

# A Flash Photolysis Method to Characterize Hexacoordinate Hemoglobin Kinetics

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**ABSTRACT** A flash photolysis method is described for analyzing ligand binding to the new and growing group of hemoglobins which are hexacoordinate in the unligated, ferrous state. Simple analysis of a two exponential fit to time courses for CO rebinding at varying CO concentrations yields rate constants for formation and dissociation of the hexacoordinate complex, and the bimolecular rate constant for CO binding. This method was tested with a nonsymbiotic plant hemoglobin from rice for which these values had not previously been determined. For this protein, dissociation and rebinding of the hexacoordinating amino acid side chain, His<sup>73</sup>, is rapid and similar to the rate of CO binding at high CO concentrations. These results indicate that hexacoordination must be taken into account when evaluating the affinity of hexacoordinate hemoglobins for ligands.

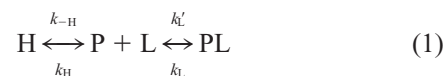
## INTRODUCTION

Kinetic measurements of the binding of oxygen and many other ligands to hemoglobins have traditionally used stopped flow techniques or laser flash photolysis to initiate the reaction (Olson, 1981; Sawicki and Morris, 1981). However, the dead time associated with mixing by stopped flow frequently prevents measurement of rapid bimolecular reactions at convenient ligand concentrations. Most direct measurements of ligand binding exploit lasers or other flashed light sources to initiate the reaction by photolysing the protein-ligand bond for a period of time long enough for the ligand to diffuse out of the protein matrix. When the light pulse is turned off, rebinding can be monitored on time scales much more rapid than those observed in stopped flow mixing. In most cases, these reactions are carried out under pseudo first order conditions with the ligand in excess, resulting in single exponential time courses associated with ligand rebinding to the five coordinate ferrous, unligated (deoxy) hemoglobin.

Several new classes of high oxygen affinity hemoglobins have recently been discovered in plants, protozoa, bacteria, and cyanobacteria which are not strictly five coordinate in the ferrous deoxy state (Duff et al., 1997; Arredondo-Peter et al., 1997; Couture et al., 1999; Delgado-Nixon et al., 2000). These hemoglobins have been the subject of much interest because they demonstrate many new (and some as of yet undiscovered) physiological functions. The group of plant proteins which are members of this general class of hexacoordinate hemoglobins are likely to be found in all plants and appear to have some role in the physiological response to hypoxia (Taylor et al., 1994; Sowa et al., 1998).

The protein studied here, rice nonsymbiotic hemoglobin 1 (rHb1), contains a His residue (His<sup>73</sup>) which coordinates the heme iron in the ferric and deoxy ferrous states (Arredondo-Peter et al., 1997). Ligand binding is hypothesized to follow the reaction scheme shown in Fig. 1. Before ligand binding, the side chain of His<sup>73</sup> must dissociate to allow the ligand to bind the heme iron. After ligand binding, it has been demonstrated that the His<sup>73</sup> side chain moves to a conformation from which it can stabilize the bound ligand, particularly in the case of oxygen (Arredondo-Peter et al., 1997). The necessity of hexacoordination for the function of these proteins is not yet clear, but it is possible that they have evolved this mechanism to adjust ligand affinity through competition with the displaceable amino acid side chain capable of coordinating the ligand binding site.

Ligand binding to hexacoordinate hemoglobins is clearly more complex than the simple bimolecular reaction that occurs in traditional hemoglobins with an open binding site. A reaction scheme for ligand binding to hexacoordinate hemoglobins is shown in Eq. 1 where H is the hexacoordinate species, P the pentacoordinate species, L the ligand, PL the protein-ligand complex,  $k_H$  and  $k_{-H}$  the rate constants for the formation and dissociation of the hexacoordinate complex, and  $k'_L$  and  $k_L$  the rate constants for formation and dissociation of the protein-ligand complex.



