Kinetic Studies on the Binding Affinity of Human Hemoglobin for the 4th Carbon Monoxide Molecule, $L_4$*

(Received for publication, March 31, 1976)

ALICE DEYOUNG, RUSSELL R. PENNELLY, ANNA L. TAN-WILSON,+ and ROBERT W. NOBLES§

From the Departments of Biochemistry and Medicine, State University of New York at Buffalo, Veterans Administration Hospital, Buffalo, New York 14215

$L_4$, the affinity of hemoglobin for the 4th CO molecule, has been determined for human adult hemoglobin (HbA) as a function of pH and the presence of organic phosphates by measuring the kinetic parameters for the reaction. $I_4$, the rate of combination of CO with the triliganded molecule, was measured by flash photolysis while $L_4$, the rate of CO dissociation for the ligand-saturated molecule, was measured by ligand replacement. $L_4$ is pH-dependent and affected by 2,3-diphosphoglycerate. Additionally, this pH dependence of the high affinity state is largely eliminated by carboxypeptidase A digestion. $L_4$ for human fetal hemoglobin (HbF) in phosphate buffers was also determined and found to be pH-dependent. These results cannot be reconciled within the framework of the two-state allosteric model. Additional structures in the conformational equilibrium due to either intermediates in the T to R transition or two or more R states must exist.

The differences in the structural and functional properties of liganded and unliganded hemoglobin are well established and have become the basis for essentially all models explaining heme-heme interaction. Of these, the Monod, Wyman, Changeux model (2) has excited the most interest. In its simplest form, it postulates the existence of only two conformational states of the hemoglobin tetramer. These are a tense (T) state in which the heme groups have a low affinity for ligand, and a relaxed (R) state in which the heme groups have a high affinity for ligand. To a first approximation the main functional properties of human adult hemoglobin, its sigmoidal ligand saturation curve, the Bohr effect, and the effects of DPG and other polyphosphates on ligand affinity, can be explained by this two-state model. It is the concerted structural transition from the low affinity state to the high affinity state that is responsible for the observed sigmoidal, rather than hyperbolic, ligand saturation curve. In addition, protons and organic polyphosphates exert their effects by preferentially binding to the tense structure, shifting the allosteric R ↔ T equilibrium, and resulting in a decrease in ligand affinity. One of the major premises of this two-state model is that the properties of the T and R states are not changed by allosteric effectors but that only the free energy of the R ↔ T transition is altered. The correctness of this premise relative to the R state has already been questioned by McDonald and Noble (3) who demonstrated a pH dependence of the rates of $O_2$ dissociation ($k_1$) and CO dissociation ($L_4$) from both fully liganded human adult and fetal hemoglobins, and by Lindstrom and Ho (4) who detected changes in the NMR spectra of liganded hemoglobin with variations of pH. However, it is conceivable that both of these parameters might vary with pH while the ligand affinity, the parameter of primary importance, remains unchanged. Ligand affinity can be computed from kinetic parameters by taking the ratio of the combination to the dissociation rate constants. Therefore, determination of $I_4$, the rate of combination of carbon monoxide with the triliganded hemoglobin molecule, when coupled with knowledge of $L_4$, permits the calculation of the affinity constant ($L_4 = I_4/L_4$) for the binding of the 4th CO molecule to the hemoglobin tetramer.

The use of flash photolysis techniques to measure the kinetics of the reaction of carbon monoxide with hemoglobin is well documented, beginning with the work of Gibson in 1956 (5). When the photodissociation of the carbon monoxymoglobin is limited to 10% or less, the predominant contribution to the observed recombination reaction comes from the binding of the 4th CO molecule to the triliganded tetramer ($I_4$). The
observed reaction is generally homogeneous and is at least 20-fold faster than the overall reaction of CO with fully deoxyhaemoglobin. This latter reaction is rate-limited by the reaction of CO with the low affinity (T) state of the hemoglobin molecule. We have measured $1'$, for HbA as a function of both pH and the presence of organic phosphates, the most common heterotropic allosteric effectors of hemoglobin. In addition, we have extended the measurements of McDonald and Noble (3) to examine the effects of these same ions on $1'$, the CO dissociation rate constant. Not surprisingly, $1'$, is greatly affected by the presence of inositol hexaphosphate. Also, the computed values of $1'$ for both HbA and HbF are highly pH-dependent. Less expected is a clear effect of 2,3-diphosphoglycerate on the pH dependence of the high affinity state observed in unmodified hemoglobin. Two of these are the isolated subunits of hemoglobin (6) and HbA-CPA (7), hemoglobin from which the COOH-terminal histidine and penultimate tyrosine residues of the $\beta$ chains have been removed by digestion with carboxypeptidase A. In an attempt to further elucidate the functional properties of the R state, flash photolysis studies of $\delta_{\text{ox}}$ chains and HbA-CPA have been carried out. The results are reported and compared to those obtained with normal HbA. Apparently, carboxypeptidase A digestion largely eliminates the pH dependence of the high affinity state observed in unmodified hemoglobin.

