A Kinetic Analysis of the Binding of Oxygen and Carbon Monoxide to Lamprey Hemoglobin

PETROMYZON MARINUS AND PETROMYZON FLUVIATILIS

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SUMMARY

We have studied the ligand-binding reactions of lamprey (Petromyzon marinus and Petromyzon fluviatilis) hemoglobin with O₂ and CO in the pH range 9.0 to 5.6 by means of both rapid reaction and equilibrium techniques. All the data can be accurately described by a model in which it is assumed that there are two forms of hemoglobin, a high affinity monomer and a lower affinity dimer, and that the formation of the dimer is pH dependent and requires the protonation on the protein of one specific site which has a pK of 6.0. For the deoxyhemoglobin monomer-dimer reactions, the dissociation equilibrium constant, $K_d$, is estimated as 0.5 μM at pH 5.6; the association rate constant as $2 \times 10^6$ M⁻¹sec⁻¹; and the dissociation rate constant as 1 per sec. The binding rates for the reaction of CO with the monomer and the dimer are $0.9 \times 10^6$ M⁻¹sec⁻¹ and $0.12 \times 10^6$ M⁻¹sec⁻¹, respectively, and for the reaction of O₂, $10 \times 10^6$ M⁻¹sec⁻¹ and $0.25 \times 10^6$ M⁻¹sec⁻¹. The dissociation rates of CO and O₂ from the monomer are 0.09 and 80 per sec, respectively, and for the monomer the partition coefficient, $M$, is 89. In the proposed mechanism the deoxyhemoglobin dimer itself is supposed to be noncooperative, and cooperativity, as seen at acid pH, results from the oxygen-linked dissociation of the liganded dimer which has a dissociation rate constant of 150 per sec. This dissociation gives rise to a high affinity species, and thus produces the observed cooperativity. The rate constants that gave the best fits to the kinetic data were used to simulate equilibrium O₂-binding curves. The model accurately predicts the equilibrium behavior of this protein without considering aggregation above the level of the dimer. Physiologically, higher aggregates may not have functional importance.

The experiments described in this paper were started in 1954 and were intended to serve as a control on the adequacy of the

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stopped flow method to deal with the more complicated mammalian hemoglobin system. It was quickly found that lamprey hemoglobin is very different from mammalian hemoglobin in its kinetic behavior, and that the large Bohr effect shown by this protein (1) appeared to be solely the result of an alteration in the combination velocity with pH. Discrepancies soon appeared, however, particularly between the results of stopped flow and flash photolysis experiments on CO binding, and the seasonal availability of fresh material greatly slowed progress in exploring these problems.

In the meantime, a reasonable explanation of the kinetic anomalies arose from detailed studies of the equilibrium with oxygen (2, 3), and from the ultracentrifuge results which showed that lamprey deoxyhemoglobin aggregated under conditions where oxyhemoglobin was monomeric (2, 4-6). It now seems possible to offer an internally consistent account of the equilibrium and kinetic behavior of lamprey hemoglobin by taking into consideration subunit association-dissociation reactions. Preliminary accounts of this work have been given (7, 8).

EXPERIMENTAL PROCEDURE

Lampreys—The first experiments were performed with the pooled blood of 48 Petromyzon fluviatilis obtained from the Marine Biological Laboratories, Plymouth, England. Blood was washed from the animals by cannulating the main vessel in the tail, incising the gills, and perfusing with 1% NaCl containing 0.4% sodium citrate. The cells were washed with 1.0% NaCl solution, packed by centrifugation, and hemolyzed by freezing and thawing. The stroma was removed by centrifugation, and the solution diluted in appropriate buffers. Later experiments were performed with blood from single specimens of Petromyzon marinus treated similarly, and a final set of experiments was performed with pooled blood from 50 spawning P. marinus taken from Lake Cayuga. This batch of hemoglobin showed six components on electrophoresis, as reported by Rumen and Love (9). For purification this hemoglobin was oxidized to the met form with KCN (1 mole per heme). The major component (≈40%) was obtained by (NH₄)₂SO₄ precipitation, taking the fraction coming down between 53 and 63% saturation. The precipitate was redissolved by dialysis against 200 volumes of 0.005 M Tris-HCl buffer, pH 8.5, and applied to a diethylaminoethyl cellulose column (8 × 40 cm) equilibrated with the same
buffer. Washing the column with 20 volumes of the Tris buffer removed the chief contaminant, and elution with 0.018 M NaCl brought down the main component in an electrophoretically pure form. This stock solution was stored as the cyanomet compound, and was prepared for use by reduction with dithionite in an atmosphere of CO. Excess dithionite and its oxidation products were removed by filtration through a column (4 × 20 cm) of Sephadex G-25.

When similar experiments were performed on both the purified major component and the hemolsate of this last batch of *P. marinus* blood, equivalent results were always obtained.

**Carbon Monoxide**—This was obtained from the British Oxygen Company and from The Matheson Company, East Rutherford, New Jersey. In the early experiments CO was measured into glass tonometers with a mercury manometer and buffers were equilibrated with the gas by gentle shaking. In the later experiments water was equilibrated with the pure gas by bubbling for 10 min, and was subsequently diluted as required with oxygen-free buffer.

**Sodium Dithionite**—This was obtained from Hardman and Holden, Miles Plating, England, as “Manox” brand. It was prepared as described by Geraci, Parkhurst, and Gibson (10).

**PMB**—p-Chloromercuribenzoic acid was obtained from Sigma, and was converted to the sodium salt as described by Boyer (11). Concentrations of stock solutions were determined by measuring the absorbance at 232 nm, and reactions of the mercurial with hemoglobin were always performed within 12 hours after preparing the neutralized PMB solution.

**Buffers**—A borate-boric acid buffer was used at pH 9.0; phosphate-citrate at pH 5.0; and phosphate buffers at all intermediate pH values.