This reaction scheme has been used to describe other reactions involving hemoglobins in which a first order event precedes bimolecular ligand binding (Brancaccio et al., 1994; Coletta et al., 1996). In this reaction, a steady state approximation for [P] can be used to provide the following equation for  $k_{\text{obs}}$ .

$$k_{\text{obs}} = \frac{k_{-H}k'_L[L] + k_Hk_L + k_{-H}k_L}{k_{-H} + k_H + k'_L[L]} \quad (2)$$

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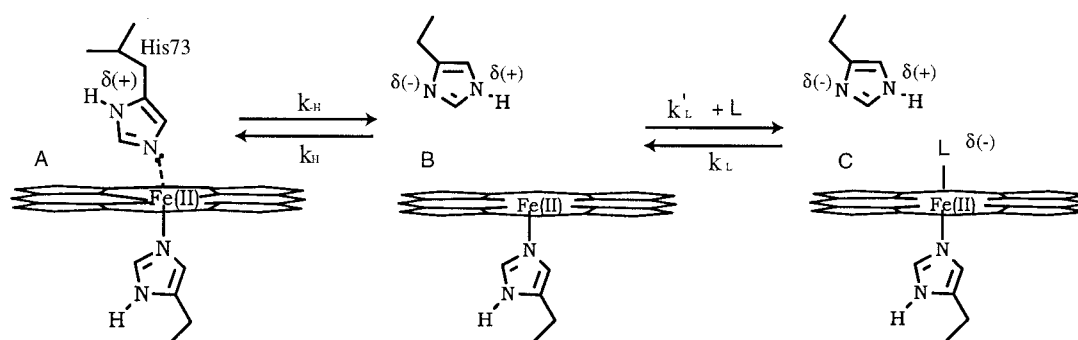


FIGURE 1 A reaction scheme for ligand binding to rHb1. (A) The ferrous deoxy form of this protein has the His73 side chain coordinated to the ligand binding site which must dissociate to give rise to the pentacoordinate species (B) to which ligands can bind (C). The rate constants for each of these reactions is titled along with the arrow indicating the reaction. The equilibrium affinity constant,  $K_H$ , is defined as the ratio of  $k_H$  and  $k_{-H}$ .

If  $k_L$  is very small, two approximations for  $k_{\text{obs}}$  are convenient: 1) If  $k_{-H}$  is very small compared to  $k'_L[L]$  and  $k_H$ , then  $k_{\text{obs}} = k_{-H}k'_L[L]/(k_H + k'_L[L])$  (Couture et al., 1999a). In the limiting case where  $k'_L[L] \gg k_{-H}$  and  $k_H$ ,  $k_{\text{obs}} = k_{-H}$  and ligand binding must wait for dissociation of the hexacoordinate complex (Coletta et al., 1996). 2) If interconversion between the five and six coordinate states ( $k_H$  and  $k_{-H}$ ) is much more rapid than ligand binding ( $k'_L[L]$ ), then  $k_{\text{obs}} = k'_L[L]/(k_H/k_{-H} + 1)$  (Delgado-Nixon et al., 2000). There are many examples of hexacoordinate hemoglobins which exhibit both kinetic scenarios (Duff et al., 1997; Arredondo-Peter et al., 1997; Couture et al., 1999a,b; Delgado-Nixon et al., 2000). Unfortunately, analysis of  $k_{\text{obs}}$  using these methods cannot provide  $k_H$ ,  $k_{-H}$ , and  $k'_L$  independently (Coletta et al., 1996; Couture et al., 1999a; Delgado-Nixon et al., 2000).

The necessity of hexacoordination for physiological function is not yet clear, but until we can evaluate the kinetic behavior of these proteins in detail we will be unable to fully link structure and function in hexacoordinate hemoglobins. A method is presented here for determining all three rate constants which influence ligand binding to these proteins using flash photolysis and monitoring the rebinding of carbon monoxide at varying ligand concentrations.

