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

Received for publication, July 25, 2008, and in revised form, September 8, 2008. Published, JBC Papers in Press, October 7, 2008, DOI 10.1074/jbc.M805727200

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The major pathway for $O_2$ binding to mammalian myoglobins (Mb) and hemoglobins (Hb) involves transient upward movement of the distal histidine (His-64(E7)), allowing ligand capture in the distal pocket. The mini-globin from Cerebratulus lacteus (CerHb) appears to have an alternative pathway between the E and H helices that is made accessible by loss of the N-terminal A helix. To test this pathway, we examined the effects of changing the size of the E7 gate and closing the end of the apolar channel in CerHb by site-directed mutagenesis. Increasing the size of Gln-44(E7) from Ala to Trp causes variation of association ($k_{o2}$) and dissociation ($k_{o2}$) rate coefficients, but the changes are not systematic. More significantly, the fractions ($F_{gem}$ = 0.05–0.19) and rates ($k_{gem}$ = 50–100 μs$^{-1}$) of gemic CO recombination in the Gln-44(E7) mutants are all similar. In contrast, blocking the entrance to the apolar channel by increasing the size of Ala-55(E18) to Phe and Trp causes the following: 1) both $k_{o2}$ and $k_{o2}$ to decrease roughly 4-fold; 2) $F_{gem}$ for CO to increase from −0.05 to 0.45; and 3) $k_{gem}$ to decrease from ~80 to ~9 μs$^{-1}$, as ligands become trapped in the channel. Crystal structures and low temperature Fourier-transform infrared spectra of Phe-55 and Trp-55 CerHb confirm that the aromatic side chains block the channel entrance, with little effect on the distal pocket. These results provide unambiguous experimental proof that diatomic ligands can enter and exit a globin through an interior channel in preference to the more direct E7 pathway.

The structural mechanisms for $O_2$ binding to myoglobins (Mb) and hemoglobins (Hb) have been the topic of much research since Kendrew (1) and Perutz et al. (2) first reported the three-dimensional structures of Mb and Hb over 45 years ago. In the case of mammalian myoglobins, ligand binding is well understood (Refs. 3–10 and references therein) and consists of four major steps. Weakly bound water is displaced to create a vacant distal pocket above the heme iron atom. Ligands migrate into the protein through a short channel that is created when the distal histidine (His-64 at the E7 helical position) transiently rotates upward and then are captured in the interior of the distal pocket. This noncovalent intermediate is often called the B state because it can also be generated by photolysis of the equilibrium bound or A state. Covalent bond formation between the internal ligand and the iron atom of the heme group then competes with ligand escape back out through the His(E7) gate. The bound ligand can be further stabilized by electrostatic interactions with surrounding polar amino acid side chains. This E7 gate pathway appears to occur in most if not all animal hemoglobins, with the classic 3-on-3 α-helical globin fold and a distal histidine, although experimental verification has only been done rigorously for vertebrate Mbs using time-resolved crystallography (4–8), site-directed mutagenesis (3, 9, 11), and time-resolved absorbance and FTIR spectroscopy (12–18).

In contrast, the pathways for ligand binding in truncated 2/2 Hbs (trHbs) appear to be distinct from the E7 gate in the classic 3-on-3 α-helical globins (19–22). These Hbs display loss of elements found in the classic globin fold, including the absence of an N-terminal A helix, significant shortening of the CD corner-D helical region, and reduction of the F helix to an extended polypeptide loop (19, 20, 23). The resulting tertiary structure has the appearance of a 2-on-2 α-helical fold. The abbreviated CD-E region results in the pulling of the E helix closer to the

The abbreviations used are: Mb, myoglobin; CerHb, C. lacteus mini-hemoglobin; $k_{o2}$, oxygen association rate coefficient; $k_{o2}$, oxygen dissociation rate coefficient; $k_{o2}$, oxygen affinity coefficient; $F_{gem}$, fraction of gemic recombination; FTIR, Fourier-transform infrared spectroscopy; Hb, hemoglobin; Hb, truncated hemoglobin; WT, wild type; $k_{gem}$, gemic recombination rate coefficient; TDS, temperature derivative spectroscopy; r.m.s.d., root mean square deviation.

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4 Amino acids have been identified with their topological site numbers as defined in the conventional globin fold.
heme distal pocket, which may preclude the use of the E7 residue as a gate for ligand entry and exit. The crystal structures of Mycobacterium tuberculosis trHb (trHbN) and other trHbs display the presence of a channel through the protein matrix between the E and H helices, which is large enough for ligand diffusion to the heme distal pocket from external regions near the EF corner and N terminus of the protein (20, 23–27).

