Hemoglobins of the Tadpole of the Bullfrog, *Rana catesbeiana*

TEMPERATURE DEPENDENCE OF OXYGEN BINDING AND pH DEPENDENCE OF SUBUNIT DISSOCIATION*

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The temperature dependence of the oxygen equilibrium of tadpole hemoglobin has been determined between 0° and 32° for the unfractionated but phosphate-free lysate and between 12° and 32° for each of the four isolated components between pH 6 and 10 in 0.05 M cacodylate, Tris, or glycine buffers containing 0.1 M NaCl and 1 mM EDTA. Under these conditions the Bohr effect (defined as Δlog \( p_{50} / \Delta p \)) of the unfractionated lysate is positive at low temperatures between pH 6 and 8.5 and is negative above pH 8.5 to 8.8 at any temperature. As the temperature rises the Bohr effect below pH 8.5 changes greatly. In the interval, pH 7.0 to 7.5, the magnitude of the Bohr effect decreases from 0.28 at 0° to zero at about 24° and becomes negative, as in mammalian hemoglobins, above this temperature. Measurements with the isolated components show that the temperature dependence of oxygen binding for Components I and II and for Components III and IV is very similar. For both sets of components the apparent overall enthalpy of oxygenation at pH 7.5 is about -16.4 kcal/mol and -12.6 kcal/mol at pH 9.5. The measured enthalpies include contributions from the active Bohr groups, the buffer ions themselves, the hemoglobin groups contributing buffering, and any pH-dependent, oxygenation-dependent binding of ions such as chloride by the hemoglobin. The apportioning of the total enthalpy among these various processes remains to be determined.

Between pH 8 and 10.5 tadpole oxyhemoglobin undergoes a pH-dependent dissociation from tetramer to dimer. The pH dependence of the apparent tetramer-dimer dissociation constant indicates that at pH 9.5 the dissociation of each tetramer is accompanied by the release of approximately 2 protons. In this pH range the oxygen equilibrium measurements indicate that about 0.5 proton is released for each oxygen molecule bound. The results are consistent with the conclusion that one acid group per αβ dimer changes its pK from about 10 to 8 or below upon dissociation of the tetramer.

The hemoglobins of the tadpole are functionally and structurally different from those of the adult (1-5). Tadpole hemoglobin, unlike the adult pigment, lacks a Bohr effect in inorganic phosphate and in certain other buffers (2, 5, 6) but organic phosphates have a marked allosteric effect (7) and induce a substantial pH dependence of the oxygen equilibrium (6). The discovery that tadpole hemoglobins possess a marked reversed Bohr effect below pH 8.5 under certain buffer conditions (5) and not under others (5, 6) prompted a further investigation of this effect. Morpurgo et al. (8) discovered that the sign of the Bohr effect of newt hemoglobin in undialyzed hemolysates was reversed at low temperatures (Δlog \( p_{50} / \Delta p \)) and “normal” at high temperatures (Δlog \( p_{50} / \Delta p \) < 0). They found, however, that stripped hemolysates showed a normal Bohr effect at all temperatures. These observations suggested to us that the sign of the Bohr effect of tadpole hemoglobin might depend not only on the buffer but also on the temperature. This consideration led to the present study of the effect of temperature on the oxygen equilibrium of tadpole hemoglobin.

**EXPERIMENTAL PROCEDURE**

Preparation—The procedures for obtaining the blood and the hemoglobins are identical with those described (5) except that all preparations were saturated with CO and the cells were lysed with 10 volumes of deionized water. After concentration, the lysate was dialyzed overnight against 0.2 M NaCl containing 0.05 M Tris-HCl buffer and 1 mM EDTA at pH 7.5 saturated with CO. Hemoglobin was stripped of organic phosphates as described (5) except that the EDTA was omitted. The efficiency of this procedure was tested by measure.
ment of residual phosphate by the procedure of Ames and Dubin (9). The result, 0.07 mol of phosphate/mol of hemoglobin (tetramer), was considered insignificant. Each of the four components was isolated by chromatography on DEAE-Sephadex as described (5).