## MATERIALS AND METHODS

### Protein production

The recombinant nonsymbiotic hemoglobin from rice and the H73L mutant of this protein were expressed and purified as described by Arredondo-Peter et al., (1997). Both proteins were expressed using the PET system from Novagen (Madison, WI). Induced BL21DE3 cells were bright red, and the soluble proteins were purified without further addition of heme. The carbon monoxide derivatives were formed by diluting  $\sim 10 \mu\text{l}$  of a stock of ferric protein ( $\sim 1 \text{ mM}$ ) into  $500 \mu\text{l}$  of buffer equilibrated in a known concentration of CO balanced by  $\text{N}_2$ , and containing an excess concentration of sodium dithionite ( $\sim 2$  times the protein concentration) which serves as the reductant. The extinction coefficients used to measure concentrations of the CO and deoxy proteins are  $153 \mu\text{M}^{-1} \text{ cm}^{-1}$  (CO, 416 nm);  $157 \mu\text{M}^{-1} \text{ cm}^{-1}$  (deoxy, 424 nm);  $93 \mu\text{M}^{-1} \text{ cm}^{-1}$  (CO, 424 nm);

and  $106 \mu\text{M}^{-1} \text{ cm}^{-1}$  (deoxy, 416 nm) with the CO and deoxy protein Soret absorbance maxima at 416 and 424, respectively (Goodman et al., unpublished).

### Flash photolysis experiments

The apparatus used for these experiments consists of a 10 Hz Continuum (Santa Clara, CA) Surelite I YAG laser frequency doubled to provide a 5-ns excitation pulse at 532 nm. Absorbance measurements were made using a 75-W xenon lamp from Photon Tech International (Monmouth, NJ) as a light source in combination with a broad band interference filter to remove all light except that in the 410- to 440-nm region. The probe light was focused on the sample and then refocused on the monochromator slit. A manual holographic monochromator from Edmund Scientific (Barrington, NJ) was mounted to a 1P28 photomultiplier tube from Hamamatsu (Bridgewater, NJ) in combination with a Hamamatsu high voltage supply mount. The voltage signal from the photomultiplier was monitored with a Techtronix digital oscilloscope which was triggered electronically by the laser. The time course from the oscilloscope was transferred and processed using the program Igor Pro (Lake Oswego, OR).

Samples for flash photolysis were prepared as follows. Perfektum gas tight syringes containing 100 mM potassium phosphate, pH 7.0, were bubbled with either CO or  $\text{N}_2$  for 20 min, and then dry sodium dithionite was added so that each syringe was  $\sim 200 \mu\text{M}$  in concentration. These solutions were then mixed in another syringe to generate the CO concentrations used in each experiment. Each sample was prepared by using each of these solutions to completely fill a glass cell with a 1 mm path length which was sealed with a rubber septum. Finally,  $\sim 10 \mu\text{l}$  of a stock protein sample ( $\sim 1 \text{ mM}$ ) was added to each cell to generate a sample with a final concentration between 20 and  $40 \mu\text{M}$ . These samples were flashed using the apparatus described above while monitoring at either 416 or 424 nm.

### Kinetic analysis

The reaction scheme given in Eq. 1 simplifies to Eq. 3 under pseudo first order conditions with CO and considering CO binding to be irreversible on these time scales. The actual CO dissociation rate constant is  $0.001 \text{ s}^{-1}$  (Arredondo-Peter et al., 1997).



An analytical solution for this scheme exists when the reaction starts with P (Capellos and Bielski, 1972). Under these conditions, the concentration of each reactant is as follows.

$$[P] = [P]_0 \left[ \left( \frac{k_{-H} - \gamma_1}{\gamma_2 - \gamma_1} \right) e^{-\gamma_1 t} + \left( \frac{k_{-H} - \gamma_2}{\gamma_1 - \gamma_2} \right) e^{-\gamma_2 t} \right] \quad (4)$$

$$[H] = k_H [P]_0 \left[ \left( \frac{1}{\gamma_2 - \gamma_1} \right) e^{-\gamma_1 t} + \left( \frac{1}{\gamma_1 - \gamma_2} \right) e^{-\gamma_2 t} \right] \quad (5)$$

$$[P_{CO}] = [P]_0 \left[ 1 - \left( \frac{k'_{CO}[CO](k_{-H} - \gamma_1)}{\gamma_1(\gamma_2 - \gamma_1)} \right) e^{-\gamma_1 t} - \left( \frac{k'_{CO}[CO](k_{-H} - \gamma_2)}{\gamma_2(\gamma_1 - \gamma_2)} \right) e^{-\gamma_2 t} \right] \quad (6)$$

