relatively high pH (e.g. pH = 10), where little or no Bohr effect or organic phosphate effects are present, one can assume that the hemoglobin has n protons bound. If a series of ATP binding experiments is performed on deoxyhemoglobin starting with n protons bound and the pH is systematically lowered until the organic phosphate-hemoglobin affinity is maximized, then the equilibrium can be described by:

§ Supported by National Institutes of Health Training Grant GM 00057.
† To whom all correspondence should be addressed.

* This work was supported by National Science Foundation Grants GB37548 and DEB76-19877. Additional support was provided by the National Geographic Society and a National Institutes of Health Biomedical Research grant to Johns Hopkins University. This is Contribution 1036 from the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. 21218. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address, University of California, San Diego, Scripps Institution of Oceanography, La Jolla, Calif. 92038.
these minor ionized species and will be presented elsewhere along with other theoretical considerations and appropriate computer simulations.²

It is reasonable that the fully (ATP₄⁻) and the partially (ATP₃⁻) ionized states would have different affinities for hemoglobin. The simplest model for these interactions is presented in Fig. 2.

FIG. 2. A schematic for the binding to deoxyhemoglobin tetramers by protons (H) and organic phosphate in the fully ionized state (ATP₄⁻) and the partially ionized state (ATP₃⁻). The association constant for the ATP₃⁻ (K₉₋) is represented below the schematic. The others are association constants for the appropriate molecular species in the scheme.

An experimentally determined organic phosphate-hemoglobin binding constant is a composite of the individual binding affinities for the various molecular species involved:

$$K_{\text{app}} = \frac{[\text{all hemoglobin-organic phosphate complexes}]}{[\Sigma \text{hemoglobin, no organic phosphates}] - [\Sigma \text{free organic phosphate species}]} \quad (1)$$

where: $K_{\text{app}}$ = the apparent or observed affinity constant.

Substituting the appropriate species of Fig. 2 into Equation 1, we obtain:

$$K_{\text{app}} = \frac{[\text{HbH}_{n} \cdot \text{ATP}^{+} + \text{HbH}_{n} \cdot \text{ATP}^{-} + \text{HbH}_{n} \cdot \text{ATP}^{0}]}{[\text{HbH}_{n} + \text{HbH}_{n} \cdot \text{ATP}^{+} + \text{HbH}_{n} \cdot \text{ATP}^{0}]} \quad (2)$$

The macroaffinity constants in Fig. 2 are defined by:

$$K_{1} = \frac{[\text{HbH}_{n} \cdot \text{ATP}^{+}]}{[\text{HbH}_{n}][\text{ATP}^{+}]}$$

$$K_{2} = \frac{[\text{HbH}_{n} \cdot \text{ATP}^{0}]}{[\text{HbH}_{n}][\text{ATP}^{0}]}$$

$$K_{3} = \frac{[\text{HbH}_{n} \cdot \text{ATP}^{-}]}{[\text{HbH}_{n}][\text{ATP}^{-}]}$$

The system (Fig. 2) can be described thermodynamically by five of the nine hemoglobin-ligand affinity constants above (e.g. $K_{1}$, $K_{2}$, $K_{3}$, $K_{4}$, $K_{5}$) plus the ATP-proton association constant ($K_{10}$).

³ M. Hobish and D. A. Powers, manuscript in preparation.
Substituting these constants into Equation 2 and factoring out \( \text{HbH}_\text{s} \), we obtain:

\[
K^\text{out} = \frac{[\text{HbH}_\text{s}][K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2] }{[\text{HbH}_\text{s}][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

Since:

\[
K_{\text{m}} = \frac{[\text{ATP}^+]}{[\text{ATP}^+] + [\text{ATP}^+] [\text{ATP}^+] + [\text{ATP}^+]^2}
\]

then

\[
[\text{ATP}^+] = K_{\text{m}} [\text{H}][\text{ATP}^+] + [\text{ATP}^+] [\text{ATP}^+] + [\text{ATP}^+]^2
\]

Substituting and factoring out \([\text{ATP}^+]\) in both the numerator and denominator the resulting equation is:

\[
K^\text{out} = \frac{[\text{HbH}_\text{s}][K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2] }{[\text{HbH}_\text{s}][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

where the \( K \) values are the macroscopic equilibrium constants in the scheme (Fig. 2), \([\text{H}]\) = proton concentration, and \( z, m, q \) and \( n \) are the number of protons bound/hemoglobin tetramer for the appropriate binding steps in Fig. 2. This is the equation that was used in fitting our data.