**Stopped Flow Apparatus**—Three different apparatus were used, two of which have already been described in detail. In the early experiments the apparatus of Gibson and Roughton (12) was used. This had a circular observation tube of 2.5-mm diameter, and roughly monochromatic light was obtained by means of an interference strip filter (Barr and Stroud Optical, Glasgow, Scotland). The oscilloscope (Cossor 1049) had no slow speeds in its time base, so for reaction times upward of 3 sec a special stopped flow apparatus was constructed which flushed a volume of 7 ml through a 1-cm spectrophotometer cell with a male ground glass joint attached. The female joint had a mixer fused to its top, and an inflow and exit tube. The cell was mounted in a Unicam SP200 spectrophotometer, and the progress of a reaction followed by starting a stop watch at the end of the flow period and noting the time required to achieve fixed increments in absorbance. The most recent experiments used a Gibson-Durrum apparatus with digital data collection devices described by DeSa and Gibson (13), and a 2-ep optical path. Light was obtained from a Bausch and Lomb 250-mm grating monochromator.

**Flash Photolysis**—The early experiments were done with the apparatus of Gibson (14), and the most recent experiments used the apparatus of Gibson and Greenwood (15).

**Oxygen Equilibrium Titration**—These curves were determined by preparing a number of solutions each of which had the same hemoglobin concentration, but contained varying amounts of oxygen. The visible spectrum of each solution was recorded and the absorbance at 540, 556, and 576 nm was measured. The difference from the absorbance of the deoxyhemoglobin solution at the same wave length was calculated, and for each O₂ concentration the sum of the absolute values of these three differences was determined. This number was then compared to the value of this sum for a completely saturated solution, the free O₂ concentration was calculated for each solution, and the saturation curve constructed as percentage saturation versus the free O₂ concentration.

**Other Apparatus**—pH measurements were made with a Radiometer model 22: in early experiments buffers were prepared with tables. Spectra were prepared with a Cary 14 spectrophotometer. Electrophoresis was performed with a Millipore strip apparatus with 0.05 M sodium barbital buffer, pH 8.5, with 100 volts for 30 min. The strips were stained with Ponceau S.

**Data Analysis**—Curve fitting of kinetic data was carried out by hybrid computation with a TR 48 analogue computer (Electronico Associates, Inc., Long Beach, New Jersey) interfaced to a PDP-8I digital computer (Digital Equipment Corporation, Maynard, Massachusetts). In some cases digital computation alone was used to fit kinetic data. Since this is a much more laborious procedure, it was only used when several of the rate constants under investigation could be independently determined.

**Notation**—The oxygen-binding rate constant = k' M⁻¹ sec⁻¹; carbon monoxide-binding rate constant = k M⁻¹ sec⁻¹; oxygen dissociation constant = k sec⁻¹; carbon monoxide dissociation constant = k sec⁻¹. All equilibrium constants are given as dissociation constants.

**RESULTS**

### Oxygen Dissociation

This reaction was measured with hemoglobin from *P. fluviatilis* in pH 6.2, 6.5, 6.8, 7.4, and 7.8 phosphate buffers 0.1 m, and in 0.05 M borate buffer, pH 9.1, at 18.7° with 0.4% dithionite and 80 µM hemoglobin (heme basis, concentrations after mixing), with observation at 475 nm. The reaction was closely first order in each case, and the rates were 150, 108, 112, 110, 114, and 96 per sec, respectively. With the possible exception of the first and the last figures, these values are considered to be the same within experimental error, and are in marked contrast to the behavior of mammalian hemoglobin (16). There was a large effect of temperature, a determination at 3.6° and pH 7.4, yielding a rate of 20 per sec. An estimate of the rate of dissociation of oxygen was also made from the rate of the replacement reaction in which CO displaces O₂ from saturated hemoglobin. As shown by Gibson and Roughton (12), if this reaction is followed at various O₂ concentrations, a plot of the reciprocal of the observed rate versus O₂ concentration should yield a straight line with intercept 1/k and slope k'/[(k)(k′)(CO)]. The results of an experiment of this kind are given in Fig. 1. This confirms that the rate of dissociation is high, and gives the ratio k′/k as 10. Both figures differ materially from those for mammalian hemoglobin, human hemoglobin giving equivalent values of 12 per sec and 3.3 for k and k′, respectively. A rapid rate of dissociation of O₂ from *P. fluviatilis* hemoglobin at 24° was also observed when ferrieryanide rather than CO was used as the replacing reagent, giving k = 110 per sec at pH 7.4. Experiments with hemoglobin from *P. marinus* have shown similar behavior. A replacement reaction with CO at 24° and at pH 5.6 gave k = 76 per sec, and the reaction of this oxyhemoglobin with 0.4% dithionite gave k = 80 per sec at both pH 5.6 and 7.5.
Fig. 1 (left). Determination of \( k \) and the ratio \( k' = \frac{k}{k_d} \) for Petromyzon fluviatilis hemoglobin. Reaction of HbO\(_2\) with CO at various O\(_2\) concentrations. Plot of the reciprocal of the observed first order rate constant in sec\(^{-1}\) against the O\(_2\) concentration. \( k = 30 \mu M; \) \( (CO) = 450 \mu M; \lambda = 450 \text{ nm}; \) and \( T = 24^\circ \). All reactions were performed in 0.1 M phosphate buffer, pH 7.4.