**MATERIALS AND METHODS**

**Hemoglobins**—The hemolysate of human adult hemoglobin was prepared from freshly drawn blood according to the method of Geraci et al. (8). After lysis and a preliminary centrifugation to remove the cell membranes, the hemolysate solution was dialyzed overnight against distilled water and again centrifuged at 48,000 x $g$ for 20 min to remove the remaining cell membranes. Fetal hemoglobin (HbF) was found to be free of contamination by HbA and Noble (3). The HbF was found to be free of contamination by the presence of inositol hexaphosphate. Also, the computed values of $1'$ for both HbA and HbF are highly pH-dependent. Less expected is a clear effect of 2,3-diphosphoglycerate on the pH dependence of the high affinity state observed in unmodified hemoglobin. Two of these are the isolated subunits of hemoglobin (6) and HbA-CPA (7), hemoglobin from which the COOH-terminal histidine and penultimate tyrosine residues of the $\beta$ chains have been removed by digestion with carboxypeptidase A. In an attempt to further elucidate the functional properties of the R state, flash photolysis studies of $\delta_{\text{ox}}$ chains and HbA-CPA have been carried out. The results are reported and compared to those obtained with normal HbA. Apparently, carboxypeptidase A digestion largely eliminates the pH dependence of the high affinity state observed in unmodified hemoglobin.

**Phosphorylase-Measurements of Rate of CO Combination**—These experiments were performed with a Xenon Corp. model 472 high energy microprobe with flash energies up to 1000 J. The flash duration was approximately 30 $\mu$s and data points were collected 120 $\mu$s after initiation of the flash. In order to shield the photomultiplier from the photolysing light the flash tubes were surrounded by quartz water jackets containing a solution of auramine hydrochloride (Eastman, T1247). A 1.2-ms delay was used for some earlier experiments since at that time the photomultiplier was not completely shielded from the flash intensity. This longer delay did not appear to affect the $1'$, determinations greatly. The reaction was followed at 420 nm where there is a large optical density difference between the deoxy and carboxy Hb spectra and auramine absorbs strongly. In order to limit dissociation of the hemoglobin to dimers, the hemoglobin concentrations varied from 40 to 65 $\mu$M in heme equivalents. This required the use of a cuvette with a 1-mm optical path length. The cell was surrounded by a wafer jacket through which water at 29.1$^\circ$C was circulated. The total CO concentration varied from 60 to 95 $\mu$M. Data collection and processing was achieved by means of an on-line digital computer essentially as described by DeSa (15).

**Measurement of Rate of CO Dissociation with NO Replacement**—The rate of CO dissociation from liganded hemoglobin ($1''$) was measured by replacement with nitric oxide. All solutions were carefully deoxygenated so that anaerobic conditions were maintained since dithionite could not be introduced into the system. A 1.5-M solution of copper perchlorate in hemoglobin was reduced, carboxylated, and denatured. The solution was then stoppered, anaerobic cuvette with a 1-cm path length. An equal volume of deoxygenated buffer that had been equilibrated with 1 atm of NO and had been kept at a temperature of 29.1$^\circ$C was added to the cuvette. The concentration of NO in was in excess of 1.0 x 10$^{-4}$ M, while that of the hemoglobin for most measurements was approximately 1.0 x 10$^{-4}$ M in heme. Since large optical density changes were observed, the HbA concentration was greater than that previously used by McDonald and Noble (3), requiring the reference cuvette to also contain carbon monoxide. The concentration of NO was determined by measuring the rate of CO combination following NO replacement, the rate measured by partial flash photolysis to the enhanced, carboxylated form, which has a more efficient turnover of bound CO. The measured rate was limited by and equal to the rate of dissociation of the first CO molecule.