The other constants in Fig. 2 can be calculated from the fitted constants (see "Experimental Procedures") of \( K_1, K_2, K_3, K_4, K_5 \) by the following equations:

\[
K_1 = \frac{K_6 K_7}{K_8} \\
K_2 = \frac{K_9 K_{10}}{K_{11}} \\
K_3 = \frac{K_6 K_7 K_8}{K_{12}}
\]

It should be noted that the fitted values of \( z, q \) result in the elimination of proton concentration from the equation for \( K_6 \) (see "Results") so that:

\[
K_6 = \frac{K_6 K_7}{K_8}
\]

but one should not assume this a priori. The more rigorous definition should be used to calculate \( K_6 \).

Factoring and rearranging Equation 3, the following is obtained:

\[
K^\text{out} = \frac{[\text{ATP}^+]^2}{[\text{ATP}^+] [\text{ATP}^+] + [\text{ATP}^+]^2} \frac{K_6 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2} {K_1 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

If one considers individual species of ATP (ATP\(^4\)- and ATP\(^2\)-) interacting with hemoglobin and protons (i.e. single faces of Fig. 2), then by the same method used above, the following can be obtained:

\[
K_{\text{ATP}^4} = \frac{K_6 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2} {K_1 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

\[
K_{\text{ATP}^2} = \frac{K_6 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2} {K_1 [1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

Consequently Equation 5 can be rewritten as:

\[
K^\text{out} = \frac{[\text{HbH}_\text{s}][K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+] + K_\text{ATP}^+][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2] }{[\text{HbH}_\text{s}][1 + K_\text{H}[\text{ATP}^+]][\text{ATP}^+] + [\text{ATP}^+]^2}
\]

where: \( K^\text{ATP}^4 \) and \( K^\text{ATP}^2 \) are the apparent or observed hemoglobin-ATP affinity constants for ATP\(^4\) and ATP\(^2\), and \( f_{\text{ATP}^4} \) and \( f_{\text{ATP}^2} \) are the fractions of ATP that are in the fully ionized (ATP\(^4\)) and partially ionized (ATP\(^2\)) states, respectively. Therefore, the experimental hemoglobin-ATP affinity \( (K^\text{out}) \) is a weight average of the respective hemoglobin affinities for ATP\(^4\) and ATP\(^2\), which are, in turn, dependent on pH.

**EXPERIMENTAL PROCEDURES**

### Determination of Hemoglobin Concentrations

Total hemoglobin and the percentage of methemoglobin were determined by the method of Evelyn and Malloy (22). Routine determinations of hemoglobin concentrations were made as follows: After appropriate dilutions into 10 mM phosphate buffer, pH 7.2, the solution was gently bubbled with distilled water-washed carbon monoxide for 2 to 3 min. The absorbance at 540 nm was measured and the concentration was calculated using an extinction coefficient for carbonmonoxyhemoglobin (HbCO) of 53.6 \( \times 10^3 \ M^{-1} \ cm^{-1} \). Concentrations are reported on a tetramer basis.

### Purification of Hemoglobins

**Human Hemoglobin A**—Human hemoglobin A was prepared by the method described by Atta and Ackers (23). The fractions containing hemoglobin A were concentrated using pressure filtration and then dialyzed for 12 h against two changes of the buffer which was to be used in the binding measurements (see below). The resulting hemoglobin was allowed to drip slowly into liquid nitrogen. The frozen droplets were stored under liquid nitrogen until ready for use.

**Carp Hemoglobin**—Carp (Cyprinus carpio) were caught from the Potomac River in gill nets and bled in the field by severing the caudal peduncle. The blood from several fish was collected and pooled in excess of 0.9% NaCl. After washing the cells, hemolysates were prepared by immersing the cell pellets directly into liquid nitrogen. After thawing, 2 volumes of cold 10 mm Tris-Cl, pH 8.0, were added to the pellet of cells. Mechanically lysing the cells by freeze-thawing minimizes the formation of chromatin complexes in the hemolysate.

The hemolysate was centrifuged at 19,000 \( \times g \) for 2 h to remove membranous and nuclear material. The hemoglobin components were separated on a column of DEAE-Sephadex A-50 according to the method of Gillen and Rigs (14). The peak fractions containing hemoglobin component I were pooled and concentrated using pressure filtration. The hemoglobin solution was dialyzed for 12 h against two changes of the experimental buffer and stored as frozen droplets in liquid nitrogen.

### Buffers Used for Binding Constant Measurements

Twenty-five millimolar bis-Tris\(^+\) was used for all binding constant determinations made at pH 7.7 and below. The buffer was made 0.1 M in chloride ion with the addition of KCl, taking care to include those chloride ions used to titrate the buffer and the fact that KCl affected the pH of the solution. Ensuring accuracy in the chloride concentrations was necessary since variations have a marked effect on the interaction of organic phosphate with hemoglobin (24). The buffers were prepared at the concentration to be used (i.e. 25 mm) and carefully adjusted to the desired pH at the temperature at which the binding measurement was being made. This was critical due to the considerable heat of ionization of the bis-Tris buffer (24). For binding constant measurements at pH values greater than 7.7, Tris-Cl, 0.1 M KCl was used. Bis-Tris and Tris were reagent grade from Sigma.

