

THE RELATION BETWEEN CARBON MONOXIDE BINDING AND THE CONFORMATIONAL CHANGE OF HEMOGLOBIN

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ABSTRACT The spectral difference between normal and rapidly reacting deoxyhemoglobin (Sawicki and Gibson [1976], *J. Biol Chem.* **251**:1533-1542) is used to study the relationship between CO binding to hemoglobin and the conformational change to the rapidly reacting form in a combined flow-laser flash experiment. In both pH 7 phosphate buffer and pH 7 bis(2-hydroxy-ethyl)imino-tris (hydroxymethyl)methane buffer (bis-Tris) with 500 μ M 2,3-diphosphoglycerate (DPG), the conformational change lags far behind CO binding; rapidly reacting hemoglobin is not observed until more than 10% of the hemoglobin is liganded. In pH 9 borate buffer the formation of rapidly reacting hemoglobin leads CO binding by a significant amount.

A simple two-state allosteric model (Monod et al. [1965], *J. Mol. Biol.* **12**:88-118) which assumed equivalence of the hemoglobin subunits in their reaction with CO was used to simulate the experimental results. In terms of the model, the conformational change lead observed at pH 9 suggests that significant conformational change has occurred after binding of only one CO molecule per tetramer. In the presence of phosphates good agreement between experimental results and simulations is obtained using parameter values suggested by previous experimental studies. The simulations suggest that the conformational change occurs after binding of three CO molecules.

INTRODUCTION

Ligand-linked conformational changes of the hemoglobin molecule are generally thought to be responsible for the sigmoidal binding curves observed for oxygen (1) and carbon monoxide (2). Several experimental techniques have been used to study the relationship between fractional ligation and conformational change in an attempt to test models describing cooperative ligand binding by hemoglobin. Equilibrium experiments have usually shown a proportionality between saturation and conformational change (3-6) as might be expected in view of the great difference (several hundredfold) in the affinity of hemoglobin for the first and last molecules of ligand. Unless some special requirements are imposed, this affinity difference means that intermediate species will be sparsely populated, with approximate proportionality as a necessary consequence. Such experiments have little value in testing specific models for hemoglobin cooperativity. Equilibrium experiments involving hemoglobin spin-labeled at the β 93 position will not be discussed in detail because, in relation to native hemoglobin, these modified hemoglobins have a very high affinity which is relatively insensi-

tive to phosphates (3, 7). Considerable disruption of functional properties by the spin label is not surprising in light of structural studies (8).

Kinetic techniques combining rapid mixing with other methods have provided a more powerful approach to the study of conformational changes in hemoglobin because nonequilibrium distributions of intermediates are present in these experiments. The release of a fluorescent analogue of 2,3-diphosphoglycerate (DPG) was shown to lag CO binding in flow-fluorescence experiments (9). Experiments combining flow with partial photolysis (10) showed that the formation of rapidly reacting hemoglobin lagged CO binding in pH 7 phosphate buffer.

In the present experiments, a new flow-laser flash technique is presented which makes use of the spectral difference between rapidly reacting deoxyhemoglobin and normal deoxyhemoglobin to measure the extent of conformational change at various times after mixing of deoxyhemoglobin and CO solutions. This rapidly reacting transient species has been interpreted as deoxyhemoglobin remaining in the carboxy-hemoglobin conformation after the sudden removal of ligand by a laser pulse. At 20°C in pH 9 borate buffer this species relaxes to normal deoxyhemoglobin at a rate of $6,500\text{ s}^{-1}$ and much more quickly in the presence of phosphates at pH 7 (11). The primary advantage of this technique is that the relation between ligand binding and conformational change can be studied for unmodified hemoglobin under a wide range of conditions both in the presence and absence of phosphates. In this study the population of rapidly reacting hemoglobin present after mixing is found by direct absorbance measurements rather than by inference from the kinetics observed after flow-partial photolysis (10). Analysis of the partial flash experiments requires the assumption that the conformational distribution present after the flash reaches equilibrium before significant ligand binding occurs at the rapid rate (10, 12). This assumption is not necessary in the analysis of the present experiments.

MATERIALS AND METHODS

Hemoglobin was prepared and freed of residual CO as previously described (11). Deoxygenated stock solutions, typically 5 mM in heme, were stored at 4°C in a tonometer and used within 5 days of preparation. All experiments were carried out at 20°C. Buffers and dilute working solutions of deoxyhemoglobin and CO were prepared as discussed previously (11). The experimental geometry (11) and apparatus (13) are similar to those used in previous studies. Aside from a brief description, only differences in experimental approach will be discussed here.