**Oxygen Equilibria**—Determinations were made in buffers and by procedures described (5) at various temperatures between 0° and 32° except that the data below pH 7.2 were obtained with the 0.05 M cacodylate buffer rather than bis-Tris (2,2'-bis(hydroxymethyl)-2,2'-dinitroethanol). Absorbance measurements at 560 and 576 nm were made from the spectra recorded between 500 and 600 nm with a Cary model 14 spectrophotometer. The cuvette holder was thermostatted with a Haake water bath coupled with a Brinkmann “Thermo-Cool” unit. The equilibration bath for the tonometers was independently regulated to the same temperature. Tests with a model 42 SC Tele-thermometer probe (Yellow Springs Instrument Co.) showed that temperature fluctuation of the tonometer upon transfer from bath to spectrophotometer could be neglected provided that the transfer was made rapidly and a re-equilibration time of 6 min was allowed for measurements at 0°, 4 min at 7° and 12°, and 2 min at 17°. At higher temperatures the measurements were made immediately. The pH determinations were done by standardizing the pH meter and making all measurements at the same temperature which was used for the determination of the oxygen equilibrium.

Methemoglobin was determined as described (10). The methemoglobin content was less than 5% at the start of the equilibrium measurements and not more than 10% at the end. These higher values were obtained only below pH 6.7.

**Ultracentrifugation**—Sedimentation velocity measurements were done on stripped tadpole hemoglobin with a Beckman model E ultracentrifuge with schlieren optics. Runs were made at 58,100 rpm using single sector cells and an AN-D rotor thermostatted at 20°. The buffers for these experiments were the same as for the oxygen equilibria. Measurements with deoxyhemoglobin were done by deoxygenating the hemoglobin in a tonometer and transferring the hemoglobin from tonometer to ultracentrifuge cell in a plastic glove bag previously purged overnight with high purity nitrogen. Absorption spectra of the hemoglobin within the ultracentrifuge cell confirmed that the hemoglobin was deoxygenated at the start and that no detectable oxygenation occurred during the sedimentation measurements. The data were corrected to s_{inert} values using density and viscosity data determined for the buffers. The densities were measured with a Paar Precision Density Meter model DMA-02C, and the viscosities were determined with a Bausch and Lomb Viscometric-1 automatic viscometer.

**RESULTS**

The data on the oxygen equilibrium of phosphate-free, unfraccionated hemoglobin from the tadpole are summarized in Fig. 1 for temperatures 0–32° and pH 6 to 10. These data confirm the earlier finding (5) that the Bohr coefficient, \( r = \Delta \log p_{so}/\Delta \text{pH} \), is positive at room temperature and below pH 8.6. The change in \( p_{so} \) with pH appears almost linear at 0° in the range pH 6 to 5.5, with an average slope of \(-0.28\). As the temperature increases the magnitude of this “reversed” Bohr effect decreases so that at room temperature the value is much smaller and at 32° it is actually negative in the region pH 7.0 to 7.5. Thus the pH of minimal oxygen affinity shifts from about 8.6 at 0° to about 7.2 at 32°. The Hill coefficient, \( n \), decreases with pH from 1.1 to 2.2 at pH 6 to 7 to 1.3 to 1.5 at pH 9 to 10. This behavior varies little with temperature.

The data in Fig. 1 can be used to estimate the apparent enthalpy at each pH for the oxygenation of hemoglobin. A van’t Hoff plot gives an apparent \( \Delta H \) of \(-16.4 \text{ kcal/mol} \) for hemoglobin oxygenation at pH 7.5 and \(-12.6 \text{ kcal/mol} \) at pH 9.5, after subtracting the heat of solution of oxygen, about \(-3.0 \text{ kcal/mol} \). These overall values appear appropriate near room temperature, but the oxygen affinities at 0° are significantly higher than expected on this basis so that the apparent heat of oxygenation at 0° is about \(-4 \text{ kcal/mol} \) larger than at the higher temperature. The meaning of this change in heat capacity is not known, but it is close to the values reported for horse (11) and human (12) hemoglobins.

Oxygen-binding experiments with each of the four individual components yielded the results in Fig. 2. Unlike the data for the unfraccionated mixture (Fig. 1) these data can be described in terms of just two segments: a low pH part in which \( \Delta \log p_{so}/\Delta \text{pH} \) is always positive and a high pH part in which this coefficient is always negative. The pH of minimal oxygen affinity of Components III and IV shifts from about 8.5 to 7.2 as the temperature increases from 12–32°, much as seen with the unfraccionated mixture. This shift appears to be much smaller for Components I and II. Components III and IV account for about two-thirds of the hemoglobin in the unfraccionated mixture; Components I and II comprise the remaining third. The behavior of these components appears adequate to account for the properties of the unfraccionated mixture. At 32° the data for Components III and IV begin to resemble the results for mammalian hemoglobin at 20° except that the minimal oxygen affinity for the latter occurs about 1 pH unit lower. The extension at high temperatures of the alkaline Bohr effect where \( \Delta \log p_{so}/\Delta \text{pH} < 0 \) to pH below 8 by Components III and IV accounts for the change in sign of the Bohr effect in the unfraccionated mixture as the temperature increases to 32°.

