Direct Measurement of Equilibrium Constants for High-Affinity Hemoglobins

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ABSTRACT The biological functions of heme proteins are linked to their rate and affinity constants for ligand binding. Kinetic experiments are commonly used to measure equilibrium constants for traditional hemoglobins comprised of pentacoordinate ligand binding sites and simple bimolecular reaction schemes. However, kinetic methods do not always yield reliable equilibrium constants with more complex hemoglobins for which reaction mechanisms are not clearly understood. Furthermore, even where reaction mechanisms are clearly understood, it is very difficult to directly measure equilibrium constants for oxygen and carbon monoxide binding to high-affinity ($K_D \ll 1$ μM) hemoglobins. This work presents a method for direct measurement of equilibrium constants for high-affinity hemoglobins that utilizes a competition for ligands between the "target" protein and an array of "scavenger" hemoglobins with known affinities. This method is described for oxygen and carbon monoxide binding to two hexacoordinate hemoglobins: rice nonsymbiotic hemoglobin and *Synechocystis* hemoglobin. Our results demonstrate that although these proteins have different mechanisms for ligand binding, their affinities for oxygen and carbon monoxide are similar. Their large affinity constants for oxygen, 285 and ~100 μM^{-1} respectively, indicate that they are not capable of facilitating oxygen transport.

INTRODUCTION

Hemoglobins (Hbs) carry out diverse and physiologically important functions that depend on appropriate affinities for ligands such as O_2, CO, and NO. Measurement of these values using kinetic and equilibrium binding methods has provided critical insight into the physiological functions of Hbs from many different organisms (Bolognesi et al., 1997; Royer et al., 2001; Weber and Vinogradov, 2001). Traditional mammalian Hbs that store and transport O_2 have dissociation equilibrium constants in the low μM range (Wittenberg, 1966). Because many spectrophotometric measurements are possible at this concentration range, and as oxygen electrodes are sensitive at these concentrations, it is convenient to measure affinity constants using either equilibrium or kinetic methods. For myoglobin and human hemoglobin, equilibrium constants resulting from either procedure are identical (Olson, 1981a,b).

However, equilibrium affinity measurements cannot be assessed for high-affinity ($K_D \ll 1$ μM) Hbs using traditional oxygen titration experiments because the low protein concentration required to avoid stoichiometric binding precludes simple spectroscopic measurement, and because it is difficult to measure free ligand concentrations accurately in this concentration range (Giardina and Amiconi, 1981). This is not a problem for some high-affinity Hbs, such as soybean leghemoglobin (Lba), that have simple bimolecular reaction schemes allowing unambiguous assignment of equilibrium constants based on measurement of kinetic rate constants. For Hbs with ligand binding reaction schemes that are much more complex or not yet fully understood, kinetic analysis cannot provide equilibrium constants with confidence (Couture et al., 2000; Dewilde et al., 2001; Hvitved et al., 2001; Trent and Hargrove, 2002; Trent et al., 2001a,b).

A good example of this difficulty is the hexacoordinate class of Hbs. Unlike myoglobin and human hemoglobin that have pentacoordinated heme iron in the unliganded or deoxygenated state, these newly discovered Hbs are hexacoordinated, with heme coordination similar to the *bis*-histidyl ligation in cytochrome *b5* (Arredondo-Peter et al., 1997; Duff et al., 1997). Surprisingly, although cytochrome *b5* is completely unreactive toward diatomic ligands, hexacoordinate hemoglobins (hxHbs) bind oxygen rapidly. These proteins were first found in higher plants and named nonsymbiotic hemoglobins (nsHbs) to distinguish them from the symbiotic leghemoglobins (Arredondo-Peter et al., 1998). Two hxHbs have recently been discovered in humans: neuroglobin (Burmester et al., 2000; Dewilde et al., 2001; Trent et al., 2001b), present in brain tissue and in the retina (Schmidt et al., 2003), and histoglobin (a.k.a. cytoglobin) (Burmester et al., 2002; Trent and Hargrove, 2002), expressed in many different tissues. HxHbs have also been found in the cyanobacterium *Synechocystis* (Scott and LeComte, 2000), and in the single celled alga *Chlamydomonas* (Couture et al., 1999). Initially hxHbs were predicted to have large affinities for oxygen (Arredondo-Peter et al., 1998), but as several of these proteins have now been investigated in more detail, these conclusions are still in question.

In hxHbs, where the kinetic reaction schemes are more complex than a simple bimolecular reaction, it is imperative that equilibrium constants estimated from kinetic values be tested against experiments that directly measure binding under equilibrium conditions. Their potentially high ligand affinities mean that a new method allowing direct

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measurement of equilibrium constants is required not only to investigate potential physiological roles, but also as an independent evaluation of kinetic reaction schemes. The method presented here allows direct measurement of equilibrium constants for any Hb regardless of its affinity. It is based on the competition for binding between the Hb whose affinity is to be measured and an array of Hbs with known ligand affinities. Theoretical and experimental details of this method are described along with measurement of equilibrium constants for O_2 and CO binding to two different hxHbs. We find that *Synechocystis* hemoglobin (*Syn*Hb) and rice nonsymbiotic hemoglobin (riceHb) have similar affinities for oxygen and carbon monoxide despite their differences in kinetic behavior.

MATERIALS AND METHODS

Protein production

Wild-type sperm whale myoglobin (swMb), Lba, and each of their respective mutant proteins were produced as described by Kundu et al. (2002) and Kundu and Hargrove (2003). RiceHb and *Syn*Hb were expressed and purified as described by Arredondo-Peter et al. (1997) and Trent et al. (2001a) (for riceHb and riceHb^{H73L}), and Hvitved et al. (2001) (for *Syn*Hb). The cDNA for *Ascaris* Hb domain 1 was kindly provided by Dr. Dan Goldberg. This domain was expressed in *Escherichia coli* BL21(DE3) cells grown in LB media at 37°C, induced with 0.5 mM IPTG at an optical density of ~0.6 at 600 nm, and incubated for an additional 15 h post induction. After cell lysis, recombinant *Ascaris* Hb was purified as described by others (De Baere et al., 1994; Kloek et al., 1993). The rate constants for oxygen and carbon monoxide binding to recombinant *Ascaris* Hb were measured using methods described by Kundu et al. (2002). Protein concentrations were measured spectrophotometrically.

