Analysis of the Kinetic Barriers for Ligand Binding to Sperm Whale Myoglobin Using Site-directed Mutagenesis and Laser Photolysis Techniques*

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Time courses for NO, O2, CO, methyl and ethyl isocyanide rebinding to native and mutant sperm whale myoglobins were measured at 20 °C following 17-ns and 35-ps laser excitation pulses. His64(E7) was replaced with Gly, Val, Leu, Phe, and Gln, and Val88(E11) was replaced with Ala, Ile, and Phe. For both NO and O2, the effective picosecond quantum yield of unliganded geminate intermediates was roughly 0.2 and independent of the amino acids at positions 64 and 68. Geminate recombination of NO was very rapid; 90% rebinding occurred within 0.5–1.0 ns for all of the myoglobins examined; and except for the Gly64 and Ile88 mutants, the fitted recombination rate parameters were little influenced by the size and polarity of the amino acid at position 64 and the size of the residue at position 68. The rates of NO recombination and ligand movement away from the iron atom in the Gly64 mutant increased 3–4-fold relative to native myoglobin. For Ile88 myoglobin, the first geminate rate constant for NO rebinding decreased ~6-fold, from $2.3 \times 10^{10}$ s$^{-1}$ for native myoglobin to $3.8 \times 10^{8}$ s$^{-1}$ for the mutant.

No picosecond rebinding processes were observed for O2, CO, and isocyanide rebinding to native myoglobins; all of the observed geminate rate constants were $\leq 5 \times 10^{4}$ s$^{-1}$. The rebinding time courses for these ligands were analyzed in terms of a two-step consecutive reaction scheme, with an outer kinetic barrier representing ligand movement into and out of the protein and an inner barrier representing binding to the heme iron atom by ligand occupying the distal portion of the heme pocket. Substitution of apolar amino acids for His64 decreased the absolute free energies of the outer and inner kinetic barriers and the well for non-covalently bound O2 and CO by 1 to 1.5 kcal/mol, regardless of size. In contrast, the His64 to Gln mutation caused little change in the barrier heights for all ligands, showing that the polar nature of His64 inhibits both the bimolecular rate of ligand entry into myoglobin and the unimolecular rate of binding to the iron atom from within the protein.

Increasing the size of the position 68(E11) residue in the series Ala to Val (native) to Ile caused little change in the rate of O2 migration into myoglobin or the equilibrium constant for noncovalent binding but did decrease the unimolecular rate for iron-O2 bond formation. Decreases in the equilibrium constants for noncovalent methyl and ethyl isocyanide binding were observed for the same series of mutants, but again the largest effect was an increase in the height of the inner kinetic barrier when Ile was substituted for Val88. These results show that the isopropyl side chain of Val88 comprises a portion of the inner steric barrier for iron-ligand bond formation. The Val88 to Phe mutation had little effect on the inner kinetic barrier and final equilibrium bound state. However, the affinities of ligands for the noncovalent binding site in the Phe88 mutant decreased 3–30-fold compared with native myoglobin, and the kinetic barrier for escape and entry into the mutant protein increased by 1–2 kcal/mol. The first result is due to decreasing the size of the distal cavity in the mutant protein. The effect of the Val88 to Phe substitution on the outer kinetic barrier may be due to inhibition of motions of the E helix that are required to open a channel between Val88 and His64 or to direct blockage of an alternative pathway for ligand entry.

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We have examined the physiological roles of the distal pocket histidine and valine in sperm whale myoglobin by measuring the overall rate and equilibrium constants for ligand binding to 14 different single and double mutants at
positions 64(E7) and 68(E11) (Springer et al., 1989; Rohlfis et al., 1990; Egeberg et al., 1990). Similar studies of these residues in the α and β subunits of R-state human hemoglobin have been completed (Olson et al., 1988; Mathews et al., 1989). The effects of site-directed mutagenesis on the equilibrium constants for O₂, CO, and alkyl isocyanide binding are readily interpreted in terms of the crystal structures of deoxymyoglobin and its corresponding liganded complexes. His₈⁴ stabilizes bound O₂ by at least ~1.4 kcal/mol through hydrogen bonding between the ε-amino nitrogen of the imidazole side chain and the second bound oxygen atom (Phillips, 1981). Bound CO is destabilized by +1.0 kcal/mol due to steric hindrance by His₈⁴.

This is expressed structurally by a bent Fe=C=O geometry and movement of the E7 imidazole side chain away from the iron atom (Kuriyan et al., 1986). Val₈⁴ does not appear to hinder O₂ binding, may serve to orient the bound ligand for more efficient hydrogen bonding to His₈⁴, but does inhibit CO binding, although the extent is significantly smaller than that due to His₈⁴. Bound methyl and ethyl isocyanide are markedly hindered by both His₈⁴ and Val₈⁴, and these steric interactions are observed structurally as disorder in the position of His₈⁴ and a highly distorted iron-isocyanide geometry (Johnson et al., 1989). A quantitative summary of these results is presented in Egeberg et al. (1990).

Interpretation of the overall association and dissociation rate constants for ligand binding to the position 64 and 68 mutants is more difficult since these parameters are determined by at least two kinetically distinct processes: 1) migration into and out of the protein and 2) binding to or dissociation from the iron atom within the distal portion of the heme pocket. Frauenfelder's group was the first to resolve these processes experimentally and to attempt to explain them in terms of specific structural features within the sperm whale myoglobin molecule (Austin et al., 1975). In their early work, unimolecular recombination from within the distal pocket was measured directly by photochemically dissociating CO-myoglobin in glycerol/water mixtures at low temperatures, conditions which prevent the ligand from leaving the protein matrix. Over the intervening 15 years, these processes have been resolved at room temperature in ordinary aqueous solutions by using short excitation pulses (see Ansari et al., 1986; Gibson et al., 1986; Henry et al., 1983; Cornellius et al., 1981; Jongeward et al., 1988; Petrich et al., 1988). Most ligand-protein complexes exhibit at least one unimolecular recombination phase which can be assigned to bond formation between the iron atom and photodissociated ligand present within the protein. In combination with the overall association and dissociation rate constants, the observed geminate rate constants can be used to define most if not all of the kinetic parameters for a linear, consecutive reaction scheme (Henry et al., 1983; Gibson et al., 1986).

Previous structural, theoretical, and kinetic work have suggested that ligands enter the distal pocket of myoglobin through a channel between the distal histidine and valine which is created by rotation of the imidazole side chain of His₈⁴ about its Cα-Cβ bond (Bolognesi et al., 1982; Ringe et al., 1986; Kottalam and Case, 1988; Johnson et al., 1989; Rohlfis et al., 1990). We have attempted to resolve the individual contributions of His₈⁴ and Val₈⁴ to the kinetic barriers involved in ligand binding by analyzing geminate recombination time courses for a series of site-directed mutants of sperm whale myoglobin. His₈⁴ was replaced by Gly, Val, Leu, Phe, and Glu to determine the size and polarity of the side chain at the E7 position; Val₈⁴ was replaced with Ala, Ile, and Phe to determine the effect of size at the E11 position. By using ligands which differ in size and chemical reactivity, we were able to examine intramolecular recombination over a wide range of time scales. The geminate recombination reactions for the NO derivatives were dominated by large, extremely rapid picosecond phases (τ₀ ≈ 40 ps), whereas only nanosecond geminate intermediates were observed for the corresponding O₂, methyl isocyanide, and ethyl isocyanide complexes. CO recombination reactions were also examined, but in most cases, the extent of recombining from within the protein was too small (±5%) to allow accurate measurements.

**MATERIALS AND METHODS**

**Preparation of Mutants**—Wild-type and mutant sperm whale myoglobins were expressed in *Escherichia coli* using the synthetic gene of Springer and S ligar (1987). Construction and purification of the position 64 and 68 mutants were described by Springer et al. (1989) and Egeberg et al. (1990), respectively. Native sperm whale myoglobin (type II) was obtained from Sigma prior to the United States ban on whale products, stored at ~20 °C, and used without further purification. Preparation of ligand solutions for kinetic measurements was described by Rohlfis et al. (1990) and Gibson et al. (1986). In their initial photolysis experiments, we found no differences between the geminate recombination time courses for native and wild-type myoglobin expressed in *E. coli* (Ringe et al., 1990) and native (Sigma) and synthetic wild-type myoglobin have identical overall association and dissociation rate constants for eight different ligands. Phillips et al. (1990) reported that the three-dimensional structure of wild-type metmyoglobin expressed in *E. coli* is identical to that of native metmyoglobin except in the immediate vicinity of the NH₃-terminal methionine. In view of these controls, we combined and averaged the results for Sigma and wild-type myoglobin and listed the parameters as applying to native protein (Tables I–IV).