Fig. 2 (center). Reaction of Petromyzon marinus hemoglobin with O\(_2\) at acid pH in the stopped flow apparatus. For all curves, \( (Hb) = 10\mu M; \) \( (O_2) = 124, 62, 31, 15.5, \) and \( 7.8 \mu M \) in order reading from the highest curve to the lowest; \( \lambda = 445 \text{ nm}; \) 0.1 M phosphate-citrate buffer, pH 5.6; and \( T = 20^\circ \). Points are the experimental data; solid curves were calculated according to the dimer model given under “Discussion” with the following set of constants: \( k'_1 = 10 \times 10^6; k_1 = 80 \text{ sec}^{-1}; k'_2 = 0.5 \times 10^4; k_2 = 32 \text{ sec}^{-1}; \) \( h'_4 = 0.25 \times 10^5; h_4 = 64 \text{ per sec}; \) \( k'_d = 2 \times 10^5; k_0 = 1 \text{ per sec}; \) and \( k'_d = 150 \text{ per sec}. \) This particular set of curves could obviously have been fitted more accurately by a slightly different set of constants. However, not only these five kinetic curves, but 10 other kinetic experiments and also the equilibrium data were considered in this fitting. Therefore, the set of constants given is that which most accurately described all of the data, not just this one set of reactions.

Fig. 3 (right). Observed rate of the approach to equilibrium for the reaction of O\(_2\) with Petromyzon marinus hemoglobin at alkaline pH in the stopped flow apparatus. \( (Hb) = 10 \mu M; \) 0.1 M phosphate buffer, pH 7.5; \( \lambda = 445 \text{ nm}; \) and \( T = 20^\circ \). The ordinate is the observed first order rate constant in sec\(^{-1}\).

Oxygen Binding

Although this reaction has been difficult to study because of its high velocity, and because lamprey deoxyHb is readily denatured upon shaking the solution, experiments have been carried out with hemoglobin from Petromyzon fluviatilis at pH 6.2 and 9.0 and 20\(^\circ\). With an air-equilibrated buffer (before mixing) the observed first order rates for the approach to equilibrium were 95 per sec and approximately 1000 per sec, respectively, the latter rate being too rapid for precise measurement. These results, although suggesting that a change in the O\(_2\)-binding reaction was the main source of the Duta effect, were not easily reconciled with a large pH-independent dissociation rate for oxygen, since the rate of approach to equilibrium, \( k'_d = 10^6; k'_d = 1 \text{ sec}^{-1}. \) Thus it seems that \( k \) as measured in the replacement reaction does not represent the actual dissociation rate of O\(_2\) from the protein in the stopped flow experiments at acid pH.

Later experiments with hemoglobin from P. marinus at acid pH confirmed this anomaly; the results of a series of reactions in which the oxygen concentration was varied at a constant hemoglobin concentration are shown in Fig. 2. At the highest O\(_2\) concentration used in these experiments, 125 \mu M after mixing (all concentrations are given after mixing unless otherwise specified), the rate of approach to equilibrium is only 40 per sec, a value substantially lower than the dissociation rate of 80 per sec. Even with O\(_2\)-saturated buffer the observed rate of O\(_2\) binding at 20\(^\circ\) is 30 \mu M hemoglobin, and at pH 5.6 was only 290 per sec.

Rates of O\(_2\) combination can also be estimated from flash photolysis experiments. When a solution of HbCO containing a 5- to 10-fold excess of O\(_2\) over CO is photolyzed, the combination of O\(_2\) with deoxyHb can be followed immediately after the flash (14). (These experiments, and all the experiments to be reported subsequently, unless otherwise specified, were performed with P. marinus hemoglobin.) Determination of \( k' \) in this way gave a value of approximately \( 12 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}, \) a number which was independent of pH, and which corresponded to the rate observed in the stopped flow experiments at pH 9.0. A similar discrepancy between the rate of ligand binding determined by these two techniques at acid pH was also observed when analogous experiments were carried out with CO as ligand. It appears that at acid pH the deoxyHb produced by flash photolysis is not the same chemical species as that present in the stopped flow experiments at this pH.

In the pH range where stopped flow and flash photolysis techniques give similar estimates of \( k' \), the oxygen-binding reaction can be treated as a simple, noncooperative combination of the ligand with protein. A plot of the rate of approach to equilibrium versus O\(_2\) concentration gives a straight line with intercept \( k' \) and slope \( k'_d \). For O\(_2\) combination as measured in the stopped flow at pH 7.5, this plot is shown in Fig. 3. The estimates of \( k \) and \( k' \) are 90 per sec and \( 12 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}, \) respectively.

Saturation curves shown in Fig. 4 were determined both from separate spectrophotometric titrations and from these stopped flow observations in which the extent of reaction and the amount of free O\(_2\) in solution at equilibrium were estimated. In a Hill plot (17), the data from the saturation curve at pH 7.5 yields an \( n \) value of 1.0. At this relatively alkaline pH lamprey hemoglobin behaves like a monomeric, noncooperative molecule. These estimated values of \( k \) and \( k' \) are more reminiscent of those for aplysia myoglobin (70 per sec and \( 1.5 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}, \) than of those for mammalian hemoglobin (18).

However, at pH 5.6 in similar stopped flow experiments the reactions are considerably more complex. The rate of approach to equilibrium versus O\(_2\) concentration for three hemoglobin concentrations is shown in Fig. 5. Curves are obtained, not straight lines such as that observed in Fig. 3 at pH 7.5, and these curves cannot be treated in a straightforward manner to yield estimates of \( k \) and \( k' \) at this pH. Stopped flow and separate spectrophotometric determinations of the saturation curves at pH 5.6, which are also given in Fig. 4, yield upon appropriate analysis \( n \) values.
first order and virtually independent of pH: at pH 9.0 and 5.6, I is measured by mixing a 10% solution of potassium ferricyanide. At pH values of 6.2 and 7.4 at room temperature the reaction was first order and had a rate constant of 0.15 per set at both pH values. In the later experiments this reaction was measured by the oxidation of a solution of I'.