Experimental conditions for carbon monoxide binding

All CO experiments were carried out in a volume of 1.5 ml in glass cuvettes (Starna model 29/G/10) with a 1-cm light path in a buffer of 0.1 M potassium phosphate, pH 7.0. Unless otherwise specified, 6 μM each of target Hb and scavenger proteins were reduced with 100 μM sodium dithionite, and the resulting spectrum was collected. A quantity of 6 μM CO was introduced to the reaction mixture by the addition of 9 μl of a saturated (1000 μM) CO stock solution. After a 30-min incubation period to ensure that the reactions were at equilibrium (no spectral changes were observed after this period of time), an absorbance spectrum was measured of the 1:1:1 ratio of Hb:scavenger:ligand. Excess CO (an additional 10 μl of the stock solution) was then added to generate the saturated sample. All absorbance spectra were measured with a Cary-50 Bio spectrophotometer from Varian (Mulgrave, Victoria, Australia), and exported as a text file for use in the SpectraSolve software (described below). Individual component spectra were measured for each protein using the same procedure but with single proteins rather than mixtures.

Experimental conditions for oxygen binding

Oxygen binding was measured in a square 1-cm quartz cuvette fitted with a male ground-glass ¼-inch top. The fundamentals of the reduction system have been described previously (Hayashi et al., 1973). Our implementation of this system was as follows: 3 ml of 0.1 M potassium phosphate (pH 7.0) containing 500 μM glucose-6-phosphate (G6P) and 20 μM $NADP^+$ (Sigma, St. Louis, MO) was sealed in the cuvette using a white rubber septum (Aldrich, Milwaukee, WI). The cuvette was then gently bubbled for 20 min with nitrogen flowing through an oxygen scrubbing column (Agilent model OT3-4) and venting through a second needle in the stopper. While maintaining positive pressure, the following were added to the cuvette:

- 2 μl of a 150-μM stock of spinach ferridoxin (Sigma) (final concentration of 0.1 μM).
- 2 μl of a 150-μM stock of ferridoxin reductase (Sigma) (final concentration of 0.1 μM).
- 0.5 μl of a 1-mM catalase stock (Sigma) (final concentration of 0.16 μM).
- 1 μl of a 5-mg/ml G6P dehydrogenase stock (Roche, Pleasanton, CA) (final concentration 1.6 μg/ml).
- Appropriate aliquots (from stocks of ~2 mM) of N_2 purged, ferric scavenger, and unknown Hbs (final concentration of 6 μM of each).

After addition of these components, the flush and vent needles were removed and the stopper was covered with parafilm. Absorbance spectra were collected at 15-min intervals until both Hbs were in the deoxy form, and no further changes occurred. This was typically 30 to 45 min, but reduction times were variable for different hemoglobins. After collecting the final deoxygenated spectrum, air-saturated buffer was added to bring the O_2 concentration to 6 μM. This spectrum was allowed to equilibrate for ~20 min (or until no further changes in absorbance occurred) before collection of the final spectrum. The cuvette was then opened to air to saturate the Hbs, and a final saturated spectrum was collected. As with the CO experiments, individual component spectra were measured using the same procedure but with single proteins rather than mixtures.

Spectral fitting

Concentrations of individual species in the mixed spectra were measured by fitting to the individual components collected under the same experimental conditions. Spectra were fit using the program SPECTRA-SOLVE (Ames Photonics), using the sequential simplex method to fit spectral shapes. In some cases (specified in the Results section), liganded and ligand-free species for both the scavenger and unknown Hb could be resolved. In other cases, (as in the CO and oxy-species of riceHb compared to Lba and its mutant proteins), the ligand-bound proteins were fit as a single species and differential ligand binding was determined from the ratio of ligand-free hemoglobins. The specific details of each fitting routine are included in the Results section for each experiment. All figures were prepared using Igor Pro (WaveMetrics).

RESULTS

Qualitative considerations

In this ''equilibrium competition'' method, two Hbs are mixed together along with a ligand, and the equilibrium populations of ligand-bound and ligand-free (also termed ''deoxy'' from here on) species are measured. Based on the relative populations, the equilibrium constant of the unknown Hb can be assigned with respect to that of the scavenger. There are two basic requirements for this method to work. First, one must have a scavenger Hb with an affinity near (within a factor of ~100) that of the unknown Hb. Second, there must be a measurable spectral difference between either the two deoxy proteins or the two ligand-bound proteins.

The first requirement is met here by the pentacoordinate Hbs listed in Table 1 along with their equilibrium constants

TABLE 1 Scavenger hemoglobins and their affinity constants

CO scavengers	K_{CO} (μM^{-1})	$K_{D,CO}$ (μM)	O_2 scavengers	K_{O2} (μM^{-1})	$K_{D,O2}$ (μM)
Lba:H61W	3	0.3	Lba:H61F	0.5	2
swMb	27	0.04	swMb	1.1	0.9
Lba:H61Y	130	0.008	Lba: wild-type	23	0.04
Lba: wild-type	2000	5×10^{-4}	Lba:H61A	93	0.01
Lba:H61A	14,000	7×10^{-5}	*Ascaris* Hb	220	0.004
Lba:H61L	70,000	1×10^{-5}	Lba:H61R	1200	8×10^{-4}

Data for Lba and its mutant proteins and swMb are from Kundu and Hargrove (2003).

for O_2 and CO. These proteins all exhibit simple bimolecular ligand binding, and their equilibrium affinity constants were calculated as the ratio of association and dissociation rate constants. Their dissociation equilibrium constants (K_D) range from the nM to the μM levels, making them convenient for competition with unknown Hbs with potentially high ligand affinities. The second requirement is met for hxHbs because their deoxy spectra are always distinct from those of the pentacoordinate scavengers (Fig. 1 *A*). In many cases, the ligand-bound spectra are also different (Fig. 1 *B*).

