The Kinetics of the Alkaline Bohr Effect of Human Hemoglobin*

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SUMMARY

Liganded hemoglobin is a stronger acid than ligand-free hemoglobin. The kinetics of the transformation between these states was studied in the pH range 6.0 to 0.0 in 0.3 M NaCl with a pH indicator (phenol red or m-cresol purple) to follow the pH changes accompanying flash photolysis of human carbon monoxide hemoglobin. At least three distinct processes involving protons were detected kinetically: a rapid uptake of $\text{H}^+$ presumably reflecting the shift from the ligand-bound to ligand-free conformation, followed by a slower biphasic release of protons accompanying the dark reaction of CO and hemoglobin.

The fast proton uptake occurred with a rate constant $k = 8000 \text{ sec}^{-1}$ (20°C, pH 7.8), whereas the rate of the transition was greater both at pH 6.9 and 9.0 ($k > 10,000 \text{ sec}^{-1}$). The transition state parameters were also obtained: $\Delta H^* = 11.2 \pm 0.4$ kcal per mole and $\Delta S^* = -3.2 \pm 1.5$ e.u.

The biphasic CO-binding and $\text{H}^+$ release reactions following the flash were analyzed by treating the results as the sum of two simple exponential expressions; this procedure revealed that 27% of the reaction involving protons occurred at the higher rate. The rapidly reacting hemoglobin observed (50%) under these conditions (21°C, pH 7.6, 0.3 M NaCl, when carboxypeptidase B, some of the Bohr effect is lost (9). The Cambridge group (6) concluded that the rate constant for the structural transition from which the decrease in proton affinity arises is greater than 80 sec$^{-1}$, the maximal rate of CO uptake studied.

Perutz et al. (6) recently elucidated the structural basis for most of the alkaline Bohr effect by correlating crystallographic data with the effect of chemical modification of hemoglobin functional groups on the magnitude of the Bohr effect. Benesch and Benesch (1) and Riggs (7) found that reaction of NEM with the $-\text{SH}$ group of cysteine 693 resulted in a substantial decrease in the alkaline Bohr effect, and Kilmartin and Rossi-Bernardi (8) noted that 25% of the pH change disappeared when the NH$_2$-terminal $\alpha$-NH$_2$ groups of the $\alpha$-chains were blocked with cyanate. Similarly, if the COOH-terminal arginine of the $\alpha$-chain is removed by carboxypeptidase B, some of the Bohr effect is lost (9). The Cambridge group (6) concluded that most of the alkaline Bohr effect thus arises from shifts induced by interaction between the amino termini and carboxyl-terminal groups of complimentary $\alpha$-chains as well as between aspartic acid 594 and histidine 5146 of the same chain.

The photolability of HbCO provides a convenient means for studying the kinetics of CO binding by Hb. By using flashes of short duration it is possible to rapidly strip HbCO of its ligand and subsequently observe the kinetic behavior of the resultant Hb in the dark as it rebinds CO. If the lifetime of any intermediate conformation is long compared to the length of the flash, it should be possible to study these states, provided that they can be detected. One means of distinguishing among these intermediates is by comparing their reaction rates with CO with the rates observed under identical conditions when Hb is rapidly mixed with CO.

Three species have been described thus far which react sub-

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Tetrameric human deoxyhemoglobin releases about 2.7 moles of proton to the medium during the process of oxygenation at physiological pH (1). This reduction in the affinity of the protein for hydrogen ions, termed the alkaline Bohr effect after its discoverer (2), is one of several ligand-linked phenomena (3) which are ascribed to conformational differences between oxygenated and deoxygenated hemoglobin (4).

Both the kinetics and structural basis of such conformation changes are obviously of prime interest for understanding the mechanisms involved in the transformations. Antonini et al. (5), by rapidly mixing unbuffered deoxyhemoglobin with CO solutions containing the pH indicator phenol red, found that the CO-binding reaction and the accompanying pH decrease exhibited identical kinetics. They concluded that the rate constant for the structural transition from which the decrease in proton affinity arises is greater than 80 sec$^{-1}$, the maximal rate of CO uptake studied.

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$^*$ The abbreviations used are NEM, N-ethylmaleimide; Hb, hemoglobin; HMB, $\beta$-hydroxymercuribenzoic acid; Mb, myoglobin.