### Preparation of Deoxyhemoglobin for Binding Measurements

One gram of frozen droplets of the hemoglobin to be tested was slowly thawed at room temperature to give 1 ml of hemoglobin solution. This jelly-like complex of nuclear material was present in large amounts when the cells were lysed conventionally with dilute buffer. Retrieving the hemoglobin was thus much more difficult.

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were bubbled with argon in a sealed plastic test tube. After 65 min, 1 mg of purified sodium dithionite (sodium hydrosulfite Na₂S₂O₄ from Fisher) was quickly added to the deoxygenated buffer under a flow of argon, and the tube was sealed again. One to two milliliters of this oxygen-free buffer were anaerobically withdrawn using a syringe and anaerobically transferred to the hemoglobin solution which by now was nearly deoxygenated. The buffer was slowly added to the flask under an argon atmosphere while the hemoglobin solution was stirred. This resulted in a deep purple solution of deoxyhemoglobin at the desired experimental concentration. Without the use of dithionite, deoxygenation of this volume of fish hemoglobin was exceedingly difficult due to its high oxygen affinity. Since the absorbance of the solution at 575 nm did not change upon addition of more dithionite, the solution was considered 100% deoxyhemoglobin.

**Synthesis of [γ-32P]ATP**

[32P]Phosphoric acid (carrier-free) was purchased from New England Nuclear. [γ-32P]ATP was synthesized by the combined reactions of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (Sigma). The ligand was purified on DEAE-cellulose (Whatman) with a linear gradient of 0.05 to 1.0 M triethylammonium bicarbonate, pH 7.5. Reagent grade triethylamine (Baker) was redisolved and a 1.0 M solution bubbled with CO₂ at pH 7.5 to prepare the triethylammonium bicarbonate buffer. Specific activity was 10⁶ cpm/μmol. The radioactive ATP was stored frozen at -20°C in 25 mM Tris-HCl, pH 8.0, in 10-μl ampules.

**Determination of Binding Constants to Hemoglobin**

The general procedure was originally described by Colowick and Womack (25, 27) and has been employed to measure organic phosphate binding to human hemoglobin (1). The apparatus consists of a flow dialysis cell (Bel-Art Products, Pequannock, N.J.) with an upper chamber, containing hemoglobin and radioactive ATP, separated by a viscose dialysis membrane (VWR Scientific, Catalogue No. 25225-248) from a lower chamber. Buffer was pumped rapidly through the lower chamber and the effluent was collected with a fraction collector and sampled for radioactivity.

We made modifications in the method to improve temperature control and deoxygenation which were critical for studies on fish hemoglobin. A 1-liter aspirator bottle, fitted with a two-hole rubber stopper, contained 500 ml of dialysis buffer and rested in a constant temperature water bath constructed from styrofoam. A magnetic stirring bar facilitated deoxygenation and temperature equilibration. A peristaltic pump was used to pump the buffer out of the aspirator bottle into the lower chamber of the dialysis cell which rested in a styrofoam temperature bath. The buffer reservoir with the dialysis cell was insulated in the sections which were not immersed in the bath. The argon was first hydrated by passage through a 1-liter flask containing deionized distilled water. The hydration flask was maintained at the temperature of the experiment by immersing it in the constant temperature water bath. The argon continued to pass through the buffer in the reservoir during the experiment. A stream of argon was also passed over the top of the hemoglobin solution in the upper chamber of the dialysis cell during the course of the experiment (see Fig. 3).

**Experimental Procedure**

For measurements involving carbonmonoxyhemoglobin, the gassing needle was used for ATP additions only, and the entire system was left open to the atmosphere by eliminating the gas flow. After bubbling the buffer with argon for 2 h, sodium dithionite was added to give a final concentration of 10⁻³ M. This insured a flow of oxygen-free buffer through the lower chamber of the buffer chamber. The hemoglobin solution, containing [γ-32P]ATP (10⁶ cpm/ml), was transferred anaerobically to the upper chamber of the dialysis cell with a syringe, and allowed to equilibrate for 20 min. The upper chamber was flushed with argon prior to the addition of the hemoglobin solution. The syringe was weighed both before and after delivery; the difference in weights and the known density permitted the exact volume of solution delivered to the upper chamber to be calculated. A series of additions of unlabeled ATP (1-L Biochemicals, disodium salt) were made in the upper chamber and the radioactivity in the effluent was monitored. When the rates for isotope entering and exiting the lower chamber were equal, the concentration in the effluent was a true measure of unbound ATP in the upper chamber (25, 27). This steady state was reached after 8 fractions of 2.5 ml each were collected at a flow rate of 7.5 ml/min out of the lower chamber. Twelve to fifteen fractions were collected between additions of unlabeled ligand and portions of the last five fractions in each set were immediately placed in an ice bath. One-half to one milliliter of dialysate was plated into a scintillation vial to which 10 ml of a complete counting mixture had been added (Research Products International, Elk Grove Village, Ill.). The total counts per min as 32P