A Gibson-Milnes stopped-flow apparatus (14) was used to mix deoxyhemoglobin and CO solutions. The photolyzing dye laser pulse (0.2 J at 540 nm) and the observation beam enter the same window of the 2.7-mm path length optical cell. The ends of this 2 mm diameter optical cell were sealed with large windows (6 mm diameter, 1.5 mm thick) epoxied to the metal surface to allow a free path for the laser pulse and sample beam. The stopped flow was positioned so that the optical cell takes the place of the sample cell in the previously described experimental geometry (11). Experiments performed by mixing myoglobin and CO solutions and fully photolyzing the mixture after 2 ms gave a mixing dead time of 1.1 ± 0.2 ms. Calculations suggest that the temperature jump associated with the absorption of laser light by the hemoglobin is $<0.1^\circ\text{C}$ (for a 0.2 J pulse and $20\ \mu\text{M}$ hemoglobin).

Changes in the absorption of light by the mixture were detected photoelectrically and voltage

changes digitized with a transient recorder (Biomation, Cupertino, Calif., model 805) and transferred to a PDP 8/E minicomputer (Digital Equipment Corp., Marlboro, Mass.) for averaging and conversion to absorbance. In the flow-flash mode, an electronic time delay started by the stopping syringe was used to fire the laser and start data collection after an adjustable delay.

Data were collected at two wavelengths. The fractional saturation of hemoglobin with CO was observed as a function of time after the stopping of flow at the isosbestic point of rapidly reacting deoxyhemoglobin and normal deoxyhemoglobin near 436 nm (11). At this wavelength the absorbance change is proportional to ligation. The formation of rapidly reacting hemoglobin was observed at the isosbestic point of deoxy and carboxyhemoglobin near 425 nm. The initial absorbance excursion at this wavelength after full photolysis is taken to be proportional to the concentration of rapidly reacting hemoglobin (11) present in the mixture just before the flash. The initial absorbance excursion in a flow-flash run was recorded as a function of the time delay. The fraction of hemoglobin in the rapidly reacting state as a function of time was obtained by dividing these absorbance excursions by the full absorbance excursion observed after the hemoglobin was fully liganded (2-s delay). A more detailed discussion of the properties of these isosbestic points and the methods used to locate them has been given previously (11).

RESULTS AND DISCUSSION

Fig. 1 presents plots of the fraction of rapidly reacting hemoglobin vs. fractional ligation for several CO concentrations in pH 9 borate and in pH 7 phosphate buffer. Within experimental error, the fraction of rapidly reacting hemoglobin depends on fractional saturation but not on CO concentration over the twofold range of concentrations studied. Under these conditions, it appears that the conformational changes

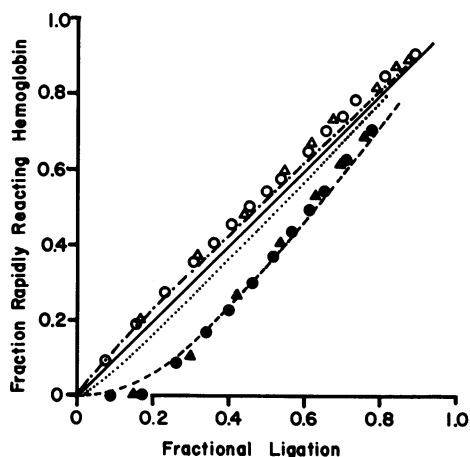


FIGURE 1 The dependence of the fraction of hemoglobin in the rapidly reacting state on fractional ligation, measured as described in the text. The solid diagonal line represents proportionality between conformational change and ligation. The open symbols correspond to experiments in pH 9 borate buffer whereas the closed symbols resulted for experiments in pH 7 phosphate buffer. The hemoglobin and CO concentrations after mixing were: ○, 21.5 and 45 μM ; △, 20.3 and 90 μM ; ●, 18.5 and 45 μM ; and ▲, 20 and 90 μM . The broken lines are the result of simulations discussed in the text.

responsible for the formation of rapidly reacting hemoglobin are quick by comparison with CO binding. Rapid mixing experiments have led to similar conclusions (15). The broken lines are the results of simulations that will be discussed later in this section.

The simplest form of two-state allosteric model (16) has proven to be a useful tool for description of a wide range of the equilibrium and kinetic properties of hemoglobin (12, 17). This model, which neglects differences between hemoglobin subunits in reactions with CO, is adopted in the analysis of the present experiments, for although there is some evidence for subunit differences in the presence of phosphates (18, 19), too little is known to justify the use of a more complex model.