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stantially faster with CO than is evident in flow experiments. The first of these is observed when only a small proportion of the CO is removed photolytically and is attributed to the species of intermediate saturation, Hb(CO)2 (10). The second rapidly reacting type, termed Hb*, is found at pH 9.0 and 10 (11). It is observed apparently because of the finite rate of transformation of Hb from the ligand-bound to the ligand-free conformation under these conditions. The proportion of this material observed depends on the concentration of CO but not on the concentration of hemoglobin.

The third type of rapidly reacting hemoglobin results from symmetrical dissociation of the ligand-bound tetramer to the αβ2 dimer. The proportion of this rapid species depends appropriately on protein concentration as well as on the concentration of added salts such as NaCl (12, 13) or NaClO4 (12) which favor the dimerization process.

The results of the present investigation provide information on the kinetics of the conformational change responsible for the alkaline Bohr effect. The observations are consistent with the structural interpretation of the Bohr effect (6) outlined above as well as the results of Edelstein and Gibson (13), which indicate that the αβ2 dimer is devoid of cooperative interaction in ligand binding.

**EXPERIMENTAL PROCEDURE**

**Materials**—Hemoglobin was prepared by lysing human erythrocytes with distilled water (14). The final stock solutions (about 4 mM heme) were dialyzed against boiled, deionized water (200 volumes, four changes) at 4°C for 48 hours. These solutions were assumed free of CO2 and were stored under argon in tomaneters at 4°C and were used within 10 days of withdrawal of the blood from the subject.

The stock solutions of phenol red (Aldrich) and m-cresol purple (Matheson, Coleman, and Bell) were also prepared with boiled, deionized water and were protected from atmospheric CO2. N-Ethylmaleimide (Sigma), p-hydroxymercurobenzoic acid (Sigma), NaClO4 (Fisher), and crystalline, salt-free sperm whale metmyoglobin (Calbiochem) were used as obtained from the manufacturers.

**Preparation of Solutions for Flash Photolysis**—The apparatus used to prepare the HbCO for the flash experiments as well as to measure the pH and absorbance changes produced in going from HbO2 to Hb to HbCO consisted of a 500-ml round-bottom flask with a 10-mm path length cuvette sealed to the bottom. The flash-cuvette arrangement had provisions for evacuation and gas flushing, as well as an injection port for adding known volumes of gases or solutions from syringes. A Radiometer GK-2021C combination pH electrode sealed in the neck of the flask allowed continuous monitoring of the pH of the solution with a Radiometer PHM 22 meter.

A typical experiment was initiated by injecting 50 ml of CO2-free 0.3 M NaCl into the evacuated apparatus. After adding argon to give a slight positive pressure, the required volume of stock HbO2 solution was injected and the absorbance of the resulting solution was determined at 548, 555, 570, and 578 nm with a Zeiss PMQ II spectrophotometer. The hemoglobin solution was then alternately degassed by suction and flushed with argon for four or five cycles, after which the optical density and pH changes were recorded. The pH was then adjusted to the desired value with dilute NaOH, and argon-purged indicator was added. The absorbance of the solution was redetermined, CO was added from a gas-tight syringe, and a final set of absorbance measurements was recorded.

The procedure described above allowed the calculation of [HbO2] (E∞ = 15.6 at 578 nm) and correction for evaporation of the solvent which occurred during the evacuation procedure (from the absorbance change at the isosbestic point for HbO2, Hb, and HbCO, 548 nm), as well as the pH changes which occurred on the loss or gain of ligand.

**Flash Photolysis Experiments**—The change in state of ionization of the indicators was measured at 548 nm. Ligand binding was followed at the isosbestic point for ionized and un-ionized dye: 482 nm for phenol red or 467 nm when m-cresol purple was used as the indicator.

The flash apparatus was essentially the one described by Parkhurst and Gibson (15) with the following modifications. The flash box contained two vertical flash tubes, each enclosed in a 5-mm thickness of alkaline o-cresolphthalein solution. This filter absorbed most of the energy at 548 nm but passed nearly all of the photochemically effective radiation around 400 nm. When combined with a Corning No. CS4-65 filter situated in front of the entrance slit of the monochromator, observation of the reaction could begin within 75 usec after dissipation of 400 joules of energy through each of the flash tubes. The range of energies available was 200 to 500 joules.

The emitter-follower circuit was replaced with a model 110A operational amplifier (Analogue Devices, Inc.) and the photomultiplier output (EMI 9592B) was fed both to a Tektronix type 3A3 differential amplifier used with a Tektronix 564 storage oscilloscope and to the on-line data acquisition and processing system of DeSa and Gibson (16).