A qualitative assessment of the feasibility of this method is shown in Fig. 1. The absorbance spectrum of hexacoordinate deoxy riceHb in Fig. 1 *A* is obviously different from the pentacoordinate scavenger (in this case Mb). The proteins are also different in their CO bound forms (Fig. 1 *B*). Fig. 1 *C* shows the absorbance spectra of 6 μM riceHb mixed with 6 μM Mb (*solid*) or 6 μM Lba^{H61L} (*dashed*) in the presence of 6 μM CO. These two mixed spectra are clearly distinguishable. The riceHb/Mb spectrum has a shoulder at 434 nm associated with deoxy Mb, and the visible region lacks a pronounced splitting associated with deoxy riceHb; in other words the CO has bound to riceHb, leaving Mb in the deoxy form (Fig. 1 *C*, *solid*). However, the riceHb/Lba^{H61L} spectrum shows the visible region peak-splitting associated with deoxy riceHb, indicating that the scavenger has won the ligand (Fig. 1 *C*, *dotted*).

Quantification of this phenomenon is achieved using the program SpectraSolve, which fits a mixed spectrum to defined component spectra and returns the relative contributions of each. Fig. 2 illustrates this procedure with riceHb competing for CO against Lba^{wt} (*A–C*) and Lba^{H61L} (*D–F*). In each case, the dashed line is the observed mixed spectrum, the dotted line is the fitted spectrum, and the solid line (*below*) is the residual difference between the fitted and observed spectra. Mixed spectra are shown at 0, 6, and 12 μM CO for both scavenger examples, and the relative contribution of each component spectrum is shown in an inset to each graph. As expected, the deoxy species predominate in Fig. 2, *A* and *D*, and the CO-bound species are the majority in Fig. 2, *C* and *F*. The component ratios in Fig. 2, *B* and *E* indicate that Lba^{H61L} can outcompete riceHb for CO, but Lba^{wt} cannot.

Quantitative considerations

Equilibria and kinetics of competitive binding have been analyzed quantitatively for a number of applications (Wedemeyer et al., 1997). For convenience, important relationships specific to the Hb competition method will be stated here. The reaction scheme for a scavenger protein (*S*) competing with a target Hb (*H*) for a ligand (*L*) is shown in Reaction 1 (R1):

$$H_L \underset{K_{HL}}{\rightleftarrows} H + L + S \overset{K_{SL}}{\rightleftarrows} S_L. \qquad \text{(R1)}$$

The relative affinities can be analyzed as the ratio of ligand-bound scavenger and ligand-bound target hemoglobin:

$$\frac{S_L}{H_L} = \frac{K_{SL} S}{K_{HL} H}. \qquad (1)$$

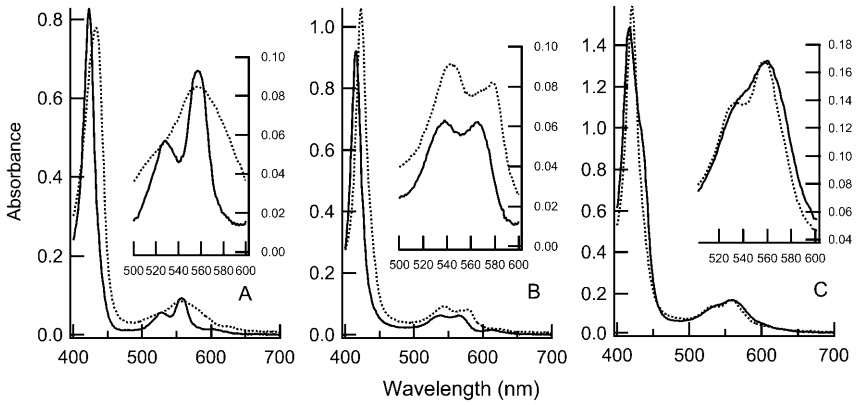

FIGURE 1 Absorbance spectra of pentacoordinate and hexacoordinate hemoglobins. (*A*) Ferrous, unligated (deoxy) riceHb (*solid*) and swMb (*dotted*). (*B*) CO-bound spectra of riceHb (*solid*) and swMb (*dotted*). (*C*) 6 μM CO mixed with 6 μM swMb and 6 μM riceHb (*solid*), and 6 μM CO mixed with 6 μM Lba^{H61L} and 6 μM riceHb (*dotted*). The differences between the spectra in each panel (*A* and *B*) allow quantification of each species in the mixtures present in *C*.

