The Position 68(E11) Side Chain in Myoglobin Regulates Ligand Capture, Bond Formation with Heme Iron, and Internal Movement into the Xenon Cavities*

Received for publication, June 10, 2005, and in revised form, August 29, 2005 Published, JBC Papers in Press, September 9, 2005, DOI 10.1074/jbc.M506333200

David Dantsker1, Camille Roche5, Uri Samuni1, George Blouin1, John S. Olson1, and Joel M. Friedman1,2

From the 1Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 and the 2Department of Biochemistry and Cell Biology and the W. M. Keck Center for Computational Biology, Rice University, Houston, Texas 77005-1892

After photodissociation, ligand rebinding to myoglobin exhibits complex kinetic patterns associated with multiple first-order geminate recombination processes occurring within the protein and a simpler bimolecular phase representing second-order ligand rebinding from the solvent. A smooth transition from cryogenic-like to solution phase properties can be obtained by using a combination of sol-gel encapsulation, addition of glycerol as a bathing medium, and temperature tuning (−15 → 65 °C). This approach was applied to a series of double mutants, myoglobin CO (H64L/V68X, where X = Ala, Val, Leu, Asn, and Phe), which were designed to examine the contributions of the position 68(E11) side chain to the appearance and disappearance of internal rebinding phases in the absence of steric and polar interactions with the distal histidine. Based on the effects of viscosity, temperature, and the stereochemistry of the E11 side chain, the three major phases, B → A, C → A, and D → A, can be assigned, respectively, to ligand rebinding from the following: (i) the distal heme pocket; (ii) the xenon cavities prior to large amplitude side chain conformational relaxation, and (iii) the xenon cavities after significant conformational relaxation of the position 68(E11) side chain. The relative amplitudes of the B → A and C → A phases depend markedly on the size and shape of the E11 side chain, which regulates sterically both ligand return to the heme iron atom and ligand migration to the xenon cavities. The internal xenon cavities provide a transient docking site that allows side chain relaxations and the entry of water into the vacated distal pocket, which in turn slows ligand recombination markedly.

Proteins are inherently complex materials, and even their simplest reactions exhibit layers of complexity that were not anticipated a few decades ago (1–3). A case in point is carbon monoxide (CO) binding to the heme iron atom in myoglobin (Mb)3 (4). Much of the work on Mb has been directed toward understanding the biophysical principles associated with both bimolecular and internal ligand rebinding after photolysis of the Fe–CO bond. Key issues include the following: (i) the roles of distal and proximal heme pocket amino acids; (ii) the roles of internal water molecules near the active site; (iii) the roles of local and global conformational relaxations that are modulated by solvent; and (iv) the roles of pre-existing internal cavities associated with xenon binding. Time-resolved spectroscopic and x-ray crystallographic studies have demonstrated that dissociated ligands can access internal cavities that arise from packing defects in the globin tertiary structure (5–26). A remaining challenge is to establish quantitatively how all these factors contribute to the multiple kinetic phases associated with internal ligand binding at cryogenic temperatures and high viscosity and to those observed at ambient temperatures and physiologically relevant viscosities (21, 27–37).

Much of the cryogenic work and the time-resolved x-ray crystallography have been carried out with MbCO derivatives. At ambient temperatures, most vertebrate MbCOs exhibit very small amplitudes for the internal rebinding after laser photolysis. Consequently, geminate CO rebinding is usually examined at low temperatures or high viscosity where bimolecular rebinding from the solvent is effectively zero, and ligand escape from the protein is slowed greatly. O2 and NO complexes are typically used to examine recombination phases in low viscosity solvents at room temperature because of their much higher intrinsic reactivity with the heme iron atom, which enhances the rate of iron-ligand bond formation 10 –1,000-fold (10, 14, 38–40). However, in contrast to the stable CO complex, the NO and O2 derivatives pose difficulties because of autoxidation of the equilibrium ligand-bound A state. Over the past several years, we have developed the use of sol-gels and glycerol solutions that enhance geminate recombination by blocking ligand escape to the solvent. These conditions provide a method to follow the evolution of the multiple kinetic phases for MbCO complexes and other hemeproteins by viscosity tuning at room temperatures in nonfrozen states (28, 30, 33).

In this study, we have focused on a set of Mb double mutants that allow direct determination of the role of the position 68(E11) side chain in partitioning photodissociated CO between the distal heme pocket and the xenon cavities. In each protein the distal histidine (E7) was replaced with leucine (H64L) to prevent water from entering the distal heme pocket and hydrogen bonding to the imidazole side chain after photodissociation of bound CO (41). The Leu(E7) side chain in H64L Mb does not shift position after ligand dissociation (42) (Fig. 1, right panel). After ligand dissociation in wild-type Mb, the imidazole side chain of His64 swings inward, occupies a position closer to the iron atom, inhibits internal rebinding, and eventually binds an internal water molecule in the equilibrium unliganded state, markedly inhibiting bimolecular rebinding from the solvent phase (Fig. 1, left panel). In contrast, the Leu64 side chain does not inhibit geminate rebinding and increases the overall second-order rate of association, k−1/2CO ∼ 60-fold.

---

1 Recipient of Fellowship GM 08280 from the Houston Area Molecular Biophysics Pre-doctoral Training Program.

2 To whom correspondence should be addressed: Dept. of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461. Tel.: 718-430-3591; Fax: 718-430-8819; E-mail: jfriedma@ae.com.yu.edu.

3 The abbreviations used are: Mb, myoglobin; MEM, maximum entropy method; DHP, distal heme pocket; CPK, Corey, Pauling, Koltun color scheme.
(TABLE ONE). Thus, when the H64L mutation is coupled to mutations at position 68(E11), all of the observed differences can be assigned to the size and stereochemistry of the E11 side chain.

The H64L mutation was combined with four different Val68(E11) substitutions, V68A, V68L, V68N, and V68F, all of which have been well characterized as single mutations with His at the E7 position (13, 14, 39, 40, 43–51). The E11 Ala, Val, Leu, and Phe series examines the effect of side chain size on ligand movement from the distal pocket to the remote xenon cavities and on access to the heme iron atom (Fig. 2). The E11 Leu and Asn pair allows an examination of the effects of side chain polarity and flexibility on both ligand recombination and migration. The crystal structures of Leu68 deoxy-Mb and MbCO show that the isobutyl side chain rotates away from the iron atom and has multiple conformations after CO is bound (Fig. 2). The reverse of these changes occurs after photolysis of bound CO, and thus extensive conformational relaxation is expected in the H64L/V68L double mutant. In contrast, the side chain of Asn68 is less free to rotate because of the planar configuration of the amide group (50).

All of the myoglobin mutants were encapsulated in porous sol-gels, and glycerol was added as a bathing medium. The observed ligand and protein conformational dynamics were followed over a wide range of viscosities (∼1 → 10^3 cP) by varying the temperature of the samples from 257 to 337 K (∼15 to 65 °C). This approach both eliminates complications associated with "glass transitions" that occur at more cryogenic temperatures and avoids the limitations of trehalose matrices that act like glasses precluding smooth transitions from intermediate to low viscosity at room temperature. In the sol-gel/glycerol matrix, solvent-coupled phenomena can be selectively enhanced or suppressed, allowing more direct determination of solvent-decoupled kinetic processes.

This viscosity "tuning" approach complements the cryogenic temperature derivative spectroscopy methods utilized by the Nienhaus group (8, 13, 22, 37, 52), which track both positional changes of dissociated CO and the barrier height changes with temperature. Eaton and co-workers (35, 53) have argued that alterations in viscosity, and not temperature per se, are responsible for the large changes in the geminate recombination patterns seen for MbCO when going from cryogenic to ambient conditions. In the sol-gel work with glycerol as the bathing medium, high viscosity kinetic patterns are seen at temperatures in the range from −15 to 0 °C and change smoothly to the low viscosity pattern upon elevation to 45 and 65 °C.

