The Effect of pH on Carbon Monoxide Binding to Menhaden Hemoglobin

ALLOSTERIC TRANSITIONS IN A ROOT EFFECT HEMOGLOBIN*

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The major hemoglobin fraction of menhaden (Brevoortia tyrannus) is a Root effect hemoglobin; at acid pH it exhibits low affinity behavior characteristic of the T state and at alkaline pH functional behavior characteristic of the R state. The rates of CO binding and dissociation have been measured by stopped flow and flash photolysis at low and high pH. At acid pH CO binding is slow and biphasic, with rate constants of 0.25 and 0.023 μM⁻¹ s⁻¹; these were assigned as the T state binding rates of the two chains. CO dissociation is also biphasic. At alkaline pH CO binding and dissociation are homogeneous, with rate constants of 1.3 μM⁻¹ s⁻¹ and 0.028 s⁻¹, respectively; these were assigned as the R state rates for both chains. Full and partial flash photolysis data were collected over the pH range 6.0 to 8.5, and CO equilibrium data from 6.0 to 7.5. These were fit to the two-state model modified to include chain differences. The T and R binding rates, and the R dissociation rate, were assigned; L and the T dissociation rates were calculated. L varies from 4 × 10⁴ at pH 6.0 to 8 at pH 8.5; the T rates vary from 0.15 and 0.25 at pH 6.0 to 0.02 at pH 8.5.

Both Hb and carbon monoxide hemoglobin (HbCO) have pH difference spectra in the Soret and visible regions. The Hb spectrum changes above pH 7.5 and the HbCO spectrum changes below pH 7.0. The transition from R to T state was observed at an Hb-HbCO isosbestic during flash photolysis of HbCO. The total absorbance change at this wavelength rises, then falls with pH, with a maximum at pH 7.0. The time course of conformation change at pH 7.0 was followed after full and partial flash photolysis and fit to the extended two-state model with an L value of 10⁵ and a 3-fold decrease in the rate of R to T transition for each additional bound CO. The magnitude of static and kinetic absorption changes was compared to the conformation state of Hb and HbCO calculated from the functional measurements. There is poor correlation; both spectral measurements indicate a too great effect of pH on the conformation of both derivatives. It is suggested that optical absorption measurements do not give a quantitative measure of quaternary conformation.

The two-state model of cooperativity proposed by Monod et al. (1) has been widely applied to the interpretation of the functional behavior of hemoglobin. The basic principle of the simple two-state model is that hemoglobin is in equilibrium, at all stages of ligation, between just two quaternary conformations, a low affinity T state and a high affinity R state. The conformational equilibrium is affected by ligand binding and by allosteric effectors such as organic phosphate. Deoxyhemoglobin assumes the T state; as ligand binds the equilibrium is moved toward the R state, until fully liganded hemoglobin is in the R state. This conformational transition from T to R states produces cooperativity in ligand binding. The functional properties of hemoglobin are determined by the properties of the two states and the position of conformational equilibrium between them. It should therefore be possible to quantitatively describe the behavior of hemoglobin from the properties of the T and R states and L, the conformational equilibrium constant.

This is a difficult task with the cooperative mammalian hemoglobins, since it is hard to isolate the two states and study them. Only the extremes near full saturation or no saturation are purely R or T; intermediate saturations are mixtures of molecules in the two conformations. Much of the work characterizing these states has been done on highly specific systems which exhibit properties attributed to either the T or R conformations, chemically modified or mutant hemoglobins with little or no cooperativity, either frozen in one state or with the conformational equilibrium greatly displaced toward one or the other state (2).

Fish hemoglobins with a Root effect (3) may be regarded as flexible systems within which to measure the parameters and test the assumptions of the two-state model. The ligand affinity of these hemoglobins is greatly affected by pH; it is high at alkaline pH, but decreases so much as the pH drops that at low pH the hemoglobin is only partially oxygenated in the presence of air. This exaggerated Bohr effect has been interpreted as being due to a large pH induced shift in the conformational equilibrium.

Carp hemoglobin is frozen in the T state at acid pH; even liganded hemoglobin is in the T state, and ligands are bound noncooperatively, with low affinity (4-6). At alkaline pH the conformational equilibrium is moved far toward the R state; deoxyhemoglobin is partly in the R state and ligands are bound with low cooperativity and high affinity. There is a
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region of high cooperativity and intermediate ligand affinity at neutral pH, reflecting conformational transition from the T to R states upon ligand binding.