FIG. 6 (right). pH relaxation experiments. Solutions of deoxyHb in 0.01 M phosphate buffer, pH 7.5, were mixed with 0.1 M phosphate-citrate buffer, pH 5.6, containing varying amounts of O₂ as noted on the curves. (Hb) for all reactions was 10 mM and T = 20°. Dotted lines on the right side of the figure represent the final values of percentage of saturation in these acid solutions after equilibrium was reached.

pH Jump Experiments

The affinity of the protein for O₂ at the hemoglobin concentrations used in these kinetic experiments is approximately 3 times higher at pH 7.5 than it is at pH 5.6. A number of pH relaxation experiments were carried out to measure the dissociation of O₂ from lamprey hemoglobin after the conversion of the molecule from the high affinity to the low affinity form. Thus, deoxyHb weakly buffered at pH 7.5 was mixed in the stopped flow apparatus with a strong pH 5.6 buffer containing a known amount of O₂. Initially as the ligand reacts with the alkaline form of the protein, combination is rapid, then, as the hemoglobin reverts to the lower affinity species, O₂ dissociates in a slow relaxation step. The time course of this relaxation reaction at several O₂ tensions is plotted in Fig. 6.

CO Reactions

CO Dissociation—In the early experiments this reaction was measured by the oxidation of a solution of P. fluvititis HbCO by a 2% solution of potassium ferricyanide. At pH values of 6.2 and 7.4 at room temperature the reaction was first order and had a rate constant of 0.15 per sec at both pH values. In the later experiments this reaction was measured by mixing a 10 μM solution of HbCO at low CO concentration with a solution containing nitric oxide, a ligand which binds even more firmly than CO does. The replacement reaction measured at 418 nm was accurately biphasic with a contribution from both the rapid and the slow phase.

CO Binding—In the stopped flow apparatus this reaction is affected by pH in the same way the O₂-binding reaction is: this dependence on pH is shown in Fig. 7. At both extremes of pH the reaction is virtually homogeneous, but at pH 9.0 the reaction is some 7 times greater than it is at pH 5.6. 0.85 × 10⁻⁸ vs. 0.12 × 10⁻⁸ M⁻¹ sec⁻¹. At intermediate pH values the reaction is markedly biphasic with a contribution from both the rapid and the slow phase.

In alkaline solution where CO binding is rapid the reaction can be treated as if it were an irreversible second order combination of CO with deoxyHb. Therefore, when the observed rate of CO binding was plotted versus CO concentration a straight line was obtained which had a slope, 0.9 × 10⁻⁸ M⁻¹ sec⁻¹, equal to I', and which, since the expected intercept, I, is only 0.09 per sec, apparently passed through the origin. A similar plot for CO binding at pH 5.6 where combination is slow is shown in Fig. 8, and yields a curve, not a straight line. Above CO concentrations of 70 μM this curve does have a linear portion which by extrapolation gives a y-intercept of 4 per sec whose significance is discussed later. This plot, unlike that for O₂ binding at this pH (Fig. 5), was not noticeably affected by protein concentration.

However, the proportion of slowly reacting hemoglobin formed at a given pH is dependent on the protein concentration. This distribution of rapidly and slowly reacting hemoglobin at 3, 8, and 30 μM (before mixing) at pH 5.6 is shown in Fig. 9. It is apparent that as protein concentration increases, the proportion of slowly reacting hemoglobin also increases. This dependence of the amount of slowly reacting hemoglobin on protein concentration suggests that the slowly reacting form of the protein present in acid solution may be a polymer.

Flash Photolysis—The results of several experiments at various CO concentrations and constant hemoglobin concentration at acid pH are given in Fig. 10. At the highest CO concentration the recombination reaction is homogeneous and occurs with a
FIG. 7 (left). The reaction of Petromyzon marinus hemoglobin with CO in the stopped flow apparatus at various pH values. For all reactions (Hb) = 7 μM; (CO) = 250 μM; T = 20°; and 0.1 M buffer with pH as indicated on the curves. The ordinate is the proportion deoxyHb unreacted on a logarithmic scale. The usual excursion was 0.5 optical density units at 435 nm.

FIG. 8 (center). The observed rate of CO binding to Petromyzon marinus hemoglobin at pH 5.6 in the stopped flow apparatus as a function of CO concentration. All reactions were carried out in 0.1 M, pH 5.6, phosphate-citrate buffer at 20° with observation at 440 nm. ▲, determinations at 11 μM hemoglobin; ■, at 5.5 μM; ●, at 2.75 μM with observation at 430 nm.

FIG. 9 (right). The effect of protein concentration on the amount of slowly reacting hemoglobin at acid pH. At 30 and 8 μM hemoglobin, λ = 435 nm; at 3 μM, λ = 420 nm; 0.1 M phosphate-citrate buffer, pH 5.6; and T = 20°. The ordinate is the proportion of deoxyHb unreacted on a logarithmic scale. The protein concentrations are given as those before mixing to emphasize the quantity which is important in calculating the dimer-monomer dissociation constant, $K_{d}$. 

rate constant similar to that observed at pH 8.0 in the stopped flow experiments (Fig. 7). At lower CO concentrations the recombination reaction becomes biphasic, developing a slow phase with a rate constant characteristic of that observed for hemoglobin at pH 5.6 in the stopped flow experiments. These observations can be explained if it is supposed that there is a time dependent formation of a slowly reacting material whose extent of reaction is directly related to the lifetime of the photo-lytically produced deoxyHb. At high CO concentrations, then, the recombination reaction is rapid, the lifetime of the deoxyHb very short, and no slowly reacting deoxyHb can be formed. As the CO concentration is lowered, however, both the lifetime of the deoxyHb and the amount of slowly reacting deoxyHb formed would be expected to increase, as is observed.

FIG. 11 shows the results of several experiments at constant CO concentration and various hemoglobin concentrations at acid pH. Increasing protein concentration accelerates the formation of the slowly reacting form. These data and those shown in Fig. 9 suggest that the slowly reacting hemoglobin observed both in the stopped flow at acid pH and in flash photolysis experiments at low CO concentrations is a polymer.