In order to determine the effects of the dimer population on these rate constants, $1''$ for stripped HbA was also measured at concentrations of 1.0 x 10$^{-8}$ M and 1.0 x 10$^{-7}$ M in heme at pH 6.5 and pH 9.0. Stopped flow techniques were used in these studies and the previously mentioned on-line computer system were used for these measurements. There is an assumption that is made for both the measurement of $1''$ and the assignment of the rate measured by partial flash photolysis to the constant, $1''$. This assumption is that the species, HbCO, which is the steady state for a particular reaction, is the species formed when an aliquot is added to a solution of HbA, such that the reaction with CO to form HbCO is in the measurement of $1''$ or the reaction with NO to form Hb(ONO)NO in the measurement of $1''$. There exists the possibility that if $1''$, the affinity of the hemoglobin for the third CO, were low enough, and if $1''$, the rate of dissociation of
Hb(CO)₃ to Hb(CO)₂ + CO, were fast enough, our assumption for one or both techniques might be incorrect. However, these circumstances would require the existence of a functional state of the hemoglobin molecule for which no evidence exists. In addition, the value of \( k \) necessary to invalidate our assumption would be orders of magnitude greater than any reported rate of CO dissociation from a hemoglobin derivative.

Measurements of \( O_2 \) Dissociation Kinetics—Both the overall rate of \( O_2 \) dissociation \((k)\) and the rate of \( O_2 \) dissociation with replacement by carbon monoxide \((k,_)\) were measured with a stopped flow apparatus as described by Gibson and Milnes (14) using the same automatic data collection system as with the flash photolysis experiments. A 2-mm optical path length was used. \( k \) was followed at 435 and 415 nm and \( k, \) was followed at 420 and 406 nm. The dithionite solution was prepared by dissolving the reagent in a carefully deoxygenated solution of 1 mM NaOH. The Hb concentration for the determination of both \( k \) and \( k, \) concentration in the \( k, \) determination was 5 x 10⁻⁶ M.

Optical path length was used. \( k \) was followed at 435 and 415 nm and \( k, \) was followed at 420 and 406 nm. The dithionite solution was prepared by dissolving the reagent in a carefully deoxygenated solution of 1 mM NaOH. The Hb concentration for the determination of both \( k \) and \( k, \) was 2.5 x 10⁻⁶ M after mixing in the stopped flow. The CO concentration in the \( k, \) determination was 5 x 10⁻⁴ M.

Oxygen Equilibrium Measurement—The oxygen affinity of HbA-CPA was measured by a modification (16) of the method of Allen et al. (17) using a Cary 14 recording spectrophotometer. Equilibration times were carefully determined at each pH measured. The times required for HbA-CPA were significantly longer than those required for unmodified HbA due to its higher ligand affinity. All measurements were made in the visible region of the spectrum from 500 to 800 nm and the heme concentration was 2.5 x 10⁻⁴ M. The \( n \) value and the pressure of half-saturation of ligand, \( p_0(O_2) \), were calculated from the Hill equation:

\[
\text{RESULTS}
\]

Flash Photolysis of Human Adult Hemoglobin—The rate constants of CO combination with stripped HbA, hemolysate, and stripped HbA + 1 mM IHP after 10% flash photolysis, \( l', \), are shown as a function of pH in Fig. 1. These values were calculated by a least squares fit of the first 60% of the reaction. Additionally, the slopes of the second order rate plots at 50% completion of the reaction were estimated and these rate constants deviated from the values shown in Fig. 1 by no more than +10%. When the observed optical density change was large enough to measure, the rates of CO combination were also calculated after 5% photodissociation of CO. These latter \( l', \) values were the same as those calculated after 10% photodissociation and it was assumed that the reaction observed at either per cent of flash was the same.

The values of \( l', \) for stripped HbA are clearly pH-dependent, ranging from 5.8 x 10⁴ M⁻¹ s⁻¹ at pH 6 to 11.0 x 10⁴ M⁻¹ s⁻¹ at pH 9. The minimum value of \( l', \) for hemolysate, 4.8 x 10⁴ M⁻¹ s⁻¹ occurs near pH 6.5, but the maximum value is the same as that observed for stripped HbA. In addition to the large alkaline Bohr effect, both systems exhibit an acid Bohr effect. At pH 5, the rate constant is approximately 30% higher than that seen at pH 6.0. What should be noted in particular in Fig. 1 is that the \( l', \) values for stripped and unstripped HbA do not differ through a restricted pH range. When the endogenous organic phosphate (DPG) is removed from HbA, the rates of CO combination with the triliganded tetramer are 20% to 40% faster. Although this effect is relatively small, particularly when compared to the effect of the more highly charged polyphosphate, IHP, the differences are consistent. In addition, these results have been duplicated in a comparison of stripped HbA and stripped HbA + 1 mM DPG. The two experiments are indistinguishable and, therefore, the values of \( l', \) for hemolysate and stripped HbA + DPG are equivalent.