The major fraction of menhaden hemoglobin has properties similar to those of carp but has a single electrophoretic band. It is therefore possible to isolate and study the T state properties at acid pH and the R state properties at alkaline pH. In this paper we assign T and R rate constants for carbon monoxide binding at the pH extremes. Both kinetic and equilibrium observations of CO binding over the entire pH range of the Root effect have been analyzed according to the two-state model, using these T and R parameters. This model, modified to include chain differences and some variation of T state properties with pH, was found to give a complete quantitative description of CO binding at all pH values. The calculated value of L ranged from 4 \times 10^{-5} at pH 6.0 to 5 at pH 8.5.

**EXPERIMENTAL PROCEDURES**

**Materials** — The buffers used were 0.05 or 0.10 M phosphate/citrate at pH 5.0 and 5.5, phosphate from pH 6.0 to 8.0, 0.05 M Tris at pH 8.5, and 0.05 M borate at pH 9.0. Inositol hexaphosphate (Sigma) and dithiothreitol (Aldrich) were used without further purification.

**Gas Solutions** — Saturated CO solutions were prepared by bubbling CO gas (Matheson) through buffers; deoxygenated solutions were prepared by bubbling prepurified N2 gas (Airco). Saturated NO (Matheson) solutions were made by shaking deoxygenated buffer in an NO-filled tonometer and withdrawal into a syringe.

**Menhaden Hemoglobin Fractions** — Washed erythrocytes were prepared by suspension and centrifugation, three times, in 1.0% NaCl. The washed cells were stored in liquid nitrogen until needed. The hemolysate was prepared by addition of 5 to 10 volumes of water to the thawed cells and centrifugation for 10 min at 5000 rpm to spin down membranes and chromosomal material. The fractions were separated by passage through a column of DEAE-Bio-Gel A (Bio-Rad) equilibrated with 0.05 M Tris, pH 8.65, run at 4°C. The minor fraction came off immediately; the major fraction was then eluted with 0.05 M Tris, pH 7.8 (Fig. 1). The major fraction comprised 80% of the total hemoglobin.

The purity of the fractions was checked by polyacrylamide disk gel electrophoresis. The gels were 7% polyacrylamide, run in a continuous Tris/EDTA/borate system (24.2 g of Tris, 3.12 g of EDTA, and 1.84 g of boric acid in 2.6 liters water) for 2 h, stained 20 min in 0.2% amido black, and destained in methanol:water:acetic acid (5:5:1).

**Stopped Flow Measurements** — The apparatus used was that described by Gibson and Milnes (7). Partial flash measurements were made by introducing a colored glass filter (Corning) between the flash lamp and sample to attenuate the photolyzing light. Another glass filter, or interference filter of appropriate wavelength, was placed between the sample and the observation lamp to reduce sample photoslysis by the observing beam. All measurements were done at 20°C.

**Equilibrium Measurements** — Equilibria were measured on a Cary 15 recording spectrophotometer, using the method of Rossi-Fanelli and Antonini (9). CO equilibria were measured on the computer-controlled spectrophotometer described by Knowles and Gibson (10). Deoxy Hb solutions for the standard spectrum were prepared by addition of Hb stock solution to a syringe of deoxygenated buffer containing 1 mM EDTA, 0.1 mM dithiothreitol, and 50 μM sodium dithionite. The Hb solution was transferred to a 1-cm pathlength stopped cuvette which had been flushed thoroughly with N2 gas. HbCO standard solutions were prepared by equilibrating the deoxy Hb solution in the cuvette with a bubble of CO gas. Partially liganded Hb solutions were prepared as for deoxy Hb, with the addition of a measured amount of CO saturated buffer to the syringe before the Hb was added. A fresh sample was prepared for each point. The hemoglobin concentration was from 0.5 to 3 μM in the HbCO standard solutions; all measurements were made in the Soret region, at 20°C.

**pH Difference Spectra** — Visible and Soret spectra were taken on the computer-controlled spectrophotometer. A stock solution of deoxy Hb at twice the sample concentration in weak buffer was prepared in a deoxygenated syringe as in the equilibrium measurements; it was then diluted into full strength deoxygenated buffer of the appropriate pH. All spectra were measured against a water blank, and the difference spectra were computer-generated by subtracting spectra from each other. The HbCO spectra were taken in the same way, from HbCO samples made from deoxy Hb samples as in equilibrium measurements.