Measurement of the O₂ recombination reactions for Gly₄⁴, Val₈⁴, Leu₈⁴, and Phe₄⁴ myoglobin were complicated by high rates of autooxidation which made quantum yield determinations difficult. The oxygen complexes of these proteins were prepared at pH 9 in 0.1 M borate buffer under 1 atm of O₂, and the flash photolysis experiments were carried out as quickly as possible (see Rohlfis et al., 1990). The geminate recombination reactions of native myoglobin were examined in 0.1 M phosphate at pH 7 and in 0.001, 0.010, 0.050, and 0.1 M borate at pH 9. No differences were found among the five conditions. Conventional rapid mixing and flash photolysis experiments were carried out to measure the overall association rate constants for O₂, CO, and methyl isocyanide binding to native and mutant myoglobins at pH 7 and 9, and again no pH dependence was observed. Consequently, we assume that the O₂ geminate recombination parameters for the position 64 mutants apply at both pH 7 and 9.

**Laser Photolysis Experiments**—For the NO, O₂, and CO reactions, protein samples were prepared in tonometers equipped with 1-mm pathlength cuvettes. Isocyanide complexes were injected into thin cells capped with a serum stopper as described by Gibson et al. (1986). The laser concentrations were 20–100 μM. Overall quantum yields for the CO and isocyanide complexes, Q₀,total, were measured using a conventional photolysis apparatus equipped with two photographic strobes containing thyristor-quenching devices. The excitation flash was set to be a rectangular pulse with a width of ~0.5 ms and rise and decay times ~0.1 ms. The extent of deoxymyoglobin formation produced by the pulse was measured as a function of relative light intensity, and the value of Q₀,total was obtained as described by Gibson et al. (1986, Miniprint). Overall quantum yields for O₂ were measured using either a 300-nm pulsed dye laser (Phase-R 2100B, see Rohlfis et al., 1990) or the 17-nm pulse system described below.
Three types of laser photolysis experiments were carried out depending on the excitation pulse and data collection systems used. For nanosecond experiments, photolysis was initiated by a rectangular 17-n pulse using a Phase-R (New Durham, NH) model 2100B dye laser operated in cavity dump mode with a switching time of less than 2 ns. Rhodamine 575 (Exciton, Inc.) in ethanol was used to produce an excitation flash of about 75 mJ at 577 nm. Transmittance changes at 436 nm (MbCO or MbO) or 445 nm (Mb-isocyanide) were followed with a Hamamatsu photomultiplier connected to a Tektronix model 7104 oscilloscope which was equipped with a video camera and interfaced to an IBM-AT computer. The data were collected, reduced to absorbance changes, and stored as files containing 400 points both during and after the flash pulse. The response time of the recording system was measured to be ~0.7 ns.

Picosecond excitation experiments were carried out using a Nd-YAG active-passive mode locked laser (Quantel model YG571) which provides 35-ps pulses at 1064 nm that were frequency doubled to 532 nm. Since none of the O2, CO, and isocyanide complexes examined showed rapid picosecond processes (t < 500 ps), transmittance changes for these derivatives were often collected using the Tektronix oscilloscope, video camera, and IBM-AT data collection system. For the NO complexes, faster response times were needed, and a probe pulse data collection system was used. The Nd-YAG pulse was split. One beam was used for photolysis; the other beam was passed through a Raman shifter and the first anti-Stokes line at 436 nm used to probe the transmittance of the sample cuvette. An automated optical delay line was used to obtain probe absorbance readings over a time period of 1.5 ns.

**Data Analysis: the Photophysical Yield Problem**—In our previous work, we assumed that the intrinsic photophysical yield of all ligand-myoglobin complexes was 1.0 and that any reduction in the overall quantum yield was due to photophysical processes in the intermediate states. The effective picosecond quantum yield is considerably less than 1 for O2 and NO heme complexes. Our results for NO and O2 re-binding to myoglobin and hemoglobin using the 35 ps pulse system agree with their conclusions (Bellelli et al., 1990; Tables I and II).

The minimum scheme required for describing ligand re-binding to myoglobin following a very short laser pulse (≤30 ps) is shown below and was adapted from Petrich et al. (1988):

\[
\begin{align*}
M^* \text{ (r ≤ 3 ps)} \\
A & \xrightarrow{k_{BA}} B_1, \ldots, B_n \\
A & \xrightarrow{k_{BA} + k_{BC}} [C_1, \ldots, C_n] \\
A & \xrightarrow{k_{BA} + k_{BC}} X + Mb
\end{align*}
\]

State A represents the equilibrium bound state. The picosecond intermediates observed in laser photolysis experiments at room temperature are thought to represent iron-bound contact pairs and the designated B states in Equation 1 (Jongeward et al., 1988; Petrich et al., 1988). The nanosecond intermediates (C states) are thought to represent ligand molecules farther removed from the iron atom and non-covalently bound in the protein. Petrich et al. (1988) have suggested that the picosecond photochemical yield of the first geminate intermediate, Qps, is determined by the ground state properties of the heme-ligand complex. For NO and O2 complexes, absorption of a large fraction of excitation quantum, 1 Qps, leads to the formation of a photochemical intermediate (M*) which exhibits an absorption of the heme-ligand complex. For NO and O2 complexes, absorption of a large fraction of excitation quantum, 1 Qps, leads to the formation of a photochemical intermediate (M*) which exhibits an absorption

\[
\begin{align*}
\frac{d[M^*]}{dt} &= k_{BA}(1-Q_{ps})[A] - k_{BA}[M^*] \\
\frac{d[B]}{dt} &= k_{BA}Q_{ps}[t][A] - (k_{BA} + k_{BC})[B]
\end{align*}
\]

\[k_{BA}\] is a proportionality constant which depends on the geometry of the optical system and the extinction coefficient of the heme-ligand complex at the wavelength of excitation. t is the relative light intensity of the pulse at time t after initiation of photexcitation and is given by a Gaussian expression with a width at half-maximum intensity equal to 35 ps. Qps represents the effective picosecond photochemical yield of the first B geminate state. The rate of decay of the photoexcited M* state was fixed at 2.3 × 10^11 s^-1, corresponding to the 3-ps half-life reported by Petrich et al. (1988).

Correlations with the observed absorbance traces were complicated by the width of the observing pulse, and the calculation of theoretical values had to be done in two steps. First, Equation 2 was integrated over the time window of the observing pulse and the results stored at 1-ps intervals. Second, the theoretical time dependences of the intermediates were converted to a transmittance time course by assigning extinction coefficients to states A (liganded myoglobin), B (deoxymyoglobin), and M* (assumed to be equivalent to liganded myoglobin at 436 nm; Petrich et al., 1988). This transmittance time course was scanned at 1-ps intervals by computing the product of the transmission and the probe light intensity at each time point and then summing the results to give an overall transmission value for the entire observing beam. The shape of the probe pulse was also assumed to be Gaussian, and the observation window was fixed at 140 ps. The theoretical absorbance for the sample was calculated as the logarithm of the ratio of the summed transmittance when photooxidation occurred to that for the fully liganded complex when the excitation beam was blocked before reaching the cuvette. This value was then assigned to the reaction time interval defined by the physical length of the optical delay line. This process was continued for each experimentally observed time point by moving the observation window along the theoretical time courses for the geminate intermediates and the ground state. The absolute value of k was determined emperically by fitting picosecond time courses for MbCO to Equation 2 assuming k_{BA} ≈ 0 and Q_{ps} = 1.0 (see Gibson et al., 1986; Bellelli et al., 1990.) Analyses of the MbCO time courses also allowed precise determinations of the time intervals between the exciting and probe pulses. Time courses for NO rebinding were then generated using Equation 2 and nonlinear least squares methods used to optimize the fitted parameters, Qps, kps, and kbc by comparing the observed and computed absorbance traces at various laser light intensities.