It is clear that the allosteric effector, IHP, has a much more pronounced depressant effect on \( l', \) than does DPG. At pH 5, 6, and 7 there are 4-, 6-, and 10-fold decreases, respectively, in the values of \( l', \) when 1 mM IHP is present. Unlike hemolysate and stripped HbA, the combination of the 4th CO molecule with HbA-IHP is an extremely heterogeneous reaction in this pH region. A second order rate plot clearly shows that there is an initial fast phase followed by a later slow phase. Consequently, the \( l', \) values for HbA-IHP which are plotted in Fig. 1 are merely representative of an averaging of these different CO combination rates and, therefore, cannot be interpreted as being characteristic of the whole reaction. In the alkaline pH region, where it is known that phosphates do not bind tightly to the more negatively charged protein and where it is believed the allosteric equilibrium favors the R conformation, the rates for HbA-IHP do approach those measured for stripped HbA and the reactions are homogeneous. In fact, at pH 9, the rate constants are essentially the same.

In the upper graph of Fig. 2 the values of \( l', \) the rate of CO dissociation from the fully liganded hemoglobin molecule, for stripped HbA and stripped HbA plus DPG, are plotted as a function of pH. In the lower graph of this same figure, the value

![Fig. 1 (left)](image)

The second order rate constants for the combination of the 4th CO molecule, \( l', \), are plotted as a function of pH at 25° C. 

![Fig. 2 (right)](image)

Upper panel, the effect of pH and the presence of 0.2 mM DPG on the CO dissociation rate, \( l' \), O, stripped HbA; ●, stripped HbA in the presence of DPG. Lower panel, the effect of pH and the presence of DPG on \( l' \), the affinity constant for the 4th CO molecule. O, stripped HbA; ●, HbA in the presence of DPG.
of $L_4$, the binding affinity of HbA for the 1st CO molecule, is plotted as a function of pH for both the phosphate-free and phosphate-containing material. The values of $L_4$ for HbA were derived from the equation $L_4 = 1''/1'$, the combination and dissociation rate constants being extrapolated from their respective curves.

As reported previously by McDonald and Noble (3), $L_4$ is pH-dependent. The direction of this dependence is such that rather than cancelling the pH dependence of $I_4'$, it adds to it to give a pH dependence for $L_4$, which is greater than that for either of the kinetic constants. For stripped HbA the value of $L_4$ varies by 2.5-fold by going from a low of $0.97 \times 10^9$ M$^{-1}$ to a high of $2.5 \times 10^9$ M$^{-1}$. There is also an obvious acid Bohr effect. Like $L_4$, $l_4$ is affected by DPG, being consistently faster in the presence of DPG from pH 5 to 7.5. The effects of DPG on the two kinetic constants combine to produce an even larger effect on $L_4$, increasing the Bohr effect to a full 3-fold affinity decrease in going from pH 9 to pH 6.5. This finding is strengthened by the results of Kilmartin (18) who showed that DPG binds to the liganded state of hemoglobin.

Fig. 3 is a composite showing the pH dependences of $l_4'$, $l_4$, and $L_4$ for HbF. The CO affinity values were calculated in the same manner as HbA. The values of $l_4'$ are those reported by McDonald and Noble (3). HbF has large pH dependences for all the parameters, the variations being 2.5-fold for $l_4'$, 2-fold for $l_4$, and 5-fold for $L_4$. For $l_4'$, the maximum rate constant is $10.0 \times 10^6$ M$^{-1}$ s$^{-1}$ and the minimum is $3.6 \times 10^6$ M$^{-1}$ s$^{-1}$. The slowest rates of CO dissociation from HbF ($5.5 \times 10^{-4}$ s$^{-1}$) are measured at pH 8, while the fastest rates are measured at pH 5.5 and are nearly twice as fast. Like HbA, the pH dependences of the values of $l_4'$ and $l_4$ are additive, and this is reflected in the 5-fold change in affinity of HbF for the 4th CO molecule over the pH range examined. It should be pointed out that any comparison between HbA and HbF must be done cautiously since the actual levels of endogenous organic phosphates in the chromatographically purified HbF are not known.

Flash Photolysis of HbA-CPA, $\alpha_{SH}$ Chains—In order to compare the pH dependence of $l_4'$ for the intact HbA molecule to that of the reaction of CO with systems postulated to be permanently in the R state, the rates of recombination of CO to isolated $\alpha_{SH}$ chains and to carboxypeptidase A-digested hemoglobin (HbA-CPA) were examined. Fig. 4 is a plot of the pH dependences of the $l_4'$ values for HbA-CPA and $l_4'$ values for $\alpha_{SH}$ chains in the presence of phosphates; the $l_4'$ values for stripped HbA are included for comparison. For both the chains and chemically modified Hb, the CO recombination rates after 10% and 20% flash photolysis were measured and were found to be the same. Since the optical density change is larger after 20% photodissociation of CO, the error in measurement is smaller; therefore, these are the rate constants which are plotted.