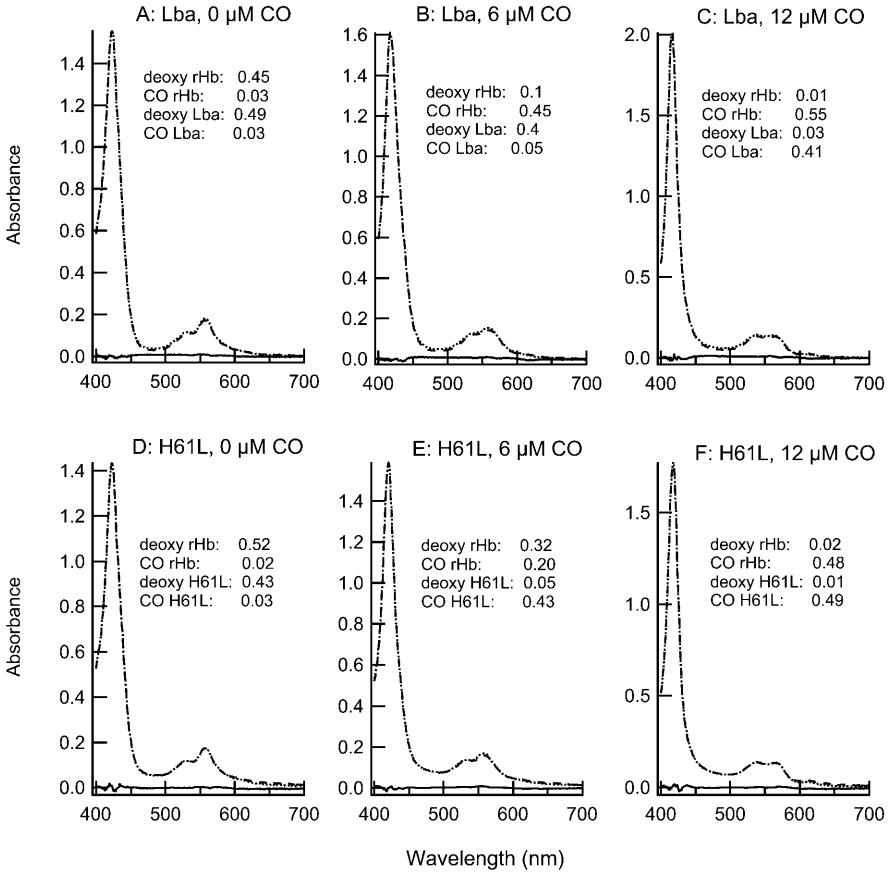

FIGURE 2 Component fitting to composite spectra. Absorbance spectra of mixtures of scavenger and hxHb are fit to their individual component spectra. The observed spectrum in each case is the dashed line, the fitted spectrum is the dotted line, and the difference between the fitted spectrum and the observed spectrum is the solid line. The inset text values are the normalized contribution of each component in the fitted spectrum. By examining the ratios of CO-bound species in *B* and *E*, it is clear that Lba^{H61L} has a higher affinity for CO than riceHb, but Lba does not.

With the goal of deriving a relationship between one ligand-bound species and the total concentration of ligand added in the experiment, we can define the total concentrations of each protein and ligand as $H_T = H + H_L$, $S_T = S + S_L$, and $L_T = L + S_L + H_L$ where the fraction of bound protein in each case is $Y_{HL} = H_L/H_T$ and $Y_{SL} = S_L/S_T$. When each affinity constant is large (that is $1/K_H$ and $1/K_S \gg L_T$) and if $L_T \leq H_T + S_T$, then L can be neglected and $L_T = Y_{HL}H_T + Y_{SL}S_T$. With these substitutions into Eq. 1, a general relationship between the fraction of ligand-bound scavenger (Y_{SL}), L_T, S_T, and H_T can be written:

$$\frac{Y_{SL}S_T}{L_T - Y_{SL}S_T} = \frac{K_{SL}(1 - Y_{SL})}{K_{HL}(H_T - L_T + Y_{SL}S_T)}. \quad (2)$$

To solve for Y_{SL}, Eq. 2 can be rearranged into a quadratic equation with the following terms.

$$\text{“}a\text{”} = K_S S_T - K_H S_T$$
$$\text{“}b\text{”} = K_H L_T - K_H H_T - K_S S_T - K_S L_T$$
$$\text{“}c\text{”} = K_S L_T.$$

Equation 1 indicates that the ratio of ligand-bound proteins is not directly equal to the ratio of affinity constants, but is also a function of the ratio of deoxy species. As one might expect intuitively, Eqs. 1 and 2 predict that if scavenger and unknown Hbs with equal affinity constants are mixed in equal concentrations, the ratio of ligand bound species will be one at each concentration of ligand. Less intuitive is the relationship when the affinity constants are unequal (as is usually the case in practice), or if the concentration of ligand is varied. These situations are explored in Fig. 3.

Fig. 3 *A* simulates the dependence of the fraction of ligand bound scavenger (Y_{SL}) on total ligand concentration at different ratios of affinity constants and equal protein concentrations (that is, $S_T = H_T$ in all cases). The x axis is total ligand concentration (L_T) relative to S_T and H_T (i.e., when $[ligand]_{total} = S_T = H_T$, $x = 1$). The curves proceeding from top to bottom in Fig. 3 *A* are calculated using Eq. 2 with $K_S/K_H = 100$, 10, 5, and 1. Alternatively, one may analyze results as the ratio of one ligand-bound species to the other, as shown in Fig. 3 *B* (only the $K_S/K_H = 10$, 5, and 1 curves are shown for convenience). Fig. 3 *B* demonstrates that as L_T approaches 0, the ratio of products approaches K_S/K_H. Experimentally this is not very useful though, as working at very low ligand concentrations generates small changes from the deoxy spectra and subsequent difficulty in accurate fitting. However, it is convenient that at $L_T = S_T = H_T$ (x axis value of 1), the ratio of affinity constants is exactly equal to the squared ratio of ligand-bound species. This is derived by setting this condition and substituting $H = S_L$ and $S = H_L$