**RESULTS**

**Menhaden Hemoglobin Fractions** — There are two hemoglobin components in menhaden blood, a major and a minor fraction with widely differing properties. The minor fraction is cooperative at all pH values, and has a very small Bohr effect. Its p50 is unchanged at 1.27 from pH 9.0 to 7.0; then rises slightly to 1.33 at pH 6.0. The n value decreases with pH, from 2.6 at pH 9.0 to 2.0 at pH 6.0.

The major fraction is a Root effect hemoglobin, showing enhancement of ligand affinity and cooperativity with pH. All subsequent experiments in this paper have been done on the purified major component.

**pH Difference Spectra** — The absorption spectra of both deoxy Hb and HbCO were found to change with pH. The pH difference spectra were recorded in the Soret and visible regions. The deoxy difference spectra from pH 5.5 to 8.5 (Fig. 2a) were calculated by subtracting the pH 5.0 spectrum from the other spectra. No changes in the deoxy spectrum are seen between pH 5.0 and 7.0; a difference spectrum with a minimum at 431 nm, an isosbestic at 437 nm, and a maximum at 442 nm starts to appear at pH 7.5 and increases up to pH 8.5. This spectrum is similar to the kinetic difference spectrum between tetrameric hemoglobin and the isolated deoxy chains seen by Brunori et al. (11), and also to that between normal deoxy Hb and Hb*, the immediate product of HbCO photolysis, observed by Sawick and Gibson (12). These have both been interpreted to be difference spectra between the T and R conformations of deoxy Hb.

The pH difference spectrum of HbCO is shown in Fig. 2b. These spectra were calculated by subtracting the pH 8.5 spectrum from the others. The liganded difference spectrum with a saturated NO solution. All measurements were done in the Soret region, at 20°C.

**Flash Photolysis** — The apparatus used was described by Parkhurst and Gibson (8). Partial flash measurements were made by introducing a colored glass filter (Corning) between the flash lamp and sample to attenuate the photolyzing light. Another glass filter, or interference filter of appropriate wavelength, was placed between the sample and the observation lamp to reduce sample photolysis by the observing beam. All measurements were done at 20°C.
has a minimum at 417 nm, isosbestic at 421 nm, and maximum at 426 nm; it starts to appear at pH 7.0 and is still increasing at pH 5.0. Similar spectra have been reported for two other Root effect hemoglobins, carp (13) and trout IV (14), upon inositol hexaphosphate addition.

The visible pH difference spectra are shown in Fig. 2, c and d. The magnitudes of the visible spectra are much smaller than those of the Soret spectra, so intermediate spectra have been omitted. The deoxy difference spectrum between pH 9.0 and 5.0 (Fig. 2c) is, again, similar to the kinetic difference spectrum between isolated chains and tetramers (11). The HbCO spectrum in the visible is similar to the inositol hexaphosphate difference spectrum for HbO2 observed by Adams and Schuster (15).

These pH-induced spectral differences possibly reflect quaternary conformational changes in the hemoglobin derivatives. Such an interpretation would indicate that at acid pH deoxy Hb is completely in the T state; a small proportion assumes the R state at pH 7.5, and this proportion increases up to 8.5. HbCO is entirely in the R state at alkaline pH; it partially assumes the T state at pH 7.0, and the T state proportion increases as the pH drops.

Kinetic Observations of Conformational Change—The transition between R and T conformations can be followed kinetically, at a single pH, after flash photolysis of HbCO. HbCO is, except at acid pH, entirely in the R state; the immediate product of photolysis is R state deoxy Hb, which then relaxes to the T state. Since the spectra of T and R state hemoglobins are different, the conformation change can be followed spectrophotometrically at the isosbestic between Hb and HbCO. There is actually more than one isosbestic, in the static spectra, the Hb-HbCO isosbestic at pH 5.0 is at 456.6 nm, while the pH 9.0 isosbestic is at 457.3 nm, a separation of 0.7 nm. The pH 5.0 wavelength is close to the HbO2-HbCO isosbestic, since much of the liganded hemoglobin is in the T state at this pH. Similarly, the pH 9.0 wavelength is close to the HbO2-HbCO isosbestic.

The absorbance changes during the photolysis of HbCO by a slow flash were observed at both the T and R isosbestics. The T isosbestic was found by looking for a wavelength at which no absorbance excursion occurred during full photolysis of a pH 5.5 HbCO sample. The R isosbestic used was the wavelength at which no absorbance change was seen after partial photolysis of a pH 9.0 sample; partial photolysis was used since it could be safely assumed that the partially saturated hemoglobin was entirely in the R state at this pH. There was some absorbance change seen during full photolysis at this wavelength, indicating that deoxy Hb at pH 9.0 is at least partly in the T state. The two isosbestics found by this method were 1.5 nm apart, rather than the 0.7 nm seen in the static spectra, another indication that deoxy Hb is not completely in the R state; the R deoxy spectrum is shifted farther to the red.

