

Determination of the Carbon Monoxide Binding Constants of Myoglobin Mutants: Comparison of Kinetic and Equilibrium Methods[†]

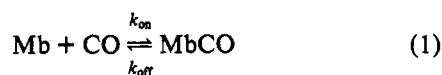
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Received April 20, 1994[®]

ABSTRACT: The carbon monoxide (CO) binding constants of human myoglobin (Mb) and several single-site mutants have been determined using two different methods. In the kinetic method, which is commonly used for this ligand, the overall association (k_{on}) and dissociation (k_{off}) rates of CO were measured by flash photolysis and NO replacement, respectively, and the ratio k_{on}/k_{off} was calculated. In the equilibrium method, the binding constant K_{eq} was measured directly using a thin-layer technique. These two measurements yield similar results for human wild-type Mb but differ significantly for some of the mutants. Possible reasons for these discrepancies are analyzed. A model assuming the presence of interconverting conformers with different association and dissociation rates is considered in light of infrared measurements on the CO stretching frequency in the MbCO forms of the same proteins [Balasubramanian *et al.* (1993a) *Proc. Natl. Acad. Sci. U.S.A.* 90, 4718]. It is suggested that in the case of some mutants which exhibit multiple conformations, this model may lead to nonequilibrium kinetics, which could produce the observed discrepancies between the kinetic and equilibrium determinations of the binding constant. These results suggest that both equilibrium and kinetic data should be obtained, even for a monomeric protein such as Mb, before the relative stabilities of mutants can be meaningfully compared.

Equilibrium binding of ligands to hemoglobin (Hb) is the primary property related to its physiological function and has been a rich source of information regarding the cooperativity and conformational changes in the binding process (Antonini & Brunori, 1971; Imai, 1982; Gill *et al.*, 1987; Di Cera *et al.*, 1987; Ackers & Johnson, 1990). The reaction with oxygen has been extensively studied, and oxygen binding constants have been determined for many myoglobins (Antonini & Brunori, 1971; Wang *et al.*, 1979; Di Cera & Gill, 1988). However, there have been relatively few direct measurements of the equilibrium binding of CO to myoglobin (Mb), due primarily to the very large affinity of Mb for this ligand ($K_{eq} > 10^7 M^{-1}$). Determinations of the CO binding constant have therefore relied on kinetic measurements of the overall association and dissociation rate constants (k_{on} and k_{off} , respectively). The reaction of a monomeric protein such as Mb is expected to be relatively simple and can be described by the following equation:



The ratio of these rate constants, k_{on}/k_{off} , then yields the apparent binding constant K for this reaction. The binding constant obtained in this manner should agree with that obtained by equilibrium techniques (K_{eq}), provided the kinetic and equilibrium data measure exactly the same reaction (Antonini & Brunori, 1971).

In general, for Mb it has been formed that the association and dissociation reactions follow single-exponential kinetics, unlike the case of Hb (Gibson, 1959; Gibson & Antonini,

1967; Antonini *et al.*, 1968). However, even for Mb or the isolated α and β chains of Hb, the ratio k_{on}/k_{off} does not always agree with K_{eq} . In fact, for oxygen binding at 20 °C, the ratio k_{on}/k_{off} is almost twice K_{eq} (Antonini, 1965; Antonini & Brunori, 1971; Di Cera & Gill, 1988). A similar comparison for CO is difficult to make due to the sparse data on K_{eq} and the relatively high error in its determination; nonetheless, available data obtained a number of years ago do indicate a disparity in this case as well (Keyes *et al.*, 1967; Antonini, 1965; Brunori *et al.*, 1968). The reasons for this disparity in the kinetic and equilibrium measurements are not well understood. One reason may be that the reaction of CO with Mb is in reality a complex one, and the observed rate constants may not refer to the same elementary process. Another possibility is suggested by infrared and Raman experiments which show that there is often more than one conformation of liganded Mb in solution even at room temperature (Makinen *et al.*, 1979; Ansari *et al.*, 1987; Morikis *et al.*, 1989). In such a case, k_{on} and k_{off} become dependent on the rates of interconversion among these conformers.