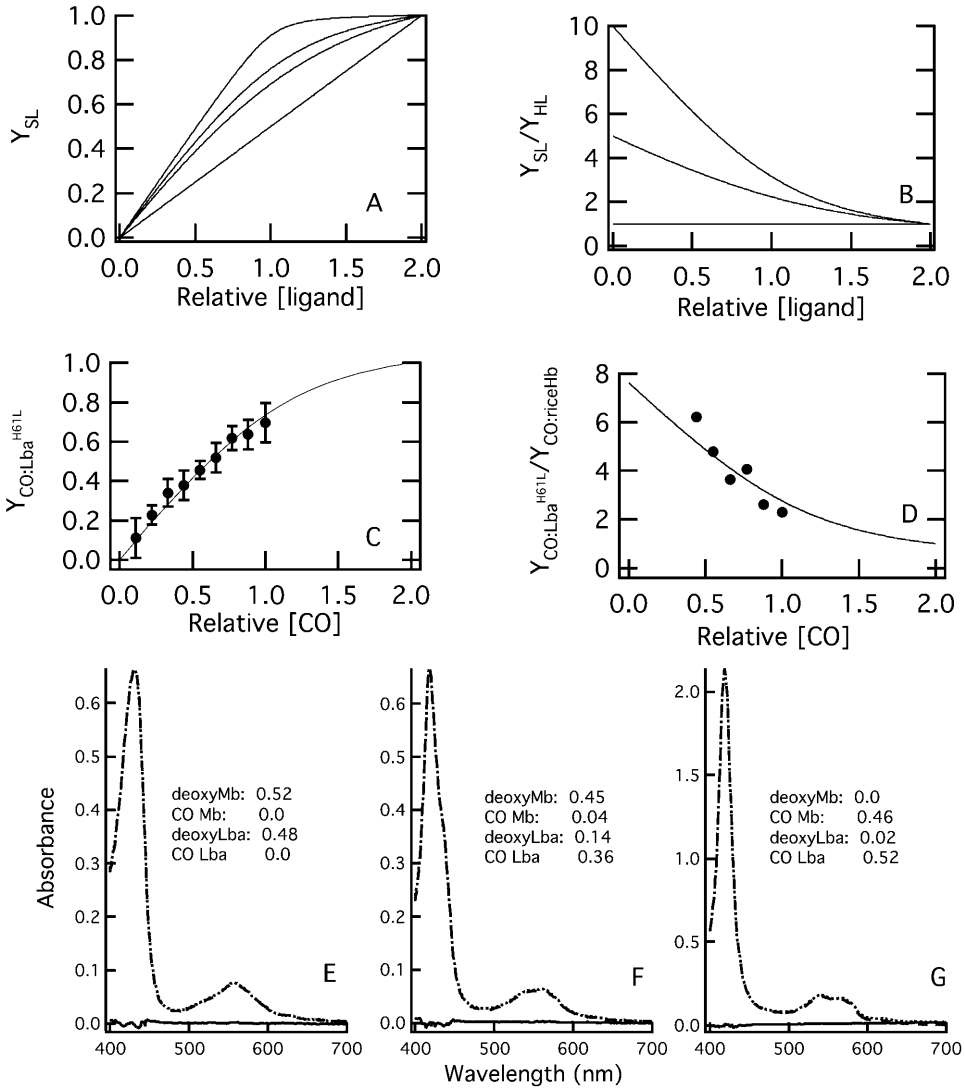

FIGURE 3 Simulation of equilibrium competition ligand binding. (*A*) The fraction of ligand bound scavenger (Y_{SL}) is plotted against relative ligand concentration with $S_T = H_T$ (as described for Eq. 2). The curves (from top to bottom) are for K_S/K_H ratios of 100, 10, 5, and 1. (*B*) With these data represented as the ratio of ligand bound species (Y_{SL}/Y_{HL}), the relationship in Eq. 3 is apparent. (*C* and *D*) An experiment contesting Lba^{H61L} against wild-type riceHb (rHb) for CO binding with data analyzed as in *A* and *B*. The solid line in each case is the fit of the observed data (*dots*) to Eq. 2 with $S_T = H_T = 6\ \mu M$, and $K_{SL} = 70{,}000\ \mu M^{-1}$ (Table 1). (*E*) The absorbance spectrum of equal concentrations of deoxy Lba and deoxy Mb in the absence of CO, (*F*) with equimolar CO (6 μM), (*G*) and with excess CO. In each case, the experimental spectrum (*dotted*) is overlaid with the fitted spectrum (*dashed*) and the residual between the two (*solid*). The normalized and fitted population of each component is inset in each figure.

into Eq. 1. This is a useful relationship when these experimental parameters are used.

$$\left(\frac{S_L}{H_L}\right)^2 = \frac{K_{SL}}{K_{HL}}. \tag{3}$$

A series of CO binding experiments were conducted to explore these models. In Fig. 3, *C* and *D*, competition for CO between riceHb and Lba^{H61L} was measured by mixing equal concentrations of these proteins (6 μM) and titrating CO into the cuvette. As in Fig. 3, *A* and *B*, the *x* axis reflects the ratio of [CO] to [H_T] and [S_T]. Fig. 3 *C* is the observed Y_{SL} fit to Eq. 2 by allowing the ratio of equilibrium constants to vary; this fit returned $K_S/K_H = 7.5$. Fig. 3 *D* represents these data as described for Fig. 3 *B*, with the value obtained for Y_{SL}/Y_{HL} at $L_T = 1$ equal to 2.5. These results indicate that the CO affinity of riceHb is ~7 times less than that of Lba^{H61L}.

As a control experiment, this method was used to evaluate the ratio of CO affinity constants for two known Hbs, Mb and Lba (Fig. 3, *E–G*). In Fig. 3 *E*, no CO has been introduced and a fit to these spectra indicates the presence of only the deoxygenated species for each protein. In Fig. 3 *F*, the concentrations of Mb, Lba, and CO are 1:1:1, and the ratio of CO:Lba to CO:Mb is measured to be 9. Using Eq. 3, this yields a ratio of affinity constants for Lba/Mb of 81. The known values of K_{CO} for Lba and Mb are 2000 and 27 μM^{-1}, respectively (Table 1). The resulting expected value for the ratio of equilibrium constants is 74, and is near that predicted from Fig. 3, *E–G*.

Equilibrium CO affinity in *Syn*Hb and riceHb

Ratios of CO-bound scavenger to CO-bound riceHb or *Syn*Hb were measured for each of the CO-scavengers listed in Table 1. In all cases, $S_T = H_T = L_T$. Results can be reported as both the ratio of CO-bound scavenger to CO-bound unknown Hb (S_L/H_L), or the ratio of deoxy species

(*H*/*S*). Experimentally, the two ratios were very similar and the numbers were averaged. The data for both riceHb (*solid*) and *Syn*Hb (*open squares*) in Fig. 4 show ratios near 1 when assayed against Lba^{H61A}, indicating that each has an affinity constant of ~14,000 μM^{-1}. The ratios for both proteins against Lba^{H61L} are ~2.3. Using this value along with Eq. 3, a ratio of affinity equilibrium constants for Lba^{H61L} and each hxHb is predicted to be ~5.3. When the affinity constant of Lba^{H61L} (Table 1) is divided by 5.3, these experiments agree on a value of ~14,000 μM^{-1} for riceHb and *Syn*Hb.