MATERIALS AND METHODS

Horse heart Mb was commercially obtained from Sigma and used without further purification other than centrifugation to eliminate particulates. Mutant Mbs were produced using an expression system originally developed by Springer and Sligar (54). Construction of the different mutant genes purification of the corresponding proteins followed protocols described previously (44, 45, 49, 54).

Overall association time courses for CO binding to the H64L mutants were measured after photolysis of MbCO samples under pseudo first-order conditions, using a 300-ns excitation pulse from a Phase-R model 2100B dye laser. The transmittance signals were collected using a Tektronix TDS 220 100-MHz digital oscilloscope and IGOR data collection software (Fig. 3B) (14, 43). Time courses for geminate recombination at 20 °C in simple phosphate buffer (Fig. 3A) were observed after excitation with a 9-ns Lumonics (Northville, MI) YM600 YAG laser. In this case, transmittance signals were obtained using a high speed Hamamatsu photomultiplier and a Tektronix TDS 3052 500-MHz digital oscilloscope (Beaverton, OR). A pulsed xenon arc lamp (Applied Photophysics models 04-122 and 03-412, Leatherhead, Surrey, UK) was used to increase the source light intensity during the time required for geminate rebinding (~50 μs). CO dissociation time courses were measured in a conventional Gibson-Dionex stopped flow apparatus by mixing MbCO samples in 100 μM CO with buffer containing 2,000 μM NO (1 atm). Under these conditions, the observed replacement rate is directly equal to the overall rate constant for CO dissociation, k_{CO}.

Geminate recombination measurements in sol-gel and glycerol solutions were carried out using 8-ns 532-nm pulses at 1 Hz from a Nd:YAG laser (Minilite, Continuum, Santa Clara, CA) as a photodissociation source and a greatly attenuated continuous wave 442-nm probe beam from a He-Cd laser to monitor time-dependent changes in absorption. Details of the apparatus, data collection, and data display can be found in...
Side Chain Dynamics Influence Myoglobin Reactivity

The kinetic traces are displayed on a log-log plot of normalized absorbance (proportional to fraction of deoxy-Mb photoproduct remaining) versus time. The use of the log-log plot helps expose multiple phases. Exponential rebinding originating from a single barrier and rate constant is seen as a near vertical line intersecting the time axis at a point that is roughly the inverse of the first-order rate constant. Kinetic phases originating from a continuous distribution of rates appear as a linear curve on the log-log plot, with a much smaller slope.

Kinetic measurements were carried out on solution samples in standard 10- or 1-mm stoppered cuvettes placed in a custom-built dry N₂ purged variable temperature cuvette holder (−15 to + 65 °C). Sol-gel-encapsulated samples were prepared as a thin layer lining the bottom portion of either 5- or 10-mm diameter NMR tubes as described previously (55).

Kinetic traces were deconvoluted using the maximum entropy method (MEM) analysis as a visual means of distinguishing kinetic phases and following their evolution as a function of viscosity/temperature. The MEM analysis was performed using an algorithm described previously (57, 58) that is now part of a commercially available package contained within the analysis module in Felix 3.2 software (Photon Technology International, Lawrenceville, NJ). Kinetic data were usually
analyzed using a maximum number of anticipated lifetimes and limiting the chi^2 value to 1. Center points of the reported distributions are mean values for the Gaussian peaks. Estimates of the noise ranged from 5 to 10%, depending on the range of the original data. The results of the MEM analysis are presented as the reciprocal of the rates in order to have the distribution displayed on the same axis as the kinetic traces. To a first approximation, the MEM peak position corresponds to the reciprocal of the rebinding rate constant for a given phase, and the peak width provides an estimate of the distribution of rate constants, which presumably represent first-order rebinding in closely related protein-ligand conformations. A single exponential process involving only two conformations and one rebinding process would yield a single, very sharp peak, whereas a large distribution of rates within a given kinetic phase would be reflected in a broad band corresponding to a stretched exponential.

RESULTS

Internal and Bimolecular CO Recombination in Solution—Time courses for both geminate and bimolecular rebinding to the H64L MbCO mutants at pH 7, 20 °C in low viscosity phosphate buffer are shown in Fig. 3 as conventional plots of fraction Mb versus time. The time courses for bimolecular rebinding from the solvent are monophasic and fit to simple, single exponential expressions with k^obs = k'_{CO} [CO] (Fig. 3A). Time courses for complete CO replacement by NO were also monophasic, and the observed rate constants equals k_{CO}. Complete sets of overall association and dissociation rate constants are given in TABLES ONE and TWO, and the CO association equilibrium constants were calculated from the ratio k'_{CO}/k_{CO}.

In general, the observed association rate constant decreases monotonically, from 110 to 3.4 μM⁻¹ s⁻¹ as the amino acid at the 68(E11) position is increased in size from Ala to Phe (TABLES ONE and TWO). The near identity of the time courses for H64L/V68N and H64L/V68L is consistent with this trend and indicates that the size of the E11 residue, and not its polarity, is the key factor regulating CO association in the absence of the distal histidine. The dissociation rate constants show a more complex dependence on the nature of the E11 residue. The H64L/V68N mutant also shows a 4-fold reduction in k_{CO} values that are ~4- and ~10-fold less, respectively, than that of wild-type MbCO, and the H64L/V68N mutant also shows a 4-fold reduction in k_{CO}. Perhaps the most remarkable result in TABLES ONE and TWO is the ultrahigh CO affinity of H64L/V68A Mb, which has a half-saturation [CO] ~40 pm.

The internal nanosecond rebinding time courses exhibit more complex behavior, even in low viscosity buffers (Fig. 3A). Both H64L/V68A and H64L/V68F MbCO show large and rapid geminate phases indicating ~80 and ~93% internal rebinding. Significantly less rebinding is observed for H64L mutants containing Val, Leu, and Asn at position 68(E11). The simplest interpretation of these low viscosity, room temperature data is a two-step binding mechanism shown in Scheme 1, where the fraction of geminate rebinding, F_{gem}, is defined as k_{OB}/(k_{BA} + k_{OB}), and the apparent first-order rate constant, k_{gem}, is the sum of the rates of internal rebinding and escape, k_{BA} + k_{OB}. Because simple monophasic behavior is observed for bimolecular binding and dissociation, a steady state assumption can be made for the B state intermediate, and the overall association and dissociation rate constants can be expressed as shown in Equation 1.

### TABLE ONE

| Mb             | k'_{CO} | k_{CO} | k_{CO} | F_{gem} | k_{gem} |
|----------------|---------|--------|--------|---------|---------|
| WT (H64/V68)   | 0.51    | 0.019  | 27     | 0.03    | 5       |
| H64L/V68A      | 110     | 0.0045 | 25,000 | 0.81    | 28      |
| H64L/V68C      | 38.0    | 0.026  | 1500   | 0.29    | 19      |
| H64L/V68L      | 22      | 0.018  | 1200   | ≥0.12   | ≥50     |
| H64L/V68N      | 15      | 0.0050 | 3000   | 0.28    | 14      |
| H64L/V68F      | 3.4     | 0.0022 | 1500   | ≥0.93   | ≥56     |

*Parameters were taken from Carver et al. (59). Fitted geminate rate constants approaching the speed of decay of the YAG excitation pulse represent lower estimates, and the observed fraction of geminate recombination also represents an underestimate of the true value because some recombination is occurring during the light pulse. These inequalities were carried through the calculations for the specific kinetic parameters.