In these equations  $\gamma_1$  and  $\gamma_2$  are:

$$\gamma_1 = \frac{(k_H + k_{-H} + k'_{CO}[CO]) + ((k_H + k_{-H} + k'_{CO}[CO])^2 - 4k_{-H}k'_{CO}[CO])^{1/2}}{2} \quad (7)$$

$$\gamma_2 = \frac{(k_H + k_{-H} + k'_{CO}[CO]) - ((k_H + k_{-H} + k'_{CO}[CO])^2 - 4k_{-H}k'_{CO}[CO])^{1/2}}{2} \quad (8)$$

All time course analyses and kinetic simulations were carried out using the program Igor Pro.

## RESULTS

The CO and deoxy derivatives of wild-type rHb1 have sharp absorbance peaks at 416 and 424 nm, respectively (Arredondo-Peter et al., 1997). Fig. 2 shows flash photolysis time courses for CO rebinding to this protein at concentrations of 200, 400, 700, and 1000  $\mu$ M CO while monitoring at 416 and 424 nm. Close inspection of the 1000  $\mu$ M CO rebinding time course at 416 nm indicates that these data do not fit well to a single exponential decay (Fig. 2, A and B), but that each time course can be fit to a sum of two exponential terms (Fig. 2 C). The 424 nm time courses clearly indicate a complex reaction, showing an initial rapid increase in absorbance followed by a decrease in absorbance (Fig. 2 D).

Eqs. 4–6 show that the change in concentration of each reactant in this kinetic scheme is described by two rate constants (Eqs. 7 and 8) each of which is a complex combination of the three rate constants involved in the reaction. Ideally, one could use the difference in extinction coefficient between each species and the CO-bound protein to fit data at any wavelength to the sum of Eqs. 4–6 multiplied by this value. In this way the amplitude constraints on  $\gamma_1$  and  $\gamma_2$  could be used to extract three rate constants from what is always a two exponential decay. However, the extinction coefficient for the pentacoordinate protein cannot be mea-