Eqs. 1–3 give the adaptation of the two-state model used to simulate the results of these experiments. L and c are, respectively, the allosteric parameter and the ratio of the microscopic dissociation constants for the R and T states. Hb^R and Hb^T represent the R and T conformations of hemoglobin. Eqs. 1 and 2 give the reactions assumed to occur between hemoglobin and CO. l'_R and l'_T are, respectively, the microscopic rate constants for CO combination with R and T state hemoglobin. Similarly, l_R and l_T are the microscopic rate constants for dissociation from the R and T states. The original statement of the two-state model (16) assumes that the affinity of a hemoglobin molecule depends only on conformational state. It has been further assumed in Eqs. 1 and 2 that the rate constants depend only on conformational state (12). Eq. 3 expresses the conformational equilibrium (17) assumed to apply during the flow part of these experiments. Dissociation of hemoglobin into dimers is not considered in this form of the two-state model.

An upper limit to the fraction of carboxyhemoglobin present as dimers may be obtained from the fraction of rapidly recombining hemoglobin observed after flash photolysis (11, 20). 10 s after mixing a 90- μ M CO solution with a 40- μ M hemoglobin

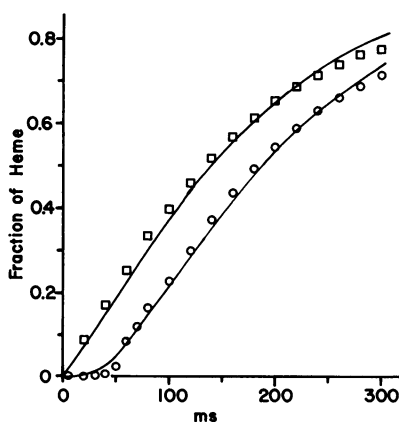
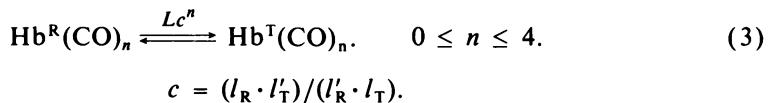
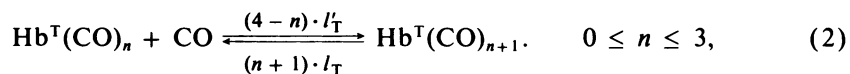
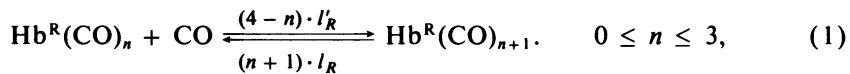


FIGURE 2 Fraction of CO-bound heme (□) and the fraction of heme in the rapidly reacting state (○) plotted against time for pH 7 phosphate buffer. The hemoglobin and CO concentrations were 18.5 and 45 μ M. The solid curves are the result of a simulation using the two-state model described in the text for the parameter values: $l_R = 0.01 \text{ S}^{-1}$, $l'_R = 6.5 \mu\text{M}^{-1} \text{ S}^{-1}$, $l_T = 0.1 \text{ S}^{-1}$, $l'_T = 0.09 \mu\text{M}^{-1} \text{ S}^{-1}$, and $L = 1.4 \times 10^7$.

solution, full photolysis produced recombination kinetics at 436 nm that were 15% rapid for pH 9 borate buffer and 10% rapid for pH 7 phosphate buffer and pH 7 bis-Tris buffer with 500 μ M DPG. Recombination of deoxy dimers to form tetramers is negligible on the time scale of the rapid CO binding reaction in these experiments (21). Flow-flash experiments begin with deoxyhemoglobin which is dimerized to a negligible extent (22) so that in the worst case <15% of the hemoglobin is in the form of dimers during these experiments. Dimers, which have a deoxy spectrum similar to that of rapidly reacting deoxyhemoglobin (23), form primarily from liganded *R* state hemoglobin so they should not have an important effect on the present experimental results.

Differential equations derived from Eqs. 1–3, with appropriate parameter values, were solved numerically with a PDP 8 computer to produce simulations for comparison with experimental data. Further discussion of the simulation process is given in the Appendix.

Fig. 2 presents flow-flash data for pH 7 phosphate buffer. The open squares give the observed fraction of heme with bound CO as a function of time,



and the open circles give the observed fraction of heme in *R* state molecules as a function of time. The solid curves are simulations using the parameter values listed in the legend. The dissociation rate constants were obtained by assuming that the dissociation rates observed for HbCO and Hb(CO)₄ (24) reflect the properties of the *T* and *R* states, respectively.