We have recently examined the presence of multiple conformers in a number of mutants of human MbCO by FTIR (Balasubramanian *et al.*, 1993a), as well as the functional consequences on both the geminate and bimolecular rebinding kinetics (Balasubramanian *et al.*, 1993b; Lambright *et al.*, 1993, 1994). In the following we present measurements of the overall on and off rates for CO binding to distal pocket mutants of human Mb along with equilibrium CO binding measurements using a highly sensitive thin-layer technique (Dolman & Gill, 1978; Gill, 1981; Gill *et al.*, 1987). These results are then compared and discrepancies between the two approaches are analyzed in terms of the conformation variations inferred from FTIR measurements.

EXPERIMENTAL PROCEDURES

Protein Preparation. The preparation of the site-specific mutants used in this study has been described previously

[†] This work was supported in part by a grant from the National Institutes of Health (GM27738).

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^{||} Dr. Stanley Gill passed away while the manuscript was in preparation.

This paper is dedicated to his memory.

[®] Abstract published in *Advance ACS Abstracts*, June 15, 1994.

(Lambright *et al.*, 1989, 1993; Balasubramanian *et al.*, 1993b). Substitutions were made at positions occupied by Lys 45, Asp 60, His 64, and Val 68 in human WT Mb. Concentrated stock solutions (1–4 mM) of the purified proteins were made and used in subsequent steps.

k_{on} by *Flash Photolysis*. The acquisition of the bimolecular on-rate data has been described elsewhere (Lambright *et al.*, 1994). Briefly, the recombination kinetics were recorded by monitoring the transient absorbance at 436 nm and 22 °C. The final exponential decay depends on CO concentration and corresponds to the bimolecular recombination (Balasubramanian *et al.*, 1993b). Under the conditions of the experiment, the reaction is pseudo first order in the concentration of Mb. Second-order rate constants were calculated from the single-exponential fits to the data using the solubility of CO in aqueous solutions (Ackerman & Berger, 1963; Weisenberg & Guinasso, 1979). The mean association rate and standard deviation were calculated from four to eight independent measurements for each protein.

k_{off} by *Replacement with NO*. The concentrated met-Mb stocks were converted to the MbCO form by stirring under CO followed by reduction with 2 equiv of sodium dithionite. NO-saturated buffer (200 mM phosphate for pH 7 and 200 mM citrate–borate–phosphate for other pHs) was prepared by bubbling NO through 2 mL of deoxygenated buffer in a sealed 1 cm path length cuvette; 2–5 μ L of the concentrated MbCO stock was then injected into the cuvette at 22 °C, and the reaction was followed by monitoring the absorbance at 424 nm (near the maximum of the MbCO–MbNO difference spectrum). The dead time in this measurement is about 2 s, in which time less than 5% of the reaction is complete even for the mutants with the fastest *k_{off}* examined in this study. It was found that the reaction is quite sensitive to pH, and thus we took extreme care to thoroughly deoxygenate the buffer before bubbling NO, as this would significantly affect the pH. The pH was also measured after the reaction was complete, and only data from runs where the pH was within ± 0.2 unit of the stated value were used for subsequent analysis.

Equilibrium Binding Constant Measurements. Stock solutions of human Mb and its mutants were deoxygenated by stirring under CO and reduced with sodium dithionite. The final conditions used in the experiments were 200 μ M Mb in 100 mM phosphate, pH 7.0, and the temperature was 22.0 °C.

The thin-layer apparatus is described in detail elsewhere (Dolman & Gill, 1978; Gill, 1981; Gill *et al.*, 1987). In this method, the sample is held between a glass window and a transparent gas-permeable membrane, and the path length is determined by the thickness of the spacer (typically 25 μ m). The protein solution is then directly equilibrated with a well-defined CO gas phase at atmospheric pressure. Experiments were initiated with 100% CO, and dilutions of the CO partial pressure were then made with nitrogen by means of a dilution valve. The resulting changes in optical density were measured at 424 nm.

RESULTS

Kinetics of Association and Dissociation. Typical CO bimolecular recombination curves are shown in Figure 1. The time axis is plotted on a logarithmic scale due to the wide variation in the association rates for these mutants. The rebinding of CO is multiphasic in human Mb and all its mutants, and the final exponential phase is the only one that depends on the partial pressure of CO (Balasubramanian *et al.*, 1993b). This exponential phase is therefore taken to be