The flash box was equipped to accept either the cuvette tip of the large volume cell described above or a jacketed cylindrical cuvette of 10-mm path length through which water from a constant temperature (±0.5°C) bath was circulated.

**Stopped Flow Experiments**—The Gibson-Durrum apparatus modified for data acquisition and processing (16) was utilized.

**Preparation of Hemoglobin Derivatives**—N-Ethyl succinimide-Hb was prepared as described by Benesch and Benesch (1). The reactive —SH titer was checked by titration with HMB (17) and found to be nil. Before performing the Bohr experiments, the N-ethylsuccinimide-Hb was exhaustively dialyzed against CO2-free deionized water; the stock solutions were stored as described above.

**Effect of Hemoglobin on pKa of Phenol Red and m-Cresol Purple**—The pK of phenol red and m cresol purple was measured spectrophotometrically both in the presence and absence of HbO2. A Beckman DK-2A or a Cary model 14 instrument was utilized.

**Calculation of Results**—First order rate constants (k) were estimated by fitting the data from flash experiments to Equation 1:

\[ v_t = v_o + (v_\infty - v_o) e^{-kt} \]  

where \( v_o, v_\infty, \) and \( v_t \) are the change in photomultiplier output (ΔAnv) at the initiation, at time \( t \), and at the completion of the pH increase. The method of Guggenheim (18) was used to estimate \( v_o, v_\infty, \) and \( k \) from \( v_t \) and \( t \); these three parameters were adjusted to give the best least squares fit to the data set by an iterative procedure (19) with a PDP/8S computer.

The biphasic reaction records obtained from the CO binding and the H+ release reactions were treated as if they resulted from the sum of two independent first order processes. The two rate
constants and the fraction of the rapid component present were estimated by fitting the data to the two-exponential expression by an iterative least squares method. The estimates of $\Delta S^*$ and $\Delta H^*$ were calculated by a least squares analysis of the temperature dependence of $k$.

RESULTS

Interaction of Hemoglobin with pH Indicators—The pK values of phenol red and m-cresol purple were determined in the presence and absence of $HbO_2$ to test the possibility of a dye-$HbO_2$ interaction. Antonini et al. (20) found that the pK of bromthymol blue shifted from 7.1 to a value greater than 8 in the presence of $HbO_2$. On the other hand, a similar study with phenol red (8) failed to provide evidence for an interaction between this indicator and oxyhemoglobin since the pK (7.55) remained the same whether or not $HbO_2$ was present. The results obtained here confirm that $HbO_2$ does not affect the ionization of phenol red; the reported pK value (21) of 8.3 for m-cresol purple was observed both in the presence and absence of $HbO_2$ as well.

Control Experiments—The following experiments were carried out in order to establish that the absorbance changes measured resulted from pH changes induced by the displacement from and subsequent binding of CO to Hb.

A solution of $HbCO$ in unbuffered 0.3 M NaCl (pH 7.80) was subjected to flash photolysis; an absorbance change was noted at 548 nm only when phenol red or m-cresol purple was present. If the $HbCO$-dye solution was made 10 mM in potassium phosphate at pH 7.8 by injecting the requisite amount of O$_2$-free buffer, the absorbance change at 548 nm was abolished. Similarly, when MbCO, which has a negligible Bohr effect (22, 23), was used in unbuffered 0.3 M NaCl, pH 7.80, with m-cresol purple, no transmittance changes were found at 550 nm, the isosbestic point for Mb and MbCO.

In addition, a comparison of the pH profile of the Bohr effect as measured in static experiments (24) and the magnitude of the absorbance changes measured after flash photolysis are given in Fig. 1. The observed curves have the shape which would be expected only if the absorbance change reflected the pH dependence of the Bohr effect.

Finally, the spectrum obtained by measuring the wavelength dependence of the absorbance change which occurred during the initial 300 usec after the flash is shown in Fig. 2. The kinetic spectrum agrees reasonably well with the pH difference spectrum of phenol red.

Effect of Flash Energy—Table I contains data illustrating the effect of partial photolysis on the magnitude of the Bohr effect. Within the error of the experiments, proton uptake is proportional to the amount of CO displaced. This proportionality appears to be maintained even at low percentages of breakdown at which the predominant tetrameric species should be $Hb(\text{CO})_3$ (10).