Equilibrium oxygen binding

Experiments with CO are vastly simpler to conduct than with oxygen because CO binds Hbs in the presence of sodium dithionite (DT). This reagent conveniently forms and maintains the Fe^{2+} state and concomitantly destroys any oxygen that might be present. Equilibrium competition experiments with oxygen must use other means to maintain a reduced heme iron and safeguard against contamination with other ligands. For a researcher familiar with Hb work, this problem can be summarized as ''How does one make deoxy Hb without chemically destroying the oxygen?'' With human hemoglobin, oxygenated protein can be treated with a few rounds of evacuation followed by equilibration with N_2 to generate the deoxy species (Giardina and Amiconi, 1981). But as the oxygen association affinity constant approaches 1 μM^{-1}, this method loses its effectiveness. Another method has been removal of all oxygen from an airtight custom-fitted burette containing a Hb, followed by titration with DT while monitoring optical absorbance. This is unpractical for the high-affinity Hbs studied here, and our experience was that more than stoichiometric DT concentrations were required for reduction. This results in a potential for reduced DT remaining in solution, and little confidence in $[O_2]$ once it is added. Stoichiometric DT addition resulted in an initial formation of deoxy Hb followed by an oxy-like spectrum, and subsequent Hb oxidation.

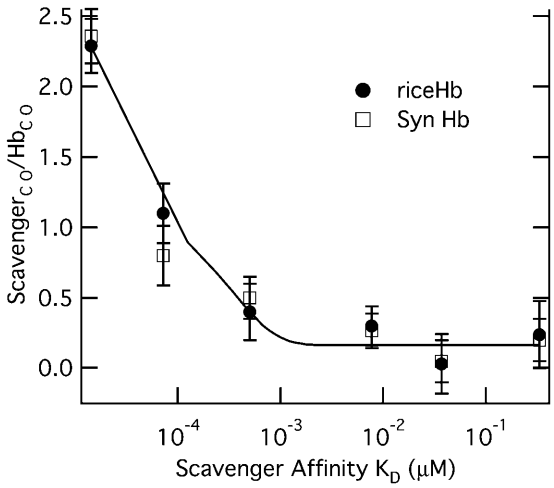

FIGURE 4 Equilibrium competition for CO. RiceHb and *Syn*Hb compete for CO with the scavengers listed in Table 1. The ratio of CO:scavenger to CO:Hb is plotted versus the CO dissociation equilibrium constants of the respective scavenger.

In the experiments defining this competition method, deoxy proteins were generated without DT by purging a cuvette containing the ferric forms of these proteins with O_2-scrubbed N_2 and then reducing the proteins with the ferridoxin/ferridoxin-reductase system (Hayashi et al., 1973). As the ferric form of the protein binds no oxygen, purging at this step allows complete O_2 removal before reduction. Using this system, we were able to generate the deoxy species of each scavenger, riceHb, and *Syn*Hb without using DT (an example is shown in Fig. 7).

Results for each O_2 scavenging experiment with riceHb are shown in Fig. 5. The oxy-species of riceHb and the Lba-based scavengers have nearly identical spectra, and were therefore fit as a single species. With the Mb and *Ascaris* Hb scavengers, all four spectra could be discerned in the fit. Each experiment contained 6 μM scavenger Hb and riceHb, and the first column of graphs in Fig. 5 show the results of fits to the deoxygenated combination of scavenger and riceHb. In each case, the deoxy species predominate. Addition of 6 μM O_2 (middle column) reduces the fraction of deoxy riceHb to the baseline against every scavenger with an affinity less than or equal to *Ascaris* Hb. Addition of oxygen generates equivalent fractions of each species of riceHb and *Ascaris* Hb, and leaves mainly deoxy riceHb in the experiments with Lba^{H61R}.

Because the respective oxy species are not resolved, scavenging ability is measured using the ratio of remaining deoxy riceHb to remaining deoxy scavenger after addition of 6 μM O_2, and normalization to the starting fractions of respective deoxy proteins. These ratios for riceHb and *Syn*Hb are shown for each scavenger in Fig. 6. Using the ratio for *Ascaris* Hb and Lba^{H61R}, the oxygen affinity of riceHb is predicted to be 270 and 300 μM^{-1}, respectively. The value for *Syn*Hb appears to be slightly lower (~100 μM^{-1}) based on competition with *Ascaris* Hb, but we were unable to make a reliable measurement against Lba^{H61R}.

The exact cause of our inability to use this assay with the specific combination of *Syn*Hb and Lba^{H61R} is unknown. However, Fig. 7 describes two unusual phenomena associated with the combination of these two proteins that prevent their use in this assay. Fig. 7 *A* shows time courses of reduction for Lba^{H61R}, *Syn*Hb, and an equal mixture of the two proteins. Lba^{H61R} is reduced by the ferridoxin/reductase system very rapidly compared to *Syn*Hb when the proteins are reduced independently, but when mixed together the reduction of both occurs at about the same rate as Lba^{H61R} alone. Fig. 7 *B* shows the combined, reduced deoxy spectra (*dots*) resulting from the time course (*open circles*) in Fig. 7 *A*. When oxygen is added, a partially oxygenated spectrum is generated initially (*solid*), but over a few minutes the spectrum becomes identical to the starting, deoxy spec-

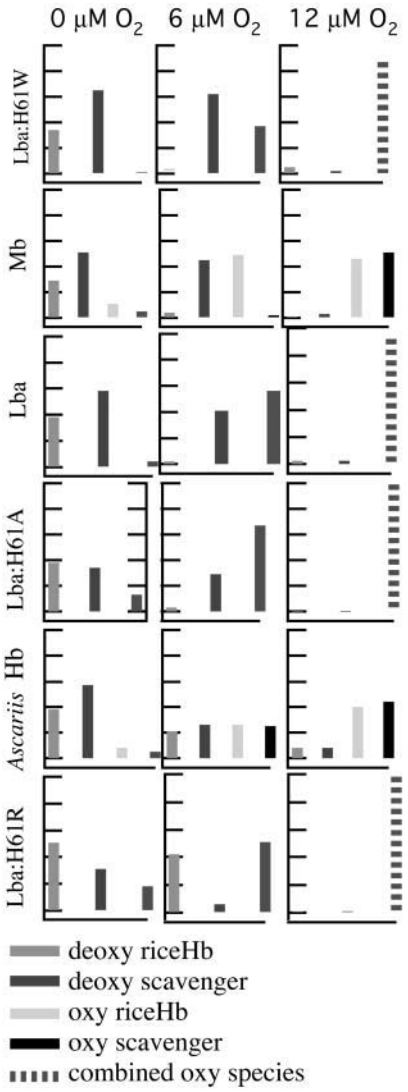

FIGURE 5 Raw data for riceHb equilibrium competition for O_2. Bar graphs show the contributions of each component spectrum to the fitted spectrum for each experiment in which riceHb competes for oxygen with the scavengers listed in Table 1.

trum (*dashed*). This process can be repeated with successive additions of oxygen for many cycles. Whatever the mechanism for combined reduction and oxygen removal (probably related to consumption of oxygen in autooxidation (Brantley et al., 1993)), this combination of scavenger and unknown Hb cannot be used in our equilibrium competition experiment. Of the 13 combinations of oxygen scavenger/unknown Hb measured in this work, this is the only one that behaved unreliably.