**[26]** Total hemoglobin and percentage of methemoglobin determinations were made on the protein before each experiment. The percent methemoglobin was negligible for human hemoglobin A preparations. The fish hemoglobin preparations varied between 5 and 15% methemoglobin. However, in addition to scavenging trace oxygen during the experiment, the presence of dithionite also served to reduce the methemoglobin to the ferrous form (26). Thus, methemoglobin formation was negligible during binding experiments involving deoxyhemoglobin in the presence of dithionite. For example, conversion to the CO-liganded form (see "Experimental Procedures") of the ferrous deoxyhemoglobin present at the end of a typical binding experiment yielded a concentration which agreed perfectly with the total hemoglobin determination made before the experiment after appropriate corrections for the dilutions of ATP additions. Total hemoglobin and percent methemoglobin determinations were made before and after each experiment. The pH of the hemoglobin solution was measured before and after experiments and found not to vary by more than 0.05 pH unit.

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*Fig. 3. Schematic of the experimental setup used for the measurement of binding constants (modified from Ref. 25). A, general setup; B, detail of dialysis chamber.*
were then measured in a Packard Tri-Carb liquid scintillation spectrometer. Two-tenths milliliter of a suspension of acid-washed Norit (Pfanstiehl Laboratories, Waukegan, Ill.), 20% packed volume, was added to 1 ml of dialysate and vortexed. After 5 min on ice, the Norit was removed by low speed centrifugation and 0.5 ml of the supernatant counted in 10 ml of the counting mixture. Since nucleotides are strongly adsorbed to the Norit, the counts per minute of the supernatant represented $^{32}$P, only. After correction for dilutions, the inorganic counts were subtracted from the total counts to give counts present in organic form, i.e. as ATP. Organic phosphate accounted for greater than 90% of the total counts in any given fraction. The correction for $^{31}$P ATP hydrolysis is critical since P$_i$ passes through a dialysis membrane much more rapidly than does the organic phosphate. This can become a severe problem during the early stages of the experiment when the concentration of free isotope is very low. Only the counts in organic form were used to calculate binding. The counts in separate fractions from each set were averaged and usually did not vary by more than 5%. The final ATP addition is very large, relative to the hemoglobin concentration, so that the fraction of labeled ATP bound becomes negligible. The concentration of isotope in the effluent then reaches its maximum value corresponding to 100% of the ATP in the free state. A control experiment was run without hemoglobin to confirm that the concentration of isotope in the effluent was identical to the maximum value obtained when hemoglobin was present. This ensured that a negligible fraction of isotope was lost from the upper chamber during the experiment (27). In all cases, the final addition of unlabeled ATP liberated essentially all of the labeled ATP. Corrections for changes in volume due to successive additions of cold ligand and accounted for no more than 2 to 3%. The number of binding sites was calculated from the Scatchard analysis using the concentration of protein as determined at the end of the experiment. Values were also calculated using successive dilutions, and the values as obtained were found to differ from the former by only 2 to 3%.

**Data Analysis and Numerical Methods**

**Dissociation Constants and Number of Binding Sites—Dissociation constants ($K_{D}$) were determined from the slopes of Scatchard-type plots of data plotted as $B$ versus $B/F$ where $B$ and $F$ are bound and free ATP, respectively (26). The slope and intercept of the various Scatchard plots were determined by linear least squares analyses. In all experiments, the $y$-intercepts of such plots had values of 90 to 100% of the deoxyhemoglobin concentration indicating one major ATP binding site per hemoglobin tetramer.**

**Fit of the Association Constants to Linkage Models—When the log of the association constants as determined from Scatchard analysis was plotted against $pH$ (see Fig. 6), data were fitted by a nonlinear minimization procedure developed by Johnson et al. (28). The method is essentially a Gauss-Newton procedure for evaluating several parameters simultaneously. It generates the best approximation of the parameters employing a weighting factor. The fitting program also provides information concerning cross-correlation between parameters and estimates the confidence limits of each estimated parameter. These confidence limits correspond to one standard deviation of the best estimate. No constraints were placed on the fitting procedure, and all parameters were allowed to float over the range $-\infty$ to $\infty$.