Kinetics of Proton Uptake by Hb—A representative experiment showing the kinetics of the pH increase following flash photolysis of $HbCO$ is illustrated in Fig. 3A; the corresponding first order kinetic plot is given in Fig. 4. The oscilloscope record in Fig. 3B shows the results of a similar flash experiment in which myoglobin was used instead of hemoglobin. The dotted lines in each panel of Fig. 3 indicate the equilibrium absorbance of the pH indicator at 548 nm both before and a few hundred milliseconds after discharging the flash tubes. The point in Fig. 3A represents the

![Fig. 1. The pH dependence of the Bohr effect. The points were obtained from the observed absorbance changes at 548 nm when 36 $\mu$M $HbCO$ in 0.3 M NaCl was subjected to flash photolysis at 25°. The changes are normalized to that at pH 7.8. The line was calculated from the data of Antonini et al. (24). The indicator was phenol red (35 $\mu$M).](http://www.jbc.org/)

![Fig. 2. Comparison of the wavelength dependence of the absorption changes induced by flash photolysis of $HbCO$ (points) with a pH difference spectrum of phenol red (line). The absorbance changes are given relative to the maximum at 556 nm. $[HbCO] = 36 \mu$M, [phenol red] = 35 $\mu$M, 0.3 M NaCl, pH 7.70, 25°.](http://www.jbc.org/)
Fig. 3. Oscilloscope records of the flash photolysis of HbCO (A, 24 μM) and MbCO (B, 29 μM). Conditions: 0.3 M NaCl, pH 7.80, 21 μM m-cresol purple, 25°. The measuring wave length was 548 nm in A, and the absorbance change was 0.018. In B, the observation was carried out at 550 nm. The initial [CO] was approximately 80 μM. See text for significance of the other symbols.

Fig. 4. First order plot of the data of Fig. 3A. The points represent the observed data, whereas the line was obtained from the least squares procedure described in the text.

Table II

<table>
<thead>
<tr>
<th>HbCO</th>
<th>pH</th>
<th>Indicator</th>
<th>k x 10^3 ± S.E.</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>8.05</td>
<td>Phenol red</td>
<td>10.2 ± 0.7</td>
<td>25°</td>
</tr>
<tr>
<td>42</td>
<td>8.05</td>
<td>Phenol red</td>
<td>10.3 ± 0.3</td>
<td>25°</td>
</tr>
<tr>
<td>36</td>
<td>7.75</td>
<td>Phenol red</td>
<td>8.4 ± 0.2</td>
<td>22°</td>
</tr>
<tr>
<td>29</td>
<td>7.80</td>
<td>m-Cresol</td>
<td>7.5 ± 0.3</td>
<td>21°</td>
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<tr>
<td>27°</td>
<td>7.74</td>
<td>m-Cresol</td>
<td>8.2 ± 0.3</td>
<td>22°</td>
</tr>
</tbody>
</table>

* N-Ethylsuccinimide derivative of carbon monoxide hemoglobin.

The values of the change in photomultiplier output calculated from Equation 1 with the data of Fig. 3A (or extrapolated from the least squares line in Fig. 4). The apparent discrepancy between the “observed” and “calculated” zero time value for the absorbance of the solution would indicate that during the dead time of the detector (approximately 75 μsec) the pH of the solution decreased somewhat (i.e., the absorbance less) compared to the equilibrium state preceding the flash. This phenomenon did not appear when myoglobin was used instead of hemoglobin, since the excursion resulting from perturbation of the detector by some of the flash energy returned to the baseline value within the 75-μsec dead time (Fig. 3B).

The values obtained for k under a variety of conditions are summarized in Table II and in Fig. 5. The rate constant for proton uptake was not affected by the concentration of phenol red over a 2-fold range as well as by the chemical nature of the indicator. However, when k was measured as a function of the equilibrium pH of the solution, a minimum of approximately 8,000 sec⁻¹ was found in the region about pH 7.8 (Fig. 5). The data at the extremes of pH 7 and pH 9 are less extensive and subject to greater uncertainty because of the diminished magni-
tude of the Bohr effect as well as the decreased sensitivity of the indicators. The rate constant, however, increases to about 12,000 sec⁻¹ at either of these extremes.

The effect of temperature on k was observed in the range 3–55°C. Values of 11.2 ± 0.4 kcal per mole and −3.2 ± 1.5 e.u. were obtained for ΔH* and ΔS*, respectively.

Kinetics of Proton Release and CO Uptake—Antonini et al. (5) noted that the pH decrease and the CO-binding reactions were congruent in time. This result was confirmed under the present conditions in stopped flow experiments in which only slowly reacting hemoglobin was observed.