**Determination of the sedimentation behavior of tadpole hemoglobin above pH 8** showed that the oxy form partially dissociates at least to dimers, but that the deoxy form shows no measurable tendency to do so. The data in Fig. 3 show that the oxyhemoglobin is almost completely dimeric at the protein concentration used in the oxygen equilibrium measurements. Although some dissociation presumably occurs below pH 8, the procedure was not sensitive enough to detect it.
**Tadpole Hemoglobins**

Fig. 2. The temperature dependence of the oxygen equilibria of the four individual components of tadpole hemoglobin. Buffers: as in Fig. 1. Symbols: O, Component I; □, Component II; ○, Component III; ●, Component IV.

Fig. 3. Sedimentation behavior of unfractionated tadpole hemoglobin as a function of hemoglobin concentration. Conditions: 0.05 M glycine-NaOH buffer, 0.1 M NaCl, 1 mM EDTA, pH 9.5, 20°C.

deoxyhemoglobin was not observed in the pH region 8 to 9.5 where oxyhemoglobin dissociates readily. The data in Fig. 4 also suggest a possible tendency for the hemoglobin to aggregate beyond tetramers at low pH: near pH 6 the $S_{20,w}$ values are slightly greater than 5.0 S, a value considerably higher than expected for tetrameric hemoglobin.

**DISCUSSION**

The pH dependence (Bohr effect) of oxygen binding by the tadpole hemoglobin components can be divided into two segments. At low pH $\Delta \log P_{50}/\Delta \mathrm{pH}$ is positive whereas it is negative at high pH. We have previously shown (5) that the Bohr effect at low pH is modulated in part by chloride. Our present experiments indicate that at high pH oxygenation is accompanied by an increased tendency for tetramers to dissociate to dimers. These processes must be included in an analysis of the temperature dependence of oxygen binding. The interpretation of the apparent enthalpies must await appropriate measurements of the enthalpies of several ancillary processes in order to account properly for the total. We can expect contributions from the oxygenation-linked acid groups and from those groups which contribute buffering: the added buffer and the groups on the hemoglobin itself which contribute buffering (13, 14). We need also to include the heat of binding by chloride and the heat of the tetramer-dimer dissociation.

The observation of a pH-dependent tetramer-dimer dissociation by tadpole oxyhemoglobin (Fig. 4) can be analyzed in the
following way. The apparent tetramer-dimer dissociation constant can be calculated from the observed sedimentation coefficient at each pH provided that we know values for the sedimentation coefficients for the tetramer and dimer. The weight fraction, \( \alpha \), of the hemoglobin in the dissociated form was estimated from \( \bar{s} \), the observed weight average sedimentation coefficient, and \( s_T \) for tetramer and \( s_d \) for dimer, respectively. We assume \( \bar{s}_{40,0} = 4.66 \) S (tetramer) and \( s_{60,0} = 2.85 \) S (dimer) as reported for liganded human hemoglobin (15). The apparent tetramer-dimer dissociation constant, \( K_{\text{app}} \), was then estimated as described (16). The variation in \( \log K_{\text{app}} \) with pH is shown in Fig. 5. The slope of the linear least squares line through these points is 1.75 ± 0.15. The slope, expressed as \( \Delta \log K_{\text{app}}/\Delta \text{pH} \), can be interpreted in terms of the linked function relationships developed by Wyman (14, 17) to give the number of protons released upon dissociation of an oxyhemoglobin tetramer to a dimer. This number, close to 2, suggests the following simple model. We propose that two identical acid groups occur at the \( \alpha \beta \) interface of the liganded tetramer, one contributed by each \( \alpha \beta \) dimer unit. We assume the presence of the \( \alpha \beta \) interface found in mammalian hemoglobins (18). The symmetry of the hemoglobin tetramer requires that at least two acid groups are present. Dissociation of tetramer to dimer is then associated with a considerable drop in the pK of these groups. If \( T \) represents the tetramer and \( D \) the dimer, the postulated relationship is:

\[
\begin{align*}
T & \Rightarrow K_1 \xrightarrow{K_3} 2D \\
\text{TH} & \Rightarrow 2DH \\
\text{THH} & \Rightarrow K_1 \\
\end{align*}
\]

We assume that the acid dissociation constants \( K_1 \) and \( K_3 \) are independent and only statistically related so that \( K_1 = 2k \) and \( K_3 = k/2 \) where \( k \) is the microscopic dissociation constant. \( K_i \) is the intrinsic tetramer-dimer dissociation constant for the unprotonated species, and \( k' \) is the acid dissociation constant for the single acid group sensitive to the dissociation in each dimer.