Both models for the high affinity conformation exhibit a slight Bohr effect in the acid pH range, but unlike stripped HbA, there is no alkaline Bohr effect. The rate constants for HbA-CPA and $\alpha_{SH}$ chains, throughout the pH range studied, do not deviate far from their average values of $10 \times 10^6$ M$^{-1}$ s$^{-1}$ and $5 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively. This 2-fold difference results in nearly the same values for $\alpha_{SH}$ chains and stripped HbA in the acid pH region, while rate constants measured for HbA-CPA agree well with the $l_4'$ values obtained for stripped HbA at pH 8 and 9.

The overall CO combination rate after full flash photolysis, $I_3'$, was also measured for $\alpha_{SH}$ chains and HbA-CPA. As expected, the rate constants for the monomeric $\alpha_{SH}$ chains after full and partial flash photolysis did not differ significantly, but those measured for the tetrameric HbA-CPA did differ. The $I_3'$ and $l_4'$ values for HbA-CPA are plotted in Fig. 5. Both measurements share a similar pH dependence but the co-
6696 Studies on the 4th Step in Ligand Binding to Hemoglobin

Combination rate of the 4th CO molecule with the triliganded HbA-CPA is 25% to 40% faster than the overall rate of CO combination to the fully unliganded HbA-CPA. Although these differences are relatively small when compared to the 20-fold changes observed in hemolysate or stripped HbA, these results are inconsistent with the belief that the functional properties of HbA-CPA are totally independent of the degree of ligand saturation.

Oxygen Equilibrium of HbA-CPA—Fig. 6 shows the Hill plots of the oxygen equilibrium curves at pH 5.7, 6.5, 7.2, and 7.5 for carboxypeptidase A digested HbA at 30°. Both an acid and alkaline Bohr effect are observed. The lowest O₂ affinity is observed at pH 6.5, where the p₅₀ is 0.63 mm, while the highest O₂ affinity measured is a p₅₀ of 0.26 mm at pH 7.5. Because of the longer time required for complete deoxygenation above pH 7.5, measurements could not be made because of denaturation and oxidation of the protein.

The O₂ affinities of HbA-CPA at pH 5.7 and 7.5 are 40 and 30 times greater, respectively, than the values previously reported for human Hb at 30° in the presence of endogenous DPG (19). Although our values for the O₂ affinity of HbA-CPA are in good agreement with earlier measurements, our values of the Hill parameter (n) are always greater than unity. In Fig. 6, this can be clearly seen in the discrepancy between the dashed line where the slope equals 1 and the solid line that is drawn through the experimental points. The n values, which vary from 1.2 to 1.4, indicate that the binding of oxygen is slightly cooperative.

Bonaventura et al. (20) have shown that IHP will at least partially restore the cooperativity of O₂ binding to HbA-CPA, thus proving that the penultimate tyrosine is not a requirement for this allosteric interaction. Our results demonstrate that even in the absence of IHP, some cooperativity persists.

Because of the very large affinity differences between HbA-CPA and unmodified HbA, this cooperativity cannot be explained by the presence of small amounts of the latter in our HbA-CPA preparations. The heterogeneity resulting from such a contaminant would effectively reduce rather than increase the apparent value of n. This would also be true of a contamination by small amounts of partially digested, des-His, hemoglobin, since hemoglobin with only the COOH-terminal histidine removed has approximately a 10-fold lower oxygen affinity than HbA-CPA. Therefore, the observed cooperativity must be a property of the fully digested material.

The purity of the samples of HbA-CPA has also been examined by starch gel and polyacrylamide disc gel electrophoresis. As expected, the mobilities of the undigested and digested hemoglobins were identical in a starch medium at pH 8.6. However, the HbA-CPA had a greater anodic mobility in the polyacrylamide medium where the upper compartment of the electrophoresis apparatus contained a Tris/glycine buffer, pH 8.9, and the lower compartment contained a Tris/HCl buffer, pH 8.1. Samples of HbA-CPA, the concentrations of which spanned a 100-fold range, A₆₆₀ = 0.4 to A₆₆₀ = 40.0, were examined. There was no trace of unmodified HbA in the HbA-CPA samples.