The pattern of the absorbance changes followed at the T and R isosbestics was that predicted by the static spectra. The absorbances of the T state species, measured at pH 5.0, and

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**Fig. 2.** pH difference spectra of Hb and HbCO in the Soret and visible regions. A, Soret Hb spectra. The spectra are plotted as absorbance at the pH value indicated minus absorbance at pH 5.0. The pH values are: ●, 8.5; ○, 8.0; ▲, 7.5. B, Soret HbCO spectra; absorbance at the pH value indicated minus absorbance at pH 8.5. The pH values are: ●, 5.0; ○, 5.5; ▲, 6.0; △, 7.0. Heme concentration was 33 µM for both series of spectra. C, visible deoxy Hb pH 9.0 spectrum minus pH 5.0 spectrum. D, visible HbCO pH 5.0 spectrum minus pH 9.0 spectrum. Heme concentration was 28 µM in both C and D.
the R state species, measured at pH 9.0, are shown schematically in Fig. 3. The order of reaction at intermediate pH is

\[ \text{HbCO} \rightarrow \text{Hb}^i \rightarrow \text{Hb}^o \]

At the R isosbestic, HbCO\textsuperscript{o} and Hb\textsuperscript{i} (pH 9.0 spectra) have the same absorbance, and \( \Pi \) (pH 5.0 Hb spectrum) has lower absorbance; the absorbance decreases monotonically during photolysis. At the T isosbestic the starting point is HbCO\textsuperscript{o}; Hb\textsuperscript{i} has a higher absorbance and Hb\textsuperscript{o} has a lower absorbance. During flash photolysis, the absorbance first rises, then falls.

The magnitude of absorbance change at the R isosbestic is shown in Fig. 4. The maximum occurs at pH 7.0, where the Hb and HbCO spectra are farthest apart. At acid pH, as the HbCO spectrum drops in absorbance at this wavelength, it moves toward the Hb spectrum, and the kinetic absorbance excursion decreases. Similarly, at alkaline pH the Hb spectrum moves toward the HbCO spectrum and the absorbance excursion again drops. Qualitatively, then, the kinetic and static spectral measurements agree, indicating large conformational differences between Hb and HbCO at intermediate pH, and small differences at acid and alkaline pH, where both liganded and unliganded hemoglobin partially share the same quaternary conformation.

The conformational transition was studied in detail at pH 7.0, where it has maximum amplitude. The time courses of conformational change, followed at the R isosbestic, and of CO photodissociation, followed at 445 nm, were observed for full and partial flash photolysis (Fig. 5). A flash of long duration was used, and the absorbance changes were followed during the flash. At all levels of breakdown the observed conformation change lagged behind the removal of CO from hemoglobin. This is partly due to the fact that transition to the T state does not occur until enough ligand has been removed from the hemoglobin to move the conformational equilibrium over to the T state. At lower pH, where L is larger, the conformation change is faster relative to HbCO photolysis, and at alkaline pH it is slower. In addition, it is suggested that the rate of conformation change decreases by a constant factor for each additional bound ligand, at high levels of saturation this step would be rate-limiting.

The solid lines in Fig. 5 were calculated according to the two-state model with a long flash length, as discussed by Sawicki and Gibson (12). The time course of the flash lamp intensity was measured, and the removal of CO from HbCO by the light was calculated as

\[ \frac{d[HbCO]}{dt} = -a[I(t)][HbCO] \]  

where a is a constant proportional to the extinction coefficient of HbCO and its quantum yield and I(t) are the tabulated values of flash intensity. The value of a was calculated for each level of photolysis by a least squares fit of the observed HbCO photodissociation to the time course of photodissociation calculated from the measured flash profile.

The conformational transition between T and R states was described as

\[ \text{Hb}^o (\text{CO})_n \xrightarrow{K_d a} \frac{K_d a}{K_w/(L_0cd^*)} \text{Hb}^i (\text{CO})_n \]

where L and c have the usual meanings of the two-state model, d is an arbitrary constant assigned by curve fitting by which the rate of conformational change decreases for each additional ligand bound, and \( K_d \) is the rate of R to T transition of unliganded hemoglobin. \( K_w \) was measured as 10,000 s\(^{-1}\) by laser photolysis.\(^2\)

The observed time course of conformational change was fit to a two-state model extended to include chain differences.