### TABLE TWO

| Mb             | k_{SB} | k_{BS} | k_{SB} | k_{BA} | k_{AB} | k_{BA} |
|----------------|--------|--------|--------|--------|--------|--------|
| WT (H64/V68)   | 17     | 4.9    | 3.5    | 0.20   | 0.020  | 7.7    |
| H64L/V68A      | 140    | 5.2    | 27     | 23     | 0.024  | 940    |
| H64L/V68L      | 131    | 14     | 9.5    | 5.6    | 0.037  | 154    |
| H64L/V68N      | 54     | 10.5   | 5.2    | 4.0    | 0.0069 | 600    |
| H64L/V68F      | 3.6    | 4.1    | 0.9    | 52     | 0.0309 | 1700   |

*Parameters were taken from Carver et al. (59). Fitted geminate rate constants approaching the speed of decay of the YAG excitation pulse represent lower estimates, and the observed fraction of geminate recombination also represents an underestimate of the true value because some recombination is occurring during the light pulse. These inequalities were carried through the calculations for the specific kinetic parameters.
Side Chain Dynamics Influence Myoglobin Reactivity

\[
k'_{CO} = k'_{SB/BA} + k_{BS} = k'_{SB/BA}F_{gem}\]

\[
k_{CO} = k_{AB} \frac{k_{BS}}{k_{BA} + k_{BS}} = k_{AB}(1 - F_{gem}) \quad \text{(Eq. 1)}
\]

The individual rate constants in Equation 1 are defined experimentally as \(k'_{SB} = k'_{CO/1 - F_{gem}}, k_{BS} = k_{gem}(1 - F_{gem}), k_{BA} = k_{gem}F_{gem},\) and \(k_{AB} = k_{CO/1 - F_{gem}}\) (59). Sets of these parameters are listed in TABLE TWO and provide an approximate interpretation of the effects of mutagenesis on CO binding to the H64L mutants at room temperature and low viscosity.

With the exception of H64L/V68N Mb, the rate of thermal bond dissociation, \(k_{AB}\), is roughly the same for all the mutants and wild-type myoglobin. In the case of the H64L/V68N double mutant, \(k_{AB}\) is reduced roughly 3-fold due hydrogen bonding between the amide NH₂ and the bound ligand, as described previously (51). This decrease in \(k_{AB}\) accounts for the decrease in the overall dissociation rate constant, \(k_{CO}\), for H64L/V68N MbCO. In contrast, the decreases in \(k_{CO}\) for the H64L/V68A and H64L/V68F mutants are due entirely to greater geminate recombination in these proteins, which decreases the frequency of escape from the initial B geminate state ~5- and 10-fold, respectively.

All of the H64L mutants show ≥25-fold higher rates of iron-ligand bond formation because the isobutyl side chain of Leu(E7) does not hinder access to the iron atom (Fig. 1, right panel). In contrast, the naturally occurring imidazole side residues to a position closer to the iron atom, sterically hindering internal rebinding (Fig. 1, left panel). The rates of internal bond formation, \(k_{BA}\), increase another 4–10-fold when Val(E6) is changed to either Ala or Phe, due to loss of the naturally occurring γ-2 CH₃ group, which restricts access to the iron atom in the wild-type protein (Fig. 3).

The results for the H64L, H64L/V68L, and H64L/V68N mutants are difficult to interpret in terms of simple two-step scheme. The poor fits to a single exponential expression of the geminate time courses to the H64L/V68A and H64L/V68N mutants indicate that more complex analyses and interpretations are needed. As has been recognized by many workers in the field, ligand movement into the internal xenon binding cavities must be considered. To examine the competition between escape, iron-ligand bond formation, and migration into these remote sites, we have examined systematically the effects of viscosity on the amplitudes and rates of CO rebinding for the set of myoglobins listed in TABLES ONE and TWO and then used the more general MEM of analysis to obtain distributions of rate constants for the various transitions (TABLE THREE).

**A Comparison of the CO Rebinding Kinetics at High Viscosity**—Complete sets of time courses for CO rebinding to the H64L mutants in buffer at 3.5 °C are shown in Fig. 4 as plots of the logarithm of fraction of deoxy-Mb remaining (normalized absorbance change at 436 nm) versus logarithm of time. These plots allow visualization of both geminate and bimolecular processes and the distribution of rate constants for each major recombination phase. Lowering the temperature from 20 to 3.5 °C increases the extent of geminate recombination in all the mutants, but the same relative features seen in Fig. 3 are observed at 3.5 °C. The log-log plot shows more clearly the distribution of rate constants for internal CO rebinding in the H64L, H64L/V68A, and H64L/V68N mutants. In this experiment with an 8-ns YAG laser pulse, it is difficult to measure bimolecular rebinding to the H64L/V68F mutant because the extent of geminate rebinding is so large (≥95%), whereas with the 300-ns dye laser pulse, there is sufficient “pumping” to produce a large bimolecular S → A phase on μs to ms time scales (Fig. 3B).

Fig. 5 shows the dramatic effects of so-gel encapsulation and high viscosity on the rebinding traces for wild-type, H64L/V68A, H64L/V68L, and H64L/V68F MbCO at ~15 °C. Except for samples embedded in trehalose-glass matrices, this condition represents the high viscosity/low temperature limit, in which rebinding from the solvent (k’_{BS}) is likely to be effectively zero (k’_{CO} ≤ 0.0005 M⁻¹ s⁻¹). Under these conditions, CO recombination in H64L/V68F and H64L/V68A MbCO is extremely fast and nearly monophasic as judged by the large slope of the nearly linear log-log plots (Fig. 4, LA and LF curves). The traces for H64L/V68L sperm whale and native horse heart MbCO, both of which display minimal geminate recombination in solution (Figs. 3 and 4), now show extended initial geminate phases that smoothly evolve into a final slow internal phase that shows either a single rate constant or a narrow distribution of constants and resembles a simple exponential. Wild-type sperm whale Mb exhibit a trace similar to that of the horse heart protein but with slightly different amplitudes for the individual phases. In the trace for horse heart MbCO, the three distinct internal phases are much more clearly delineated in time and are designated B → A (10–1000 ns), C → A (10–1000 μs), and D → A (~10 ms).

Figs. 4 and 5 show the extremes in kinetic behavior associated with the viscosity dependence of CO recombination in myoglobin. There is wide range of patterns and multiple internal rebinding phases. To dissect out the structural origin and kinetic parameters for the individual processes, we focused initially on mutants exhibiting the least complicated patterns (H64L/V68F and H64L/V68A) and then moved progressively to those proteins with more complex recombination patterns.

---

**TABLE THREE**

MEM-derived CO rebinding parameters in sol-gel + 100% glycerol at 25 °C

The relative amplitudes for phases are given in parentheses. WT indicates wild type.

| Mb                  | k_{B→A} (major) | k_{B→A} (minor) | k_{C→A} | k_{D→A} |
|---------------------|-----------------|-----------------|---------|---------|
| WT (H64L/V68)       | 1.3 × 10⁴ (25)  | 1.0 × 10⁶ (15)  | 7 × 10⁴ (25) | 4 × 10⁵ (35) |
| H64L/V68A           | 2.0 × 10⁴ (85)  | 3.7 × 10⁴ (2)   | 5.2 × 10⁴ (13) |         |
| H64L/V68            | 3.2 × 10⁴ (60)  | 3.3/0.6 × 10⁴ (15) | 2.0 × 10⁵ (15) | 1 × 10⁶ (10) |
| H64L/V68L           | 2.0 × 10⁴ (60)  | 2.2 × 10⁴ (2)   | 2.2 × 10⁵ (20) | 2.7 × 10⁶ (18) |
| H64L/V68N           | 1.6 × 10⁴ (60)  | 1.3 × 10⁵ (35)  | 1.6 × 10⁵ (4)  | 3.3 × 10⁷ (2)  |
| H64L/V68F           | 3.8 × 10⁴ (98)  |                   | 2.2 × 10⁵ (2)  |         |

*Data were generated at 65 °C (lower temperatures were insufficient to yield a measurable population for C → A).*
Side Chain Dynamics Influence Myoglobin Reactivity

(\text{H64L/V68L}, \text{H64L/V68N}, \text{H64L}, \text{and wild-type}). In each case we started at the low temperature/high viscosity limit and then systematically tuned the system to a temperature/viscosity that yielded kinetic traces resembling more closely those seen in simple solution. TABLE THREE contains the MEM determined rate constants for the different recombination phases obtained for the encapsulated samples bathed in 100% glycerol at 25 °C, except where noted. Higher temperature (65 °C) was needed to generate a measurable population of the slowest phase for two samples (\text{H64L/V68F} and \text{H64L/V68A} Mb; TABLE THREE).