**RESULTS**

**Binding of ATP to Human Hemoglobin A—Fig. 4 shows representative Scatchard plots for the binding of ATP to human deoxyhemoglobin A at 20°C and 30°C, pH 7.2. Table I lists the mean dissociation constants determined by four separate experiments at 20°C and three experiments at 30°C. Our $pH$ 7.2 values are similar to those determined by Bunn et al. (29) at $pH$ 7.3 as well as those of Gupta et al. (30) at $pH$ 7.2. These similarities are particularly important because their determinations were made by oxygen equilibrium and NMR spectroscopy, respectively, which are independent methods confirming our results. Hamasaki and Rose (1) measured ATP binding to human HbA by a method similar to that described in this paper. They reported a $K_{D} = 8.54 \times 10^{-5}$ M at $pH$ 7.2 for human deoxyhemoglobin A (Table I). However, since temperature was not rigorously controlled and experimental error not reported, it is impossible to determine if their results are similar to or different from our data.**

The van't Hoff enthalpy ($\Delta H$) calculated from our values at 20°C and 30°C for ATP binding to human deoxyhemoglobin A at $pH$ 7.2 was $-13.9$ kcal/mole. This value of $\Delta H$ is in agreement with that obtained for 2,3-DPG binding to human hemoglobin ($\Delta H = -12.2$ kcal/mol) under the same conditions (1).

**Binding of ATP to Carp Hemoglobin I (HbI)—Fig. 5, A and B show representative Scatchard plots for the binding of ATP to carp deoxyhemoglobin I at 20°C and 30°C, respectively, at $pH$ 7.20. Four determinations were made at 20°C and three at 30°C. The appropriate mean dissociation constants are reported in Table I. When the ATP binding constants for human HbA and carp HbI are compared (Table I), it is clear that the fish hemoglobin binds ATP with an affinity about 10 times higher than human hemoglobin under identical conditions.**

The binding of ATP to carp carbomonoxyhemoglobin I at $pH$ 7.2 was essentially a Gauss-Newton procedure for evaluating several parameters and estimating the confidence limits of each estimated parameter. The $y$-intercepts of such plots had values of 90 to 100% of the deoxyhemoglobin concentration, so that the fraction of labeled ATP bound becomes negligible. The concentration of isotope in the effluent then reaches its maximum value corresponding to 100% of the ATP in the free state. A control experiment was run without hemoglobin to confirm that the concentration of isotope in the effluent was identical to the maximum value obtained when hemoglobin was present. This ensured that a negligible fraction of isotope was lost from the upper chamber during the experiment (27). In all cases, the final addition of unlabeled ATP liberated essentially all of the labeled ATP. Corrections for changes in volume due to successive additions of cold ligand and accounted for no more than 2 to 3%. The number of binding sites was calculated from the Scatchard analysis using the concentration of protein as determined at the end of the experiment. Values were also calculated using successive dilutions, and the values as obtained were found to differ from the former by only 2 to 3%.

**Table I**

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>$T$</th>
<th>$pH$</th>
<th>$K_{D} \times 10^{3}$ M</th>
<th>$n$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp (HbI)</td>
<td>20</td>
<td>7.2</td>
<td>0.253 $\pm$ 0.062</td>
<td>4</td>
<td>Present</td>
</tr>
<tr>
<td>Human (HbA)</td>
<td>30</td>
<td>7.2</td>
<td>1.000 $\pm$ 0.065</td>
<td>3</td>
<td>Study</td>
</tr>
<tr>
<td>Human (HbA)</td>
<td>20</td>
<td>7.2</td>
<td>3.32 $\pm$ 0.41</td>
<td>4</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.2</td>
<td>6.47 $\pm$ 0.37</td>
<td>3</td>
<td>Study</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.3</td>
<td>4.07 $\pm$ 0.30</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.3</td>
<td>6.6   $\pm$ 0.30</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20-25</td>
<td>7.2</td>
<td>8.5   $\pm$ 0.30</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 $\pm$ 1</td>
<td>7.2</td>
<td>6.6 $\pm$ 0.30</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

Since magnetic stirring motors can generate considerable heat during a binding experiment, it is necessary to immerse the dialysis cell in a constant temperature bath. A higher temperature inside the cell during ATP measurements would yield an erroneously high dissociation constant.
ATP Binding to Carp Hemoglobin I

pH 7.2, 20°C, was too weak to be detected under the conditions of the experiment. This was true even when hemoglobin concentration was raised to millimolar levels (0.9 \times 10^{-4} \text{ M}). The technique employed in this paper cannot detect binding which is weaker than that represented by a \( K_{\text{app}} \) greater than 3 \times 10^{-7} \text{ M}. Therefore, the binding of the liganded hemoglobin tetramer of the carp is at least 1000-fold weaker than that of the deoxygenated form. This finding is expected on the basis of results obtained from studies on the binding of 2,3-DPG to human hemoglobin. However, the magnitude of the difference between the binding of organic phosphate to liganded and unliganded hemoglobin appears much greater for the Carp hemoglobin.

In our comparisons so far we have employed dissociation constants (\( K_{\text{app}} \)) because literature values for human HbA were reported as such. However, from this point on we will address only the association constants (i.e. \( 1/K_{\text{app}} \)).

