Thus, the results for mutagenesis of Gln-44(E7) are equivocal with respect to defining the pathway for ligand movement into CerHb. Rather than blocking entry and exit, the decreases in k'_{O2} caused by the His-44, Phe-44, and Trp-44 mutations are more easily interpreted as due to direct hindrance of bond formation with the iron atom by the large aromatic side chains. This idea is supported by the 10-fold decreases in the bimolecular rates for CO binding caused by the Q44H, Q44F, and Q44W mutations (k'_{CO} values in Table 1). The limiting step for CO binding to most heme proteins is the rate of internal bond formation, which is at least 10 to 1000-fold smaller than the rates of internal bond formation for O_2 and NO, respectively (48). Thus, the large ~10-fold decreases in k'_{CO} observed for the His-44, Phe-44, and Trp-44 mutants are consistent with sterically hindered access to the iron atom. This interpretation is further supported by the small fractions of geminate recombination observed for WT CerHbCO and all the position 44(E7) mutants shown in Fig. 3*A*. If the large aromatic side chains were blocking escape, the fraction of internal rebinding should increase as the size of the amino acid at the E7 position increases. However, the Phe-44 and His-44 mutants show the least amount of geminate CO recombination; the Leu-44 and Val-44 mutants show the greatest amount; and none of the mutants are significantly different from WT CerHbCO (Fig. 3*A*).

In contrast to the E7 replacements, the Ala-55(E18) mutations show a pattern of kinetic effects remarkably consistent with this position being at the entrance to ligand movement into CerHb. Both the association and dissociation rate coefficients for O_2 binding decrease uniformly with increasing side chain size, and there is little change in overall ligand affinity (Table 2 and Fig. 4). As shown in Fig. 3, *B* and *A*, and Table 2, the fraction of geminate recombination increases with increasing size of the amino acid at position 55, whereas the rate of geminate recombination decreases. These results demonstrate qualitatively that the increase in internal recombination is because of a decrease in the rate of ligand escape as would be expected if the exit to solvent were blocked by the larger amino acids at the E18 position.

Simple Mechanism for Ligand Binding to CerHb—The two-step mechanism shown in Fig. 9 was adopted to determine more quantitatively the effects of the 55 mutations on the rates of ligand entry and exit. Following a strategy that was developed earlier to examine secondary ligand docking sites in Mb variants (12, 57), Deng *et al.* (21) have shown that multiple transient docking sites also exist in the distal pocket of CerHb and are separated by relatively small enthalpy barriers against ligand migration. These discrete photoproduct intermediates are easily visualized in FTIR-TDS experiments at cryogenic temperatures (3–160 K); the locations of these states shown in Fig. 9 were assigned by the effects of mutagenesis on the transitions between them (21). However, at room temperature, simple exponential geminate rebinding is observed, suggesting that only a single intermediate needs to be considered on time scales of 10–2000 ns.

At ambient temperatures, both geminate and overall ligand binding to CerHb can be analyzed in terms of three reactions as follows: 1) photolysis to the intermediate state, where the ligand is located in transient equilibrium between the C, B, and channel positions; 2) rebinding to the iron atom from this intermediate state at a rate equal to k_{bond}; and 3) escape from the intermediate state out through the channel past the Ala-55(E18) position at a rate equal to k_{escape}. The fraction and rate of geminate rebinding, F_{gem} and k_{gem}, are given by Equation 3,

$$F_{gem} = \frac{k_{bond}}{k_{bond} + k_{escape}},\ k_{gem} = k_{bond} + k_{escape} \qquad \text{(Eq. 3)}$$

$$k_{bond} = k_{gem}F_{gem},\ k_{escape} = k_{gem}(1 - F_{gem})$$

The fractions and rates of internal recombination are obtained by fitting the observed geminate time courses to a single exponential expression, where the first-order rate is k_{gem}, and the normalized amplitude for rebinding and the offset are equal to F_{gem} and $1 - F_{gem}$, respectively (48). These parameters can then be used to compute k_{bond} and k_{escape} as shown in Equation 3. An expression for the overall bimolecular rate coefficient for ligand binding, k'_X, where X is the ligand (O_2, CO, NO, etc.), can be obtained by assuming a steady-state approximation ($d\langle I\rangle/dt = 0$) for the intermediate on μs and ms time scales. Under these conditions, k'_X is given by Equation 4,