$pH$ Jump with CO—Several attempts were made to measure the rate of conversion of the slowly reacting form of deoxyHb to the more rapidly reacting form. In the stopped flow apparatus, a 20 μM solution of deoxyHb weakly buffered at pH 5.6 (0.01 M phosphate-citrate) was mixed with a strongly buffered pH 9.0 solution (0.1 M borate) containing various amounts of CO. It was expected that the rate of ligand binding observed at pH 9.0 would initially be slow as is observed at pH 5.6, and that it would increase as the reaction proceeded because of the conversion of the slowly reacting deoxyHb to the rapidly reacting, alkaline form of this protein. However, in all experiments, even at the highest CO concentration, the reaction was homogeneous and rapid (200 per sec at 250 μM CO). Thus, the change from a slowly reacting to a rapidly reacting species at pH 9.0 must occur with a rate of greater than 1000 per sec in order to be complete in the dead time of the stopped flow apparatus.

Experiments in which the pH was dropped from pH 9.0 to pH 5.6 were also performed, and in this case, the alkaline solution was weakly buffered and the acid solution was strongly buffered and contained CO. These experiments are in principle analogous to the flash photolysis experiments at varying CO concentrations at pH 5.6 (Fig. 10). In direct analogy to the results shown in Fig. 9, the second order rate constant for the reaction de-
FIG. 11 (left). The effect of protein concentration on the CO recombination reaction at acid pH in flash photolysis experiments. (Hb) for each reaction is noted on the curves; (CO) = 92 μM for all reactions; λ = 372 nm; T = 22°; 0.1 M phosphate-citrate buffer, pH 5.6; and path length = 1 cm. Flash energy was 600 joules and in all cases 100% breakdown of the HbCO was achieved. The ordinate is the observed optical density at a given time minus the optical density when recombination is complete plotted on a logarithmic scale.

FIG. 12 (center). The effect of pH on the formation of the slowly reacting hemoglobin. Ordinate, 1 - α, is the observed proportion of slowly reacting hemoglobin at 14 μM protein. Experimental points calculated by fitting the curves shown in Fig. 7 to two exponentials. The smooth curve is calculated for the scheme shown in the figure with the equilibrium constants $K_p = 1 \mu M$ and $K_d = 0.5 \mu M$.

FIG. 13 (right). Hill plots of theoretical O₂ binding curves simulated from the dimer model for pH 7.5 and at various heme concentrations. Saturation curves were calculated from the model presented under "Discussion" with the following constants: $K_1 = 8 \mu M$; $K_{2,1} = 500 \mu M$; $K_2 = 64 \mu M$; and $K_4 = 256 \mu M$. Then these saturation curves were plotted according to Hill (17). The hemoglobin concentration for each theoretical curve is given in the figure. The value chosen for $K_{2,1}$ (500 μM) represents an α of 0.95 at 14 μM. Thus, from Fig. 12 it is estimated that this percentage monomer corresponds to a pH in the vicinity of 7.5.

$M$ was also experimentally determined. A number of solutions were prepared which had similar hemoglobin and O₂ concentrations (30 and 1150 μM, respectively), and varying CO concentrations in order to give variable O₂:CO ratios. The amounts of HbCO and HbO₂ present were determined by measuring the absorption at 578 nm, and then using the extinction coefficients of these two species at this wave length to determine the distribution of the hemoglobin in these solutions. A plot of the ratio HbO₂:HbCO versus O₂:CO then yields a straight line with slope $M$. In this way $M$ was determined to be 89 for $P$. marinus hemoglobin, a value in good agreement with calculation. This constant again is similar to $M$ determined for aplysia myoglobin, 106, and it is quite different from the $M$ of horse myoglobin, 37, or from that of the isolated α or β chains of human hemoglobin, 180 and 250, respectively (18).

PMB-treated Hemoglobin—The reaction between $P$. marinus HbCO (50 μM) and neutralized PMB (80 μM) was followed at 250 nm where an increase in absorbance is observed. Analysis of this reaction on the basis of second order kinetics suggested that the reaction was biphasic, 25% fast and 75% slow. The slower phase had a half-time of 15 min, and the rapid phase was some 2 to 3 times faster. Heterogeneity was probably caused by the different molecular species in the hemolysate which was used in this reaction. On a heme basis the change in extinction coefficient observed for this reaction at 250 nm was $7.9 \times 10^4$ M⁻¹ cm⁻¹, which, when compared to Boyer's value of $7.6 \times 10^4$ for the reaction of PMB with cysteine (11), gave a value of 1.04 sulfhydryl residues reacted per heme (or per peptide chain).

The ligand-binding reactions of $P$. marinus HbCO treated with a stoichiometric amount of PMB were investigated in flash photolysis experiments. At high pH, $k$ and $l$ were doubled.
compared to the native protein. At low pH in a series of reactions similar to those shown in Fig. 10, the CO recombination reaction at low CO concentrations was biphasic, and the rates of both the fast and slow phases were approximately doubled. Although these enhanced ligand-binding rates hinder a direct comparison between the rate of formation of the slowly reacting material for the PMR-treated versus the native protein, it appeared that the slowly reacting PMR-treated protein did accumulate at approximately the same rate as the slowly reacting native protein.

**DISCUSSION**

Before we can describe these data by a general scheme which incorporates association-dissociation reactions, it is necessary to estimate the extent to which both the liganded and the deoxy form of lamprey hemoglobin will be associated under the conditions used in these kinetic studies. Although several investigators using sedimentation velocity techniques have shown that the liganded forms of *P. marinus* and *Lampetra fluviatilis* hemoglobin are monomeric over a wide range of pH (2, 4, 5, 19), at low pH and at high protein concentration Behlke and Scheler were able to measure association of both HbO₂ and HbCO (6). However, with sedimentation equilibrium techniques, Andersen (20) has shown that the dimer-monomer dissociation constant for HbCO is quite large, 0.4 mM below pH 6.0. Therefore, in these kinetic experiments at low hemoglobin concentrations the liganded forms of lamprey hemoglobin will be monomeric even at pH 6.6.