Unhindered Iron-Ligand Bond Formation from within the Distal Heme Pocket (B → A)—As shown in Fig. 2, the \text{Ala}^{68} and \text{Phe}^{68} side chains do not sterically hinder access of CO to the iron from within the distal heme pocket (state B) nor do they change position when comparing deoxy-Mb and MbCO structures of the single mutants. Because the \text{Leu}^{64}(\text{E7}) side chain also does not change position or hinder bond formation, the onset of any new kinetic phases for the \text{H64L/V68A} and \text{H64L/V68F} double mutants cannot be attributed to a distal side chain-associated relaxations that increase the inner barrier for the B → A transition.

Fig. 6, a and b, shows time courses for CO recombination in sol-gel-encapsulated \text{H64L/V68F} MbCO and the corresponding MEM-derived kinetic populations. At temperatures ranging from −15 to 45 °C, encapsulated \text{H64L/V68F} MbCO in 100% glycerol shows essentially a single rapid phase with a major rate constant peak at ~40 μs$^{-1}$ and little or no secondary geminate recombination processes (TABLE THREE). The only variation over this temperature regime is a slight increase in the peak of the rate constant distribution with increasing temperature. Below 45 °C, this high viscosity sample exhibits neither the solution phase re-binding shown in Fig. 6c (and Fig. 3) nor any clear cut break indicative of the onset of a second well defined phase. The near linear trace seen in Fig. 6a is clearly due to the B → A transition, and the MEM analysis resolves this phase into a major and very minor component (small peak at ~1.0 μs$^{-1}$), as reported previously (21).

X-ray studies (49) have shown that the single replacement of \text{Val}^{68} with \text{Phe}^{68} has two structural consequences. First, there is a loss of steric hindrance with respect to ligand access to the heme iron that arises in the wild-type protein from the C-y of \text{Val}^{68}. Second, the aromatic side chain occupies a fixed position that hinders access from the distal pocket, state B, to the Xe4 cavity (see Fig. 2 of Ref. 49) (13).

Several simulations (23, 24, 26) and time-resolved x-ray crystallography studies (17, 18) have shown that ligand trajectories from the distal pocket to Xe4 are the most probable route of ligand movement into the protein interior after photodissociation. For the \text{H64L/V68F} double mutant, the combination of reduced steric hindrance at the iron and partial filling of the Xe4 cavity combine to cause ~100% geminate recombination from the initial B state, and the observed kinetic phase can be assigned with certainty to geminate recombination from the initial docking site labeled as “B” in the structure of wild-type MbCO shown in Fig. 2. The internal barrier for this B → A transition is low, whereas the barrier to internal ligand movement is large because of the benzyl side chain hindering access to the adjacent Xe4 cavity.

Recombination from Remote Sites; Side Chain-mediated Motion and the Onset of the C → A Recombination Phase—Heating of encapsulated \text{H64L/V68F} MbCO to 65 °C in glycerol results in the appearance of a second, much slower re-bonding phase, which exhibits rate constants on the order of 1000 s$^{-1}$ in 100% glycerol (TABLE THREE). Under these high viscosity conditions, the slowest observed phases show no detectable dependence on external [CO] but, as will be discussed below, are comparable with the pseudo first-order rates seen in solution. The distinct second slower phase in the sol-gel experiment is usually designated the C → A transition and is associated with ligands that have migrated to and then returned from a more remote site in the protein by analogy to low temperature CO geminate results (28, 60) and room temperature geminate O$_2$ re-bonding studies (7, 14, 61).

In the \text{H64L/V68F} MbCO mutant, the onset of this second phase is unlikely to be due to protein side chain relaxations that alter steric hindrance to ligand re-bonding at the iron atom. The \text{Leu}^{64} and \text{Phe}^{68} side chain positions are the same in the equilibrium deoxy-Mb and MbCO.
FIGURE 6. CO recombination traces (in black) and associated MEM-derived distribution of lifetimes (in gray) for Mb(H64L/V68F) encapsulated in a sol-gel ([L/F]) bathed in glycerol at 3.5 °C (a) and 65 °C (b), and free in aqueous solution at 3.5 °C (c) and Mb(H64L/V68A) encapsulated in a sol-gel ([L/A]) bathed in glycerol at 3.5 °C (d), and 65 °C (e) and free in aqueous solution at 3.5 °C (f). The MEM populations are presented as the reciprocal of the rate constants for the populations and are displayed on a time axis that is approximately the same as for the kinetic traces in order to help visualize the connection between the population and the feature in the kinetic trace.
Side Chain Dynamics Influence Myoglobin Reactivity

structures of the corresponding single mutants (Fig. 2). However, the high temperature required for the onset of the C → A transition in the sol-gel-encapsulated sample suggests that there is a requirement for very large side chain fluctuations in the protein, including the Phe86 side chain to allow ligand movement into the adjacent Xe4 cavity and eventually to the proximal Xe1 site.

This interpretation is supported by the observation that in a dry trehalose glass, where fluctuations are further dampened, there is no evidence of the C → A phase in H64L/V68F MbCO, even at 65 °C (not shown). The lack of any slow secondary geminate phase in the solution samples (Figs. 3A and 6c) also suggests a large steric barrier to ligand movement into the protein interior. Under these low viscosity conditions, the rate of internal movement of ligand escape (~1,000 s⁻¹) is too slow to compete with the high rate of ligand escape (5,000,000 s⁻¹) (TABLE TWO). In general, the onset of the C → A phase in the sol-gel samples can be assigned to slower rebinding of CO molecules that have diffused from the initial B site into the remote xenon-binding sites, under conditions where movement between the distal pocket and the protein interior is strongly hindered by the Phe86(E11) side chain. As a result, the absolute rate for the C → A transition is the same order of magnitude as the pseudo first-order rate of ligand rebinding from the solvent (k₃ₐ/C[CO] = 3,000 s⁻¹ for H64L/V68F Mb), and the time course for the sol-gel sample at high temperature (Fig. 6b) resembles that seen in solution at 3.5 °C (Fig. 6c).

The kinetic traces for H64L/V68A MbCO in Fig. 6, d and e, also show only two recombination phases. The rate associated with the first B → A transition in both sol-gel and solution samples is 2–3-fold slower than that for the H64L/V68F mutant (Figs. 3 and 6), whereas the rate for the second C → A transition seen at 65 °C in the sol-gel is 100-fold larger (~0.1 μs⁻¹).

Replacement of Val₆⁶ with Ala eliminates the steric contribution of the naturally occurring isopropyl side chain to the recombination barrier as in the case of the V68F substitution. In both mutants, the amplitude of the B → A transition dominates the observed time courses, whereas in wild-type Mb, the amplitude of these initial phases is very small, even in the glycerol. However, the alanine substitution also increases the free volume of the distal pocket by making the Xe4 cavity continuous with the B site (Fig. 2). Thus, the ligand in H64L/V68A Mb has more space to occupy in an enlarged B state space, which raises the activation entropy for the B → A process. In the H64L/V68F mutant, the ligand is sequestered in a smaller volume closer to the iron atom, which enhances kₐ₃ by lowering in the entropy barrier to iron-ligand bond formation. These effects are seen as faster recombination in the initial nanosecond recombination phases for H64L/V68F MbCO compared with those for the H64L/V68A mutant (see Figs. 3–6). In aqueous solutions, the H64L/V68A mutant also shows a larger distribution of B → A rates. This heterogeneity is manifest as a broader MEM peak in Fig. 6f and a poorer fit to single exponential function in Fig. 3A. The distribution of rates is probably a result of ligand rebinding from a variety of positions in the space associated with the distal pocket and the Xe4 cavity, which are now contiguous. State B in this mutant represents CO rebinding from a variety of ligand origins and the high viscosity, ambient temperature measurements described here is that at the higher temperatures, low amplitude solvent decoupled fluctuations are considerably less dampened. As a result, the C → A transition represents CO rebinding from a distribution of remote sites that in some cases may reflect the equilibrium distribution among the accessible xenon cavities.