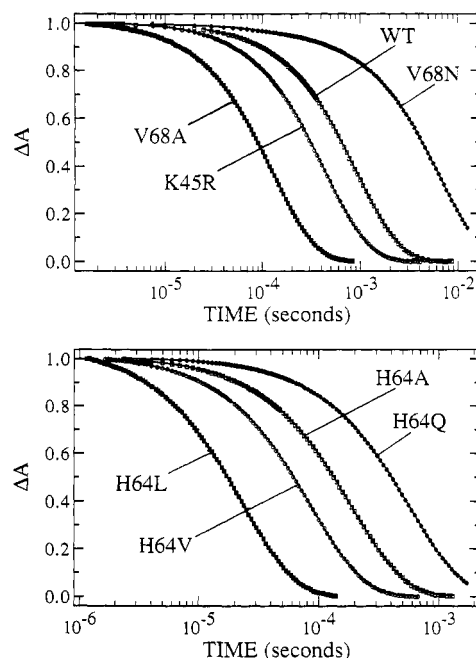


FIGURE 1: Bimolecular association kinetics of CO to human Mb mutants at room temperature. Time is plotted on a logarithmic scale along the x-axis to facilitate comparison among the mutants. (Top) Human Mb WT is shown along with Lys 45 \rightarrow Arg, and Val 68 \rightarrow Ala, Asn mutants. (Bottom) His 64 \rightarrow Ala, Val, Leu, and Gln mutants are shown; note the expanded time scale. The change in absorption has been normalized to unity at the end of the geminate processes to facilitate comparison of the bimolecular recombination (see text); the solid lines are exponential fits.

due to bimolecular recombination and is well separated in most of these proteins from the earlier geminate processes which are generally complete by about 10 μ s under these experimental conditions. The value of ΔA at the end of the geminate phase is scaled to unity for all mutants to facilitate comparison of the bimolecular process. The top panel shows the results for human WT Mb compared with two Val 68 mutants and the surface mutant K45R. As can be clearly seen, the two Val 68 mutants V68A and V68N show radically different behaviors: compared to WT, the recombination of V68A is a factor of 6 faster, while that of V68N is almost an order of magnitude slower. The unexpectedly fast recombination of the mutant K45R has been previously noted (Lambright *et al.*, 1989, 1994). The lower panel of Figure 1 shows the bimolecular recombination kinetics for the His 64 mutants. All of the His 64 mutants recombine more rapidly than WT, with H64Q being the most similar to WT. Fits of the bimolecular recombination data to single exponentials are shown as solid lines in Figure 1, and the second-order association rate constants *k_{on}* obtained from these pseudo-first-order rate constants are listed in Table 1. It is interesting that these rate constants span more than 2 orders of magnitude (the fastest and slowest rates—H64L and V68N, respectively—differ by a factor of 220).

Typical curves for the CO dissociation reaction are shown in Figure 2. It is apparent that the kinetics of dissociation vary less than those of association. Table 1 lists the rate constants *k_{off}* obtained by fitting these curves to a single exponential. For two of the mutants, V68N and H64Q, a satisfactory fit to the data requires two exponentials. For these two mutants, the parameters from a biexponential fit were as follows: V68N $\phi = 0.6$, $R_1 = 0.0112$ s⁻¹, and $R_2 = 0.0602$ s⁻¹; H64Q $\phi = 0.66$, $R_1 = .0137$ s⁻¹, and $R_2 = 0.0817$ s⁻¹ (ϕ is the fractional amplitude of the first process, and R_1 and R_2 are the rate constants for the two processes). For

Table 1: Kinetic Constants for CO Binding to Human Mb Mutants

protein	$k_{on} \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$	$k_{off} \times 10^2 \text{ s}^{-1}$	$k_{on}/k_{off} \times 10^{-7} \text{ M}^{-1}$
WT	0.986 ± 0.011	3.61 ± 0.2	2.73 ± 0.15
D60E	1.07 ± 0.06	3.56 ± 0.11	3.09 ± 0.11
D60A	0.88 ± 0.03	<i>a</i>	
K45R	1.80 ± 0.02	2.50 ± 0.11	7.20 ± 0.32
K45A	1.04 ± 0.02	4.31 ± 0.4	2.50 ± 0.24
K45Q	0.917 ± 0.017	6.03 ± 0.5	1.54 ± 0.13
H64Q	1.71 ± 0.01	1.98 ± 0.1^b	8.64 ± 0.44
H64A	3.75 ± 0.14	11.1 ± 0.6	3.38 ± 0.22
H64V	7.25 ± 0.35	10.1 ± 0.5	7.18 ± 0.5
H64L	33.1 ± 0.15	5.45 ± 0.2	60.7 ± 2.24
V68A	6.00 ± 0.18	3.75 ± 0.13	16.0 ± 0.73
V68N	0.150 ± 0.006	1.87 ± 0.1^b	0.802 ± 0.05

^a The dissociation rate was not determined. ^b For these mutants the dissociation kinetics were observed to be best fit with two exponentials (see text). The table entry is the best single-exponential fit to facilitate comparison.