An internal apolar channel is also found in the neuronal mini-Hb of the Nemertean sea worm, Cerebratulus lacteus (28, 29), which, like the trHbs, is missing an A helix. CerHb also has shortened B and H helices (29, 30) making it among the smallest known globins, with only 109 amino acids. CerHb is considered to be in a unique mini-globin subcategory within the larger globin families (29) and has evolved a Mb-like O2 storage function in the nerve system of the sea worm (30). Like many microbial and invertebrate globins, CerHb has a ligand-binding site containing Tyr(B10) and Gln(E7), which typically evokes an extremely high oxygen affinity because of formation of multiple hydrogen bonds with the bound ligand (28, 31). However, CerHb contains a Thr at the E11 position, and the Thr-48(E11) hydroxyl O-atom pulls the Tyr-11(B10) phenol hydrogen away from the bound ligand. As a result, the nonbonding electrons on the Tyr(B10) O-atom are pointing toward the bound ligand, and this negative partial charge causes the oxygen affinity of WT CerHb to be similar to that of vertebrate Msbs ($K_{O2} \approx 1 \mu M^{-1}$) (31, 32).

CerHb also displays very rapid rates of O2 association ($k'_{O2} = 240 \mu M^{-1} s^{-1}$) and dissociation ($k_{O2} = 180 s^{-1}$), which appear to be necessary for rapid gas uptake from oxygenated seawater during surface activity and release to neuronal tissue during periods of hypoxia. These large rate coefficients seem surprising considering the crowded distal pocket and multiple hydrogen bonding interactions that appear to “tie” down the amide side chain of Gln-44, which forms the E7 gate in CerHb. Bolognesi and co-workers (29, 32) have suggested that the large apolar channel between the E and H helices is the pathway for ligand movement into the heme pocket and accounts for the high rates of ligand binding and release. Thus, the loss of the A helix, which blocks access to the channel, may have been an adaptive strategy to allow for rapid rates of O2 exchange while retaining the Tyr(B10)/Gln(E7) motif.

To test this idea, we have adopted the general mapping strategy described by Scott et al. (3) for Mb to examine the extent of ligand movement through the apolar channel in CerHb. The two alternative pathways for ligand movement into and out of CerHbO2 are shown in Fig. 1. The shortest route to solvent would be through an E7 gate, which in this case would involve outward and upward movement of the Gln-44 side chain. The alternative pathway involves movement in the opposite direction through an apolar channel bounded by Val-7(B6), Ala-55(E18), Ala-82(G8), Leu-86(G12), and Leu-98(H9). We have begun this mapping study by systematically changing the sizes of the amino acids at the entry point of the E7 gate and at the entrance to the apolar channel. Gln-44(E7) has been mutated to Ala, Val, Leu, His, Phe, and Trp, and Ala-55(E18) has been mutated to Val, Leu, Phe, and Trp. The effects of these mutations on overall and germinate rates of O2 and CO binding have been measured and used to determine the route of ligand entry into CerHb. Low temperature FTIR spectra of the Phe-55 and Trp-55 CerHbCO complexes were measured to determine whether these exterior mutations significantly alter internal rebinding within the distal pocket. Finally, structures of the Phe-55 and Trp-55 CerHbO2 mutants were determined by x-ray crystallography to ensure that these replacements caused physical blockage of the apolar channel.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Wild-type (wt) and mutant recombinant CerHbs were expressed and purified as described previously using a synthetic gene with codon usage optimized for expression in Escherichia coli (29, 32). Most recombinant CerHb samples were isolated in the reduced state and used directly. All experiments to determine rate coefficients were carried out in 0.1 M phosphate buffer, pH 7.0, 1.0 mM EDTA, 20 °C. CerHbCO and CerHbO2 protein samples were prepared by equilibration with 1 atm CO and 1 atm O2, respectively. When necessary, the unstable Gln-44(E7) mutants were pre-reduced with dithionite, quickly passed through a G-25 column, and eluted with buffer equilibrated with 1 atm of CO. As noted below, O2 binding kinetics were often measured in samples containing mixtures of CO and O2. For those experiments, the starting CerHb sample was in the reduced CO form, which is extremely stable.

**Measurement of Overall Rates of Ligand Association and Dissociation**—CO association time courses were measured after complete laser photolysis of 50 $\mu M$ CerHbCO samples containing various [CO] under pseudo first-order conditions.