With deoxyHb from *L. fluviatilis* association is already evident at pH 7.5 and 150 μM (6). At higher protein concentration and pH 6.0 and Behlke and Scheler reported an <i>α₀₂₋₀₂</i> value of 4.0.

\[
2\text{CO} + 2\text{Hb} \overset{k_a}{\underset{k_d}{\rightleftharpoons}} (\text{HbCO}) + \text{CO} \overset{k_a}{\underset{k_d}{\rightleftharpoons}} (\text{Hb}_2) \overset{k_a}{\underset{k_d}{\rightleftharpoons}} \text{HbCO} + \text{CO} \overset{k_a}{\underset{k_d}{\rightleftharpoons}} (\text{Hb}_2) \overset{k_a}{\underset{k_d}{\rightleftharpoons}} \text{HbCO} + \text{CO} \]

**Scheme 1**

\[
\begin{align*}
\text{Hb} + \text{O}_2 & \overset{k_1}{\underset{k_1'}{\rightleftharpoons}} \text{HbO}_2 ; K_1 = \frac{(\text{HbO}_2)}{(\text{Hb} \times \text{O}_2)} = \frac{k_1}{k_1'}, \\
\text{Hb}_2 + \text{O}_2 & \overset{k_3}{\underset{k_3'}{\rightleftharpoons}} \text{Hb}_2\text{O}_2 ; K_3 = \frac{(\text{Hb}_2\text{O}_2)}{(\text{Hb}_2 \times \text{O}_2)} = \frac{k_3}{k_3'}, \\
\text{Hb}_2\text{O}_2 + \text{O}_2 & \overset{k_4}{\underset{k_4'}{=} } \text{Hb}_2\text{O}_2\text{O}_2; K_4 = \frac{(\text{Hb}_2\text{O}_2\text{O}_2)}{(\text{Hb}_2\text{O}_2 \times \text{O}_2)} = \frac{k_4}{k_4'}, \\
2\text{HbO}_2 & \overset{k_5}{\underset{k_5'}{=} } \text{Hb}_2\text{O}_2; K_5 = \frac{(\text{Hb}_2\text{O}_2)}{(\text{HbO}_2 \times \text{O}_2)} = \frac{k_5}{k_5'}, \\
\text{HbO}_2 + \text{Hb} & \overset{k_6}{\underset{k_6'}{=} } \text{Hb}_2\text{O}_2; K_6 = \frac{(\text{Hb}_2\text{O}_2)}{(\text{HbO}_2 \times \text{Hb})} = \frac{k_6}{k_6'}.
\end{align*}
\]

**Scheme 2**

Which corresponds to that value expected for a trimer (6). Indeed, with *P. marinus* deoxyHb Andersen has shown both a dimer-monomer and a tetramer-dimer equilibrium with dissociation constants of 0.4 and 600 μM, respectively (20). From the magnitude of these constants it appears that the polymer present in these dilute solutions of deoxyHb must be the dimer. The dissociation reaction then is assumed to follow Equation 1:

\[
2\text{Hb} \overset{k_a}{\underset{k_d}{\rightleftharpoons}} (\text{Hb}_2) \quad (1)
\]

Here, <i>kₐ</i> is the association rate constant, and <i>k_d</i> is the dissociation rate constant. The dimer-monomer equilibrium constant is defined as follows:

\[
K_{2,1} = \frac{(\text{Hb}_2)^2}{(\text{Hb})^2} = \frac{k_d}{k_a}.
\]

Fitting procedures can be used to see how closely this model which considers the dimer to be the slowly reacting species approximates the actual experimental data. The simplest scheme reproducing CO binding is given in Scheme 1. In this mechanism the dimer is considered to be both slowly reacting, and noncooperative. The rate constants for CO binding to the dimer then are statistically related (4, 19), and the other rate constants refer to the association and dissociation rates of the three dimeric species, Hb, HbCO, and Hb₂CO₂. The fully liganded dimer must be in equilibrium with HbCO, but since CO binding can be treated as if it were irreversible, the two rate constants, <i>kₐ</i> and <i>k_d</i>, which govern this interconversion will have no influence on the CO-binding reaction. The best fit (±1%) to the CO-binding data is given in Fig. 10 by the smooth curves which were calculated with the set of rate constants listed in the legend. Stopped flow experiments at varying CO and hemoglobin concentrations, similar to the O₂-binding reactions described in Figs. 2 and 5, were also fitted by mechanism (±3.5%) with a similar set of rate constants.

Simulating the kinetic O₂-binding data is somewhat more complicated. The total scheme for this series of reactions is described by the equations below (Scheme 2) and also the two reactions given in Equation 1. The values of <i>k₁</i> and <i>k₁'</i> are known (Fig. 3), and <i>k₄</i> and <i>k₄'</i> were estimated in fitting the CO reactions. For this fitting, however, both the binding of O₂ to and the dissociation of O₂ from the noncooperative dimer, as well as the dissociation rate of the liganded dimer must be calculated. However, neither the last equilibrium, Equation 6, nor the association reaction of Equation 5 was directly included in the calculations of the simulated O₂-binding curves. These omissions will be mentioned in "Discussion." The curves in Fig. 2 were calculated with this scheme with the rate constants noted in the legend. Again, the mechanism reproduces the data with reasonably accuracy. The standard deviation of the presentage saturation is ±3.4%.

At low pH for these O₂-binding experiments in the stopped flow apparatus, it is difficult to establish the initial conditions to be used in the fitting procedures. The amount of deoxyHb monomer and dimer present at zero time must be calculated from the value of <i>K₂₁</i> which, of course, is determined from the values of <i>k₅</i> and <i>k₆</i> in the fitting. Since the starting material in flash photolysis experiments is monomeric deoxyHb, treatment of this data is greatly simplified.