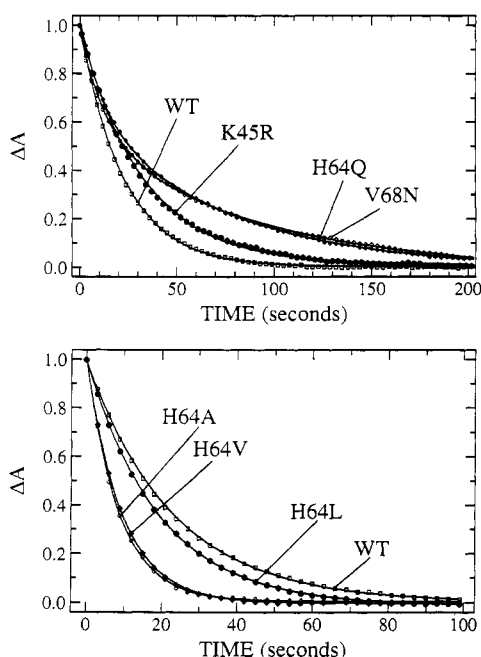


FIGURE 2: Kinetics of CO dissociation by replacement with NO to human Mb mutants at room temperature. (Top) Human Mb WT is shown with Lys 45 → Arg, Val 68 → Asn, and His 64 → Gln mutants. (Bottom) WT and His 64 → Ala, Val, and Leu mutants are shown; the time axis is expanded by a factor of 2 (note: this axis is linear). The data have been normalized to one at zero time. The solid lines are fits to a single exponential, except for V68N and H64Q, where a biexponential fit is used (see text for details).

these two mutants, the solid lines in Figure 2 are from the biexponential fit; for all of the others, the fits obtained by using a single exponential are shown. The best fit rate parameters are shown in Table 2 for all of the mutants. The fastest and slowest rate constants differ by a factor of 6. Interestingly, the His 64 mutants show rates both faster (H64A, H64L, H64V) and slower (H64Q) than that of WT, unlike the case of the association reaction. The Val 68 mutants show the same trends as in the association reaction, i.e., V68A is faster and V68N is slower than WT.

These changes lead to significant differences in the apparent binding constant $K_{app} = k_{on}/k_{off}$, which are also listed in Table 1. The kinetic constants are the averages of at least three runs on each mutant, and the errors from these experiments were propagated to obtain the errors in the apparent binding constant. The largest increases in the apparent binding constants are seen for the mutants H64L and V68A; these are followed by H64Q, H64V, and K45R. The greatest decrease

Table 2: Comparison of Equilibrium Constants and Apparent Binding Constants Obtained by Kinetic Measurements for CO Binding to Human Mb Mutants

protein	β (Torr ⁻¹)	$K_{eq} \times 10^{-7} \text{ M}^{-1}$	$k_{on}/k_{off} \times 10^{-7} \text{ M}^{-1}$
WT	36.0 ± 0.92	2.66 ± 0.07	2.76 ± 0.15
D60E	31.6 ± 2.0	2.34 ± 0.15	3.09 ± 0.11
D60A	39.1 ± 2.0	2.89 ± 0.12	<i>a</i>
K45R	53.7 ± 1.6	3.98 ± 0.12	7.20 ± 0.32
K45A	31.9 ± 2.0	2.36 ± 0.15	2.50 ± 0.24
K45Q	32.1 ± 2.0	2.38 ± 0.15	1.54 ± 0.13
H64Q	91.4 ± 0.4	6.76 ± 0.47	8.64 ± 0.44
H64A	73.5 ± 5.3	5.44 ± 0.39	3.38 ± 0.22

^a Not determined.