Since most of the myoglobin-NO complexes examined exhibited heterogeneous picosecond re-binding time courses, the rate equations were expanded to include additional B states assuming a linear consecutive reaction scheme: d[B]/dt = k_{BA}Q_{ps}[t][A] - (k_{BA} + k_{BC})[B]; d[B]/dt = k_{BA}Q_{ps}[t][A] - (k_{BA} + k_{BC})[B], etc. In most cases, two B states were sufficient to fit the observed data (Fig. 1). This interpretation is not unique, and parallel reaction schemes can fit the
experimental results equally well. Kuriyan et al. (1986) and others have reported evidence for multiple conformations of bound CO each of which could generate different initial B states. Thus, parallel schemes are structurally plausible and have been used extensively by Frauenfelder and co-workers to describe low temperature rebinding phenomena (Austin et al., 1975; Doster et al., 1982; Ansari et al., 1986). At present, it is difficult to distinguish between these possibilities experimentally, and thus, the fitted parameters in Table I should be viewed as an empirical description of the observed time courses.

Analysis of Nanosecond Recombination Time Courses—In contrast to the NO results, we and Petrich et al. (1988) have not observed any rapid picosecond rebinding processes for the O2, CO, methyl, and ethyl isocyanide complexes of sperm whale myoglobin, indicating that the extent of rapid rebinding from contact pairs (B states) must be very small.2 Normally, only a single geminate recombination phase with a half-time of 200 ns was observed. Under these conditions, Equation 1 can be reduced to a simple two-step reaction mechanism:

\[
\text{MbX or A} \overset{k_{\text{c}}}{\longrightarrow} \overset{k_{\text{c}}}{\longrightarrow} \text{MbX or A} \overset{k_{\text{c}}}{\longrightarrow} \overset{k_{\text{c}}}{\longrightarrow} \text{MbX or A}
\]

Equation 3). For O2, CO, and isocyanide-myoglobin complexes listed in Tables I-IV; k'xc, kcx, Kxc, kca, and KM were displayed in Tables I-IV. k'xc, kcx, Kxc, kca, and KM were calculated from these averages using the expressions in Equation 6.

As footnoted in Tables II-IV, certain ligand-mutant pairs showed heterogeneous rebinding time courses even on nanosecond time scales. These data were also fitted to a two-step consecutive reaction scheme involving multiple C states, and again, it was difficult to distinguish between sequential and parallel reaction schemes. For comparison with the majority of the ligand-myoglobin complexes, only the parameters for fits to the simple intermediate scheme were listed in Tables II-IV and used to compute the free energy barriers and wells in Figs. 7 and 8.

RESULTS

O2 Rebinding to Native and Mutant Myoglobins—Time courses for native, Ile64, and Phe66 NO-myoglobin are shown in Fig. 1. Even at the highest light intensities, only 20-40% of the expected absorbance change for total photodissociation into deoxymyoglobin was obtained.3 This result demonstrates qualitatively that the effective picosecond quantum yield is considerably less than 1.0, as shown by Petrich et al. (1988). Sets of recombination time courses at different light intensities were analyzed by integrating Equation 2 and fitting for the optimum values of Qns and the appropriate number of geminate recombination rate parameters. In most cases, two B substates were needed to obtain satisfactory fits to the observed data, and the resultant fitted rate constants are given in Table I.

The effective picosecond quantum yield of the first geminate intermediate, B1, was relatively invariant for all nine of the myoglobin-NO complexes and ranged from 0.22 to 0.36. The first geminate recombination rate constant, kB1A, was little affected by most of the mutations. The two major exceptions were the His64 to Gly and the Val64 to Ile substitutions. Replacing the distal histidine with glycine increased kB1A 2.5-fold. The rate constant describing the formation of the second B state, kB2B, also increased for this mutation, suggesting that a more open distal pocket facilitates both rebinding and movement away from the initial photodissociated contact pair. Replacing Val64 with Ile caused a 6-fold decrease in the first order rate constant for the geminate rebinding of nitric oxide, presumably because the larger Ile side chain limits access to the iron atom even in the contact pair (Table I).

O2 Rebinding—Sample time courses for the photolysis of oxyhemoglobin are shown in Fig. 2, and the corresponding kinetic parameters are listed in Table II for all of the mutant proteins. No evidence of rapid geminate recombination on picosecond time scales was observed, and again, only 20-30% of the MbO2 molecules could be photodissociated at the highest light levels used with a 35-ps excitation pulse. Qm values were obtained by fitting observed time courses to Equation 2 as a function of light intensity, and similar values (0.1-0.3) were obtained when the nanosecond quantum yield, Qns, was calculated as Qns/(1-Qns) (last column of Table III). The position 64 mutants appear to have higher photophysical yields; however, the uncertainties in Qm were large (±40-60%).

Multiple photons were absorbed at high light levels using the 35-ps excitation pulse. At full light intensity when the beam was focused down to a small cross-sectional area, "hole burning" occurred with complete bleaching of the heme pigment. These processes decreased with the square of the excitation pulse intensity, and by dispersing the beam over a larger area and using relative light levels less than full intensity, little or no photodestruction occurred.
that the low overall quantum yield of oxymyoglobin is due primarily to the
Another consequence of the low picosecond photophysical
geminate recombination of O₂ from state C further reduces
the quantum yield by only a factor of 2 or less (Table II).

Leu'j₄, and PheG₄ mutants. Even with this variation, it is clear
due to the high rates of autooxidation of the Gly'j₄, ValG₄,
were, from top to bottom A, 0.75; B, 0.78, C, 0.58. The relative laser
light intensities were from top to bottom A, l/r, l/g, %G; B, %, l/1, %; C,
by numerical integration of Equation 2. The maximum possible
absorbance changes (Mb versus MbNO) for the samples in each panel
bin in 0.1 M potassium phosphate, pH 7.0, 20 °C. The open circles
represent observed absorbance changes measured at different relative
photolysing pulse intensities. The solid lines are fitted curves obtained
during and after a 35-ps light pulse; samples were 20-100 μM myoglobin.

FIG. 1. Time courses for picosecond rebinding of nitric ox-

Kinetic parameters for NO rebinding to native and mutant sperm
whale myoglobin at 20 °C, pH 7

Picosecond recombination time courses were fitted to the linear
consecutive reaction scheme by numerically integrating the rate
expressions in Equation 2 as described in the text. Examples of the
fits are shown in Fig. 1.

| Protein | k_{HIA} | k_{HIB} | k_{HIC} | k_{HIC} | Q_{0a} | Q_{0a} |
|---------|---------|---------|---------|---------|-------|-------|
| Gly₆⁴ | 58  | 25  | 7.4  | (<0.1) | 0.98  | (0.001) |
| Val₆⁴ | 18  | 2.9  | 3.0  | 0.19  | 0.29  | 0.002  |
| Leu₆⁴ | 26  | 2.0  | 1.4  | 0.11  | 0.25  | 0.001  |
| Phe₆⁴ | 10  | 3.6  | 3.4  | 0.60  | 0.28  | 0.007  |
| Gly₆⁴ | 12  | 6.3  | 2.6  | (0.1) | 0.21  | ~0    |
| Native | 23  | 6.0  | 3.0  | 0.35  | 0.22  | 0.005  |
| Ala₆⁴ | 20  | 5.4  | 5.1  | (0.1) | 0.36  | ~0    |
| Ile₆⁴ | 3.8  | 4.0  | 0.9  | 0.1   | 0.20  | 0.001  |
| Phe₆⁴ | 25  | 1.0  | 4.4  | (0.1) | 0.31  | ~0    |

Larger changes in the geminate recombination rate param-
eters were observed for the position 68 mutations. Although
the rates of O₂ escape from the protein were roughly the same,
the rate of binding to the iron atom from within the distal pocket, k_{CA},
was roughly equal to the rate of ligand escape from the protein, k_{CX},
and neither changed more than 3-fold. In contrast, the overall
association and dissociation rate constants and the corre-
sponding rate parameters describing O₂ migration into the
protein, k'_{CX}, and thermal iron-O₂ bond breakage, k_{CA},
changed 10-1000-fold for the same set of mutations (Table II).