Recombination from Remote Sites after E-helix Side Chain Relaxations: the D → A Transition—In contrast to the V68A and V68F Mb mutants, significant differences are observed between the position of the E11 side chain in the deoxy-Mb and MbCO structures of V68L sperm whale myoglobin (Fig. 2) (49, 51). Greater steric hindrance of access to the iron atom occurs in the deoxy conformation. Under high viscosity, sol-gel conditions, it is probable that rotation of the isobutyl side chains is dampened to greater degree by the sol-gel and glycerol, because of its close proximity to the solvent phase, than the side chain dynamics that regulate diffusion of the CO between interior of the distal heme pocket and the xenon cavities. Under such conditions, the following two geminate phases from the remote sites occur in the H64L/V68L mutant: one prior to the onset of the large amplitude relaxations of the E11 side chain; and a second, even slower internal rebinding phase occurring after relaxations that place one of the Leu C-δ atoms directly over the iron atom, inhibiting the B → A transition. A similar large relaxation of the His₄₄ side chain to a position over the iron atom occurs in wild-type Mb after photodissociation of bound CO (Fig. 1, left panel).

The temperature/viscosity-dependent geminate traces are shown in Fig. 7 for H64L/V68L MbCO. Two phases, designated B → A and C → A transitions, are seen even at the highest viscosity sol-gel condition at ~15 °C. The overall distributions of rates associated with both the B → A and the C → A phases are very similar to those seen for H64L/V68A, below 45 °C. The amplitude for this second geminate phase increases at the expense of B → A as the temperature of the sol-gel/glycerol sample increases. The absence of the C → A phase under high viscosity conditions (100% glycerol, sol-gel, 3.5 °C, Fig. 6d), indicates that, even though the B and Xe4 sites are contiguous, side chain fluctuations are still needed for the ligand to migrate further into the protein interior and into the Xe1 site, from which ligand rebinding is then seen as a separate recombination phase. However, rebinding from these internal positions is significantly more rapid in the H64L/V68A mutant compared with H64L/V68F because of the lack of a steric barrier between the Xe4 cavity and the distal pocket. As can be seen from TABLE THREE, the C → A process for H64L/V68A is comparable with that of the wild type, consistent with the early time wild-type photoproduct not as yet having water in the distal pocket and retaining a conformation allowing for an unrestricted trajectory between Xe4 and the distal heme pocket.

The time courses for CO rebinding to the H64L/V68F and H64L/V68A mutants suggest strongly that the onset of the C → A process occurs with the diffusion of the CO out of the distal pocket into remote sites in the protein interior, presumably through a pathway that includes the Xe4 cavity. The observed rapid transit of the ligand among the xenon cavities at ambient temperatures precludes associating the C → A transition in the sol-gels with recombination originating from the ligand being completely localized in any one specific xenon cavity (17, 18). This conclusion is in contrast to cryogenic measurements below 200 K where CO occupancy within specific xenon cavities can be monitored spectroscopically and crystallographically (8, 11, 15, 20, 22, 37, 62–64). Under these cryogenic conditions, a clear progression of CO occupancy from the adjacent Xe4 to remote Xe1 sites can be followed as a function of temperature. As a result, specific kinetic phases can be associated with rebinding from the individual cavities (8, 11, 13, 15, 20, 22, 37, 63, 64). The major difference between these cryogenic experiments and the high viscosity, ambient temperature measurements described here is that at the higher temperatures, low amplitude-solvent decoupled fluctuations are considerably less dampened. As a result, the C → A transition represents CO rebinding from a distribution of remote sites that in some cases may reflect the equilibrium distribution among the accessible xenon cavities (65).
but the amplitude of the slower processes are much larger at all viscosities and temperatures for the H64L/V68L mutant (Figs. 6 and 7). Based on the similarity of the kinetic patterns, we attributed the B → A and C → A phases for the H64L/V68L mutant at high viscosity (−15 to 3.5 °C, sol-gel) to rebinding from the distal pocket B site and from internal xenon cavities, respectively.

The larger amplitude for secondary recombination in the H64L/V68L mutant seems in conflict with the structure of the mutant MbCO complex, which shows that the large isobutyl side chain blocks access to the Xe4 channel (Fig. 2). However, unlike the benzyl side chain in H64L/V68F Mb, which penetrates into the Xe4 site but does not block access to the iron atom, the Leu(E111) side chain only acts as a gate to the...
Side Chain Dynamics Influence Myoglobin Reactivity

The progressive loss of the C→A and appearance of the D→A phases for the His64 side chain in wild-type MbCO structure that accounts for relaxation from the C→A to the slower D→A transition in the H64L/V68L mutant. However, once rotation to the deoxy conformation has occurred, the H64L/V68L mutant shows a rate constant for slow internal rebinding from remote sites that are very similar to that for the V68N double mutant, ~3,000 s⁻¹ (TABLE THREE and Figs. 7 and 8). The similarity of the equilibrium deoxy-Mb Asn68 and Leu68 containing double mutants reflects the similarity of the conformations seen in the equilibrium deoxy-Mb structures of the single mutants (49, 51). In both cases, the side chain adopts a conformation where the terminal atoms, C-61 and C-62 in Leu68 and O-δ and N-δ in Asn68, lie almost parallel to the heme plane. One δ atom blocks access to Xe4, and the other hinders binding to the heme iron from the B site (Fig. 2, V68L deoxy-Mb structure). It is the complete rotation of the Leu68 side chain from the MbCO to the deoxy-Mb conformation that accounts for relaxation from the C→A to the slower D→A transition in the H64L/V68L mutant. However, once rotation to the deoxy conformation has occurred, the H64L/V68L mutant shows a rate constant for slow internal rebinding from remote sites that are very similar to that for the V68N double mutant, ~3,000 s⁻¹ (TABLE THREE and Figs. 7 and 8). The similarity of the equilibrium deoxy-Mb Asn68 and Leu68 conformations also accounts for why the bimolecular rate constants for the H64L/V68L and H64L/V68N double mutants are almost the same, 15–22 μm⁻¹s⁻¹ (TABLE ONE).

The Temperature/Viscosity Dependence of CO and O₂—The temperature/viscosity dependence of CO re-binding in the single H64L mutant is compared with that in wild-type sperm whale Mb in Fig. 9. In sol-gels at the high viscosity limit (100% glycerol, ~3.5 °C), log-log plots are linear with the slope for the wild-type protein being much smaller than that for the faster reacting H64L mutant. The peak positions and distributions of the B→A rate parameters for the single H64L mutant are similar to those for the H64L/V68N double mutant, implying that the naturally occurring Val68 side chain confers almost as much resistance to iron-ligand bond re-formation as Asn68.

In contrast, the rate of initial CO rebinding in wild-type sperm whale Mb is much smaller both in solution and in sol-gels in 100% glycerol at low temperature because of a marked decrease in the amplitude of the B→A transitions (Fig. 3A; TABLES ONE and TWO; and Fig. 9d). This effect is clearly due to the His68 side chain in wild-type Mb. In the bound A state, the imidazole ring is donating a proton to the bound ligand, and this electrostatic interaction places the N-e atom within 3.0 Å of the

Entrance of the Xe4 pathway and does not fill the cavity. However, as the C-δ atoms of Leu(E11) relax to the deoxy conformation, they reduce access to the iron atom and the size of the B site pocket.