\(^{2}\) C. Sawicki, personal communication.
The removal of CO was calculated according to Equation 1, assuming equal quantum yields for the two chains. Rebinding of CO was calculated according to the kinetic scheme discussed below (Fig. 9), using the measured rate constants. The conformational transitions were calculated according to Equation 2, using the values of a for pH 7.0 calculated as described above. The value of L was fixed at 100,000, and d was varied until a best fit was achieved with a value of 3.2. The observed time course of conformation change was well described by this procedure; however, equally good fits were obtained by fixing L at other values and letting d vary. There is no unique solution to the problem, and L cannot be calculated from this information, since we have no independent method of determining d.

**Kinetic Measurements**—According to the strict two-state model, hemoglobin can assume just two conformations, each with invariant characteristics. Although the equilibrium between T and R states may be changed by ligation or allosteric effectors, their intrinsic properties remain unchanged. It therefore follows that any T or R state properties observed under one set of conditions should be applicable to any other set of conditions. Thus, the T state constants measured for menhaden hemoglobin at low pH, where the hemoglobin is frozen in the T state, should describe the behavior of T state hemoglobin at higher pH, where conformational change does occur. Similarly, the R state constants measured at high pH should describe R state hemoglobin under all conditions. This assumes that protons act as allosteric effectors, i.e. that the only effect of proton binding to hemoglobin is to change L, and not the intrinsic properties of the T and R states. The rate constants of CO binding and dissociation were therefore measured at the pH extremes, and the model tested for its ability to represent CO binding at intermediate pH.

**Binding Constants**—The T state CO binding was observed by both stopped flow and flash photolysis at pH 6.0 (Fig. 6). The stopped flow time courses of CO binding to deoxy and to partially saturated hemoglobin are shown in Fig. 6a. These curves are biphasic, with rate constants of 0.25 μM⁻¹ s⁻¹ and 0.033 μM⁻¹ s⁻¹. There are precisely equal proportions of the two components in the full CO binding to deoxy Hb. The proportion of the fast component decreases as the initial saturation of the hemoglobin increases, since the CO binds preferentially to the high affinity component and the unliganded hemes are those of the low affinity component.

The proportions of the two components remain equal at all levels of flash photolysis at pH 6.0 (Fig. 6b) and the rates of the two components are the same as the stopped flow measurements, except at the smallest levels of breakdown. At 10% photolysis there is slightly more fast than slow binding, and the initial fast rate of CO binding rises to 0.30 μM⁻¹ s⁻¹. The major starting intermediate at this level of breakdown is Hb₂(CO)₅; the increased rate of CO binding indicates that this species is partly in the R state.

The two components seen in the stopped flow and full photolysis experiments have been interpreted here as being the two chains of hemoglobin, binding with different rates. The tetramer-dimer dissociation constant of menhaden hemoglobin is about 10⁻⁹ M (16), too small for the rapidly reacting species to be identified as dimers.

The R state rate constants were measured at pH 8.5. CO binding as observed by flash photolysis is shown in Fig. 7. After full photolysis, the CO binding time course is noticeably autocatalytic. The hemoglobin is not frozen in the R state at this pH: ligand binds to deoxy Hb at least partly in the T state, which rapidly switches to the R state. This accelerating time course is also seen at higher pH; the deoxy Hb never completely attains the high affinity conformation.

The rate of CO binding increases with initial saturation of the hemoglobin, and loses its autocatalytic character. At 3 and 8% breakdown, the principal initial intermediate is Hb₂(CO)₅, which at this alkaline pH was assumed to be in the R state. These two curves are homogeneous, with a rate constant of 1.3 μM⁻¹ s⁻¹. At pH 8.0 as well the lowest breakdown yields one rate constant of the same size; this was taken as the R state rate.

**Off Constants**—The CO off rates for both T and R states

![Figure 6](https://example.com/fig6.png)

**Figure 6** (left and center). CO binding to hemoglobin at pH 6.0 by stopped flow and flash photolysis. A, Stopped flow measurements of CO binding to fully deoxy and partially saturated hemoglobin. **Home** concentration was 5 μM, and **CO** concentration was 93 μM before mixing. Cuvette path length was 2 cm. The solid lines are drawn through the points. B, full and partial flash photolysis of HbCO. **Home** concentration was 5 μM; **CO** concentration was 230 μM. Cuvette path length was 1 cm. The solid lines are fits of the flash photolysis data to the extended two-state model, as discussed in the text.