DISCUSSION

This equilibrium competition method is the first report of direct measurement of equilibrium constants for O_2 and CO binding to Hbs with dissociation equilibrium constants in the

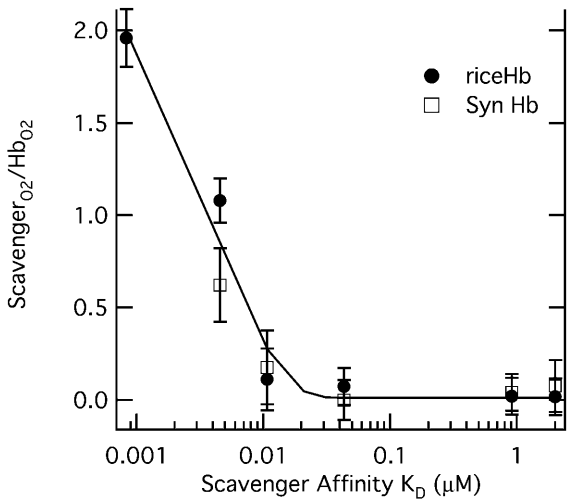

FIGURE 6 Equilibrium competition for O_2. RiceHb and *Syn*Hb compete for O_2 with the scavengers listed in Table 1. The ratio of O_2:scavenger to O_2:Hb is plotted versus the O_2 dissociation equilibrium constants of the respective scavenger.

nM range. Due to the inability of traditional equilibrium methods to measure these values, previous estimates have relied upon kinetic measurements to provide equilibrium constants. With hxHbs and other hemoglobins that display more complex reaction schemes, the kinetic binding models must be accurate to have confidence in equilibrium constants extracted from the kinetic data. The equilibrium competition method can provide an independent measurement of affinity constants for any hemoglobin with spectral properties different from the scavenger proteins. It is therefore ideally suited for hexacoordinate hemoglobins but could also be used with any hemoglobin whose kinetically derived affinity constants are in question.

Extensive kinetic analysis of the reaction scheme for riceHb has led to a model for ligand binding and prediction of equilibrium constants based on kinetic rate constants (Trent et al., 2001a). But in the case of *Syn*Hb (and other Hbs with complex reaction schemes), confident estimates of ligand affinities have not been proposed (Couture et al., 2000; Hvitved et al., 2001). Presented here, in addition to a description of the equilibrium competition method, are equilibrium constants for O_2 and CO binding to these two hxHbs. The following discussion examines equilibrium versus kinetic measurements of affinity in riceHb and the biological implications of the equilibrium constants measured for both hxHbs.

Equilibrium versus kinetic measurements of affinity constants

The reaction scheme for ligand binding to hxHbs involves ''open'' and ''closed'' protein conformations in addition to the hexacoordinate and pentacoordinate states (Trent et al., 2001a).

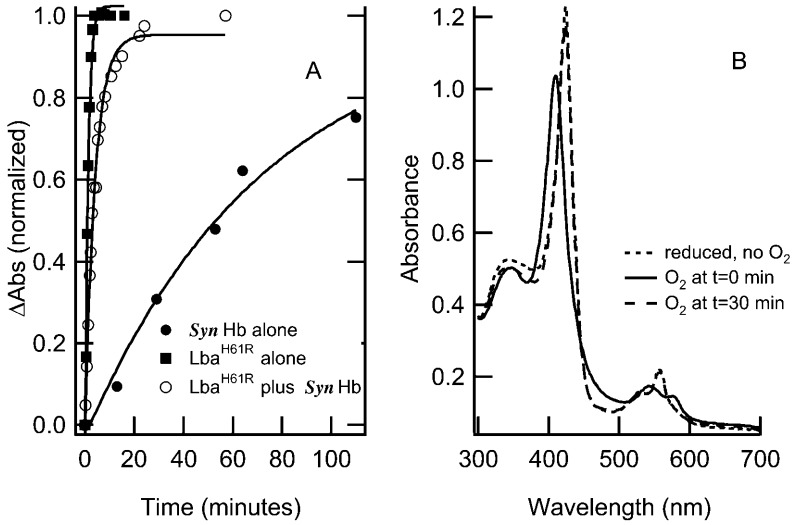

FIGURE 7 Time courses for reduction of *Syn*Hb and Lba^{H61R} individually and together. (*A*) Time courses for reduction as measured at the deoxy Soret maximum for each protein. (*B*) Over time, the combination of *Syn*Hb with Lba^{H61R} and the reduction system consumes oxygen. The dotted spectrum is the reduced, deoxygenated protein mixture. Immediately after O_2 addition, a partially oxygenated spectrum is generated (*solid*). After 30 min, the protein mixture again returns a deoxygenated spectrum (*dashed*).

$$Hb_C \underset{K_C}{\rightleftarrows} Hb_{O,H} \underset{K_H}{\rightleftarrows} Hb_{O,P} + L \overset{K_L}{\rightarrow} Hb_{O,L}. \qquad (R2)$$

This reaction scheme was developed to describe slow phases of ligand binding to hxHbs that are observed in rapid mixing experiments. An effective affinity constant that takes into account the effects of hexacoordination (K_H) and closed, slow phases in binding (K_C) can be derived (as described in detail by Trent et al. (2001b)) from R2.

$$K_{L,Effective} = \frac{K_L}{1 + K_H + K_C}. \qquad (4)$$

Affinity constants for O_2 and CO binding to riceHb were calculated using this model based on kinetic measurements of $K_{O2} = 1800\ \mu M^{-1}$ (Arredondo-Peter et al., 1997), $K_{CO} = 6000\ \mu M^{-1}$, $K_H = 0.38$ (Hargrove, 2000), and $K_C = 10$ (Trent et al., 2001a). These corrected affinity constants are reported in Table 2 along with the uncorrected (i.e., k'_L/k_L) values and the values measured using the equilibrium competition method.