The equilibrium constant for non-covalent O₂ binding, K_{XC},
was obtained from k'_{XC}/k_{CX} where k'_{XC} was computed as
K_{XC} was roughly equal to 1.5 × 10⁸ s⁻¹.

The equilibrium constant for non-covalent O₂ binding, K_{XC},
was obtained from k'_{XC}/k_{CX} where k_{CX} was computed as
k'_{CX}/(k_{CA} + k_{CX}) (Equation 6). The largest values of K_{XC}
were found for Gly₆⁴ and Val₆⁴ myoglobin and the smallest for
Ile₆⁴ and Phe₆⁴ myoglobin (Table II). Increasing the polarity
and size of the position 64 amino acid and the size of the
position 68 residue inhibited non-covalent binding, and these
trends were observed for all of the ligand molecules examined
(K_{XC} values in Tables II-IV).

CO Rebinding—Geminate rate parameters are reported for only those CO-myoglobin complexes which exhibited overall
quantum yields ≤0.9 and Θ > 0.1. The rate parameters for
native sperm whale myoglobin were taken from Henry et al.
(1983). In agreement with the results of Petrich et al. (1988),
Q_{0a} appears to be ~1.0 for all of the CO complexes examined.
The rate of escape from the protein, k_{CX}, was at least 2-fold
less than that observed for O₂ when direct comparisons were
made (native, Leu₆⁴, and Phe₆⁴ myoglobin). Similar small
differences were observed between k_{CX} values for O₂ and CO
escape from the distal pockets of isolated α and β subunits of
human hemoglobin (Olson et al., 1987). The biggest differ-
Fig. 2. Time courses for nanosecond rebinding of oxygen to myoglobin mutants. Reactions were monitored at 436 nm during and after an attenuated 17-ns light pulse. Conditions: 0.1 M borate, pH 9.1, 20 °C for the position 64 (E7) mutants and 0.1 M phosphate, pH 7.0, 20 °C for the position 68 (E11) mutants. Panels A and B show normalized time courses for O2 rebinding to E7 and E11 mutants of myoglobin. The observed absorbance changes were represented as open circles connected by thin lines. Panels C and D show time courses and fitted curves for O2 rebinding to native and Phe68 myoglobins. The open circles represent observed data; the solid lines are fitted curves obtained by numerical integration of the differential equations describing Equation 3. The rightmost time course in panel C represents data collected in 160 ns (% of the y axis scale) and was fitted simultaneously with the data collected in 400 ns. The inset in panel D represents data collected on a longer time scale and, again, this time course was fitted simultaneously with the others. Relative laser light intensities for the traces in C and D were from top to bottom C, 1/6, 1/4, 1/3, 1/2; D, 1, 1/2, 1/4, 1/6, and 1/8 for the inset.

ences between O2 and CO were observed for the rates of binding from within the distal pocket: kca for O2 rebinding was 5–30-fold greater than that observed for CO.

Braunstein et al. (1988) examined the low temperature recombination kinetics of Gly64 CO-myoglobin. Extrapolation of 200 K suggested that the kca values for CO rebinding to Gly64 and His64 (native) myoglobin are similar. The relative insensitivity of the NO picosecond rebinding process to mutations at position 64 is consistent with this observation. Braunstein et al. (1988) reported a 15-fold increase in the pocket occupancy factor for CO rebinding when His64 was replaced with Gly, in agreement with the increases in Kxc which we observed for O2 and CO binding to mutants containing Gly or apolar amino acids at residue 64 (Tables II and III). A direct comparison is not possible since the pocket occupancy factor corresponds to Kxc/Kca in Equation 1 and cannot be measured experimentally at room temperature (Doster et al., 1982; Henry et al., 1983; Gibson et al., 1986).

Isocyanide Rebinding and the Importance of Pocket Size—The geminate recombination time courses for methyl isocyanide showed a greater dependence on protein structure than those for O2 rebinding, particularly for the position 64 mutants (Fig. 3, Table IV). The extent of intramolecular rebounding (Qo) increased with increasing size of the E7 residue for the series Val64, Leu64, and Phe64. The Val64 to Ile mutation increased the extent of methyl isocyanide escape from the distal pocket, whereas the Val64 to Phe mutation effectively prevented ligand movement out of the protein (Qoverall ≤ 0.01). Kxc for non-covalent methyl isocyanide binding depended markedly on the size of both the position 64 and 68 amino acids, decreasing from 3.8 M⁻¹ for Gly64 to 0.0094 M⁻¹ for Phe68 myoglobin (Table IV).

The ethyl and methyl isocyanide rebinding parameters exhibited similar dependences on the position 64 and 68 amino acids (Table IV). The major difference was that the rate and extent of geminate recombination were uniformly greater for the larger ligand (Fig. 4A). We previously interpreted this result in terms of the limited size of the distal pocket (Gibson et al., 1986). Large translations or rotations away from the iron atom after photodissociation cannot occur for ethyl isocyanide without substantial steric interactions with surrounding amino acid side chains. Although less stable in state C, as judged by a 3-fold lower value of Kxc for non-covalent ethyl isocyanide binding compared with that for the
methyl compound, the larger ligand is held in place for more rapid recombination. This idea is supported by three independent observations. First, little or no nanosecond geminate recombination was observed for the methyl and ethyl isocyanide complexes of soybean leghemoglobin, which is known to have tightly packed Leu$^\ddag_3$, Phe$^{33}$, Phe$^{43}$, and Gly$^{46}$ molecules located in the distal cavity. Second, the x-ray crystallographic structure of ethyl isocyanide-myoglobin shows tight packing of Leu$^\ddag_3$, Phe$^{33}$, and Phe$^{43}$ that state C can be assigned to non-covalently bound ligand molecules (Fig. 4). A large picosecond geminate rebinding phase is observed for this ligand, presumably because the bulky tert-butyl group prevents movement of the isocyanide group away from the iron atom (Gibson et al., 1986; Jongeward et al., 1989). These data and observations also suggest strongly that state C can be assigned to non-covalently bound ligand molecules in the distal cavity.

Further evidence for the importance of the size of the distal cavity is provided by the results for the Val$^{68}$ to Phe mutation caused marked increases in the rates and extents of geminate recombination for all ligands, including CO. $K_{\text{XC}}$ decreased 15-30-fold for CO and O$_2$ binding and 3-fold for methyl and ethyl isocyanide binding. The similarities between the effects of increasing the size of the ligand molecule for a given protein and those of the Val$^{68}$ to Phe mutation for a given ligand are striking and argue for a similar underlying cause, a decrease in the ratio of the size of the distal pocket to the size of the ligand molecule (Fig. 4).

### DISCUSSION

**Structural Interpretations**—Ortep drawings of the heme pockets of the O$_2$ and ethyl isocyanide complexes of sperm whale myoglobin are shown in Fig. 5. The view is from the back of the distal pocket, looking out toward the solvent through the proposed channel between Val$^{68}$ and His$^{46}$ in the ethyl isocyanide complex, the His$^{46}$ imidazole side chain was drawn in the open conformation (Fig. 5B; Johnson et al., 1989). In Fig. 6, top views of the distal pockets are presented using space filling models. The iron atom and porphyrin ring (dark blue atoms) are located underneath the distal residues and in the plane of the photograph, and Leu$^{68}$(B10), which forms the top of the ligand-binding site, has been removed to reveal the sides and back of the distal pocket. The first two atoms of the ligand molecules (red) are located underneath the distal residues and directly adjacent to the $\gamma$-CH$_3$ group of Val$^{68}$(V68, light blue).