The pH Dependence of ATP Binding to Carp HbI—Hemoglobin-ATP association constants were measured at several pH values at 30°C. These data were plotted as a function of pH (Fig. 6) and the points were fitted to Equation 4 (see "Experimental Procedures").

The resultant fitted constants along with their confidence limits are tabulated in Table II. The other listed parameters were calculated from the estimated values which were obtained by the nonlinear fitting program (see: "Definitions and Assumptions").

The parameters (Table II) were used to generate a fitted curve to the 30°C experimental data (Fig. 6). This pH dependence of ATP binding to carp deoxyhemoglobin I (Fig. 6) resembles a titration curve with an apparent pK = 7.6. When the oxygen equilibria data of Gillen and Riggs (14) are plotted,
a similar apparent pK is observed. The oxygenation data of Gillen and Riggs, together with the pH dependence of ATP binding (present study), are consistent with an interpretation that the major pH effect on ATP binding to Carp deoxyhemoglobin I is due to the titration of the \( \alpha \)-amino protons of the NH\(_2\)-terminal valines on the \( \beta \) chains (14).\(^7\)

Apparent enthalpies (\( \Delta H \)) of binding were calculated from the experimental values of \( K^{\text{app}} \) at 30°C and 20°C for various pH values, whereas the \( \Delta H \) at pH 6.9 were calculated employing three temperatures (Table III). As shown in Table III, the \( \Delta H \) values are minimized at the high and low pH ranges employed in our study. The apparent enthalpy change reaches a maximum in the pH range 7.2 to 7.6.

**DISCUSSION**

The oxygen equilibria of vertebrate hemoglobins are regulated, in part, by the preferential binding of organic phosphates (3, 4, 32) to the deoxytetramer. A number of studies have shown that 1 mol of organic phosphate binds for each mol of hemoglobin tetramer and that the NH\(_2\) termini of the \( \beta \) chains are involved in ionic interactions with the organic phosphate (see Ref. 32 for review).

Our results show that ATP binds with a strong affinity to Carp hemoglobin I. The binding is considerably tighter than that reported for 2,3-DPG binding to human hemoglobin A under the same conditions (1). As is the case for the interaction of 2,3-DPG with mammalian hemoglobins, the binding is preferential for the deoxytetramer and the stoichiometry is one binding site per hemoglobin tetrameric molecule. Since the addition of ATP to a mixture of human deoxyhemoglobin and 2,3-DPG has been shown to increase the level of free, or unbound, 2,3-DPG (1), it is presumed that the same binding site is involved in the binding of both organic phosphates.

X-ray diffraction studies have confirmed that 1 molecule of organic phosphate (i.e. 2,3-DPG) binds to human deoxyhemoglobin on the diad axis of the tetramer (2, 33). The stereochemistry of the bound 2,3-DPG appears to complement certain basic residues in the central cavity of the deoxytetramer. Presumably, salt bridges are formed with the NH\(_2\) terminal chains (Val, \( \beta 1 \)), and the imidazole side chains (His, \( \beta 2 \), \( \beta 143 \)) of both \( \beta \) chains, as well as the \( \epsilon \)-amino group of Lys, \( \beta 82 \), from one of the \( \beta \) chains. Homologous residues for fish hemoglobins are: Val (\( \beta 1 \)), Lys (\( \beta 82 \)), Glu (\( \beta 2 \)), and Arg (\( \beta 143 \)) (13, 14).

Assuming that the same amino acid residues are involved in the binding of organic phosphates by fish hemoglobins, the only residues that could be titrated over the pH range of our experiment are the 2 valyl residues of the \( \beta \) chain NH\(_2\) termini and the \( \beta 82 \) lysyl residues.

The fitted parameters (Table II) indicate that the Carp deoxyhemoglobin I binds three protons over the pH range of our experiment when ATP\(^+\) is bound, but only two protons when ATP\(^-\) is bound. These data are consistent with three titratable amino acid side chains. Presumably, two of the three are the \( \alpha \)-amino groups of the \( \beta 1 \) valyl residues. The third could be one of the \( \beta 82 \) lysyl \( \epsilon \)-amino groups (actually, an averaged contribution of one-half proton from each of the \( \beta 82 \) lysyl groups, as postulated by Arnone (2)), or perhaps a different residue whose involvement has not yet been elucidated.

Since the binding of ATP\(^+\) involves a greater ionic interaction than the binding of ATP\(^-\), one would expect significant differences in the respective hemoglobin-ATP affinities. Table II indicates that hemoglobin’s affinity for the fully ionized species (ATP\(^+\)) is approximately 6 orders of magnitude greater than that for the partially ionized form (ATP\(^-\)). It would, therefore, seem appropriate to consider the various ionic species of organic phosphates when oxygen equilibria studies are designed. However, as has been the case for the ligand-linked effect of subunit dissociation, these and other important parameters are too often neglected.