Relaxation of the Leu68 side chain not only inhibits rebinding from site B but, at the same time, also appears to “push” the photodissociated ligands either toward the protein interior or out of the protein through the histidine gate (Fig. 2, lower left). The latter effect accounts for the 4–8-fold higher calculated rate of CO escape from this mutant (k₆₈ ≈ 4.4 μs⁻¹; TABLES ONE and TWO). Note that a similar increase in the rate of escape of photodissociated O₂ was observed for the single V68L mutant (14). In the V68L MbCO structure, multiple orientations of the C-δ atoms are seen in the electron density maps, differing in rotation about the C-β–C-γ bond (Fig. 2, green circles). The alternative conformations indicate steric pressure on the bound ligand and presumably on the ligand in the initial site B “locking” site. Thus, ligand diffusion into the Xe4 channel and toward the Xe1 site is, like direct ligand escape, “activated” by the motions of the Leu(E11) side chain. Above ~15 °C, these rotations reduce the fraction of rapid initial rebinding (B→A) to ≤30% in the sol-gel/glycerol samples and to ≤10% in solution (Figs. 3A and 6f). In the latter case, no slow secondary geminate phases are observed indicating that CO either rebinds from site B in the first 50 ns after photolysis or escapes to the solvent phase.

Further increases in temperature and decreases in viscosity of the sol-gel/H46L/V68L sample (Fig. 7, c–e) lead to the appearance of a third, even slower geminate rebinding phase, which is designated D→A. This third phase increases in amplitude with increasing temperature at the expense of the C→A transition. The amplitude of the first B→A transition remains relatively unchanged from 25 to 65 °C. The simplest interpretation is that the onset of the D→A phase corresponds to full relaxation of the E11 leucine side chain, which causes a large increase in the barrier to iron-ligand bond formation and a small rate for internal ligand recombination. The increases in amplitude of the slower B→A process shown in the high temperature, low viscosity sol-gel time courses (Fig. 7, d and e) support this interpretation. The observed progression of phases suggests that complete E11 side chain relaxation only occurs after photodissociated CO diffuses out of the B site. The assumption is that the fluctuations required for ligand movement into the xenon sites are less dependent on solvent viscosity than those associated with relaxation of the E11 side chain, which are closer to the solvent phase.

In solution the time course for geminate rebinding to H64L/V68L Mb is remarkably simple. Only a small (~10%), rapid B→A phase (~10–50 μs⁻¹) is observed at either 20 or 3.5 °C. Relaxation of the E11 side chain occurs on the same time scale, pushing the ligand further into the protein interior or out of the protein and, at the same time, inhibiting rebinding from all remote sites, including the solvent phase. The net result is that no geminate rebinding is observed after ~50 ns, and ~90% of the photodissociated ligands escape from the protein (Fig. 3A). Similar behavior has been observed for CO, O₂, and NO geminate rebinding to V68L Mb. In this case, the C-δ atom of the sec-butyl side chain rotates directly over the iron atom after photolysis, pushing the dissociated ligand out of the protein or into the Xe4 site and markedly inhibiting iron-ligand bond re-formation. The net result is the absence of geminate CO rebinding, only 12% O₂ nanosecond recombination, and 5–10-fold slower rates of picosecond NO rebinding in the V68L single mutant (14, 49, 59).

The progressive loss of the C→A and appearance of the D→A phases for the H64L/V68L double mutant with decreasing viscosity is the result of the rate of relaxation of the E11 side chain becoming competitive with the rate of C→A rebinding transition. However, even the D→A phase is composed of at least two kinetic components based on MEM analysis, with peaks at 15,000 and 5,000 s⁻¹. These results indicate that, even at 65 °C, some protein conformational inter-conversions are still slower than the remote rebinding process in this double mutant.

Less well defined multiple phases are seen for geminate CO recombination in H64L/V68N Mb (Fig. 8). At the high viscosity limit (~15 to 3.5 °C, Fig. 8, a and b), a broad and slow B→A process dominates the rebinding time course. MEM analysis indicates multiple barriers with two rate distribution peaks, the first of which is still significantly smaller than that for the H64L/V68A, H64L/V68F, and H64L/V68L mutants. Similar large barriers to internal rebinding were observed for the single V68N sperm whale and pig Mb mutants (51) and are attributed to direct hindrance from the relatively inflexible planar amide side chain, which sterically hinders both access to the iron atom and to the Xe4 pocket. Log-log plots for the sol-gel/100% glycerol samples are roughly linear with a small slope in the temperature range from ~15 to 25 °C. There is no clear “plateau” separating the B→A and C→A transition. Only at elevated temperatures, ~≥45 °C, are two phases clearly seen: the first being the broad initial B→A transition, and the second being a simple exponential but slow process resembling the D→A transition seen for the H64L/V68L mutant (Fig. 8e).

The correspondence of the slower geminate phases for the Leu68 and Asn68 containing double mutants reflects the similarity of the conformations seen in the equilibrium deoxy-Mb structures of the single mutants (49, 51). In both cases, the side chain adopts a conformation where the terminal atoms, C-61 and C-62 in Leu68 and O-δ and N-δ in Asn68, lie almost parallel to the heme plane. One δ atom blocks access to Xe4, and the other hinders binding to the heme iron from the B site (Fig. 2, V68L deoxy-Mb structure). It is the complete rotation of the Leu68 side chain from the MbCO to the deoxy-Mb conformation that accounts for relaxation from the C→A to the slower D→A transition in the H64L/V68L mutant. However, once rotation to the deoxy conformation has occurred, the H64L/V68L mutant shows a rate constant for slow internal rebinding from remote sites that are very similar to that for the V68N double mutant, ~3,000 s⁻¹ (TABLE THREE and Figs. 7 and 8). The similarity of the equilibrium deoxy-Mb Asn68 and Leu68 conformations also accounts for why the bimolecular rate constants for the H64L/V68L and H64L/V68N double mutants are almost the same, 15–22 μm⁻¹s⁻¹ (TABLE ONE).

The correspondence of the slower geminate phases for the Leu68 and Asn68 containing double mutants reflects the similarity of the conformations seen in the equilibrium deoxy-Mb structures of the single mutants (49, 51). In both cases, the side chain adopts a conformation where the terminal atoms, C-61 and C-62 in Leu68 and O-δ and N-δ in Asn68, lie almost parallel to the heme plane. One δ atom blocks access to Xe4, and the other hinders binding to the heme iron from the B site (Fig. 2, V68L deoxy-Mb structure). It is the complete rotation of the Leu68 side chain from the MbCO to the deoxy-Mb conformation that accounts for relaxation from the C→A to the slower D→A transition in the H64L/V68L mutant. However, once rotation to the deoxy conformation has occurred, the H64L/V68L mutant shows a rate constant for slow internal rebinding from remote sites that are very similar to that for the V68N double mutant, ~3,000 s⁻¹ (TABLE THREE and Figs. 7 and 8). The similarity of the equilibrium deoxy-Mb Asn68 and Leu68 conformations also accounts for why the bimolecular rate constants for the H64L/V68L and H64L/V68N double mutants are almost the same, 15–22 μm⁻¹s⁻¹ (TABLE ONE).
ligand O atom. After photolysis, the His⁶⁴ side chain actively pushes the dissociated ligand away from the iron as it swings inward to occupy a position over the iron atom. The final conformation of the imidazole ring restricts CO rebinding from any position in the protein (Fig. 1). This inward movement of His⁶⁴ is seen when comparing equilibrium MbCO to deoxy-Mb structures, in recent time-resolved, x-ray crystallographic studies at room temperature with wild-type sperm whale MbCO (18, 66), and accounts for the Stark splitting of the IR bands of photodissociated CO (B states) (67).