Oxygen Dissociation from HbA-CPA—In order to explore the effect of this cooperativity on the kinetics of ligand dissociation, the overall rate of O₂ dissociation, k, was compared to k₄, the dissociation rate of oxygen from the fully liganded Hb tetramer. Measurements of these two first order reactions were made at 20° and the rate constants are plotted as a function of pH in Fig. 7. The values of k and k₄ for HbA hemolysate, also at 20°, are included for comparison. Although not presented here, these parameters were also measured for α₂β₂ chains, and these values were identical. k and k₄ for HbA-CPA have identical values, giving no hint of the cooperativity observed in the oxygen equilibrium experiments. The O₂ dissociation rates for HbA-CPA are 10 s⁻¹ from pH 5.2 to pH 9.0 and are nearly the same as the values of k and k₄ obtained for HbA hemolysate above pH 8.5. Of greater interest is the absence of any pH dependence in these

* R. W. Noble, unpublished results.
From the data presented, it appears that the affinity of binding of the 4th CO molecule to triliganded hemoglobin, Hb(CO)$_4$, varies with pH. However, before reaching this conclusion, it is important to consider alternative explanations of the observed results. The conclusion assumes that we are actually examining tetrameric hemoglobin, yet human hemoglobin in the liganded state is known to dissociate to dimer dimers with an equilibrium constant of approximately 2 mM (21). At a heme concentration of 50 μM, 80% of the heme groups should be in tetrameric molecules. Unless the dimers have very different properties from the high affinity state, which available data do not suggest (21-23), the measured rate of l', should be a good estimate of the properties of the tetrameric molecule. In addition, a series of measurements was carried out at a hemoglobin concentration of 10 μM and was indistinguishable from those obtained at the higher concentration. Most of our measurements of l', were carried out at a hemoglobin concentration of 10 μM and these are subject to the criticism that under these conditions almost 40% of the heme groups are contained in dimer dimers. However, measurements were carried out at pH 6 and pH 9 using a hemoglobin concentration of 100 μM. The results obtained differed by less than 10% from those at 10 μM in hemoglobin. A significant variation of l' only appeared when the hemoglobin was diluted to 1 μM. This was caused by a marked increase in the dissociation rate constant by as much as 30%, but these measurements were probably compromised by problems of protein stability at this low concentration. All concentrations of pH dependence of the observed l', persisted. These results make it highly unlikely that the pH dependence of l, is the result of the dimer-dimer equilibrium. The apparent insensitivity of the measured rate constants to the protein concentration in a concentration range in which the fraction of dimeric hemoglobin is expected to vary considerably is itself puzzling. The effects that the presence of dimers might be expected to have on measured values of K$, the affinity of triliganded hemoglobin for the 4th O$_2$ molecule, have recently been discussed by Ackers et al. (24) and should be the same for l'. However, such calculations must assume a set of properties for the hemoglobin dimer for which there is a paucity of data. Indeed, if the properties of the dimer and those of the fully liganded and triliganded tetramer were very similar or identical, no concentration dependence of the apparent values of l' and l' would be expected. Our results suggest that this might be the case. However, these experiments were not designed to test this hypothesis, and it would be a mistake to reach a firm conclusion from the results presented here.

A second important question is whether the reaction observed after partial flash photolysis is truly l'. Assuming random photolysis, 73% of the triliganded heme groups are found on triliganded molecules after removal of 10% of the CO from carbonmonoxoyhemoglobin. The remaining are predominantly on diliganded molecules and a small fraction is on moniliganded molecules. This 27% of the reaction which is not l' should be as rapid as l' if the diliganded molecule is predominantly in the R state or it will have a much slower rate if HbHb(CO)$_3$ is predominantly in the T state. If it is much slower than l', a heterogeneous, biphase reaction should result, but this is not observed. Even if there were a 27% slow phase, the initial rate observed would be slower than the true l', by only that percentage. The fact that homogeneous reactions were observed for stripped HbA, hemolysate, and HbA + DPG suggests that the contribution of l' to these reactions compromises the calculated values of l', very little. This conclusion is further strengthened by the observation that when examined 5% photolysis gave results identical with those obtained with 10% photolysis. Specifically, in the presence of phosphates at pH 6 and pH 7, where the likelihood of the existence of T state in the diliganded molecule is maximal, the values of l' for 10% and 5% photolysis differ by less than 10%, the limit of the experimental method. In addition, the sign of the difference was random. After 5% photolysis, 86% of the unliganded heme groups are on triliganded molecules.

Thus, it appears that one can validly conclude that l', is pH-dependent and is affected by the presence of organic phosphates. This pH dependence is quite unlike the properties of the two high affinity derivatives of hemoglobin, isolated α chains and carboxypeptidase A-digested hemoglobin, which display little or no pH dependence in the different kinetic parameters studied.