These fitted rate constants determine the first four equilibrium
constants of the scheme at pH 5.6 ($K_1 = 8 \mu M; K_{1,1} = 0.5 \mu M; K_3 = 64 \mu M; \text{and } K_4 = 256 \mu M$), and these four constants can be used to predict the equilibrium behavior of the protein. The solid curves drawn in Fig. 4 are calculated from these constants and as these curves show, the mechanism also accurately predicts the equilibrium behavior of the protein. These four equilibrium constants also prescribe the values of the other two constants, $K_3$ and $K_4$. Thus,

$$K_3 = \frac{(K_d)(K_4)}{(K_3)^3} (K_{1,1})$$

and,

$$K_4 = \frac{(K_d)}{(K_1)} (K_{1,1})$$

These two dissociation constants can then be described as a function of the deoxyHb dimer-monomer dissociation constant: $K_3 = 256 (K_{1,1})$, and $K_4 = 8 (K_{1,1})$. Since the ligand-binding rates to the monomer and dimer are pH independent, these relationships should hold at all pH values.

This mechanism readily explains the two apparent anomalies, the discrepancy between a large, pH-independent value of $k$ and a low rate of approach to equilibrium at acid pH, and that between the different estimates of $k'$ (or $k'$) obtained by flash photolysis and stopped flow determinations at low pH. Both of these discrepancies arose by inadvertently equating the properties of the dimer with those of the monomer. In the first case, $k$ refers to the dissociation rate of $O_2$ from Hb$O_2$, while the approach to equilibrium rate depends on the kinetic constants of Hb$O_2$. In the second, at high ligand concentrations, $k'$ measured in flash photolysis experiments is the rate of recombination of $O_2$ with the deoxyHb monomer, whereas $k'$ measured in stopped flow experiments is the rate of $O_2$ binding to the deoxyHb dimer.

Within the framework of this scheme it is also possible to explain the curious shapes of the curves in Figs. 5 and 8. For CO binding (Fig. 8), at low ligand concentrations the rate of CO binding to the dimer is slower than dissociation of Hb$O_2$, and the kinetic properties observed are those of the monomer. At higher CO concentrations ligand binding to Hb$O_2$ is rapid compared to polymer dissociation, and the observed rate of CO binding is expected to be that of the dimer plus a constant increment, $k_d + k_d'/2$, whose value is given by the intercept in Fig. 8 as 4 per sec. This estimate is very close to that sum, 3.5 per sec, calculated from the rate constants used to fit the kinetic data of Fig. 10. Furthermore, the slope of the linear portion of the curve in Fig. 8 gives an estimate of $k_d'$ as 0.12 $\times 10^6$ M$^{-1}$ sec$^{-1}$.

With $O_2$ binding the upward curvature of the plots in Fig. 5 at low $O_2$ concentrations occurs because dissociation of Hb$O_2$ is more rapid than $O_2$ binding by the dimer, and, thus, the kinetic properties observed are those of the monomer which has a large dissociation rate, $k_1$ (i.e. a large y intercept). The slope of these plots at high ligand concentration, 0.25 $\times 10^6$ M$^{-1}$ sec$^{-1}$, is equal to $k_4'$, and this estimate is the same as that which was obtained from the fitting procedures (Fig. 3).
per sec, is still approximately proportional to the O₂ concentration, and therefore, the dimer must react with ligand unless \( k'_d \) is substantially greater than 280 per sec, which is highly unlikely. The present model, which does consider ligand binding to the dimer, also predicts all those features of the \( O_2 \)-binding curves reported previously. Hill plots of simulated saturation curves for pH 7.5 and various protein concentrations are shown in Fig. 10. These Hill plots have the same shape and changes in affinity with concentration that were previously noted by Briehl (2).

Wyman (22) has discussed these equilibrium properties of lamprey hemoglobin, and has shown from thermodynamic arguments how the linkage between ligand binding and subunit dissociation would produce cooperativity although the polymer lacked heme-heme interaction. In this concise treatment Wyman assumed that the affinities of the polymer and the monomer were widely separated, with the monomer possessing the higher affinity, and, thus, that ligand binding induced a transition from 100% polymer to 100% monomer. This limiting case is, of course, very similar to Briehl's scheme which was described above. For this extreme situation Wyman calculated that the maximum \( n \) value at 50% saturation is 1.33 with a dimer-monomer dissociation, and, obviously, when the affinities of the monomer and the dimer approach each other, the \( n \) value becomes appreciably lower.

In our mechanism which analyzes the kinetic basis of these linkage relationships, the interconversion between the low and the high affinity forms does not occur through the dissociation of \((\text{Hb})_3\), but through the dissociation of \(\text{Hb}_2(\text{O}_2)_2\). In this respect the rate of this dissociation reaction, \( k'_d \), is very important. It must be large to provide rapid equilibration such as that which is observed in the kinetic experiments. If it were slow, the approach to equilibrium at high \( \text{O}_2 \) concentrations would be biphasic; a rapid binding to the dimer would be followed by the slower dissociation of \(\text{Hb}_2(\text{O}_2)_2\), and an ensuing increase in the affinity of the solution. No drift phase was observed, and the estimate of \( k'_d \) is quite large, 150 per sec. This is not unreasonable since \( K_3 \) is some 256 times greater than \( K_2 \). Thus, \( k'_d \) could be increased to 256 per sec with no difference between the values of \( k_3 \) and \( k'_d \). Indeed, the estimated rate of dissociation of \((\text{Hb})_3\) at pH 9.0 is itself very large, at least 1000 per sec, as measured by pH jump experiments in the stopped flow apparatus.