**FIGURE 1. Structure of WT CerHbO2 (Protein Data Bank code 1KR7) showing side and back views of the molecule.** Gln-44(E7) is labeled in cyan and located directly above the heme propionates. Ala-55(E18) is labeled in green and located at the end of the E helix forming part of the open entrance to the apolar channel between the E and H helices.
O$_2$ Binding to Cerebratulus Hb through an Apolar Tunnel

An $\sim$500-ns excitation pulse from a Phase-R model 2100B dye laser was used for photoexcitation (33, 34), and transmittance changes were recorded with a Tektronix TDS220 digitizing oscilloscope. The time courses were fitted to a simple exponential expression, and $k'_{\text{CO}}$ was calculated from the slope of plots of $k_{\text{obs}}$, versus [CO].

The association rate coefficient for NO binding to deoxy-CerHb was measured using a flow-flash apparatus and the 500-ns dye laser. The flow part of the instrument is composed of a BioLogic SFM400 Multi-Mixer controlled by the MPS-60 Microprocessor. The Multi-Mixer output is connected to the final mixer by a 2-foot water-jacketed umbilical cord containing two flow tubes. These final mixer is part of the base holding a 2-mm fluorescence flow-through cuvette. The excitation and observing beams are focused to intersect at a point in the middle of the quartz cuvette. An anaerobic sample of CerHbCO containing little or no free ligand was mixed with 3/4, 1/2, 1/4, and 1/10 atm NO (1500, 1000, 500, and 200 $\mu$M, respectively). Photolysis of the CerHbCO complex was initiated 100 ms after flow stopped and before any significant displacement of CO. Then the complete absorbance change for NO binding to the newly generated deoxy-CerHb was followed at 436 nm. At least four time courses representing new mixtures of CerHbCO and NO were averaged to reduce the signal to noise ratio. This approach avoids the difficulty of photolyzing CerHbNO, which has an extremely small quantum yield. The value of $k'_{\text{NO}}$ was determined from the slope of $k_{\text{obs}}$, versus [NO].

O$_2$ association and dissociation time courses were determined after complete laser photolysis of CerHbCO samples using the 500-ns dye laser (34, 35). In these reactions, CerHbCO is prepared in buffer containing $\sim$50 $\mu$M CO and 1250 $\mu$M O$_2$, 750 $\mu$M CO and 250 $\mu$M O$_2$, 500 $\mu$M CO and 500 $\mu$M O$_2$, and 250 $\mu$M CO and 750 $\mu$M O$_2$. The laser pulse photolyzes the CerHbCO complex, generating deoxy-CerHb, which can then react rapidly with either ligand. The rate of this initial rapid bimolecular rebinding phase, $k_{\text{fast}}$, is monitored by a large decrease in absorbance of unliganded CerHb at 436 nm and is dominated by O$_2$ rebinding because of its larger association rate coefficient. The value of $k_{\text{fast}}$ is given by Equation 1,

$$k_{\text{fast}} = k'_{\text{O2}}[O_2] + k_{\text{O2}} + k'_{\text{CO}}[CO] \quad \text{(Eq. 1)}$$

CO dissociation can be neglected because the absolute value of $k_{\text{CO}}$ is $\sim$0.01 s$^{-1}$, whereas under the conditions of the laser experiments, $k_{\text{fast}} = 10,000-300,000$ s$^{-1}$. The O$_2$ dissociation rates, $k_{\text{O2}}$, for the WT and mutant CerHbO$_2$ samples (10–300 s$^{-1}$, Tables 1 and 2) also make a negligible contribution to the observed rate coefficients for the fast phase.

The slow phase observed after photolysis of CerHbCO in buffer containing mixtures of CO and O$_2$ represents displacement of the transiently bound O$_2$ by CO, which, although kinetically less reactive, has a higher affinity for heme (34, 35). The time course of this replacement reaction is best monitored by a large increase in absorbance of CerHbCO at 425 nm. The rate of this process, $k_{\text{slow}}$, is given by Equation 2,

$$k_{\text{slow}} = \frac{k'_{\text{CO}}[CO]}{k'_{\text{CO}}[CO] + k_{\text{O2}}[O_2]} \quad \text{(Eq. 2)}$$

An iterative, nonlinear, least squares fitting routine (Solver in Microsoft Excel) was used to optimize the values of $k'_{\text{O2}}, k_{\text{O2}},$ and $k'_{\text{CO}}$ to give the best fit to the dependence of $k_{\text{fast}}$ and $k_{\text{slow}}$ on [O$_2$]/[CO], including conditions where [O$_2$] = 0 and where [CO] $\approx$ 0 (34). In previous work, we have shown that this method of using photolysis and O$_2$/CO mixtures gives results identical to those obtained in more conventional rapid mixing and photolysis experiments, and eliminates problems because of autooxidation of unstable mutants and low HbO$_2$ quantum yields (35).