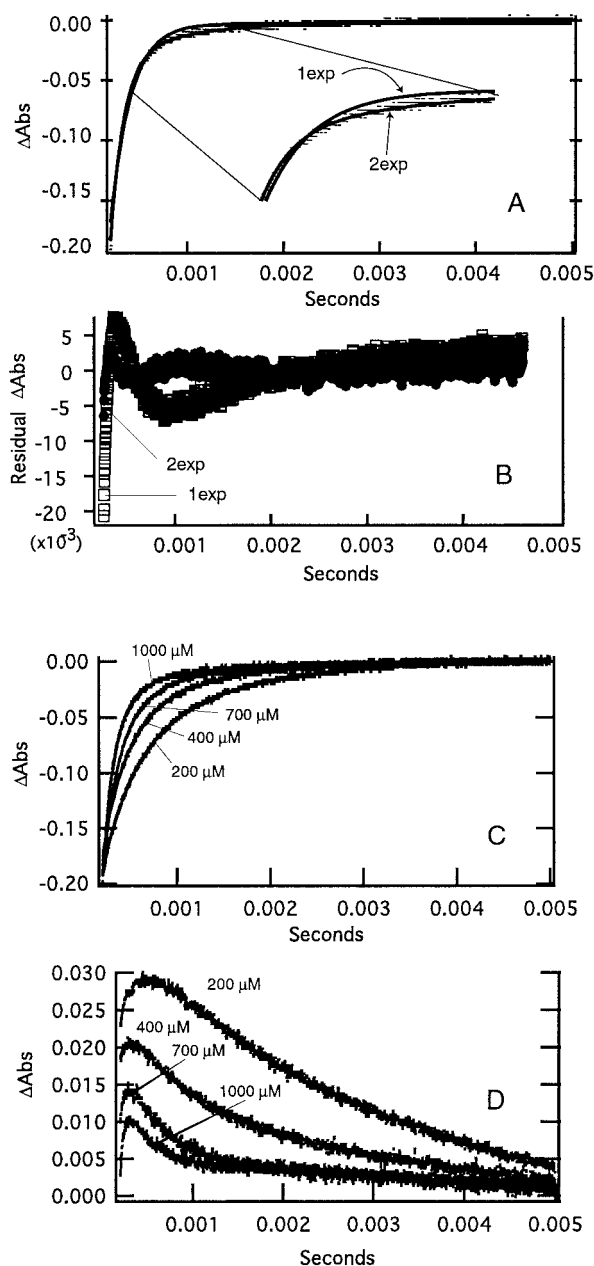


FIGURE 2 Time courses for CO binding to rHb1 following flash photolysis. (A) The time course for CO rebinding at 416 nm and 1000  $\mu$ M CO does not fit to a single exponential decay. The fitted curves are solid lines and the raw data are dots. (B) The residual values for the single (open squares) and double (closed circles) exponential fits show that the double exponential fit is significantly better than the fit to one exponential, which supports the conclusion from A. (C) Time courses for CO rebinding at different CO concentrations monitored at 416 nm, each fit to a simple two exponential decay equation. (D) Time courses for CO rebinding at different CO concentrations monitored at 424 nm. CO binding to rHb1 is clearly not described by a single exponential decay, but is described by the sum of two exponentials.

sured easily and allowing this parameter to vary in nonlinear least squares analysis proved deleterious in combination with the complexity of the fitting equations.

**TABLE 1** Rate constants for CO binding to rHb1

[CO] ( $\mu\text{M}$ )	$\gamma_1$ ( $\text{s}^{-1}$ )	$\gamma_2$ ( $\text{s}^{-1}$ )	$\gamma_1 + \gamma_2$ ( $\text{s}^{-1}$ )	$\gamma_1 \times \gamma_2$ ( $\text{s}^{-2}$ )	$k_{-H}$ ( $\pm 10\%$ ) ( $\text{s}^{-1}$ )	$k_H$ ( $\pm 10\%$ ) ( $\text{s}^{-1}$ )	$k'_{CO}[\text{CO}]^*$ ( $\pm 6\%$ ) ( $\text{s}^{-1}$ )
695	4,937	1,724	6,661	8,511,388	1,911	517	4,170
594	4,192	1,669	5,861	6,996,448	1,911	517	3,564
495	3,811	1,630	5,441	6,211,930	1,911	517	2,970
396	3,371	1,531	4,902	5,161,001	1,911	517	2,376
297	2,845	1,311	4,156	3,729,795	1,911	517	1,782

\*The bimolecular rate constant for CO binding,  $6.0 \mu\text{M}^{-1}\text{s}^{-1}$ , is obtained by dividing  $k'_{CO}[\text{CO}]$  by  $[\text{CO}]$ .

These problems can be overcome by noting that regardless of which species dominate the time course at the wavelength of interest, all time courses at a particular CO concentration and wavelength must fit to the same two exponential terms,  $\gamma_1$  and  $\gamma_2$ . This was found to be true experimentally. In fact, each of the 416 nm time courses in Fig. 2 C can be fit to Eq. 6 to yield  $\gamma_1$  and  $\gamma_2$ . However, a simple two exponential expression provides the same two values for these terms. The simple two exponential fit is shown with the data in Fig. 2 C, and the fitted rate constants are provided in Table 1.