$$k'_x = k'_{entry}\frac{k_{bond}}{k_{bond} + k_{escape}} = k'_{entry}F_{gem} \qquad \text{(Eq. 4)}$$

$$k'_{entry} = \frac{k'_x}{F_{gem}}$$

Equations 3 and 4 and the measured kinetic parameters for O_2 and CO binding in Table 2 were used to compute estimates of k'_{entry}, k_{escape}, and k_{bond} for ligand binding to WT CerHb and the four position 55 mutants. The results are shown in Table 3. These parameters provide a self-consistent interpretation of both O_2 and CO binding. The estimated value of k_{bond} for O_2 rebinding is, as expected, roughly 10 times greater than that for CO, ~50 μs^{-1} *versus* ~4 μs^{-1}. Both values of k_{bond} are independent of changes at the position 55(E18), demonstrating that the amino acid at this remote location does not influence the reaction at the iron atom. In contrast, the values of k'_{entry} and k_{escape} are roughly independent of the ligand, but both rates decrease over 10-fold as the size of the position 55 amino acid is increased (Table 3). The correspondence of the absolute values of k'_{entry} and k_{escape} calculated using the O_2 and CO data is remarkably good, considering that it is difficult to determine the exact values of k_{gem} and F_{gem} for O_2. For this ligand, significant geminate rebinding is occurring during the laser excitation pulse, and the fitted rate is approaching the decay rate of the pulse light intensity.

Bimolecular rates of NO binding were measured to verify the computed values of k'_{entry}. NO is expected to show F_{gem} values $\geq$0.95, and we verified that CerHbNO has an extremely small quantum yield for complete photodissociation to the solvent (data not shown). Thus k'_{NO} should be approximately equal to k'_{entry}. As shown in Table 3 and the Fig. 5, *inset*, there is a strong linear correlation between k'_{NO} and k'_{entry}. However, the calculated values of k'_{entry} appear to be ~2-fold greater than k'_{NO} for WT and Val-55 CerHb. The cause of this discrepancy is

probably the oversimplification inherent in the two-step model. For WT and Val-55 CerHb, the extent of geminate recombination is small and the rate is large and difficult to define. In addition, there are probably multiple steps that cannot be easily visualized using a 9-ns excitation pulse. In these proteins, the internal channel is open to solvent, and, as a result, the photodissociated ligands are not easily trapped in an intermediate state. More complex expressions define the overall rate coefficient when contributions from the rates of interconversion of the B and C states are incorporated into the steady-state expression for k'_{entry}. Evidence for these inner ligand transitions is seen in the kinetic data for mutations in the B and C sites (data not shown), complicating our larger mapping study of the entire pathway. However, regardless of the exact interpretation, there is a very good correspondence between k'_{NO} and the calculated values of k'_{entry} for the larger 55 mutants (Table 3), which supports our view that ligands enter CerHb primarily through the apolar channel.

Perhaps the strongest argument in favor of the mechanism in Fig. 9 for interpreting the effects of the position 55 mutants is the independence of the estimated equilibrium constant, K_{entry}, for noncovalent capture of ligands in the intermediate state (*i.e.* in the channel and the space associated with state B and C). The average computed value is $\sim$9.1 $\pm$ 2.0 M^{-1} for WT and the four 55 mutant CerHbs and does not vary with either ligand or the size of the residue at the entrance to the apolar channel (Table 3). The lack of dependence on the amino acid at position 55(E18) fits with the structures of WT, Phe-55, and Trp-55, all of which show equivalent channel volumes of $\sim$56 Å^3. The value of K_{entry} for ligand capture in WT sperm whale Mb is $\sim$5.5 $\pm$ 1.8 M^{-1}, implying that the apolar channel in CerHb provides almost twice the volume for noncovalent ligand binding than the distal pocket and xenon cavities in mammalian Mb (3). However, in WT sperm whale Mb, a water molecule is present in the distal pocket reducing the effective volume for noncovalent capture. When this water is removed by replacing His-64(E7) with Leu, the value of K_{entry} for sperm whale Mb increases to $\sim$10 M^{-1}, and similar values were estimated for Val-64 and Phe-64 Mb (3). Thus, when the water is removed, the functional capture volume in Mb, as measured by the equilibrium constant for noncovalent ligand binding, is similar to that in the CerHb channel.