![Figure 7](https://example.com/fig7.png)

**Figure 7** (right). Full and partial flash photolysis of HbCO at pH 8.5. **Home** concentration was 5 μM; **CO** concentration was 25 μM. The solid lines are fits of the flash photolysis data to the model.
were measured by NO replacement of CO at acid and alkaline pH. Since the hemoglobin is fully liganded throughout this experiment, the T state measurements have to be made under conditions where completely saturated hemoglobin assumes the T state. They were therefore done at pH 5.5, in the presence of inositol hexaphosphate. The time course of NO replacement at pH 5.5 is slightly heterogeneous and can be resolved into two components with rate constants of 0.1 and 0.13 s⁻¹. As with the CO binding rates, these two components were assigned to the two chains.

The NO replacement of CO at pH 7.0 was measured to obtain the R state dissociation rates. The R state time course is again homogeneous, with a rate constant of 0.028 s⁻¹, and both chains were given this value as their R off rate.

Measurements at Intermediate pH—The rate of the first 5% of CO binding was measured at intermediate pH values by observing recombination after full photolysis of a hemoglobin solution containing enough CO to saturate only 5% of the hemes. Hemoglobin at this low level of saturation is in the T state since the CO is distributed evenly over the entire population in a kinetic experiment. The measured rates of binding were 0.24 and 0.26 μM⁻¹ s⁻¹ at pH 7.0 and 7.5, respectively, unchanged from the pH 6.0 rate. Measurements were not made at higher pH, since the assumption that only T state hemoglobin is binding loses its validity when the T to R switch occurs early in the ligand binding reaction. The binding rate of the slow component could not be measured at high pH, since CO binds first to the rapid component and by the time hemoglobin is half-saturated, much of it is in the R state. Similarly, direct measurements of pH effects on the T dissociation rates cannot be made, since liganded hemoglobin, on which the measurement is made, is in the T state only at very low pH.

Indirect measurements can be made at intermediate pH in the form of CO equilibrium curves, which are functions of both binding and dissociation rates, and contain more information about CO dissociation than the flash photolysis experiments. CO equilibrium curves were obtained between pH 6.0 and 7.5 (Fig. 8). At pH 6.0, binding is noncooperative. The affinity is very low; pₐ is 2.1 μM and n at 50% saturation is 0.9, reflecting the heterogeneity of binding by the two chains. Cooperativity and affinity both increase with pH; n rises to 2.6 and pₐ drops to 0.1 at pH 7.5. The affinity becomes too high at more alkaline pH to measure accurately.

CO binding was also observed by full and partial flash photolysis across the entire pH range from 6.0 to 8.5.

![Fig. 8](image_url)

**Fig. 8.** The effect of pH on the CO binding equilibrium. Heme concentration was 0.3 to 3.9 μM. The pH values are: 0, 6.0; O, 6.5; ■, 6.75; ●, 7.0; ▲, 7.5. The solid lines are fits to the extended two-state model, using both equilibrium and flash photolysis data for each pH.

**DISCUSSION**

Curve Fitting—The equilibrium and flash photolysis data for each pH value were fit, simultaneously when both were available, to a two-state model modified to include chain differences. Although the two ligand binding components are designated here as α and β chains, in analogy to those of human hemoglobin, it should be noted that no structural determinations have been made on menhaden hemoglobin, and there is no evidence that it has α and β homologs.

The scheme, for one conformation alone, is shown in Fig. 9. Ligands are assumed to bind randomly to α and β chains, with different rates. Although α chains have been assigned the rates of the high affinity component 1, there was no attempt at identification of the components, and the opposite situation may apply equally well. This scheme includes four intrinsic rate constants: the binding and dissociation constants for each of the two chains. The appropriate statistical factors are included in the scheme. Another, parallel, scheme was used to represent binding to the other conformation. Each of the nine intermediates was then partitioned between the T and R states in order to calculate the apparent rate constants.

For instance, the rate of disappearance of deoxy Hb, using the T state scheme alone, is

$$\frac{dαβ}{dt} = -[2λ_{T1} + 2λ_{T2} + \alpha_1(x) β_1] + λ_{R1} [α_1(x) β_1] + λ_{R2} [α_1(x) β_2]$$