The corrected K_{O2} value is within a factor of two of the value measured by the competition method, and both methods predict a lower affinity compared to the uncorrected value. However, the corrected and competition K_{CO} values are very different, and the competition measurement indicates a higher CO affinity compared to the uncorrected, kinetic estimate. Equation 4 predicts that the effects of hexacoordination and protein conformational changes on ligand affinity should be ligand-independent, so it was anticipated that the uncorrected values of K_{O2} and K_{CO} would be decreased by the same factor (that is, $1/(1 + K_H + K_C)$). The observed values of K_{O2} and K_{CO} measured by the competition method are therefore not consistent with the reaction scheme proposed in R2 and Eq. 4.

There are at least three reasons to have confidence in the results of the equilibrium competition method over those of the kinetic method: 1), Competition for CO between Lba and Mb indicate that the equilibrium competition method can accurately measure the relative CO affinity constant for these two proteins. 2), With both *Syn*Hb and riceHb, competition with each scavenger is internally consistent. In no case did a scavenger with lower affinity show appreciable ligand binding in competition with the hxHb. Only when the scavenger affinity reached that of *Ascaris* Hb (for oxygen) or Lba^{H61A} (for CO) were appreciable concentrations of either deoxy hxHb observed. If there was a problem with a particular scavenger protein, or with the method, one might expect spurious results with at least one of the

TABLE 2 A comparison of affinity constants for Mb, Lba, riceHb, and *Syn*Hb

	*Kinetic, uncorrected		†Kinetic, corrected		Equilibrium competition	
Protein	K_{O2}	K_{CO}	K_{O2}	K_{CO}	K_{O2}	K_{CO}
Mb	1.1	27	–	–	–	25‡
Lba	23	2000	–	–	–	2200‡
RiceHb	1800	6000	160	530	285	14,000
SynHb	17,100	90,000	–	–	~100	14,000

*These values are the ratio of the bimolecular rate constant for ligand binding to the pentacoordinate form of the protein to the dissociation rate constant.
†These values for riceHb were calculated from Eq. 4 as described in the text.
‡The equilibrium constant for Lba was measured relative to Mb, and vice versa, as a control for this method.

experiments. In the case of Lba^{H61R} and *Syn*Hb, reliable data were not collected, but this reaction did not violate the continuity of the results in the same way that would occur if, for example, wild-type Mb were to outcompete *Syn*Hb for O_2 but Lba could not. 3), R2 is not a complete model for ligand binding, and only accounts for some of the slow phases of ligand binding after rapid mixing. Several hxHbs were examined by Trent et al. (2001a), and riceHb had the smallest degree of slow-phase ligand binding. For this reason, kinetic estimates of affinity constants would seem to have the greatest chance of being accurate with riceHb. Ligand binding to *Syn*Hb is so complex that affinity values have still not been assigned by kinetics (thus the lack of these values in Table 2) (Couture et al., 2000; Hvitved et al., 2001). Although riceHb provides the best chance of measuring accurate affinity constants from kinetic methods, the kinetic model is still not complete enough to challenge a direct affinity measurement.

Biological implications

Numerous physiological functions have been proposed for hxHbs including oxygen transport, ligand sensing, scavenging and destruction of ligands such as oxygen or nitric oxide, and alternative respiratory biochemistry (Dordas et al., 2003; Hunt et al., 2002; Trent and Hargrove, 2002; Van Doorslaer et al., 2003). If the role involves oxygen binding, a fundamental question is whether scavenging or transport is the biochemical objective. Transport by facilitated diffusion for purposes of respiration requires: 1), Hb concentration high enough to augment nonfacilitated diffusion. The solubility of pure O_2 in water is ~1 mM. For a Hb to facilitate diffusion, its concentration must be in this range or higher. 2), The affinity constant and exogenous ligand concentrations must be such that the facilitating Hb is partially saturated across a concentration gradient. 3), The dissociation rate constants must not limit ligand diffusion (Wittenberg, 1966, 1965; Wyman, 1966). For riceHb and *Syn*Hb, these parameters are not within ranges that would enable a role in facilitated diffusion. RiceHb concentrations are thought to be very low in plants (Arredondo-Peter et al., 1997; Duff et al., 1997), and high concentrations of *Syn*Hb have not been observed in vivo. Furthermore, both proteins have oxygen dissociation rate constants that are very slow. In the case of riceHb, oxygen dissociation is slowed by a hydrogen bond with the distal histidine (His^{73}); in *Syn*Hb, the structural factors causing slow dissociation are less clear and involved multiple amino acids (Arredondo-Peter et al., 1997; Couture et al., 2000; Hvitved et al., 2001).

The results presented here indicate that although oxygen affinities are lower than those predicted from kinetic experiments, they are still too large to allow facilitated diffusion to occur under any known physiological conditions. Both proteins possess affinity constants for oxygen that are similar to that of *Ascaris* perienteric Hb, a protein that does not facilitate oxygen diffusion even under micro aerobic conditions (Wittenberg, 1966; Wittenberg et al., 1974). Our results also indicate that the current kinetic model for ligand binding to hxHbs is not sophisticated enough to accurately predict equilibrium constants from kinetic rate constants. It is possible that the kinetic rate constants for the slow phases described for hxHbs (Dewilde et al., 2001; Trent and Hargrove, 2002; Trent et al., 2001b) are ligand specific, or that the dissociation rate constants have an additional component that is extremely slow which has not yet been accounted for. Whatever the reason is for the lack of agreement between kinetics predictions and equilibrium measurements, it almost certainly arises from the limited kinetic model. A combination of kinetic and equilibrium methods is therefore required to evaluate the biophysical behaviors of these proteins.

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