Both H64L and wild-type Mb show a build up of a slow final rebinding phase. In the case of the single H64L mutant, this last phase should probably be defined as a C → A transition because of the high speed of the rebinding process, ~30,000 s⁻¹ and its similarity to the C → A transition in the H64L/V68A mutant (Fig. 6f). In contrast, the D → A transition in the wild-type Mb is much slower (~1000 s⁻¹).
transition for the sol-gel sample of wild-type Mb is remarkably slow, with a peak rate constant of $\sim 1,000 \text{ s}^{-1}$ at $65^\circ \text{C}$. This value is close to that for the slow transition observed for H64L/V68F Mb under similar conditions, which is assigned to rebinding from remote internal xenon cavities when the Xe4 $\rightarrow$ B channel is blocked by the E11 benzyl side chain (Fig. 2, V68F structures). The most plausible explanation for the remarkable slowness of the D $\rightarrow$ A phase in wild-type Mb is water penetration into the distal pocket and formation of a hydrogen bond to the His64 side chain, which blocks both internal ligand rebinding and bimolecular entry from the solvent phase by filling the B site (Figs. 1 and 2, wild-type deoxy-Mb structures).

Strong support for the idea of water penetration into wild-type Mb is shown in Fig. 10. These time courses show how the various geminate phases evolve in a trehalose glass as the water content in the material is increased. In the dry glass, there is almost no contribution from ultra-slow internal rebinding, and a clear C $\rightarrow$ A transition is observed with a
rate on the order of $10^5$ s$^{-1}$ (Fig. 10a). As the extent of hydration is increased further, a remarkably slow geminate phase appears for the wild-type protein (Fig. 10, c and d) that is never seen for the H64L series of mutants. This ultra-slow geminate phase only occurs in the sol-gel/100% glycerol experiments and the humidity-exposed trehalose glass at high temperatures, when water penetration can compete with rebinding from the remote sites (41).

The bimolecular rate constant for CO binding to wild-type Mb is also remarkably slow compared with any of the H64L mutants listed in TABLES ONE and TWO because of the presence of distal pocket water in the equilibrium deoxy-Mb structure (42, 68). Thus, the same barrier that limits rebinding from remote sites in the protein interior after complete relaxation also limits ligand entry into the distal pocket.

**DISCUSSION**

Assignment and Nomenclature for Recombination Phases; Combining Localization Sites and Protein Relaxation—Our results at ambient temperatures, where the distinction among the remote Xe4 and Xe1 sites cannot be easily made, led us to propose a slightly different nomenclature that takes into account both conformational and CO coordinates. In our interpretations, the A–D states now refer to the CO localized in the following: A, the distal heme pocket, covalently bound to the heme; B, the distal heme pocket (and sometimes the Xe4 site) but dissociated from the iron; C, the remote xenon cavities (primarily Xe1) under conditions when the major relaxations leading to equilibrium deoxy-Mb structure are not complete; and D, the remote xenon cavities under conditions when the relaxations back to the equilibrium deoxy-Mb population have occurred (Fig. 11). The relaxations that result in the D state can include not only conformational relaxations but also water re-entering a polar distal heme pocket. Thus the A to B transition corresponds to dissociation with the ligand remaining in the distal heme pocket environment; the B to C transition corresponds to the diffusion of the ligand from the heme pocket to remote sites before large amplitude relaxations, and C to D transitions correspond to large amplitude relaxations occurring with the ligand in the remote sites. In Fig 11 we differentiate between B and [B], which designate states where the dissociated CO is still in the distal heme pocket/Xe4 under conditions where the distribution of local sites and rebinding rates either are not being modulated or are being modulated by low amplitude fluctuations of the DHP side chains, respectively. Similarly, Fig. 11, $B_{relax}$ designates the state in which the ligand is in the DHP under conditions where full relaxation back to the equilibrium deoxy-Mb conformational distribution has occurred.
Factors Influencing Rebinding from within the Distal Heme Pocket, $B \to A$—All of the H64L mutants used in this study display what appear to be two distinct $B_3A$ phases, which we designate as major and minor for the faster and slower $B_3A$ phases, respectively. The faster $B$ component is assigned to geminate recombination under conditions where there is minimal steric hindrance at the binding site, which occurs immediately after photolysis but before the onset of side chain relaxations (Fig. 11, top tier of the reaction coordinate diagram and top structure). The minor $B_3A$ transition appears to be due to a slow down of $B_3A$ due to side chain fluctuations that either mediate movement of the ligand into the interior portion of the distal pocket and/or sterically slow the return of the ligand to the iron atom (Fig. 11, middle tier structure).

Crystallography (17, 18), spectroscopy (8, 9, 11, 37), and simulations (24) show that photodissociated ligands occupy multiple sites within the distal heme pocket. The major and minor $B \to A$ phases likely arise from ligand recombination from closer unrestricted and more distant sites, respectively, within the active site. The latter sites probably include the $Xe4$ cavity. Assignment of the minor $B \to A$ phase to CO rebinding from $Xe4$ is consistent with the reduction of this second MEM peak in the H64L/V68F and H64L/V68A mutants where the $Xe4$ space is either filled with a benzyl side chain or is continuous with the DHP site (Fig. 1, V68F and V68A structures). The two $B \to A$ phases are most prominent when movement between the distal pocket and the $Xe4$ site is partially restricted by Val, Leu, and Asn side chains at position 68 (E11) (Figs. 1 and 7–9).

Recent fitting of the temperature dependence of the kinetic traces of many of the present mutants (69) reveals a low activation energy protein relaxation occurring on the same time scale as the minor $B_3A$ phase. This time scale coincides with the slowing of the $B_3A$ process prior to the onset of the plateau that leads to the slower $C_3A$ phase. In addition, in these mutants the $Xe4$ site is either completely blocked (H64L/V68F Mb) or already open and accessible (H64L/V68A Mb) so that small scale protein relaxations have no effect on ligand movement further into the protein interior. However, as described above, those mutants, which have intermediate-sized side chains, display a clear slowing of the $B \to A$ phase with increasing temperature (decreasing viscosity), just prior to the onset of the $C \to A$
Side Chain Dynamics Influence Myoglobin Reactivity

phase. In these mutants, the side chains can potentially hinder but not completely block access to the Xe4 site. This temperature slowing effect is seen most clearly as increases in the amplitude of the minor B → A phase and the appearance of multiple minor B → A MEM populations for the single H64L/V68 and the double H64L/V68F mutants. Based on these observations, we attribute the onset of the minor B → A phase to low amplitude side chain motions that facilitate ligand movement out of the B site into the Xe4 cavity. In cases where the side chains are large enough and positioned appropriately, these relaxations may also hinder access to the iron, slowing bond formation directly.

Recombination from Remote Sites; the C → A and D → A Transitions—In our nomenclature, the C → A process is associated with recombination of CO from the remote xenon sites before the completion of large amplitude protein relaxations that restrict the size of site B and iron-ligand bond formation. The rates of the C → A transition are very similar (50,000–200,000 s\(^{-1}\); TABLE THREE) for all the H64L mutants except H64L/V68F Mb, presumably because the sizes of the xenon sites are unaltered and movement between them is governed by more general but internal protein fluctuations that are less influenced by external viscosity. In the case of the H64L/V68F mutant, the Xe4 site is blocked and, as a result, rates of movement into and rebinding from Xe1 are very slow, ≲2,000 s\(^{-1}\). This latter result strongly supports our assumption that the Xe4 cavity is part of the pathway for ligand movement into the major remote site, Xe1.