Partitioning the intermediates between T and R states according to the two-state model, it is recalculated as

$$\frac{dαβ}{dt} = \left[ L \left[ \frac{λ_{T1}}{1 + L} + \frac{λ_{T2}}{1 + L} \right] \left[ α_1(x) β_1 \right] \right]$$

where L is the conformational equilibrium constant for deoxy Hb binding to hemoglobin; x, the ligand. λ₁ and λ₂ are the intrinsic rates of association and dissociation, respectively, to the high affinity component; λ₃ and λ₄ are the intrinsic binding rates to the low affinity component. No attempt at identification of the chains was made; these were assigned arbitrarily. The complete rates, including statistical factors, are given for each step.
Distribution of intermediates calculated from the measured for all species, and the initial conditions were the binomial percentage breakdown. The fit of the data to the model was optimized by a nonlinear least squares iterative procedure. Runs an equal quantum yield for CO photolysis was assumed species were initially unpopulated. In the partial photolysis order Runge-Kutta process (17). The concentrations of the nine intermediates were calculated at each integration step by summing the appropriate family of equations. In the full photolysis runs, all intermediates but the fully liganded species were initially unpopulated. In the partial photolysis runs an equal quantum yield for CO photolysis was assumed for all species, and the initial conditions were the binomial distribution of intermediates calculated from the measured percentage breakdown. The fit of the data to the model was optimized by a nonlinear least squares iterative procedure.

Both kinetic and equilibrium experiments were analyzed according to this model. The first curve-fitting operation, in which the rate constants for T and R states determined at the pH extremes were fixed and only L was allowed to vary, resulted in systematic misme of the data. When the T dissociation constants were allowed to vary as well as L the fit to the model improved greatly. The calculated T off rates are shown in Fig. 10. Both high and low affinity component rate constants decrease at alkaline pH. This decrease indicates that the intrinsic affinity of T state hemoglobin increases with pH. The high affinity chain increases its affinity 7-fold from pH 6.0 to pH 8.5. The T on rate of the low affinity chain was assumed to remain the same; its affinity increases 13-fold. The simple form of the two-state model, with invariant properties of T and R states, is insufficient to account for the CO binding data across a range of pH. Proton binding appears to alter the intrinsic properties of the T state.

With these alterations in the model, the calculated curves fit the data well at intermediate pH, in the highly cooperative region of ligand binding. The fits to equilibrium curves are shown as solid lines in Fig. 8. The solid lines in Figs. 6b and 7 are fits to flash photolysis data at pH 6.0 and 8.5, respectively. At pH 8.5 there were no equilibrium data available and so the photolysis curves only were used to calculate L. Fig. 11 shows the fit to the cooperative case of CO binding at pH 7.0.

The calculated L values are given in Fig. 12 as log L versus pH. L varies from 4 x 10^6 at pH 6.0 to 8 at pH 8.5. At pH 6.0 the calculated values of c₁ and c₂ are 0.036 and 0.002, respectively. The ratio of T to R state in fully liganded hemoglobin is therefore

\[ \frac{c}{c_T} = 0.26 \]

and Hb(CO)₉ is about 20% in the T state at pH 6.0. At pH 8.5 L = 6 x 10^8 and HbCO is only 4% in the T state; at pH 7.0 virtually all of the HbCO is in the R state.

At pH 8.5 the ratio of T to R state deoxy Hb is

\[ \frac{T}{R} = L = 8 \]

and deoxy Hb is about 10% in the R state. The L value at pH 8.0 is 40, and about 2% of deoxy Hb is in the R state.

pH-induced Spectral Changes—These calculations from functional observations can be compared to the amplitude of the pH difference spectrum, as a possible source of conformational information. The HbCO difference spectrum has begun to appear at pH 7.0, where it has 1/3 the amplitude of the pH 6.0 peak. This is much more than would be expected if this spectrum is a quantitative indicator of the amount of T state hemoglobin.

The deoxy Hb difference spectrum at pH 8.0 has about 1/6 the amplitude of the pH 8.5 spectrum, again more than that calculated from functional measurements. There is a small spectrum at pH 7.5, where virtually no deoxy Hb assumes the R state. These difference spectra therefore do not seem to quantitatively reflect conformational changes in hemoglobin as defined by function.

The pH difference spectrum of carp hemoglobin shows discrepancies like these at acid pH (18). The magnitude of the
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HbCO difference spectrum is still increasing at pH 4.5, while that of the IHP difference spectrum has already declined by pH 5.5. Functional studies also indicate that the conformational equilibrium of HbCO does not continue to move toward the T state below pH 5.5 in phosphate buffer; in fact, an acid Bohr effect appears and the CO affinity of carp hemoglobin increases (6).