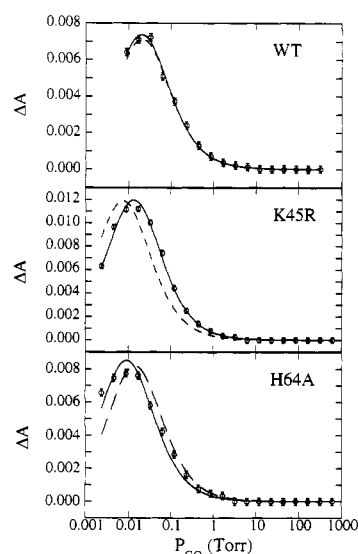


FIGURE 3: Absorption changes with stepwise changes in CO partial pressure for WT and two mutants during measurement of the equilibrium binding constant in the thin-layer cell. The data are plotted as the change in absorbance at each step versus the logarithm of the CO partial pressure. The solid lines are fits to a single binding site model according to eqs 4 and 5, and the binding constants are reported in Table 2. For comparison, the dashed curves are those obtained from the measured k_{on}/k_{off} using the same equations.

in the apparent binding constant is seen for the mutant V68N. In this case, a large change in the association rate constant appears to dominate the change in the apparent binding constant; while this is true for many others, for example H64L, there are some, for example H64Q, where the change in the apparent binding constant is due to substantial changes in both the association and dissociation rates.

Equilibrium Binding Measurements. Typical datasets obtained using the thin-layer method are shown in Figure 3 for human WT MbCO and two mutants. The data are plotted as the change in absorbance (ΔA) at each dilution step versus the logarithm of the partial pressure at that step. The points appear equally spaced on a log scale because the partial pressure changes by a constant dilution factor at each step. The partial pressure of CO after the i th dilution step is given by

$$p_i = p_0 D^i \quad (2)$$

where p_0 is the initial partial pressure of CO and D is the dilution factor ($D = 0.51954$). The absorbance change at the i th step, ΔA_i , is given by

$$A_i - A_{i-1} = \Delta A_i = \Delta A_T (\theta_i - \theta_{i-1}) \quad (3)$$

where θ_i is the fractional saturation defined as

$$\theta_i = \beta p_i / (1 + \beta p_i) \quad (4)$$

and β is the equilibrium binding constant in units of Torr⁻¹. The data were fit to eqs 2–4 using a nonlinear least-squares fitting program. The fits are shown as solid lines in Figure 3, and the resulting parameters are displayed in Table 2. The data below about 1 mTorr are subject to considerable experimental error and were not used in the analysis. The error bars are the estimated errors in measurement of the absorbance on the spectrophotometer. The parameters in Table 2 are typically averages of two runs, and the errors in the fitted parameters are quoted at the 1 σ level. To illustrate the sensitivity of the fitting to the value of the equilibrium constants, curves derived from the measured value of k_{on}/k_{off} are shown with dashed lines in Figure 3.

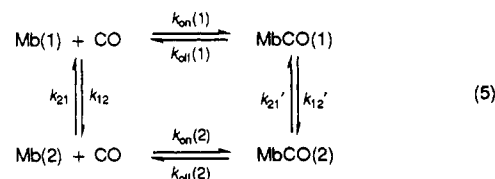
DISCUSSION

It is clear from the results presented above that, even for Mb, the binding process is more complex than the simple reaction scheme of eq 1. There are significant differences between the kinetic and equilibrium measurements of the binding constant for several mutants, even in aqueous solution at room temperature. A possible understanding of this difference requires the synthesis of information from a variety of sources in addition to the data described here.

It has been known for a number of years that the low-temperature rebinding kinetics of CO rebinding to Mb are nonexponential, and intermediate states are required to describe the kinetics (Austin *et al.*, 1975). It was shown by Eaton, Hofrichter, and co-workers that this is true even in aqueous solution at room temperature (Henry *et al.*, 1983). We have recently demonstrated that this is also the case for several mutants of human Mb, including the ones examined in this study (Lambright *et al.*, 1993, 1994). These results suggest a simple rationale for the discrepancy between the kinetic and equilibrium results presented in Tables 1 and 2 if the rate constants k_{on} and k_{off} do not refer to the same process. In the case of SW MbCO under similar conditions, it has been argued that the rate-determining step for both the association and dissociation steps occurs during bond formation and cleavage at the heme iron (Gibson *et al.*, 1986). Similar arguments should apply for human WT MbCO. In the case of some of the mutants in Table 1, however, it is possible that the barrier for bond formation may be considerably lower, and the rate-determining step in association may be the diffusion of ligands to the binding site. We have studied the recombination of CO to these mutants with nanosecond time resolution. Under these conditions, the geminate yield for these proteins remains less than 15%, with the exceptions of H64L and V68A (Lambright *et al.*, 1994). Thus, the rate-determining step for the proteins in Table 2 remains, to a good approximation, the final bond formation step at the iron, and other explanations must be found for the discrepancy between the kinetic and equilibrium results.