The kinetic results obtained by flash photolysis (Fig. 6) were, in contrast, distinctly biphasic in both the CO-binding and the H⁺ release reactions. The fraction of the total reaction arising from the rapid process was 51.0 ± 1.3% in the case of the CO reaction, whereas only 27.2 ± 1.8% of the protons were released rapidly. While the pseudo-first order rate constant for the CO reaction (14.3 ± 0.6 sec⁻¹) was nearly identical with the apparent first order rate constant for proton release (15.7 ± 0.5 sec⁻¹), the corresponding rate constants describing the rapid process differed significantly: 291 ± 16 sec⁻¹ for CO uptake and 216 ± 25 for H⁺ release.

The proportion of CO bound in the rapid phase depended on the concentration of HbCO present before flash photolysis. This relationship is depicted in Fig. 7, in which the curve was calculated assuming a value of 12.5 μM for the dissociation constant K₄.2 for the process [α(CO)(β(CO))]₂ → 2α(CO)β(CO). The reversibility of this reaction was established by preparing the solutions by adding successive aliquots of 3.5 mM stock HbCO to the same 0.3 M NaCl solution. The most concentrated of these was then diluted by adding successive aliquots of deoxygenated 0.3 M NaCl. The pH was checked after each addition and maintained by adding acid or base as required.

The value of K₄.2 estimated from the data of several similar experimental series of the type shown in Fig. 7 varied within the range 8 to 15 μM. K₄.2 was, however, always greater than the value 1.5 μM calculated by Edelstein and Gibson (15) from ultracentrifuge and kinetic data obtained in 0.1 M phosphate, pH 7.0. The higher proportion of rapid material is not unique to these dialyzed hemoglobin preparations. A fresh sample of
effect was studied at high ionic strength in 1,2,2-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol, pH 7.0. Most of the rapidly reacting material reverted to the slower form when free hemoglobin was subjected to flash photolysis in 0.05 M NaClO4, a salt which promotes the dissociation of hemoglobin dimers to tetramers.

The range of velocities of ligand binding can be considerably increased by subjecting HbCO to photolysis in the presence of O2 (10, 11) because of the greater reactivity of O2 with deoxy-hemoglobin compared to CO. Thus, if the apparent lag in proton release noted above was the result of a rate-limiting conformational change, an even clearer separation between ligand binding and H+ release should be evident when the velocity of ligand binding is considerably increased by including O2 in the system. Fig. 8 illustrates the results of three such experiments. At pH 7.8 or 7.9 the release of protons induced by O2 binding appears to lag behind the latter reaction. At pH 6.95, however, the pH change followed the same time course as the O2 reaction.

\[
\text{HIn} \rightleftharpoons \text{H}^+ + \text{In}^- \\
\text{HIn} + \text{OH}^- \rightleftharpoons \text{In}^- + \text{H}_2\text{O} \\
\text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^- 
\]

The response time of the reaction represented by this scheme to an instantaneous pulse of acid or base is given by the equations of Eigen (27) which describe the relaxation spectrum of the system. For the conditions of pH and indicator concentration used, a time constant on the order of 10 μsec may be calculated, a response which is fully adequate for tracking the pH changes reported here. An analogous argument indicates that proton binding (following photolysis) or proton release (accompanying CO or O2 binding) by the Bohr groups is also not rate-limiting.

**Proton Uptake by Photolytically Produced Hemoglobin**—The arguments given above strongly suggest that the rate of the pH increase is a true indication of the rate of the conformational change which is responsible for the Bohr effect. It is difficult with the information presently at hand to establish any relationship, however, between the kinetics of proton binding by photolytically produced Hb and the conversion of Gibson's (11) Hb* to conventional Hb. Both processes presumably involve conformational changes in the ligand-free hemoglobin molecule.

The implications of the experiment in which only a small proportion of the HbCO is photolyzed are consistent with recent studies with conformation-sensitive spin labels. When bonded to the -SH group of cysteine β3, these labels were sensitive only to the state of ligation of the particular β-chain (28).

**FIG. 8.** Plot of percentage remaining Hb (measured at 487 nm) and H+ released (measured at 548 nm) after photolysis of HbCO. Filled symbols refer to the pH change and open symbols to the CO-binding reaction. A (circles): [HbCO] = 21 μM, [m-cresol purple] = 31 μM, pH 7.90; B (triangles): [HbCO] = 29 μM, [phenol red] = 10 μM, pH 7.90; C (squares): [HbCO] = 30 μM, [phenol red] = 10 μM, pH 6.95. (In each experiment, pCO = 30 mm Hg, pO2 = 60 mm Hg, 0.3 M NaCl, 20°C.)