Similar spectra have indeed been observed in systems in which the conformation certainly does not change. Soni and Kiesow (19) have reported pH difference spectra of human adult hemoglobin in the Soret region. They have assigned the deoxy Hb spectral changes to Bohr effect residues. Since human deoxy Hb remains in the T state throughout the pH range studied, 7.0 to 9.0, this is not a T-R difference spectrum. Similarly, human HbCO remains in the R state, and its difference spectrum cannot be attributed to conformation changes.

The visible absorbance changes of menhaden HbCO are similar to the IHP difference spectrum observed by Adams and Schuster (15). However, Knowles et al. (13) have reported that such a spectrum can be generated by a slight shift in the position of the absorbance bands; they have observed this difference spectrum between α and β chains, HbO₂ solutions of different temperatures, and HbO₂ with and without manganic phosphate, systems in which no conformation change occurs.

There is also a poor correlation between the absorbance change at the Hb-HbCO isosbestic, observed during photolysis, and the expected total conformational difference between Hb and HbCO, calculated from the functional studies. Instead of a broad plateau between pH 6.5 and 8.0, where there is nearly complete T to R transition between Hb and HbCO, we observed a maximum at pH 7.0 and large drops in the absorbance excursions at both acid and alkaline pH.

The time course of these R → T absorbance changes at a single pH has been successfully described by the two-state model for both menhaden and human (12) hemoglobin, but their extent is at variance with the predictions of the model. The extent of absorbance change can be used as a measure of total conformation differences between Hb and HbCO, calculated from the functional studies. Instead of a broad plateau between pH 6.5 and 8.0, where there is nearly complete T to R transition between Hb and HbCO, we observed a maximum at pH 7.0 and large drops in the absorbance excursions at both acid and alkaline pH.

The Bohr effect in human hemoglobin in some aspects appears to be inconsistent with the two-state model. Protons decrease the ligand affinity of hemoglobin by binding more strongly to the low affinity deoxy Hb than to the high affinity HbO₂ or HbCO (21). Ligand binding to Hb is accompanied by proton release. If the effect of proton binding is to change L, this proton release should be associated with conformational change from the T to R states. The level of saturation sufficient to switch the conformation of hemoglobin depends on L; if L is a small number, the switch occurs early in the ligand binding reaction, and if L is large, the switch occurs relatively late in the reaction. However, in kinetic observations of CO binding and proton release, the proton release was found to be synchronous with ligand binding between pH 7.0 and 9.0 (22, 23), instead of faster or slower than CO binding, depending on the value of L. More recently, in equilibrium measurements Tyuma and Ueda (24) found that at high pH, proton release did in fact lead O₂ binding. This contradictory evidence has not been resolved, but for human hemoglobin, apparently a major portion, if not all, of the Bohr effect is due to change in the intrinsic affinity of the T state chains.

The Bohr effect of menhaden hemoglobin has a much larger contribution from proton-induced changes in quaternary conformation. Protonic observations of proton release on CO binding showed large differences between the time courses of proton release and ligand binding (25). At low pH, proton release lags behind CO binding, as would be expected in a system where L is large and the T to R conformation switch occurs relatively late in ligand binding. As the pH is increased, proton release becomes relatively faster until, at the alkaline edge of the Bohr effect, proton release slightly leads CO binding, as would be expected for a small value of L, and conformation change early in the ligand binding reaction.

With values of L and of the T state affinities calculated as a function of pH, we can estimate the numbers of protons per heme arising from conformational change and from CO binding within the T state. We assume here that there is no pH effect on the R state affinities. The total protons are calculated as (21)

\[ \Delta H_{\text{obs}} = -\frac{d \log p_H}{d \text{pH}} \]  

Proton differences between T and R states are

\[ \Delta H_{\text{c}} = -\frac{1}{4} \frac{d \log L}{d \text{pH}} \]  

and between deoxy and liganded T state hemoglobin

\[ \Delta H_{\text{r}} = -\frac{d \log K_T}{d \text{pH}} \]

The relative amounts of T state and quaternary protons vary with pH. At pH 7.5 \( \Delta H_{\text{obs}} = 1.6, \Delta H_{\text{c}} = 1.1, \text{and} \Delta H_{\text{r}} = 0.6; \) about 60% of the difference in protonation between deoxy and liganded hemoglobin is due to the conformation difference. That proportion rises to about 85% at pH 7.0, where \( \Delta H_{\text{obs}} = 1.1, \Delta H_{\text{c}} = 1.0 \text{ and } \Delta H_{\text{r}} = 0.2. \)

More precise measurements of the protonation changes associated with ligand binding and conformation transition can be made by direct studies, now in progress, of the proton...
release kinetics, which are sensitive to changes in pH and conformation.

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