Another possibility is based on the results of infrared spectroscopy of the CO stretch of MbCO, where multiple bands attest to the presence of more than one conformation of the bound ligand and/or the protein (Caughey *et al.*, 1981). We have recently studied the temperature and pH dependence of these IR bands for the mutants listed here (Balasubramanian *et al.*, 1993a). These experiments show that there are two distinct bands for human WT MbCO and many of the other mutants; a few, such as H64L and H64V, show only one.

Assuming that these bands correspond to different conformations of the protein with different association and dissociation rates, the reaction scheme of eq 1 can be expanded as



where (1) and (2) represent the two conformers. If a scheme like this makes a significant contribution, the experimentally measured on and off rates become dependent upon the populations of the two conformers in both the ligand-free and the ligand-bound forms and the rates of interconversion between them. A complete analysis requires the knowledge of all the rate constants in eq 5; however, the scheme may be substantially simplified for different mutants if additional experimental evidence about the magnitude of the interconversion rates k_{12} , k_{21} , k_{12}' , and k_{21}' among these conformers is available. For instance, in the case of SW MbCO at 300 K, experiments from the Frauenfelder (Young *et al.*, 1991) and Champion (Tian *et al.*, 1992) groups have demonstrated that the interconversion of these conformers in both the deoxy and CO-bound forms occurs on a time scale shorter than about 10–50 μ s, which is much faster than the rates of the association and dissociation reactions. Making the reasonable assumption that a similar situation prevails for human WT MbCO, we can consider the two conformers to be essentially in equilibrium during the course of the experiments conducted in this study. It can then be shown that the experimentally measured association and dissociation rate constants are weighted averages of the rate constants of the individual conformers:

$$\bar{k}_{on} = \phi_{\text{Mb}}(1)k_{on}(1) + \phi_{\text{Mb}}(2)k_{on}(2) \quad (6)$$

$$\bar{k}_{off} = \phi_{\text{MbCO}}(1)k_{off}(1) + \phi_{\text{MbCO}}(2)k_{off}(2) \quad (7)$$

ϕ_{Mb} and ϕ_{MbCO} represent the fractional populations of the conformers 1 and 2 in the deoxy and CO-bound forms, respectively. The ratio of these experimentally measured rate constants yields the apparent binding constant. These expressions can be extended to include any number of conformations as appropriate.

The equilibrium binding constant measured in the thin-layer method can be written in terms of the total concentrations of the liganded and deoxy species as

$$\bar{K}_{eq} = [\text{MbCO}]_{\text{total}} / [\text{Mb}]_{\text{total}} [\text{CO}] \quad (8)$$

It can be shown that as long as the equilibrium between the conformers is maintained in the association and dissociation experiments, the ratio of the average kinetic constants given by eqs 6 and 7 is equal to \bar{K}_{eq} .¹ Comparison of the results in Tables 1 and 2 would then suggest that this situation prevails for human WT MbCO in phosphate buffer at pH 7 around 300 K, as the ratio k_{on}/k_{off} is quite similar to K_{eq} . On the other hand, the difference between these results for mutants such as K45R, H64Q, and H64A would suggest that the

¹ Attempts to fit the equilibrium data to the two conformer model (eq 8, using eqs 3 and 4 for each conformer) did not result in an appreciable decrease in χ^2 , except for the mutants K45R and H64A, which may be because part of the binding curve at very low partial pressures of CO could not be collected; even in those cases, the value of one of the binding constants was much too high to be realistic. Thus, all of the data were fit to a single site model and the parameters are given in Table 2.

assumptions made in this model do not hold under these experimental conditions.

Returning therefore to the scheme shown in eq 5, if these conformers have different association and dissociation rates, as indicated, then depending on the time scale of the interconversion between them, a number of possibilities arise:

If either of the interconversion rates in the deoxy and MbCO forms is less than the corresponding association or dissociation rate, respectively, then either ϕ_{Mb} or ϕ_{MbCO} will not be equal to its equilibrium value. In these cases, the observed kinetics will still be exponential but different from the rates at equilibrium. For instance, in the photolysis experiment, the ϕ_{Mb} will actually reflect the ϕ_{MbCO} present upon photodissociation, further distorted by the fact that the faster rebinding population will be selectively diminished during the geminate rebinding phase. Thus, it becomes important to know the geminate rebinding fraction in this experiment as well.