The Apolar Channel Is the Route of Entry—Taken together the kinetic and structural data for the position 55(E18) mutants demonstrate unambiguously that the major pathway for ligand entry and exit in CerHb is the apolar channel between the E and H helices, which is shown in Figs. 1, 6, and 9. Increasing the size of the amino acid at this position increases the free energy barrier to ligand movement from the solvent interface into the apolar channel. Changing the size of the barrier only affects the rates of entry and exit and not the rate of iron-ligand bond formation, the equilibrium constant for noncovalent ligand capture, and the overall affinity of CerHb for ligands. The lack of further decreases in either k'_{entry} or k_{escape} as the size of residue 55 is increased from Leu to Phe to Trp suggests that the small, limiting values of these parameters, $\sim$50 $\mu M^{-1} s^{-1}$ and $\sim$6 μs^{-1}, respectively (Table 3), may represent the rates of ligand movement through the E7 gate. The rates for the Trp-55 and Phe-55 mutants are remarkably similar to the values estimated for entry into and exit from WT sperm whale Mb which clearly uses the E7 channel ($k'_{entry} \approx 34 \pm 7\ \mu M^{-1} s^{-1}$ and 6.3 $\pm$ 1 μs^{-1} (3)). However, our current sets of single mutants and FTIR data cannot define the location of alternative pathways.

Physiology and Evolution—CerHb is part of a group of invertebrate and microbial globins, which have ligand-binding sites containing a Tyr(B10) and Gln(E7) motif (58). Domain 1 of *Ascaris suum* hemoglobin is a representative of the invertebrate Tyr(B10)/Gln(E7) proteins, which normally have ultra-high affinities for O_2 ($P_{50} \approx$ 0.003 to 0.1 μM) due both to favorable proximal geometry and strong hydrogen bonds between the Tyr(B10) and Gln(E7) side chains and the bound O_2 (31, 59–61). However, CerHb is a neuronal globin that has an oxygen storage function (30, 62). It takes up and stores O_2 when the worm is in seawater and then releases it to nerve tissue when the organism burrows into mud flats and becomes anoxic (30). Thus, CerHb evolved under strong selective pressure to have a Mb-like O_2 affinity, with a $P_{50} \approx 1\ \mu M$, and large rates of O_2 uptake and release. We showed previously that the moderate O_2 affinity is because of the presence of Thr-48 at the E11 position, which is an apolar amino acid in most other animal globins (32). The need for rapid rates of O_2 exchange during transient periods of hypoxia appears to have led to the loss of the A helix and concomitant creation of a large apolar channel between the E and H helices coupled with the loss of the A helix. This channel allows dramatically high rates of O_2 binding to and release from CerHb compared with other proteins containing the Tyr(B10)/Gln(E7) motif. For example, Tyr-29(B10)/Gln-64(E7) Mb has an O_2 affinity ($K_{O2} \approx 1.9\ \mu M^{-1}$) similar to CerHb but shows rate coefficients that are 100-fold smaller, k'_{O2} and k_{O2} = 2.8 $\mu M^{-1} s^{-1}$ and 1.6 s^{-1}, respectively, and *A. suum* Hb shows even smaller rate coefficients for O_2 binding (31). Thus, CerHb provides a clear example of convergent evolution to obtain an Mb-like function using electrostatic destabilization of bound O_2 to decrease O_2 affinity (32) and loss of the A helix to create a new, more efficient pathway for ultra fast ligand entry and exit.

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The Apolar Channel in *Cerebratulus lacteus* Hemoglobin Is the Route for O_2 Entry and Exit

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