The picosecond intermediates observed in laser photolysis experiments are thought to represent ligand molecules in the

### TABLE II

**Kinetic parameters for O$_2$ rebinding to position 64(E7) and 68(E11) mutants of sperm whale myoglobin at 20 °C**

Symbols are described in Equations 3-6. The errors for native myoglobin were computed as the standard deviation from the mean from nine independent experiments and are assumed to apply to the mutant parameters. All experiments with the mutants were carried out at least twice. The overall association and dissociation rate constants were taken from Rohlfs et al. (1990) and Egeberg et al. (1990).

| Mutant | $k'$ | $k$ | $k_2$ | $\phi_5$ | $Q_{\text{overd}}$ | $Q_5$ |
|--------|------|-----|-------|----------|-----------------|-------|
| Gly$^{68}$ | 140 | 1,600 | 31 | 0.29 | $-0.2$ | $-0.3$ |
| Val$^{68}$ | 250 | 23,000 | 39 | 0.34 | $-0.3$ | $-0.4$ |
| Leu$^{68}$ | 98 | 4,100 | 42$^a$ | 0.46$^a$ | $-0.2$ | $-0.4$ |
| Phe$^{68}$ | 75 | 10,000 | 18$^a$ | 0.58$^a$ | 0.16 | 0.37 |
| Gly$^{68}$ | 24 | 130 | 17$^a$ | 0.38$^a$ | 0.23 | 0.37 |
| Native | 14 ± 3 | 12 ± 2 | 19 ± 5 | 0.37 ± 0.03 | 0.12 ± 0.04 | 0.19 ± 0.07 |
| Ala$^{68}$ | 22 | 18 | 47 | 0.53 | 0.07 | 0.15 |
| Ile$^{68}$ | 3.2 | 14 | 24 | 0.12 | 0.09 | 0.10 |
| Phe$^{68}$ | 1.2 | 2.5 | 140 | 0.91 | (±0.01) | (0.12) |

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* The geminate recombination time courses for the Leu$^{68}$ mutation showed significant heterogeneity and fitted better to either a two-exponential or a three-step rebinding scheme ($k_1 = 39$ ps$^{-1}$, $k_2 = 4.1$ ps$^{-1}$; $\phi_5 = 0.18$). This problem is discussed in the text.

* The geminate recombination time courses for Phe$^{68}$ myoglobin were also heterogeneous ($k_1 = 32$ ps$^{-1}$, $k_2 = 3.5$ ps$^{-1}$; $\phi_5 = 0.16$).

* The ps and ns geminate recombination time course for Gly$^{68}$ myoglobin indicated an additional rapidly rebinding intermediate and fitted better to a three-step scheme or two-exponential expression ($k_1 = 133$ ps$^{-1}$, $k_2 = 39$ ps$^{-1}$; $\phi_5 = 0.40$ and $k_2 = 4.1$ ps$^{-1}$; $\phi_5 = 0.18$).

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B. Calculated parameters for O$_2$ rebinding

| Mutant | $k'_{\text{XC}}$ | $k_{\text{CX}}$ | $K_{\text{XC}}$ | $k'_{\text{CA}}$ | $k_{\text{CA}}$ | $K_{\text{CA}}$ | $K$ |
|--------|----------------|----------------|--------------|----------------|---------------|----------------|-----|
| Gly$^{68}$ | 480 | 22 | 21 | 9.3 | 2,300 | 0.0041 | 0.088 |
| Val$^{68}$ | 730 | 26 | 29 | 13 | 7,300 | 0.00098 | 0.011 |
| Leu$^{68}$ | 222 | 22 | 9.8 | 19 | 7,500 | 0.0025 | 0.023 |
| Phe$^{68}$ | 130 | 7.5 | 17 | 10 | 24,000 | 0.00043 | 0.0074 |
| Gly$^{68}$ | 62 | 11 | 5.8 | 6.7 | 210 | 0.032 | 0.18 |
| Native | 38 ± 9 | 12 ± 2 | 3.1 ± 0.9 | 7.2 ± 1.8 | 19 ± 5 | 0.38 ± 0.14 | 1.2 ± 0.3 |
| Ala$^{68}$ | 42 | 22 | 1.9 | 25 | 38 | 0.65 | 1.2 |
| Ile$^{68}$ | 36 | 21 | 1.2 | 2.9 | 16 | 0.18 | 0.22 |
| Phe$^{68}$ | 1.3 | 12 | 0.11 | 130 | 29 | 4.4 | 0.15 |

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$^a$ The geminate recombination time courses for the Leu$^{68}$ mutant showed significant heterogeneity and fitted better to either a two-exponential or a three-step rebinding scheme ($k_1 = 39$ ps$^{-1}$, $k_2 = 4.1$ ps$^{-1}$; $\phi_5 = 0.18$). This problem is discussed in the text.

$^a$ The geminate recombination time courses for Phe$^{68}$ myoglobin were also heterogeneous ($k_1 = 32$ ps$^{-1}$, $k_2 = 3.5$ ps$^{-1}$; $\phi_5 = 0.16$).

$^a$ The ps and ns geminate recombination time course for Gly$^{68}$ myoglobin indicated an additional rapidly rebinding intermediate and fitted better to a three-step scheme or two-exponential expression ($k_1 = 133$ ps$^{-1}$, $k_2 = 39$ ps$^{-1}$; $\phi_5 = 0.40$ and $k_2 = 4.1$ ps$^{-1}$; $\phi_5 = 0.18$).
Symbols are described in Equations 3-6. The overall association and dissociation rate constants were taken from Rohlf’s et al. (1990) and Egerberg et al. (1990). The geminate recombination parameters for native myoglobin were taken from Henry et al. (1983). Since our apparatus were designed to maximize the extent of photolysis of relatively insensitive O₂, NO, and isocyanide complexes, we found it difficult to measure reliably geminate CO recombination reactions for native myoglobin and the Gly⁶⁴-Gln⁶⁴ and Ile⁶⁸⁶⁴ mutants. The values listed below for the Phe⁶⁴ and Ala⁶⁴ mutants are rough estimates since the fractions of geminate recombination following a 30 ps were ≤0.1. Only in the cases of the Leu⁶⁴ and Phe⁶⁴⁶⁴ geminate reactions well-defined and Q_outer less than 0.90.

### A. Observed kinetic parameters for CO binding

| Mutant   | k' (s⁻¹) | k (s⁻¹) | k₁ (s⁻¹) | k₂ (s⁻¹) | K₁ (µM⁻¹) | K₂ (µM⁻¹) | Q_outer | Q_inner |
|----------|----------|---------|----------|----------|-----------|-----------|---------|---------|
| Native   | 0.51     | 0.018   | 7.1      | 0.32     | 0.91      | 0.90      | 0.1     | 0.01    |
| Phe⁶⁴    | 4.5      | 0.054   | 21.9     | 0.043    | 0.96      | 0.96      | 0.1     | 0.01    |
| Ala⁶⁴⁶⁴  | 1.2      | 0.021   | 4.6      | 0.11     | 0.91      | 0.91      | 0.1     | 0.01    |
| Phe⁶⁴⁶⁴  | 0.25     | 0.018   | 7.1      | 0.32     | 0.91      | 0.90      | 0.1     | 0.01    |

### B. Calculated parameters for CO recombination

| Mutant   | k' (s⁻¹) | k (s⁻¹) | k₁ (s⁻¹) | k₂ (s⁻¹) | K₁ (µM⁻¹) | K₂ (µM⁻¹) | K (µM⁻¹) |
|----------|----------|---------|----------|----------|-----------|-----------|----------|
| Native   | 0.51     | 0.018   | 7.1      | 0.32     | 0.91      | 0.90      | 0.1     |
| Phe⁶⁴    | 4.5      | 0.054   | 21.9     | 0.043    | 0.96      | 0.96      | 0.1     |
| Ala⁶⁴⁶⁴  | 1.2      | 0.021   | 4.6      | 0.11     | 0.91      | 0.91      | 0.1     |
| Phe⁶⁴⁶⁴  | 0.25     | 0.018   | 7.1      | 0.32     | 0.91      | 0.90      | 0.1     |

### C. Native MbMNC - 17 ns

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**Fig. 3.** Time courses for nanosecond rebinding of methyl isocyanide (MNC) to myoglobin mutants. Reactions were carried out in 0.1 M phosphate, pH 7.0, 20 °C, and monitored at 445 nm. A and B show normalized time courses for methyl isocyanide rebinding to F7 and E11 myoglobin mutants during and after a 35-ps light pulse. The open circles represent observed absorbance changes. The solid lines represent single exponential fits to Equation 5. C shows methyl isocyanide rebinding to native myoglobin after a 17-ns laser pulse. The solid lines in this panel represent fitted curves generated by numerical integration of the rate expressions defined by Equation 3. Relative laser light intensities for each trace in panel C were from top to bottom: V₁, V₂, V₃, V₄.