One must next consider the implications of this variation of l' with pH on the two-state model. A pH dependence of l', need not require, in all cases, that the properties of the R state be pH-dependent. If there are but two structural states of the protein, 'l' and R, each with a fixed carbon monoxide affinity, L_T and L_R, then the median CO affinity, L_m, in the Monod, Wyman, Changeux (2) formulation results from or is controlled by the conformational equilibrium between these two states. Alternatively, one can visualize the situation by considering the average number of ligands that bind to each of the two structures of the tetramer during the liganding process. If $M_T$ is the average number of ligands that bind to the T state then $M_R = 4 - M_T$ is the average number binding to the R state. The median ligand affinity (L_m) is then given by

$$\log L_m = \frac{M_T \log L_T + M_R \log L_R}{4}$$

Clearly, as L_m approaches L_T, M_T must approach 4. If the conformational transition from T to R occurred as a step function with respect to ligand saturation, then M_T would be the point during liganding at which the structure shifts from T to R. In the real situation, so long as one avoids the boundary states where M_T approaches either 0 or 4, then M_T represents the level of saturation of the tetramer at which the number of molecules in the T state is equal to the number in the R state, $|T| = |R|$. For the triliganded state this will hold true if $M_T = 3$. Therefore, one can select values of L_T and L_R such that the minimum affinity of stripped HbA requires a significant level of T state in the conformational equilibrium of the triliganded molecule. The reader is referred to the recent article by Baldwin (25) for an extensive mathematical treatment of such two-state systems.

If one assumes that the triliganded molecule is entirely in the R state at pH 9 and recalls that the rate of CO combination to the T state is much slower than to the R state, then the observed 50% reduction in l', in going from pH 9 to pH 6 would require that the triliganded molecule be 50% in the T state at pH 6. This model is attractive, since by assuming that l'/L_T for carbon monoxide equals approximately 100 one would require that the fully liganded hemoglobin molecule be 1% in the T state. If the ratio of the rates of CO dissociation from the T and R states ($l'/l_R$) is similar to that observed by Gibson (26) for oxygen; then it is possible to account for most of the pH
dependence of $k_4$ for stripped hemoglobin. Indeed, no glaring contradictions appear when this model is applied to the $O_2$ binding properties of stripped HbA. In part this may be due to the uncertainty in the experimental values of $K_T$ and $K_R$, particularly the large range of $K_T$ values reported by Imai and co-workers (27-29) under various pH and phosphate conditions. However, the presence of 50% T state in the Hb molecules with three oxygen ligands bound can be accommodated by values of $K_T$ and $K_R$ within the published ranges.

In contrast, the effect of DPG on ligand affinity cannot be reconciled within this framework. By binding preferentially to the T state, the effect of DPG at pH 6 would be to shift drastically the relative concentrations of the T and R conformations and thus to cause large changes in $k_4$. Indeed, the fourth binding step would have to be the most affected by DPG at pH 6. This is not the case. Thus, there cannot be large amounts of T state in the conformational equilibrium of the triliganded molecule and in order to reconcile the effect of pH and DPG on $k_4$ with their known effects on ligand affinity, one must invoke the existence of additional structures in the conformational equilibrium, either intermediates in the T to R transition or two or more R states. The states must differ in their ligand and proton affinities and apparently in their DPG affinities as well. The removal of the COOH-terminal histidine and the penultimate tyrosine from the $\beta$ chains by carboxypeptidase A digestion largely eliminates the pH dependence of both $k_4$ and $k_4$, suggesting a direct involvement of these residues in the phenomenon we are observing.

The observed effects of IHP on $k_4$ are in agreement with previously reported effects of this organic phosphate on the ligand binding properties of hemoglobin (11, 30, 31). Below pH 8, IHP causes a very large reduction in $k_4$. At pH 7 this reduction is 10-fold, while at pH 5 and 6 it is somewhat smaller. Similar effects of IHP on oxygenation were observed by Gray and Gibson (31). They calculated the binding affinity for the 4th $O_2$ molecule, $K_4$, and found that in the presence of IHP at pH 7 the value was 13-fold less than for stripped hemoglobin. Although these results can be interpreted as a modification of the R structure, the occurrence of appreciable amounts of the T state at high levels of saturation appears likely. The biphasic kinetic data observed after partial photolysis under these conditions have more than one potential explanation. The two phases could be attributed to the hemoglobin populations in the R and in the T conformations only if the R-T relaxation time were slow relative to the CO combination reaction. Alternatively, they could represent subunit heterogeneity in the IHP-modified R state. The data presented here are inadequate to choose between these and other possibilities.