At high pH values, \( f_{\text{ATP}} \) is small compared to \( f_{\text{ATP}} \) so that \( K^{\text{app}} \) approaches \( K^{\text{app}} \) (see Equation 8). Consequently, at high pH, the major ligand species that binds to the hemoglobin is ATP\(^-\). However, at high pH values, the \( \alpha \)-amino protons (\( \beta 1 \)) are only partially protonated (pK \( \approx 7.6 \)). Since it is well established that organic phosphates bind to the protonated \( \alpha \)-amino groups of the \( \beta \) chains (33), one would expect the observed hemoglobin-ATP affinity to be reduced as pH increases. Our data are consistent with that expectation (Fig. 6). In fact, as pH increases the experimental hemoglobin-ATP binding constant approaches \( K_\text{s} \), which is approximately 10\(^6\) times less than \( K_\text{a} \). This can be seen by taking the limit as [H\(^+\)] \( \rightarrow 0 \) of Equations 4 and 5.

On the other hand, as pH is lowered, increasing amounts of ATP\(^+\) are bound, but the fraction in the ATP\(^+\) state decreases. Consequently, although the hemoglobin affinity for ATP\(^+\) is much larger than that for ATP\(^-\), the observed ligand-binding constant (\( K^{\text{app}} \)) is increasingly shifted as the pH is lowered and approaches a value of \( (K_\text{a} + K_\text{w}) \). This can be expressed mathematically by taking the limit of Equation 4 (as [H\(^+\)] \( \rightarrow \infty \)) and substituting in the fitted values (Table II) for \( z, q, \) and \( m \), respectively, as follows:

\[
\lim_{[H^+] \rightarrow \infty} K^{\text{app}} = \frac{K_d(1 + K_d[H^+])}{K_d[H]_d(1 + K_d[H^+] + K_d[H])} \quad (9)
\]

At the limit, [H\(^+\)] \( \gg 1 \) so Equation 9 essentially reduces to:

\[
\lim_{[H^+] \rightarrow \infty} K^{\text{app}} = \frac{K_d[H]_d^+}{K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+} \quad (10)
\]

The fitted values of \( z, q, \) and \( m \) (see Table II) allow us to factor out [H\(^+\)] as follows:

\[
\lim_{[H^+] \rightarrow \infty} K^{\text{app}} = \frac{K_\text{d}K_\text{w} + K_\text{d}K_\text{w} + K_\text{d}K_\text{w}[H]^+}{K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+ + K_\text{d}K_\text{w}[H]^+} \quad (10)
\]

because \( z \equiv (q + 1) \equiv (m + 1) \) (see Table II). Therefore:

\[
\lim_{[H^+] \rightarrow \infty} K^{\text{app}} = \frac{K_\text{d}K_\text{w} + K_\text{d}K_\text{w} + K_\text{d}K_\text{w}[H]^+}{K_\text{d}K_\text{w}[H]^+} \quad (10)
\]

Substituting \( K_\text{d} \) for \( K_\text{d}K_\text{w} \) and \( K_\text{d} \) for \( K_\text{d}K_\text{w} \), it follows that:

\[
\lim_{[H^+] \rightarrow \infty} K^{\text{app}} = \frac{K_\text{d}K_\text{w} + K_\text{d}K_\text{w} + K_\text{d}K_\text{w}[H]^+}{K_\text{d}K_\text{w}[H]^+} \quad (11)
\]

While this result is derived from our observations, it should be noted that no such constraints were placed on the fitting routine (see “Experimental Procedures”). All variables were allowed to float simultaneously, and values were allowed to vary as required to obtain the best fit of the data. Finally, these limits are only conceptually useful because at both high and low pH other equilibria have to be considered.

The importance of considering the various ionic species of organic phosphate modulators is equally valid for other organophosphates such as 2,3-DPG, CTP, and IHP. A comprehensive thermodynamic analysis is currently underway that considers the binding of 2,3-DPG, protons, and oxygen to human hemoglobin A, \( \text{HbA}_\text{a} \) at physiological protein concentrations.\(^8\)

\(^{8}\) A preliminary report of these findings was presented at the Federation of American Societies for Experimental Biology meetings and published as an abstract (M. Hobish, G. S. Greaney, and D. A. Powers (1979) Fed. Proc. 38, 460.).
Our data (Table III) indicate that the temperature dependence (i.e. $\Delta H$) of hemoglobin-ATP binding is low at both high and low pH values while it is maximized at the intermediate pH range. Riggs (34, 35) concluded that the temperature dependence, $\Delta H$, of the binding of 2,3-DPG to human deoxyhemoglobin is primarily due to the heat of ionization of the $\alpha$-amino group protons of the NH$_2$ termini of the $\beta$ chains. The close agreement between our value of $\sim$11.9 kcal/mol for the apparent enthalpy of ATP binding to human hemoglobin and the value of $\sim$11.2 kcal/mol which has been reported for 2,3-DPG binding, supports this hypothesis. Furthermore, Riggs' hypothesis would predict a smaller enthalpy change for binding at pH values for which the $\alpha$-amino groups are fully protonated over the measured temperature ranges.