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initial stages of moving away from the iron atom (Jongeward et al., 1988; Petrich et al., 1988). Those contact pair intermediates should resemble closely the original ground state. During the very rapid NO rebinding reactions, there is little time for ligand movement and distal structural features to influence the observed geminate rate constants. As shown in Table I, the rate of NO rebinding from state B is little affected by the size and polarity of the position 64 residue. Only in the case of Gly⁶⁴ were the rates of rebinding and escape from the first geminate intermediate increased. The largest effect was observed for the Val⁶⁴ to Ile mutation which caused a 6-fold decrease in k_BA. This substitution also markedly increases equilibrium binding (K values in Tables IIA, IIIA, IVA, and IVC), and this inhibition appears to be due to steric hindrance of the 6-CH₃ group of Ile⁶⁴ over the iron atom due to global alterations in protein folding. The relative uniformity of the NO recombination parameters also indicates that the mutations are fairly conservative and do not cause large changes in the reactivity of the iron atom due to global alterations in protein folding.

In our view, the nanosecond intermediates observed for O₂, CO, and isocyanide re-binding represent ligand molecules non-covalently bound in the distal cavity circumscribed by Leu⁶⁴(B10), Phe⁶⁴(CD1), His⁶⁴(E7), Val⁶⁴(E11), and Ile⁶⁴⁶⁴(G8) (Fig. 6). This interpretation is consistent with molecular dynamics calculations which have defined an energy minimum for diatomic ligands in this region of the protein (Sas-saroli and Rousseau, 1986; Kottalam and Case, 1988). As shown in Fig. 6B, the alkyl side chain of covalently bound ethyl isocyanide is also located in this cavity. Kottalam and Case (1988) further suggested that the space surrounded by Val⁶⁴(E11), Leu⁶⁴(E16), and Ile⁶⁴⁶⁴(G8) may either represent a less stable binding site for small ligands or be continuous with the main cavity due to thermal motions of the aliphatic side chains lining the back of the distal pocket, particularly in aqueous solutions at high temperatures. Thus, the trajectory for ligand dissociation is thought to be non-linear, involving initial movement toward the back of the
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### TABLE IV

| Mutant   | k' | k | k_0 | Q_0 | Q_{corr} | Q_0 |
|----------|----|---|-----|-----|----------|-----|
| Gly^{64} | 10 | 6.3 | 12 | 0.34 | 0.42 | 0.64 |
| Val^{64} | 0.71 | 12 | 14 | 0.10 | 0.63 | 0.70 |
| Leu^{64} | 1.8 | 2.1 | 54 | 0.33 | ~0.4 | ~0.6 |
| Phe^{64} | 0.18 | 2.4 | 20 | 0.65 | 0.09 | 0.26 |
| Gln^{64} | 0.20 | 5.6 | 3.6 | 0.85 | 0.12 | 0.80 |
| Native   | 0.12 ± 0.02 | 4.3 ± 0.3 | 29 ± 5 | 0.85 ± 0.03 | 0.14 ± 0.02 | 0.93 ± 0.23 |
| Ala^{68} | 0.38 | 0.76 | 20 | 0.74* | 0.08 | 0.31 |
| Ile^{68} | 0.050 | 21 | 19 | 0.57 | 0.37 | 0.86 |
| Phe^{68} | 0.013 | 0.030 | 83 | 0.98* (≤0.01) | (0.5) |

A. Observed kinetic parameters for methyl isocyanide binding

| Mutant   | k' | k | k_0 | Q_0 | Q_{corr} | Q_0 |
|----------|----|---|-----|-----|----------|-----|
| Gly^{64} | 30 | 7.9 | 2.8 | 4.1 | 9.6 | 0.43 |
| Val^{64} | 7.0 | 13 | 0.50 | 1.4 | 13 | 0.11 |
| Leu^{64} | 5.4 | 36 | 0.15 | 18 | 3.1 | 5.7 |
| Phe^{64} | 0.28 | 6.9 | 0.041 | 13 | 6.9 | 1.9 |
| Gln^{64} | 0.24 | 0.54 | 0.44 | 3.0 | 37 | 0.081 |
| Native   | 0.14 ± 0.02 | 4.4 ± 0.8 | 0.032 ± 0.002 | 25 ± 2 | 29 ± 6 | 0.86 ± 0.19 | 0.028 ± 0.006 |
| Ala^{68} | 0.51 | 2 | 5.2 | 0.168 | 15 | 2.9 |
| Ile^{68} | 0.087 | 8.0 | 0.011 | 11 | 49 | 0.22 |
| Phe^{68} | 0.013 | 1.4 | 0.0094 | 81 | 1.8 |

B. Calculated parameters for methyl isocyanide binding

| Mutant   | k' | k | k_0 | Q_0 | Q_{corr} | Q_0 |
|----------|----|---|-----|-----|----------|-----|
| Gly^{64} | 3.7 | 0.8 | 2.7 | 4.1 | 9.6 | 0.43 |
| Val^{64} | 2.2 | 4.0 | 5.5 | 0.09 | 0.44 | 0.48 |
| Leu^{64} | 1.0 | 0.15 | 72 | 0.85 | 0.06 | 0.40 |
| Phe^{64} | 0.099 | 0.17 | 33 | 0.74 | 0.03 | 0.13 |
| Gln^{64} | 0.071 | 0.12 | 42 | 0.97 | 0.02 | 0.70 |
| Native   | 0.069 ± 0.010 | 0.30 ± 0.03 | 110 ± 15 | 0.94 ± 0.03 | 0.06 ± 0.02 | 0.83 ± 0.53 |
| Ala^{68} | 0.18 | 0.070 | 23 | 0.88 | 2.02 | (<0.01)* |
| Ile^{68} | 0.047 | 3.4 | 57 | 0.63 | 0.24 | (<0.01)* |
| Phe^{68} | 0.0061 | 0.0035 | 210 | 0.98 | (<0.01)* |

C. Observed kinetic parameters for ethyl isocyanide binding

| Mutant   | k' | k | k_0 | Q_0 | Q_{corr} | Q_0 |
|----------|----|---|-----|-----|----------|-----|
| Gly^{64} | 15.0 | 2.0 | 8.2 | 0.67 | 0.27 | 0.83 |
| Val^{64} | 2.2 | 4.0 | 5.5 | 0.09 | 0.44 | 0.48 |
| Leu^{64} | 1.0 | 0.15 | 72 | 0.85 | 0.06 | 0.40 |
| Phe^{64} | 0.099 | 0.17 | 33 | 0.74 | 0.03 | 0.13 |
| Gln^{64} | 0.071 | 0.12 | 42 | 0.97 | 0.02 | 0.70 |
| Native   | 0.069 ± 0.010 | 0.30 ± 0.03 | 110 ± 15 | 0.94 ± 0.03 | 0.06 ± 0.02 | 0.83 ± 0.53 |
| Ala^{68} | 0.18 | 0.070 | 23 | 0.88 | 2.02 | (<0.01)* |
| Ile^{68} | 0.047 | 3.4 | 57 | 0.63 | 0.24 | (<0.01)* |
| Phe^{68} | 0.0061 | 0.0035 | 210 | 0.98 | (<0.01)* |

D. Calculated parameters for ethyl isocyanide binding

| Mutant   | k' | k | k_0 | Q_0 | Q_{corr} | Q_0 |
|----------|----|---|-----|-----|----------|-----|
| Gly^{64} | 22 | 2.7 | 8.4 | 5.5 | 6.1 | 0.90 |
| Val^{64} | 24 | 5.0 | 4.8 | 0.5 | 4.4 | 0.11 |
| Leu^{64} | 1.2 | 11 | 0.11 | 61 | 0.97 | 63 |
| Phe^{64} | 0.12 | 8.6 | 0.015 | 25 | 0.66 | 37 |
| Gln^{64} | 0.073 | 1.3 | 0.056 | 40 | 4.8 | 8.4 |
| Native   | 0.073 ± 0.011 | 6.2 ± 0.7 | 0.012 ± 0.002 | 110 ± 15 | 5.8 ± 1.5 | 20 ± 6 |
| Ala^{68} | 0.20 | 2.7 | 0.076 | 20 | 0.58 | 36 |
| Ile^{68} | 0.074 | 21 | 0.0068 | 36 | 9.3 | 3.9 |
| Phe^{68} | 0.0062 | 3.6 | 0.0017 | 200 | 0.21 | 990 |

* Poorly defined slow phases were observed for methyl isocyanide rebinding to Ala^{68} and Phe^{68} myoglobin. Fits to two exponential expressions or a three step mechanism gave k_1 = 19.3 μs^{-1}, k_2 = 0.67, and k_3 = 1.0 μs^{-1} and φ_2 = 0.27 for the Ala^{68} mutant and k_1 = 88 μs^{-1}, φ_1 = 0.81, and k_2 = 14, φ_2 = 0.17 for the Phe^{68} mutant.