Extraction of  $k_H$ ,  $k_{-H}$ , and  $k'_{CO}$  from  $\gamma_1$  and  $\gamma_2$  can be accomplished using a method similar to that described by Zhu and Espenson (1995) for a different reaction. The sum and product of  $\gamma_1$  and  $\gamma_2$  yield the following relationships:

$$\gamma_1 + \gamma_2 = k_{-H} + k_H + k'_{CO}[\text{CO}] \quad (9)$$

$$\gamma_1\gamma_2 = k_{-H}k'_{CO}[\text{CO}] \quad (10)$$

Plots of the sum and product of  $\gamma_1$  and  $\gamma_2$  versus  $[\text{CO}]$  are shown in Fig. 3, A and B. The slope of the sum of these two rate constants versus  $[\text{CO}]$  is  $k'_{CO}$ , and was measured to be  $6.0 \mu\text{M}^{-1}\text{s}^{-1}$ . This value is similar to the value of  $7.2 \mu\text{M}^{-1}\text{s}^{-1}$  estimated by Arredondo-Peter *et al.*, (1997). The intercept of this plot is  $2428 \text{ s}^{-1}$  and represents the sum of  $k_H$  and  $k_{-H}$ .

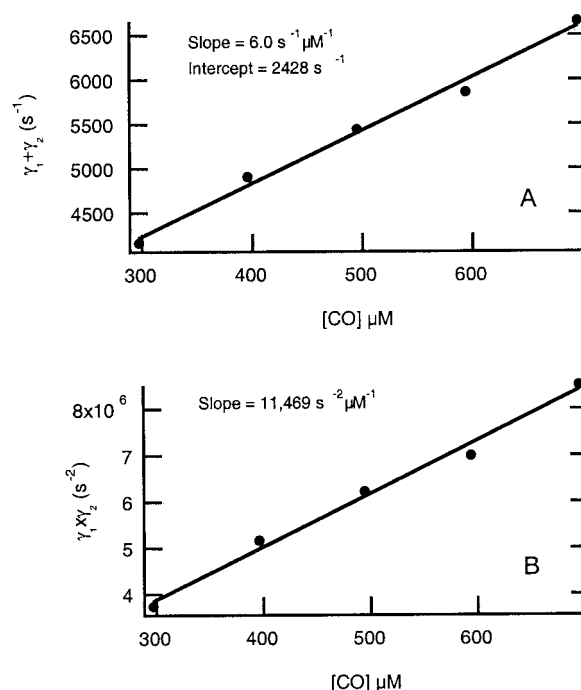
From the variation of the product of  $\gamma_1$  and  $\gamma_2$  with  $[\text{CO}]$ , a value of  $k_{-H}k'_{CO}$  was determined to be  $11,469 \text{ s}^{-2}$ . Dividing by  $6.0 \text{ s}^{-1}$  for  $k'_{CO}$  leaves a value of  $1911 \text{ s}^{-1}$  for  $k_{-H}$ . Subtracting this value from  $2428 \text{ s}^{-1}$  provides a value for  $k_H$  of  $517 \text{ s}^{-1}$ . These values along with  $\gamma_1$  and  $\gamma_2$  at each CO concentration are provided in Table 1. The error estimates reported in Table 1 represent the absolute percent variation of five independent measurements of these values. To give support to this kinetic interpretation, the values in Table 1 along with the extinction coefficients for the CO and deoxy derivatives were used to simulate time courses for  $[\text{H}]$  at different CO concentrations using Eq. 5 at a protein concentration of  $46 \mu\text{M}$  (the same as that used for the experiments resulting in Fig. 2). These simulated time courses are shown in Fig. 4.

To ensure that these kinetic phenomena are due to reversible coordination of His<sup>73</sup> in rHb1, time courses for CO rebinding to the H73L mutant protein were measured at 416 and 424 nm using the same procedure as that used for the

wild type protein. The data from the mutant protein fit well to single exponential decays at both wavelengths. The observed rate constants were dependant on  $[\text{CO}]$  as shown in Fig. 5. The value of  $k'_{CO}$  for the H73L mutant protein determined from the slope of Fig. 5 was  $161 \mu\text{M}^{-1}\text{s}^{-1}$  regardless of the wavelength of measurement.