The value used for l'_{T} is reasonable in relation to measurements of the second-order rate constant for CO binding as a function of fractional ligation (25, 26). l'_{R} was determined from partial photolysis experiments as previously described (12). Accurate determination of equilibrium curves for CO binding to hemoglobin has not been possible due to the high binding affinity (2), so that the allosteric parameter L , which is ligand independent, is taken from a laser photolysis study in which both equilibrium and kinetic data were obtained simultaneously for partially oxygenated hemoglobin solutions (13). The spectra and relaxational properties of rapidly reacting deoxyhemoglobin formed by photolysis of oxy and carboxyhemoglobin are very similar (27), in agreement with two-state model predictions of ligand independence.

The broken lines in Fig. 1 present two-state model simulations using parameter values taken from the legend to Fig. 2. The dotted line is derived for equilibrium con-

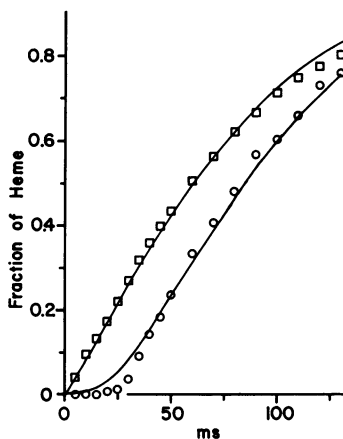


FIGURE 3 Fraction of CO-bound heme (\square) and fraction of heme in R state molecules (\circ) plotted against time for pH 7 bis-Tris buffer with 500 μM DPG. The hemoglobin and CO concentrations after mixing were 20.3 and 90 μM . The solid curves are the result of a simulation using the two-state model described in the text for the parameter values: $l_{\text{R}} = 0.01 \text{ S}^{-1}$, $l_{\text{R}} = 6.5 \mu\text{M}^{-1} \text{ S}^{-1}$, $l_{\text{T}} = 0.1 \text{ S}^{-1}$, $l_{\text{T}} = 0.1 \mu\text{M}^{-1} \text{ S}^{-1}$, and $L = 3 \times 10^7$.

ditions corresponding to Eqs. 1–3, whereas the dashed curve results from the simulation of the flow-laser flash experiment presented in Fig. 2. These simulations suggest that it should be difficult under equilibrium conditions to discriminate experimentally between a spectral change linked to ligation and one linked to quaternary structural changes. Experimental studies have generally been consistent with these conclusions (3–6, 28). In terms of the two-state model, the large lag in the formation of R state hemoglobin in the present experiments results because of the slow dissociation rate of CO. Thus redistribution of ligand to form more high affinity R state molecules after initial statistical binding to T state molecules is a very slow process by comparison with the time scale of these experiments.

Fig. 3 presents flow-flash data for hemoglobin in pH 7 bis-Tris buffer with 500 μM DPG. The solid curves are the result of a simulation with parameter values identical to those in the legend for Fig. 2 but l_{T}^{\prime} was taken to be $0.1 \mu\text{M}^{-1} \text{ S}^{-1}$ and L was taken to be 3×10^7 from laser-flash experiments similar to those for hemoglobin in pH 7 phosphate buffer (13).¹ In the presence of phosphates the simulations agree reasonably well with the experimental data; however, systematic deviations may be seen in Figs. 2 and 3. In particular, the population of rapidly reacting hemoglobin develops more slowly at short times than predicted by the model. It should be noted that the agreement between simulation and experiment is better in Fig. 1 than in Fig. 2 only because the explicit time dependence is removed in a plot of fractional conformational change vs. fractional ligation.

Fig. 4 presents flow-flash results for hemoglobin in pH 9 borate buffer. In this case

¹Sawicki, C. A., and Q. H. Gibson. 1977. Unpublished experiments.

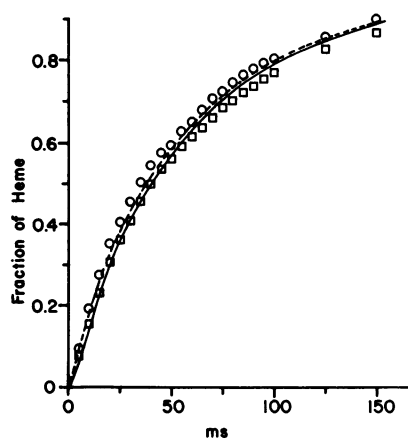


FIGURE 4 Fraction of CO-bound heme (\square) and fraction of heme in R state molecules (\circ) plotted against time for pH 9 borate buffer. The hemoglobin and CO concentrations after mixing were 21.5 and 45 μM . The curves are the result of a simulation using the two-state model described in the text for the parameter values: $l_R = 0.01 \text{ S}^{-1}$, $l'_R = 11 \mu\text{M}^{-1}\text{S}^{-1}$, $l_T = 0.1 \text{ S}^{-1}$, $l'_T = 0.1 \mu\text{M}^{-1}\text{S}^{-1}$, and $L = 550$. The dashed curve gives the calculated fraction of heme in R state molecules, whereas the solid curve presents the calculated fraction of CO-bound heme.