The overall quantum yields of these complexes were very small and the Q_{corr} values very large. As a result, Q_{corr} is poorly defined in terms of Q_{corr}/(1-φ_2).

Pocket and then migration out of the protein when the channel between Val^{68} and His^{64} is open. When the channel is closed, geminate recombination occurs. Ligand association is thought to involve a reversal of this trajectory. The fitted nanosecond geminate recombination parameters should be direct measures of the rates of these processes, and the effects of mutagenesis should allow an evaluation of these proposed trajectories and the physical location of state C in the protein.

Energy Barrier Diagrams—Complete sets of rate constants for O_2, CO, and isocyanide binding to the native and mutant myoglobins are presented in Tables II-IV. Structural interpretations of these rate parameters are facilitated by the...
preparation of free energy level diagrams based on Equation 3. This is probably the best empirical approach until molecular dynamics calculations can be used routinely to simulate kinetic phenomena on nanosecond time scales. Traditional diagrams for O₂ and CO binding to native sperm whale myoglobin are shown in Fig. 7A. The free energy of the Mb+X state was defined as 0, and those for wells C and A were computed as: 
\[ G_C = -RT \ln K_{XC} \] and 
\[ G_A = -RT \ln K, \] where \( K \) is the overall association equilibrium constant. 

The observed geminate rate constants were defined as 
\[ k_{CA} = A_{CA} \exp(-\Delta G_{CA}/RT) \] and 
\[ k_{CX} = A_{CX} \exp(-\Delta G_{CX}/RT). \] Both pre-exponential factors were set equal to \( 1 \times 10^{10} \) s⁻¹. Although somewhat arbitrary, this value is roughly equal to the largest picosecond rate constants observed for NO rebinding from contact pairs (B states in Equation 1) and also approximates the largest possible rate constant for ligand escape from the protein (i.e. \( A_{CX} = 3D_x/R^2 \), where \( R \) is the radius of the distal pocket and \( D_x \) is the ligand diffusion constant in water). The absolute values of \( A_{CX} \) and \( A_{CA} \) do not affect the differences between the barrier heights for the mutant and native proteins. Setting \( A_{CX} = A_{CA} \) makes it easier to visualize the rate-limiting step in the overall reaction and to estimate the extent of geminate recombination by comparing the relative heights of the kinetic barriers. Reduction of the observed values of \( k_{CA} \) and \( k_{CX} \) from \( 10^{10} \) s⁻¹ was expressed by positive values of \( \Delta G_{CA}^{\#} \) and \( \Delta G_{CX}^{\#} \), respectively. The free energies of the C→A and C→X kinetic barriers were calculated as 
\[ [-RT \ln(K_{XC}) + RT \ln(10^{10}/k_{CA})] \] and 
\[ [-RT \ln(K_{XC}) + RT \ln(10^{10}/k_{CX})], \] respectively. Comparisons between the energy barriers and wells for the different ligand-myoglobin complexes are shown in Fig. 7B using a bar graph format and the reaction coordinates defined in Equation 3. The X+Mb state is not shown in the bar graphs since its free energy is defined as 0.

For CO binding, 95% of the ligand molecules escape from state C after photolysis and the overall quantum yield is approximately 1 (Table III). This experimental observation requires that the inner C→A barrier be roughly 2 kcal/mol...
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Fig. 6. Top views of the distal pockets of the O2 and ethyl isocyanide (ENC) complexes of sperm whale myoglobin. Space filling images were generated by the program ANIMOL AED with coordinates from the structures cited in Fig. 5. The upper panel shows the residues circumscribing the distal pocket of oxymyoglobin using single-letter abbreviations for the specific amino acids (i.e. Hisn is labeled H64; Valn, V68, etc.). The porphyrin ring (dark blue atoms) is below these distal residues in the plane of the paper, and the ligand atoms are shown in red and labeled O. The lower panel shows the same view for the ethyl isocyanide complex. The ethyl side chain of the ligand is also shown in red and labeled C.

The rate-limiting step for bimolecular CO binding from the solvent phase is iron-ligand bond formation, and the overall association rate constant is given by $k_{OC}$ (Doster et al., 1983; Gibson et al., 1986; Jongeward et al., 1988). For O2 binding, 40-50% of the ligand molecules in state C escape from the heme pocket, and thus, the heights of the inner and outer barriers must be roughly equal. This accounts in part for the low overall quantum yield of oxymyoglobin; however, the major cause is a low picosecond quantum yield of state C. Migration into the protein and bond formation from within the pocket limit the overall association rate constant to roughly the same extent so that $k'$ for O2 binding must be computed as $k'_{OC} = k_{OC}/(k_{OC} + k_{CX})$.

The bimolecular rate constant for O2 binding is greater than that for CO binding because the inner barrier for O2 is greater than the outer $X \rightarrow C$ barrier. Thus, the rate-limiting step for bimolecular CO binding from the solvent phase is iron-ligand bond formation, and the overall association rate constant is given by $k_{OC}$ (Doster et al., 1983; Gibson et al., 1986; Jongeward et al., 1988). For O2 binding, 40-50% of the ligand molecules in state C escape from the heme pocket, and thus, the heights of the inner and outer barriers must be roughly equal. This accounts in part for the low overall quantum yield of oxymyoglobin; however, the major cause is a low picosecond quantum yield of state C. Migration into the protein and bond formation from within the pocket limit the overall association rate constant to roughly the same extent so that $k'$ for O2 binding must be computed as $k'_{OC} = k_{OC}/(k_{OC} + k_{CX})$.

The results presented in Tables II-IV show the power of combining protein engineering with laser photolysis techniques. Equation 3 is clearly a simplification of the real mechanism which probably involves multiple protein conformations (C states), side chain motions, and ligand rotations and translations. However, the empirical analysis in Figs. 8 and 9 has allowed us to resolve effects of size and polarity at the Hisn(E7) and Valn(E11) positions on the inner and outer kinetic barriers.

Polarity at Residue 64—The effects of substitutions at the E7 helical position on the free energy barriers for O2 binding are shown in Fig. 8A. A detailed discussion of the overall equilibrium changes has been presented in previous publications (Springer et al., 1989 and Rohlfs et al., 1990). The inner and outer kinetic barriers and the free energy of state C for O2 binding were lowered by 1.0 to 1.5 kcal/mol when Hisn was replaced with spolar amino acids, regardless of their size. In contrast, the Hisn to Gln substitution produced only small decreases (≤0.3 kcal/mol) in the free energies of the barriers.
FIG. 8. Contributions of residue 64(E7) to the inner and outer barriers for ligand binding. Free energy profiles like those in Fig. 7B were calculated for the reaction of O2 (panel A), CO (panel B), methyl isocyanide (MNC, panel C), and ethyl isocyanide (ENC, panel D) binding to myoglobins containing different residues at position 64. ■ native; □ Gln64; △ Gly64, Val64, Leu64, Phe64, respectively, going from left to right after the shaded bar in panels A, C, and D. In panel B, ◊ represents Leu64(left) and Phe64(right) following the solid bar for native myoglobin.

and well C. These results suggest that the polarity of His64 is more important than its size in inhibiting the kinetic processes and the non-covalent binding of oxygen.

In previous work, the effects of polarity at the position 64 amino acid side chain were explained in terms of the stabilization of water molecules within the distal pocket of deoxymyoglobin (Rohlfs et al., 1990). When comparing the properties of myoglobin and the α and β subunits of R-state hemoglobin, there appears to be an inverse relationship between the occupancy levels of distal pocket water molecules near His(E7) and the overall CO and O2 association constants (Rohlfs et al., 1990). The results in Fig. 8 are consistent with this interpretation. Even if only transiently hydrogen bonded to His64 within deoxymyoglobin, water should inhibit movement of the imidazole side chain by increasing its effective size. His64 may also be held in place by a hydrogen bonding lattice involving the heme propionates, Arg67, and Thr57 (Figs. 5 and 6). Both effects would increase the outer kinetic barrier to CO binding.