The third and slowest recombination phase, D → A, is highly slaved to solvent viscosity as is evident by its being completely inhibited under the high viscosity conditions found at 3.5 °C in sol-gel/100% glycerol or in a dry glass matrix at ambient temperatures. At higher temperatures, when large scale protein fluctuations can occur, this phase can become dominant if distal pocket side chains relax to positions that sterically hinder access to the iron atom. In the case of wild-type Mb, these relaxations can include penetration of the vacated distal heme pocket by a solvent or surface water molecule. Thus, in our interpretation, the D → A process involves rebinding of a ligand localized within the network of xenon cavities (protein matrix) under conditions where the local environment of the active site has a distribution of conformational substrates that are equivalent to those of the equilibrium deoxy-Mb structure. This situation is similar to that encountered in rapid-mixing experiments designed to measure the overall rate constants for ligand binding to the deoxy-Mb derivatives that have been unliganded for a very long time (minutes to hours). In the case of wild-type deoxy-Mb, a water molecule is hydrogen-bonded to the His(E7) side chain and inhibits equally ligand return from the remote xenon sites and ligand entry into the distal pocket from the solvent phase (Fig. 1).

Conclusions and Structural Interpretation—The results and analyses presented in TABLES ONE to THREE and Figs. 3–10 are consistent with the branched scheme described by Gibson and co-workers (7, 14), Magde and co-workers (61), LeMaster (65), and others. An expanded version of this “side path” scheme is shown in Fig. 11 and is based primarily on the kinetic consequences of mutating key amino acids located along proposed pathways, in the distal pocket, and adjacent to the Xe1 and Xe4 cavities (14). These data indicate that the imidazole side chain of His(E7) acts as a gate separating ligand molecules in solvent from the B site. Outward and upward movement of His(E7) creates the pathway for ligand entry and exit (11, 12, 14).

In geminate recombination experiments, photodissociated CO is first localized in an unhindered B state with ready access to the iron atom (Fig. 11, upper tier of diagram). Rebinding from this B site, prior to the onset of low amplitude side chain motions, is very rapid and accounts for the initial B → A (major) MEM peak at very short times. This phase should correlate with the rebinding phase at low cryogenic temperatures (process I) where there is no interconversion of conformational substrates (27, 30, 60), and kinetic hole burning (70, 71) is observable. Rebinding on the time scale of low amplitude side chain fluctuation/relaxations gives rise to the slower B → A (minor) MEM peak, depicted as the [B] → A transitions in Fig. 11 (middle tier of reaction coordinate diagram). [B] is used to designate the state in which the dissociated ligand is still localized in the extended distal heme pocket on a time scale where it is experiencing the consequences of low amplitude fluctuations of the DHP side chains. The B → [B] transition should correlate with the loss of kinetic hole burning and the onset of dynamic hole filling (72). The extent of slowing associated with the [B] → A (minor) transition is highly dependent on the amino acids present at the E7 and B10 positions. In the case of wild-type myoglobin, relaxation of the His(E7) side chain over the iron atom causes a markedly reduced rate for the B → A transition. Relaxations of the Leu(68) and Asn(68) side chains also appear to sterically restrict rebinding to the iron from either the back portion of the distal pocket or the remote xenon sites. These low amplitude motions of the position 64 and 68 side chains also appear facilitate the [B] → C transition whereby the dissociated ligand diffuses into the remote site such as Xe4. As these side chains move toward their equilibrium positions above the iron atom, they decrease the size of the B site and seemingly push the dissociated ligand into the Xe4 cavity from which the ligand can access the more remote internal sites (Fig. 11, middle structure).

Rebinding from the remote sites is slower than either B → A transition. However, in the absence of full relaxation of the side chains and overall tertiary structure, this kinetic process, designated the C → A transition, is still fairly rapidly exhibiting rates on the order of 10–50,000 s\(^{-1}\) (TABLE THREE). The slower rate of C → A compared with [B] → A is likely due in part to the entropic stabilization of the C versus[B] state because of the decrease in accessible volume for the former. In our interpretation, the even slower D → A transitions represent ligand rebinding from these same remote sites but after complete relaxation of the distal pocket to the equilibrium deoxy conformation. In the case of wild-type myoglobin, an internal water molecule attached to His(E7) completely hinders both access to the iron atom and return to the B site, giving rise to a slow geminate rate of ~400 s\(^{-1}\) in sol-gels/100% glycerol at 65 °C (Fig. 11, lower structure).

The scheme in Fig. 11 also explains why there is strong correlation between the overall bimolecular rates of CO binding to the mutants and the rate of the D → A transition. In the case of binding from solvent, both the rate and equilibrium association constants for noncovalent ligand entry into the distal pocket are governed by the fraction of empty or unhindered B sites available to the incoming ligand. For example, in the case of wild-type Mb, ~90% of the equilibrium deoxy molecules have internal water. Thus, the fraction of protein molecules with empty sites is only 0.10, and the observed rate for bimolecular entry is ~10 times smaller than that for entry into a mutant with no internal water, i.e. H64L Mb. The same extent of inhibition will occur for the ligand return to the distal pocket from the remote xenon sites because in both cases ligand entry into site B cannot occur if internal water is present. Thus, the relaxation from the unhindered B state to [B] to [B]hydrated/water is expected to have roughly the same quantitative effect on both ligand entry from solvent and ligand return from the xenon cavities.

The slowing of geminate rebinding with increasing temperature/decreasing viscosity, which seemed at first to be incongruous, is readily explained by both enhanced ligand migration to remote sites, from which return is slow, and conformational relaxations in the B site that
increase markedly the barrier to internal rebinding to the iron atom. The importance of an evolving reaction coordinate as a factor in the control of rebinding rates in Mb was first put forth by Agmon and co-workers (73–75). Scott and Friedman (76) have shown this effect experimentally in hemoglobin where the fast tertiary relaxation does control geminate ligand rebinding at ambient temperatures. The present sol-gel study shows that specific E7 and E11 side chain relaxations and water penetration are the major causes of Agmon-Hopfield slowing of geminate recombination in Mb at higher temperatures and lower external viscosities.

REFERENCES

1. Frauenfelder, H., Sliam, S. G., and Wolynes, P. G. (1991) Science 254, 1598–1603
2. Frauenfelder, H. (1995) Nat. Struct. Biol. 2, 821–823
3. Frauenfelder, H., McMahon, B. H., Austin, R. H., Chu, K., and Groves, J. T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2370–2374
4. Antonini, E., and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland Publishing Co., Amsterdam
5. Schoenborn, B. P., Watson, H. C., and Kendrew, J. C. (1965) Nature 207, 28–30
6. Tilton, R. F., Kunz, I. D., and Petko, A. G. (1984) Biochemistry 23, 2849–2857
7. Scott, E. E., and Gibson, Q. H. (1997) Biochemistry 36, 11909–11917
8. Lamb, D. C., Nienhaus, K., Arcovito, A., Draghi, F., Miele, A. E., Brunori, M., and Nienhaus, G. U. (2002) J. Biol. Chem. 277, 11636–11644
9. Kriegl, M. N., Nienhaus, K., Deng, P., Fuchs, J., and Nienhaus, G. U. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7069–7074
10. Draghi, F., Miele, A. E., Travaglini-Allocatelli, C., Vallone, B., Brunori, M., Gibson, Q. H., and Olson, J. S. (2002) J. Biol. Chem. 277, 7509–7519
11. Nienhaus, K., Deng, P., Kriegl, J. M., and Nienhaus, G. U. (2003) Biochemistry 42, 9647–9658
12. Nienhaus, K., Deng, P., Kriegl, J. M., and Nienhaus, G. U. (2003) Biochemistry 42, 9633–9646
13. Nienhaus, K., Deng, P., Olson, J. S., Warren, J. J., and Nienhaus, G. U. (2003) J. Biol. Chem. 278, 42532–42544
14. Scott, E. E., Gibson, Q. H., and Olson, J. S. (2001) J. Biol. Chem. 276, 5177–5188
15. Ostermann, A., Waschipky, R., Parak, F. G., and Nienhaus, G. U. (2000) Nature 404, 205–208
16. Vojtechovsky, I., Chu, K., Berendzen, J., Schlichting, I., and Vojtechovsky, J. (1999) Bio-

Side Chain Dynamics Influence Myoglobin Reactivity