If the interconversion rates are on a time scale similar to the corresponding association or dissociation rate, then the fractions of the species present change during the course of the experiment, and nonexponential kinetics will be observed. In our experiments, this could be the case for H64Q and V68N in the dissociation experiments.

Some of these ambiguities could be resolved by comparing the rates obtained by flash photolysis to those obtained by stopped-flow measurements. In the photolysis method, even if the interconversion in the deoxy form is fast compared to the on rates, the proteins may still be in the ligand-bound fractions and thus nonequilibrium kinetics are observed. In the latter method, the initial ϕ_{Mb} will indeed be the equilibrium fractions, but some perturbation may still be introduced if the interconversion in the newly formed MbCO state is slow, and the ϕ_{MbCO} are not equal to their equilibrium values.

A major problem in resolving these issues is that the most common technique used to study heme protein ligand dynamics, visible spectroscopy, is insensitive to the differences among these conformers and only measures the total population in the deoxy and ligand-bound states. Therefore, to resolve the kinetics of each conformer, vibrational spectroscopy with nanosecond-to-millisecond time resolution is required. Such studies have already contributed to understanding the interconversion process in wild-type SWMb (Young *et al.*, 1991). Parallel experiments on human Mb and its mutants are in progress in our laboratory.

Comparisons with Previous Work. It is striking that the binding constants in the upper half of Table 1 do not vary much (with the exception of K45R) as compared to those in the lower half. Some of these mutants were made in SW Mb, and increases in the ratio k_{on}/k_{off} were seen for the His 64 mutants, and also for V68A (Egeberg *et al.*, 1990; Rohlf's *et al.*, 1990); the equilibrium binding constants were not determined independently.² It is interesting that the apparent

² These authors also made the interesting observation that the kinetically measured apparent oxygen binding constants of the hydrophobic His 64 mutants were much lower than their reference SW MbO₂; the binding constant was not measured by equilibrium methods. The available data on horse and SW MbO₂ (Antonini, 1965; Antonini & Brunori *et al.*, 1971; Di Cera & Gill, 1988) indicate that variations between kinetic and equilibrium measurements are likely in the case of the oxygen binding reaction as well. The lowered binding constant was attributed to the loss of the hydrogen bond between the bound O₂ and N₄ of His 64 seen in neutron diffraction data (Phillips, 1980). No evidence of a hydrogen bond between bound CO and His 64 is seen in neutron diffraction data on MbCO crystals (Cheng & Schoenborn, 1991); thus, this factor probably does not play a major role in the case of the CO binding reaction.

binding constant for H64A in Table 1 is quite similar to that of WT, whereas that for the SW mutant H64G was nearly 5-fold higher than that of the reference SW Mb. Conversely, the mutant V68A in human Mb shows a 6-fold increase in k_{on}/k_{off} , but the corresponding mutation in SW Mb produces only a 2-fold increase. Thus, not all of the trends are reproduced between the two proteins, even though the overall structures are very similar (Takano, 1977; Hubbard *et al.*, 1990). This shows that there are small structural perturbations due to the other sequence differences between the two proteins that contribute to the stability of the bound ligand. Another curious observation is that while k_{on} for V68N is greatly decreased in comparison to that of WT, resulting in a decreased binding constant and indicating a destabilization of the bound state relative to the deoxy state, the k_{off} is decreased as well. This is in contrast to the Ile 68 mutant of SW Mb, in which a much reduced on rate was accompanied by a slightly increased dissociation rate (Egeberg *et al.*, 1990). Thus, in the case of V68N there may be a small stabilizing interaction of the asparagine with the bound ligand, which somewhat offsets the increased steric barrier. Finally, we note that even though the ratios k_{on}/k_{off} for H64Q and H64A appear to be quite different, their equilibrium binding constants are much more similar. Thus, in the light of the previous discussion regarding the differences between equilibrium and kinetic measurements, particularly when the kinetics are no longer single exponential, it is necessary to obtain the true equilibrium constants before relative stabilizations can be compared.

ACKNOWLEDGMENT

This work was supported in part by a grant from the National Institutes of Health.

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