The marked inhibitory effect of His64 and Gln64 on the inner kinetic barrier for oxygen binding is more difficult to interpret. It is possible that water molecules rapidly enter the protein and become associated with His64 after the photolysed ligand moves to the back of the distal pocket. Assuming a $k_{NC}$ rate for water equal to roughly $1 \times 10^8$ M$^{-1}$ s$^{-1}$ and a concentration of 55 M, the half-time for water movement into the distal pocket would be ~130 ps, which is short enough to affect nanosecond-rebinding processes. Alternatively, re-binding of O2 from state C may require small net movements of His64 away from the iron atom, and these motions may be restricted by participation of the imidazole side chain in an extended hydrogen-bonding lattice.

The effects of position 64 substitutions on the barriers to CO binding were similar to those observed for O2 binding, although data could only be obtained for those derivatives with overall quantum yields less than 0.9 (Fig. 8B, Table IIIB). Again, both barrier heights were lowered by about 1 kcal/mol when His64 was replaced with an apolar side chain, and this is consistent with the roughly parallel effects of position 64 mutations on the overall association rate constants for CO and O2 binding (see Rohlfs et al., 1990). The size of residue 64 plays a more dominant role in determining the rate of isocyanide entry into the protein and the stability of these ligands in the distal pocket (Fig. 8, C and D). Substantial increases in the free energy of the $X \to C$ barrier and well C were observed for the series Gly64 $\leq$ Val64 $< $ Leu64 $< $ Phe64 myoglobin. The inner kinetic barrier for isocyanide binding is governed by more specific stereochemical interactions with the position 64 amino acid. The overall size of the side chain and freedom of rotation about the β-carbon appear to be the key factors since the lowest C$\to$A barriers were observed for Gly64 and Leu64 myoglobin and the highest were observed for His64, Val64, and Phe64 myoglobin.
Kinetic Barriers in Myoglobin

Val^68 and the Inner Kinetic Barrier—The major effect of increasing the size of the E11 residue for O$_2$ binding to Ala^68, Val^68 (native), and Ile^68 myoglobin is a selective increase in the C$\rightarrow$A kinetic barrier (Fig. 9A). Little or no change was observed for the free energy of state C or the height of the outer kinetic barrier. Thus, for these substitutions, the E11 residue has almost no effect on the rate of O$_2$ entry into the distal pocket, but does restrict access to the heme iron atom. This restriction is quite large for the Ile^68 mutant, is manifested by a 1-kcal/mol increase in the C$\rightarrow$A barrier compared with native myoglobin, and is consistent with the 6-fold decrease in the rate constant for geminate recombination of NO produced by the same amino acid change (Table I). The lack of effect on the outer kinetic barrier suggests that O$_2$ may enter the distal pocket by passing over Val^68; however, steric interaction with this residue does occur when the ligand approaches the iron atom for bond formation (Figs. 5 and 6).

The results in Figs. 8 and 9 show that both His^64 and Val^68 form part of the inner kinetic barrier to ligand binding. Our previous measurements with double mutants have shown that these contributions appear to be roughly additive (Egeberg et al., 1990). The overall association rate constants for O$_2$ and CO binding decreased when the E11 residue was increased in size from Ala^68 to Val^68 to Ile^68, even when His^64 was replaced with Gly^64. However, the effects of these E11 substitutions were significantly smaller than those observed for the His^64 to Gly mutation. The latter observation is also consistent with the pathway proposed in Figs. 5 and 6 since the size and polarity of His^64 are postulated to regulate both the outer and the inner kinetic barriers.

For methyl and ethyl isocyanide binding, both kinetic barriers and the free energy of state C depend significantly on the size of the E11 residue (Fig. 9C and D). As was the case for the apolar position 64 residues (Fig. 8, C and D), the free energy of state C for isocyanide binding was roughly proportional to the size of the E11 side chain. The largest mutational effect was a 1-1.5 kcal/mol increase in the C$\rightarrow$A barrier when Val^68 was replaced with Ile.

Pocket Size Effects and Ligand Pathways—The key role played by the volume of the distal pocket in regulating both the overall and geminate kinetic properties of myoglobin was first discussed in detail by Frauenfelder and co-workers (Doster et al., 1982 and references therein). The data in Figs. 4, 7-9 emphasize the importance of this factor. For native myoglobin, increasing the size of the ligand from methyl to ethyl isocyanide raised the outer kinetic barrier and the free energy of state C by roughly the same amount, 1 kcal/mol, but had little or no effect on the inner barrier (Fig. 9B). The net result was a 4-fold increase in the nanosecond geminate recombination rate (Fig. 4A) and a 2-3-fold decrease in the overall quantum yield (Table IV). The larger ligand is less stable in the distal pocket because of its size, which limits the number of conformations and degrees of freedom in the distal pocket and which also causes unfavorable steric interactions with the surrounding amino acids (Fig. 6D).

Similar phenomenological changes were observed for each ligand when the size of the distal pocket was decreased by substituting Phe for Val^68. As shown in Fig. 9, this mutation increased the free energy of state C and the outer X$\rightarrow$C barrier by 1-2 kcal/mol for all ligands but produced much smaller effects on the inner C$\rightarrow$A barrier. The net results of these changes were 1) decreases in the overall association rate constants, 2) marked decreases in the overall quantum yields because the effective rate of rebinding from within the pocket increased relative to that for escape, and 3) decreases in the overall dissociation rate constants because thermally dissociated ligand molecules also rebind more rapidly from within the distal pocket (Tables II-IV). These effects may even extend to the initial contact pair since the rate for NO movement away from the iron atom in Phe^68 myoglobin, $k_{m23}$, is roughly 6-fold smaller than that for native protein (Table I).

The simplest interpretation of the decrease in $K_{OC}$ for the Val^68 to Phe substitution is that the volume of the non-covalent binding site is reduced when the space between Leu^72, Ile^111, and Ile^107 is filled by the phenyl side chain (Ardini et al., 1990). Even if this space is not contiguous with the larger distal cavity, the presence of the Phe^68 side chain in this region of the protein should reduce the number of possible orientations of the ligand and the Leu^72, Leu^111, and Ile^107 side chains in the main pocket (Fig. 6). Only a small effect was observed on the inner kinetic barrier since the phenyl group is pointing away from the iron atom. The increase in the outer kinetic barrier is more difficult to interpret. It is possible that the phenyl side chain may serve to restrict motions of the E-helix, which in turn could prevent movement of His^64 and/or other residues involved in creating a channel for ligand movement into the distal pocket. It is also possible that the space between Leu^72 and Ile^107 may represent an alternative channel for the entry of small ligands into the distal pocket, and filling this gap with the side chain of Phe^68 blocks this route. This pathway cannot be ruled out by our experimental observations; however, entry into the cavity between Leu^72 and Ile^107 appears to be blocked by the E and G helices (Fig. 6). Further mutagenesis studies in this region of the protein, refinement of the structure of Phe^68 myoglobin, and molecular dynamics calculations are needed to examine this point.

Conclusions—The functional roles of the distal histidine and valine are now well-defined. The polarity of the imidazole side chain inhibits the rate of entry into the distal pocket, decreases the equilibrium constant for the non-covalent binding of apolar ligands, and raises the inner kinetic barrier for bond formation with the heme iron atom. Although inhibitory for these kinetic processes, the polarity of His^64 is required to stabilize bound O$_2$ by hydrogen bonding. Val^68 does not significantly limit the rate of entry of small ligand molecules into the heme pocket; however, this residue does inhibit the final approach to the iron atom and equilibrium binding, particularly for CO and isocyanides, which prefer linear Fe-C-O or Fe-C-N geometries. The nanosecond kinetic intermediate observed for O$_2$, CO, and isocyanide rebinding to myoglobin, state C in our reaction scheme, can be associated with ligand located in the distal cavity (Fig. 6). The size and polarity of this non-covalent binding site is partly determined by His^64 and Val^68, and these distal pocket characteristics play an important role in determining the overall rate constants for ligand binding, even when no effect is observed on the equilibrium constant.

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