HbCO diluted to 40 μM in oxygen free 0.3 M NaCl, pH 7.5, showed approximately 40% of the fast CO reaction on flash photolysis. When O2-free phosphate buffer was added to give a final phosphate concentration of 0.1 M, pH 7.4, the proportion of rapid material dropped to less than 10% of the total CO-binding process.

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Apparently, the displacement of a single CO is sufficient to induce the uptake of a proportional number of Bohr protons as well.

The reasons for the inability to make the observed and calculated zero time values of the absorbance at 548 nm coincide remain obscure. Since the effect did not occur when MeCO was substituted for HbCO, the conclusion seems inescapable that the "discrepancy" represents a real contribution of hemoglobin to the chemistry of the system and does not result from some unknown artifact associated with the flash apparatus or its attendant electronics. One possible explanation is that the ligand displacement is accompanied by an ultrarapid production of some acid. It may be pertinent in this regard to note that some pH-dependent relaxations have been observed in the nanosecond time range (29).

The effect of temperature on the rate constant for the change in conformation allows an estimation of the entropy changes involved in the formation of an activated complex. The picture of an activated complex generally used in describing the mechanism of protein denaturation is one in which the simultaneous rupture of a number of hydrogen bonds has occurred. \( \Delta S^* \) is large and positive, as expected in view of the generally unfolded nature of the alkaline Bohr effect, as well as the experiments involving N-ethylsuccinimide, described above. The latter fraction is very close to the observed value of 30\% (Fig. 6). The concentration dependence (Fig. 7) of the rapid phase almost unequivocally labels it as dimeric species.

This report is not the first in which a substantial part of the total photochemically produced Hb is rapidly reacting in the absence of phosphates. Gibson and Parkhurst (32) found that hemoglobin stripped of organic phosphate and examined by flash photolysis in 0.1 M NaCl was kinetically heterogeneous. Recent work in at least two laboratories (33, 34) confirms that ligand-bound hemoglobin, as well as deoxyhemoglobin, binds organic phosphates, despite an earlier report that the former had no affinity for 2,3-diphosphoglycerate (and, presumably, inorganic phosphate). In view of the linkage (3) between ligand binding, phosphate binding, and protein polymerization, it is not surprising that the value of the apparent dissociation constant \( K_{d,1} \) depends on phosphate.

The flash Bohr experiments also lead to the conclusion that the dimeric species derived from ligand-bound hemoglobin (i.e. the \( \alpha_2\beta_2 \) species (35)) is devoid of cooperative interactions. The argument may be outlined as follows. Hemoglobin chains do not exhibit a Bohr effect (36). Hemoglobin which displays rapid kinetic behavior in its reaction with \( O_2 \) or CO (i.e. isolated chains (37, 38) or HbH (39)) lacks the type of so-called heme-heme interaction necessary for the sigmoidal ligand saturation curve. Because the fast material of Fig. 6 has a Bohr effect associated with it, it cannot consist of chains and therefore must be dimeric. If it is dimeric and fast, it cannot exhibit cooperative behavior. It must therefore be concluded that tetramers are necessary for the functional integrity of the hemoglobin species generated under the conditions of these photolysis experiments. Other kinetic and structural evidence concerning the tetramer hypothesis has already been presented by Gibson and Parkhurst (32) and Edelstein and Gibson (13).

The meaning of the CO-\( O_2 \) competition experiments of Fig. 8 is difficult to ascertain. Unfortunately, the interpretation is not simple because the distribution of the Bohr effect among the possible species present immediately after the flash is not known. At sufficiently high concentrations of \( O_2 \), no pH change followed the flash, presumably because the \( O_2 \) present reacted with the Hb before the latter could be transformed to the species characterized by an increased proton affinity.

The rate at which ligand-bound hemoglobin forms the species with lower proton affinity at \( \text{pH} 6.95 \) must exceed 1850 sec\(^{-1} \), which was the rate found for \( O_2 \) binding (Fig. 8). This conclusion follows from the observation that both the pH change and ligand-binding reaction were kinetically identical.

In conclusion, the studies described in this communication, in addition to showing that it is possible to measure the kinetics of the fast conformational changes responsible for the alkaline Bohr effect in hemoglobin, provide a clear example of the relation between structure, as determined by x-ray crystallography and chemical modification, and function, as determined kinetically. It also provides dramatic illustration of the importance of structural information for the complete interpretation of kinetic results in complex systems.

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