the conformational change leads ligand binding by a significant amount. Relatively little is known about the parameters of the two-state model that apply at pH 9. l'_R was determined by partial photolysis (12). L was taken to be 550. Other parameters were arbitrarily set to the values given in the legend to Fig. 3. Further simulation suggests that in terms of the simple two-state model the conformational change will only lead ligand binding when significant conformational change occurs after the binding of one CO molecule. The alternately dashed and dotted line in Fig. 1 plots the simulations of Fig. 4 as: fraction of hemoglobin in the R state vs. fractional ligation.

To summarize, flow-laser flash experiments produce much larger effects than those seen in equilibrium experiments, and so permit a more demanding test of models describing cooperativity in hemoglobin. The conformational changes responsible for the formation of rapidly reacting hemoglobin after mixing of deoxyhemoglobin and CO appear to occur much faster than CO binding under the conditions employed here. In pH 7 phosphate buffer and in pH 7 bis-Tris buffer with 500 μM DPG, the formation of rapidly reacting hemoglobin lags far behind ligand binding. In these cases simulations using the two-state model with parameter values suggested by independent experiments are in reasonable agreement with the experimental observations. These simulations suggest that hemoglobin, under these conditions, is essentially completely switched to the R state after binding three CO molecules, in contrast to the situation at pH 9, where it appears that significant conformational change occurs after binding of one CO molecule.

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APPENDIX

Simulations

The two-state model used is specified by Eqs. 1-3. Kinetic simulations (Figs. 1-4) were produced by numerically solving the corresponding differential equations, incorporating the parameter values listed earlier, using a second order Runge-Kutta algorithm (29). The program keeps account of the population of the 10 R and T state species R_n and T_n , where n is the number of bound CO molecules. In these experiments the CO concentration was kept relatively low so that very little ligand binding (<2%) occurs during the flow dead time. Thus the initial conditions when flow is stopped are $T_0 = Hb_0/4$ (Hb_0 is the total heme concentration) whereas all other species are initially unpopulated. The time increments used to produce the simulated curves were equal to or less than one-fifth of the time separation of the experimental points.

The dependence of the fraction of hemoglobin in the R state on fractional ligation under equilibrium conditions (dotted line in Fig. 1) was calculated by solution of the equilibrium expressions corresponding to Eqs. 1-3 for a set of CO concentrations.

DISCUSSION

OGAWA: In the curve-fitting of the allosteric model, how much can the parameters be allowed to vary in these measurements? The real unknown parameter is l_T (off rate) and all other parameters are essentially known. (a) It is known that K_1 (first oxygen binding constant) is pH-dependent. If this pH dependence (l_T' and l_T should be pH-sensitive) does not fit the present results, the consequence is extremely interesting. It could be that Bohr proton release is quaternary linked and the salt bridge breakage is not ligand linked. (b) Similar points apply when DPG is present. The so-called R-T difference of the deoxyheme absorption spectra comes from the alpha subunit heme only, although ligand binding properties of the alpha and beta subunits may not differ appreciably. Since the Cornell group has been strong on alpha-beta difference in the past, I am curious to know whether measurements at other wavelengths can deviate from the present analysis.

SAWICKI: In response to your first question, we have not tried to determine l_T and l_T' in these experiments. One of the aims of this work was to use values for the allosteric constant L and the rate constants taken from the literature rather than allowing these parameters to vary rather arbitrarily to obtain a best fit to our data. As you point out, the greatest uncertainty is in l_T ; however, in the case of pH 7 phosphate buffer this rate constant can be obtained from the stopped flow experiments of Sharma et al.(24).

For simulation of the pH 9 data, we have arbitrarily assumed that l_T' and l_T have values similar to those at pH 7, because it appears that significant conformational change occurs after binding of one molecule of carbon monoxide, so that it may be difficult to separate the properties of the R and T states. For example, the binding of the first ligand to a hemoglobin molecule may occur with a slow T rate while the dissociation of ligand from $Hb(CO)_1$, occurs with a rate characteristic of the R state.