In addition to the ionic interactions between organic phosphates and hemoglobin, the possibility for other types of binding also exists. Powers and Edmundson (13) postulated that the placement of an aromatic residue at position $\beta 3$ in fish hemoglobin may promote hydrophobic interaction with a ring structure like the adenine base in ATP. Competition experiments carried out with adenosine (i.e. ATP without the electrostatic interaction with the negatively charged organophosphate bonding in the interaction of fish hemoglobins with dialysis, we have been unable to measure the binding of adenosine to Carp deoxyhemoglobin even at higher temperatures (40°C) which would favor hydrophobic interactions.9 In addition, the decrease in ATP binding affinity at 40°C (see Table III) is evidence against a major involvement of hydrophobic bonding in the interaction of fish hemoglobins with ATP. The lower affinity at 40°C probably results from a shift to a lower pK for the amino acid residue(s) responsible for the electrostatic interaction with the negatively charged organophosphate. If the adenine base in ATP binds to tryptophan ($\beta 3$), as suggested by Powers and Edmundson (13), then it lies outside the detection limits of our method (i.e. $K_{\text{diss}} > 10^{-3}$ M). Finally, examination of hemoglobin models suggests that $\beta 3$ would not be readily available for an interaction with ATP. After this paper was prepared, Riggs (36) independently discussed the same possibility, and also concluded that $\beta 3$ Trp was not implicated in organic phosphate binding.

Possible Physiological and Ecological Significance—The variation in the association between hemoglobin and ATP with temperature and pH may have an adaptive value in the evolution of poikilothermic hemoglobins. For example, consider the case of hypoxia. A decrease in erythrocyte oxidative phosphorylation during anoxia leads to a reduction in total red blood cell ATP (19, 20). This response results in an increase in red cell pH by way of the indirect effect of ATP on the Donnan distribution of protons across the red blood cell membrane (16). Furthermore, less ATP is in the unbound state during hypoxia because the percentage of deoxyhemoglobin is increased and binding is preferentially to the deoxy form. The increased binding of ATP during hypoxia amplifies the pH increase that is elicited by the in vivo decrease in total intracellular ATP. This amplification is due to the fact that the Donnan influence of ATP on red cell pH depends only upon the concentration of free, or unbound, ATP. However, as pH increases, the binding affinity decreases. More ATP is released from the hemoglobin which, in turn, decreases intracellular pH by the way of the Donnan effect. Thus, the pH dependency in the binding of ATP to hemoglobin may serve as a negative feedback control. Without such a mechanism, red cell pH may rise to values which are too high to permit unloading of oxygen during hypoxia (Bohr effect). The mechanism of the increase in hemoglobin-oxygen affinity during acclimation to hypoxia is then a combination of both the direct allosteric effect (i.e. ATP binding) and the increase in cellular pH which occurs when the in vivo erythrocyte ATP concentrations are lowered. Thus, the direct and indirect effects of ATP on hemoglobin oxygenation are complementary.

Some of these interrelationships are summarized schematically by a series of thermodynamic equilibria (Fig. 1). Each of these equilibria is characterized by a negative enthalpy change. Therefore, an increase in temperature will favor the dissociation of all complexes. In this regard, it is perhaps not a coincidence that the influence of temperature on ATP binding is minimized at intracellular pH ranges which approximate the lower values encountered at the respiring tissues (8, 16). Thus, the homeostatic response to temperature change involving lowered in vivo red blood cell ATP pools described previously (37) can be extended to the molecular interaction of the ATP with the hemoglobin as well. Moreover, our recent finding (38) that genetic background influences these ATP levels suggests that this system may be useful in studying mechanisms of natural selection at the molecular level.

Acknowledgments—We are indebted to Dr. Gary Ackers of The Johns Hopkins University, Dr. Austen Riggs of the University of Texas, Dr. Franklin Bunn of Harvard Medical School, and Dr. George Somero of the Scripps’s Institute of Oceanography.

REFERENCES

8 For the adenosine binding experiments, 2,8-[$^3$H]adenosine, 36 Ci/mmol (New England Nuclear), was used with a deoxyhemoglobin concentration of 1.3 mm. The ratio of adenosine to Hb ranged from 0.1 to 2.0 for the binding studies as well as for competition experiments in the presence of [$\gamma$-32P]ATP. Due to the limited solubility of adenosine, stock solutions of this compound were maintained at 65°C in order to achieve the required high concentrations necessary for dilution into the upper chamber of the dialysis cell.
The effects of temperature and pH on the binding of ATP to carp (Cyprinus carpio) deoxyhemoglobin.
G S Greaney, M K Hobish and D A Powers


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