The kinetic fitting procedures have ignored the dissociation of the \(\text{Hb}_2(\text{O}_2)_2 \) intermediate. This dissociation constant, \( K_8 \), was estimated to be approximately 6 \( \times \) \( (K_{2,1}) \) at pH 5.6. At low ligand concentrations this omission will complicate the fittings, since by arguments analogous to those presented for the dissociation of \(\text{Hb}_2(\text{O}_2)_2\), the dissociation rate for \(\text{Hb}_2\text{O}_2\) could be 8 per sec. Because this dissociation also produces high affinity species, it would give rise to cooperativity, and would enhance the rate of ligand binding, especially at low ligand concentrations. In fitting the flash photolysis experiments shown in Fig. 10 the dissociation of the \(\text{Hb}_2\text{CO} \) intermediate was considered, and consequently the fittings produced the most accurate fits of all the various attempts.

Our analysis has also neglected the association of the deoxy-Hb dimers to tetramers. Yet, the dimer model adequately describes the behavior of this protein at low pH and at low concentrations, and the functional characteristics observed under these conditions are similar to those seen at neutral pH at higher protein concentrations (2). Furthermore, from the ultracentrifuge examination of \( P. \text{marinus} \) deoxyHb at a variety of pH values, it appears unlikely that substantial amounts of tetrameric deoxyHb exist at neutral pH even at the protein concentrations found in the erythrocyte (20). Tetramers then may have little physiological function in the animal. Indeed, increased association only occurs at very low, nonphysiological pH values (6), pH values which are very close to the protein's isoelectric point, 5.9 for the major component (9). At this pH the protein-protein interactions will be maximized, and any formation of a functionally unimportant polymer will also be at a maximum.

Although the tetramer may not be physiologically important, it is still a potentially interfering species in our experiments at pH 5.6. A small percentage of tetramers will likely be present in these experiments, especially at high hemoglobin concentration, and the kinetic behavior of this molecule would be expected to differ from that of the dimer. The difficulties introduced into the fittings by this polymer would be most pronounced in the stopped-flow experiments where an equilibrium mixture of monomers, dimers, and tetramers would exist at the initiation of the ligand-binding reaction. However, according to the value of the tetramer-dimer dissociation constant, 660 \( \mu \text{M} \) (20), there will only be 3% tetramer present even in the most concentrated solution used, 20 \( \mu \text{M} \) hemoglobin before mixing.

Another probable source of error in these fittings arises from the chemical composition of the protein solution. The use of the hemolsate in many of the experiments makes the presence of some kinetic heterogeneity inevitable. We could have used the purified major component to avoid this problem, but it was found that the purified material oxidized much more readily than the hemolsate. This marked instability of the deoxygenated form of the purified protein makes it difficult to assess the exact chemical composition of solutions containing this protein. A certain improvement in the goodness of fit was obtained by assuming a 10% rapidly reacting contaminant but since there is no way to estimate accurately the amount of any impurity or its expected kinetic behavior, this correction has not been applied in those fittings presented.

Li and Riggs (23) have recently sequenced the main component of \( P. \text{marinus} \) hemoglobin, and have found, as Rudolf, Zelenik, and Braunitzer (24) and Braunitzer and Fujiki (25) had with \( L. \text{flavilias} \), that this protein has only 1 cysteine residue per peptide chain. The reaction of this HbCO with PMB then is quite different from many other protein modification procedures in being both specific and stoichiometric. The rate of reaction of this cysteine is slow compared to the rate at which the \( \beta_6 \) cysteine residue of human hemoglobin reacts with this reagent (26). Rossi-Fanelli, Antonini, and Caputo (27), quoting unpublished results of A. Riggs, reported that the reaction of Hb\(^{2+} \) with lamprey deoxyHb is slower than with HbCO. Thus, this cysteine which is not readily accessible in HbCO is more hindered in deoxyHb. The observed effect of PMB on \( k' \) and \( P \) indicates that distortions of the environment of this cysteine caused by the mercurial make the heme site more accessible to ligand. However, the protein still forms the slowly reacting material. The mercurial therefore does not directly interfere with the interfacial contacts, and, according to the present model, it should not inhibit cooperativity.

One report has appeared whose conclusions are contrary to ours. Rumen and Chance (28) studied CO binding to slurries of crystalline lamprey hemoglobin in which the HbCO and the deoxyHb crystals were virtually isomorphous (29). The bind-
ing of CO by these slurries appeared cooperative, and because of this, the authors concluded that since neither structural changes nor dissociation occurred, some heretofore unrecognized mechanism must be operative in producing cooperativity in these slurries. However, this conclusion must be regarded as speculative, especially considering the notorious difficulties in interpreting ligand-binding reactions to a solid reactant. A complete interpretation of these data must await a full description of the crystal structure of both lamprey deoxyHb and HbCO; these crystal structures are now under investigation (29, 30).

This dimer model gives an excellent approximation of the behavior of lamprey hemoglobin. Yet, for several reasons mentioned it is somewhat incomplete. The use of low pH is an admitted artificiality introduced to make the dimerization reaction more accessible at low hemoglobin concentrations, and the low pH in turn has given us the suspected problem of the association of the dimers to tetramers. Judging from the accuracy of the various fitting efforts, however, these complications appear to be of only minor significance. This mechanism shows how lamprey hemoglobin, a molecule which in the strictest sense lacks heme-heme interactions, is able to exhibit cooperative behavior through linkage of oxygenation to the rapid dissociation of Hb2(O2)2. From the point of view of comparative biochemistry it would be interesting to determine whether other cooperative enzymes found in the lamprey exhibit this unusual mechanism as the basis of cooperative interaction, or whether a more advanced mammalian-like mechanism also exists. For lamprey hemoglobin the proposed mechanism can work because of the compartmentation of the protein in the erythrocytes, thus allowing dissociation and association to occur readily. With intracellular enzymes, which are present at much lower concentrations than hemoglobin, this mechanism would be inefficient since the second order reassociation rate would probably be very slow at the normal levels of enzyme concentration found in the cells.

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