## DISCUSSION

The photolysis method described here is a relatively simple procedure for extracting rate constants from a complex reaction with several reactants present simultaneously. The analysis is simplified because the integrated rate expressions for each reactant share the same two compound rate constants,  $\gamma_1$  and  $\gamma_2$ . As long as these binding experiments are carried out under pseudo first order conditions for the



**FIGURE 3** Extraction of  $k'_{CO}$ ,  $k_H$  and  $k_{-H}$  from  $\gamma_1$  and  $\gamma_2$ . (A) The plot of the sum of  $\gamma_1$  and  $\gamma_2$  versus  $[\text{CO}]$  gives a slope equal to  $k'_{CO}$ , and an intercept equal to  $k_H + k_{-H}$  (Eqs. 9 and 10). (B) The plot of the product of  $\gamma_1$  and  $\gamma_2$  versus  $[\text{CO}]$  gives a slope of  $k_{-H}k'_{CO}$ . The combination of these values allow calculation of all three rate constants,  $k'_{CO}$ ,  $k_H$ , and  $k_{-H}$  (Table 1).



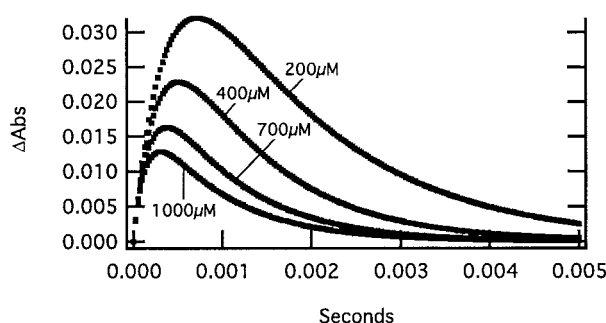


FIGURE 4 Simulations of hexacoordination. Time courses for the formation of the hexacoordinate species simulated using the values in Table 1 and Eq. 5, and a protein concentration of 46  $\mu\text{M}$  (that of Fig. 2 D). These data (both the line shapes and the changes in absorbance) are very similar to Fig. 2 D, supporting the kinetic scheme proposed here.

ligand, a two exponential fit followed by analysis of the product and sum of these constants, as a function of ligand concentration, will yield the three rate constants of interest.

It is reassuring to see the similarity between the observed time courses for CO binding at 424 nm (Fig. 2 D) and those predicted from the rate constants in Table 1 and Eq. 5 (Fig. 4). The fact that the change in absorbance calculated for this reaction by Eq. 5 is so near the observed value indicates that the predominant species observed early in the reaction at this wavelength is the hexacoordinate hemoglobin. Also reassuring is the fact that replacing His<sup>73</sup> with a residue incapable of coordinating the ligand binding site extinguishes the hexacoordinate behavior of this protein, providing further support for our hypothesis regarding the heme pocket coordination state of rHb1 (Fig. 1).

Measurement of  $k_{\text{obs}}$  by stopped flow most closely mimics the behavior of the protein as it probably functions in vivo because ligand binding is forced to compete with hexacoordination. It is this competition for the ligand binding site that makes displaceable hexacoordination a plausible

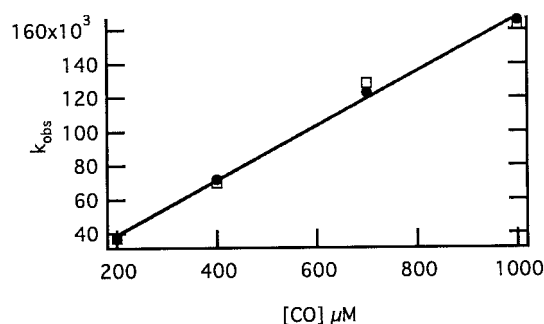


FIGURE 5 CO binding to rHb1:H73L. CO binding to the H73L mutant protein is a single exponential process at all CO concentrations and yields a bimolecular rate constant,  $k'_{\text{CO}}$ , of 161  $\mu\text{M}^{-1} \text{s}^{-1}$ . The closed circles and open boxes are values of  $k_{\text{obs}}$  monitored at 416 and 424 nm, respectively. These simulations and the behavior of the mutant protein support our hypothesis for the kinetics of CO binding to rHb1.