The inadequacy of a model invoking only two conformations to explain the properties of the hemoglobin molecule has been apparent for some time. In 1970 Perutz (32) distinguished between tertiary and quaternary conformational states in order to permit a sequential release of protons during ligand binding. Lau et al. (33) have clearly shown a pH dependence in the properties of the T state of a trout hemoglobin, and such a dependence of the properties of the T state of human hemoglobin is clear from the results of Imai (27). It now appears that the R state cannot be assumed to be invariant. Thus, a significant contribution to the Bohr effect may come from pH-dependent equilibria between R states and between T states, as well as from the pH-dependent transition from the T to the R conformation.

Acknowledgment—The authors wish to thank Dr. K. M. Wang for the amino acid analyses of HbA-CPA.

REFERENCES

1. DeYoung, A., Tan Wilson, A. L., Pennelly, R. R., and Noble, R. W. (1975) Biophys. J. 15, 80
2. Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol. 12, 66-118
3. McDonald, M. J., and Noble, R. W. (1972) J. Biol. Chem. 247, 4282-4287
4. Lindstrom, T. B., and Hu, C. (1975) Biochemistry 12, 194-199
5. Gibson, Q. H. (1956) J. Physiol. 134, 123-134
6. Noble, R. W. (1969) J. Mol. Biol. 39, 479-491
7. Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., and Caputto, A. (1961) J. Biol. Chem. 236, PC60-PC63
8. Geraci, G., Parkhurst, L. H., and Gibson, Q. H. (1968) J. Biol. Chem. 244, 4984-4987
9. Huehns, E. R., Dance, R., Shooter, E. M., Beaven, G. H., and Gratzer, W. B. (1962) J. Mol. Biol. 4, 329-337
10. Bucci, E., and Fronticelli, C. (1965) J. Biol. Chem. 240, PC531-PC532
11. Benesch, B., Benesch, R. E., and Yu, C. I. (1968) Proc. Natl. Acad. Sci. U. S. A. 59, 526-532
12. Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 769-775
13. Moffat, J. K. (1971) J. Mol. Biol. 58, 79-88
14. Gibson, Q. H., and Gray, R. D. (1970) Biochem. J. 124, 2796-2807
15. DeSa, R. J. (1972) Compt. Rend. 274, 2563-2564
16. Bonaventura, J., Bonaventura, C., Brunori, M., Giardina, B., Antonini, E., Bossa, F., and Wyman, J. (1974) J. Mol. Biol. 82, 499-511
17. Allen, D. W., Guthe, K. F., and Wyman, J., Jr. (1950) J. Biol. Chem. 187, 393-410
18. Kilmartin, J. V. (1974) FEBS Lett. 38, 147-148
19. Antonini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E., and Rossi-Fanelli, A. (1965) J. Biol. Chem. 240, 1069-1103
20. Bonaventura, J., Bonaventura, C., Brunori, M., Giardina, B., Antonini, E., Bossa, F., and Wyman, J. (1974) J. Mol. Biol. 82, 499-511
21. Edelstein, S. J., Rehmar, M. J., Olson, J. S., and Gibson, Q. H. (1970) J. Biol. Chem. 245, 4372-4381
22. Andersen, M. E., Moffat, J. K., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 2796-2807
23. Hewitt, J. A., Kilmartin, J. V., Ten Evck, L. F., and Perutz, M. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 203-207
24. Ackers, G. K., Johnson, M. L., Mills, F. C., Halvorsen, H. R., and Sharpino, S. (1975) Biochemistry 14, 5128-5134
25. Baldwin, J. M. (1975) Prog. Biophys. Mol. Biol. 29, 225-320
26. Gibson, Q. H. (1970) J. Biol. Chem. 245, 3285-3288
27. Imai, K. (1974) J. Biol. Chem. 249, 7607-7612
28. Tynan, L., Iessi, K., and Shinzu, K. (1975) Biochemistry 12, 1491-1498
29. Imai, K. (1970) Biochemistry 29, 798-800
30. Gibson, Q. H. (1971) Biochim. Biophys. Res. Commun. 41, 415-420
31. Gray, R. D., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 7168-7174
32. Perutz, M. F. (1970) Nature 228, 72-739
33. Lau, H. K. F., Walsch, D. E., Pennelly, R. R., and Noble, R. W. (1973) J. Biol. Chem. 249, 1400-1494
Kinetic studies on the binding affinity of human hemoglobin for the 4th carbon monoxide molecule, L4.

A DeYoung, R R Pennelly, A L Tan-Wilson and R W Noble

J. Biol. Chem. 1976, 251:6692-6698.

Access the most updated version of this article at http://www.jbc.org/content/251/21/6692

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/21/6692.full.html#ref-list-1