This model results in the following relationship between \( K_i \) and \( K_{\text{app}} \), the observed apparent constant:

\[
K_{\text{app}} = K_i \left( \frac{1 + H}{k'} \right)^2
\]

\( H \) is the hydrogen ion concentration. The data can be satisfactorily fitted with the assumption that the two acid groups on the tetramer each have a pK = 10.2 and that this drops to about 7.7 in the dimer; \( K_i \), then becomes \( 6.9 \times 10^{-3} \) M. Differentiation of Equation 2 gives:

\[
\frac{d \log K_{\text{app}}}{d \text{pH}} = \frac{2H}{H + k} - \frac{2H}{H + k'}
\]

The two terms on the right, \( 2H/(H + k) \) and \( 2H/(H + k') \), give the number of protons bound by tetramer and two dimers, respectively. The difference gives the number of protons released upon dissociation of tetramer to two dimers. Substitution of the values of \( k \) and \( k' \) \((7 \times 10^{-4} \text{ M} and 2.1 \times 10^{-4} \text{ M}, \text{respectively}) \) at pH 9.5 gives a value of 1.61 for the slope, which is close to that observed. The limiting values for \( K_{\text{app}} \) at low and high pH are given by \( K_i (k/k')^2 \) and \( K_i \) or 7.7 \times 10^{-4} \text{ M and } 6.9 \times 10^{-3} \text{ M, respectively.} \) In view of the low precision of the data points these values must be considered only as rough estimates. The value at low pH is an order of magnitude lower than that found for liganded human hemoglobin (19, 20). The asymptotes shown in Fig. 5 have considerable uncertainty because adequate data points do not exist below pH 8.5 nor above 10.5.

This model differs from that devised by Andersen and Gibson (21, 22) for the pH dependence of dimer formation from monomeric lamprey hemoglobin because they assumed that dimer formation requires protonation of one site on the monomer; ionization of this group within the dimer was assumed not to occur. The tetramer-dimer dissociation process in tadpole hemoglobin appears quite different from that reported for cat hemoglobin (23, 24) in which the Hill coefficient, \( n \), rises with pH and this rise is associated with enhanced dissociation of the liganded hemoglobin. In contrast, \( n \) in tadpole hemoglobin decreases with pH and appears to parallel the enhanced dissociation of the tetramer.

The finding that in the pH region near pH 9.5 nearly 2 protons are released upon dissociation of tetramer to dimer is of considerable interest because the tetramer-dimer dissociation itself is linked to oxygenation. The deoxyhemoglobin does not dissociate significantly at the concentrations used in these experiments. About 2 protons are dissociated per tetramer when the hemoglobin becomes oxygenated. It therefore might be that the alkaline Bohr effect could be describable in terms of a pH dependent tetramer-dimer dissociation.

What groups might be responsible for the observed pH dependence of oxygen binding and of the tetramer-dimer dissociation? An acid group with pK of 10.2 in the tetramer and 7.7 in the dimer suggests either tyrosyl or lysyl residues; no cysteinyl residues are present in tadpole hemoglobin (5). Studies of human hemoglobin (25) indicate that a tyrosyl residue in each dimer within the tetramer changes its pK from 10.6 to about 10.1 upon dissociation of tetramer to dimer. The lower pK presumably indicates a tyrosyl residue freely exposed to solvent (26). The very much lower apparent value of the pK in the dimer in the present model might occur if a profound
electrostatic effect existed to shift a tyrosyl pH from 10 to below 8. It is difficult to see how this might happen unless dimer formation were associated with the movement of a positively charged group such as the guanidino group close to a tyrosyl residue. Alternatively, it is conceivable that the apparent pH values arise from weighted contributions of different groups, the weighting depending on the pH.

The dissociation of tetramer to dimer increases greatly above pH 10 for both liganded and unliganded mammalian hemoglobins (25, 27, 28). The present studies suggest that a similar process occurs in the tadpole but at a much lower pH. Andersen et al. (25) found no significant monomer formation by human hemoglobin below pH 11. Our data are insufficient above pH 10 to exclude completely the possible presence of monomer.

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