mechanism for regulating ligand affinity. Unfortunately stopped flow analysis does not unambiguously provide each of the rate constants characterizing this reaction. Measurement of  $k_{\text{obs}}$  for CO binding to rHb1 by stopped flow can provide  $k'_L[L]/(k_H/k_{-H} + 1)$ . Or, if  $k_{-H}$  were slow compared to ligand binding as is the case for some other hexacoordinate hemoglobins, stopped flow measurements of  $k_{\text{obs}}$  can provide  $k_{-H}k'_L[L]/(k_H + k'_L[L])$ . Only by using flash photolysis to start the reaction in the pentacoordinate state can we extract values for  $k_{-H}$ ,  $k_H$ , and  $k'_L$ .

The rate constants measured here for the formation and dissociation of the hexacoordinate complex of rHb1 are rapid, on the scale of the observed rate of CO binding at these CO concentrations. The value of the affinity equilibrium constant for the hexacoordinate complex,  $K_H$ , is 0.27 for this protein. This is in the range predicted for a homologous plant hemoglobin from *Arabidopsis* (Trevaskis et al., 1997; Watts et al., unpublished).

Arredondo-Peter et al. (1997) used flash photolysis to measure a bimolecular rate constant of 68  $\mu\text{M}^{-1} \text{s}^{-1}$  for oxygen binding to rHb1. In their experiments, no oxygen concentration dependant heterogeneity was observed in the rebinding time courses. These results are also predicted from the analysis described here. If the bimolecular rate constant is very large compared to  $k_H$  and  $k_{-H}$ , Eqs. 7 and 8 become  $\gamma_1 = k'_L[L]$  and  $\gamma_2 = 0$ , respectively (with CO = L). Therefore, if ligand binding is very rapid, the time course for rebinding following photolysis is a single exponential process with  $k_{\text{obs}} = k'_L[L]$ . For this reason, the relatively slow reactivity of CO compared to O<sub>2</sub> makes CO a very convenient ligand for the experiments described here. Its pseudo first order rate constants at convenient ligand concentrations are in the right range to allow observation of the kinetic events associated with hexacoordination by simply monitoring the time course for CO binding.

The values measured here for  $k_{-H}$  and  $k_H$  are necessary to estimate the affinity of hexacoordinate hemoglobins for ligands. For example, the bimolecular rate constant for oxygen binding to rHb1 is 68  $\mu\text{M}^{-1} \text{s}^{-1}$  and the oxygen dissociation rate constant is 0.038  $\text{s}^{-1}$  (Arredondo-Peter et al., 1997). For a pentacoordinate hemoglobin, these values would yield an equilibrium affinity constant  $K_{\text{O}_2} = 1800 \mu\text{M}^{-1}$ . The measurement of  $k_{-H}$  presented here indicates that the maximum rate of ligand binding cannot exceed a value of 1911  $\text{s}^{-1}$ , so the maximum association rate for oxygen is obtained at  $\sim 28 \mu\text{M}$  O<sub>2</sub>. At this and higher oxygen concentrations, the affinity constant (disregarding the effects of  $k_H$ ) is  $\sim 1911 \text{s}^{-1}/k_{\text{off,L}}$ . Therefore, in a solution equilibrated in air (262  $\mu\text{M}$  O<sub>2</sub>), the actual equilibrium affinity of rHb1 for oxygen is  $\sim 50,000$  rather than  $\sim 470,000$  predicted for this protein in the absence of hexacoordination. Taking  $k_H$  into account only increases the discrepancy between these values because the hexacoordinating amino acid side chain competes with the exogenous ligand for coordination.

If the physiological function of these proteins is binding oxygen or some other ligand, it would be important that the hexacoordinating amino acid side chain not bind too tightly to the heme iron. Therefore, it is likely that other residues in the heme pocket exist to inhibit hexacoordination and generate the rapid rate constants observed for this protein. A mutagenesis study of the residues surrounding the hexacoordinating ligand in this and other hexacoordinate hemoglobins should exploit the analysis described here to determine the effects of different amino acids on all three rate constants characterizing this reaction.

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