CO dissociation time courses were determined by stopped-flow analysis in which the heme-bound CO was displaced by a high concentration of the higher affinity NO ligand. In these experiments, 5–10 $\mu$M CerHbCO samples are mixed with 1 atm NO (2000 $\mu$M), and the decrease in CerHbCO is monitored at 420 nm (33, 36). Under these conditions, the observed first-order rate constant is directly equal to $k_{\text{CO}}$.

Measurement of Geminate Recombination—Time courses for internal rebinding within CerHb were measured at 436 nm after excitation with a 9-ns excitation pulse from a Lumonics YAG-laser system, using a Tektronix TDS3052 digitizing oscilloscope on nanosecond time scales. The geminate rebinding time courses were fitted to one or two exponential expressions with an offset reflecting the amount of escape to solvent (3). For a simple exponential rebinding process, the fraction of geminate recombination, $F_{\text{gem}}$, was calculated as $F_{\text{gem}} = (\Delta A_{\text{gem}}/\Delta A_{\text{gem}} + y_0)$, where $\Delta A_{\text{gem}}$ represents the absorbance change associated with internal geminate rebinding (one or two phases), and $y_0$ (the offset) is the difference between the absorbance after complete geminate recombination and the absorbance of the original ground state, which is observed prior to photolysis or at long times after bimolecular rebinding from solvent is complete (i.e., fully liganded CerHb). The observed rates of geminate rebinding, $k_{\text{gem}}$, were independent of ligand concentration.

Crystallization and X-ray Diffraction Data Collection—Puriﬁed Phe-55 and Trp-55 CerHb mutants were crystallized by vapor diffusion techniques (protein concentration 27 mg/ml) under conditions matching those for crystallization of the WT protein (28). In both cases, elongated prismatic crystals (about 0.04 x 0.04 x 0.2 mm$^3$) grew within 2 weeks. The crystals were stored in 2.8 M ammonium sulfate, 50 mM sodium acetate, pH 6.0, and transferred to the same solution supplemented with 15% glycerol (v/v) (cryo-solution) immediately before data collection at 100 K. All x-ray diffraction data were collected at the ESRF synchrotron facility (beam line ID14-1, Grenoble, France) and processed using MOSFLM and SCALA (40, 41). Phases were calculated by molecular replacement methods with the program MOLREP (42), as implemented in the CCP4 program package (43, 44), using the WT oxygenated structure as a starting model (Protein Data Bank accession code 1KRT (29)). The mutant CerHb crystal structures were reﬁned using REFMAC (45) and models inspected and modiﬁed using COOT (46). B-factors have been reﬁned isotropically for the 1.8 Å data of the Phe-55 mutant and anisotropically for the high resolution 1.6 Å data of the Trp-55 mutant. Atomic coordinates and structure factors for Phe-55 and Trp-55 CerHb mutants have been
argue against this amino acid being part of the pathway for entry and exit because opposite effects, increases in $k'_{\text{O}_2}$ and $k_{\text{O}_2}$, would be expected if the E7 channel were enlarged and made apolar, and the side chain gate was removed. As shown in Fig. 2A, there are significant decreases in the bimolecular rate for O$_2$ binding when the size of the E7 amino acid is increased from Gln to His, Phe, and Trp. However, there are no large concomitant decreases in the O$_2$ dissociation rate coefficient, and as a result, there are significant ~10-fold decreases in O$_2$ affinity for the larger Phe-44 and Trp-44 CerHb mutants (Table 1). These effects for the larger 44(E7) amino acids appear to be due to direct side chain interactions with the bound ligand and not alterations in the speed of entry and escape.

The Trp-44 CerHb mutant shows two distinct and roughly equal phases for O$_2$ association, O$_2$ dissociation, and CO dissociation (Table 1). The slower phase for O$_2$ association shows a rate similar to that for binding to the Phe-44 mutant, whereas the faster phase is similar to that of Ala-44 CerHb. The simplest interpretation is that the indole side chain can occupy an inward orientation that directly hinders binding to the iron atom and an outward orientation that leaves the iron more accessible, regardless of the pathway for ligand entry and exit. The low $K'_{\text{O}_2}$ value would correspond to the inward conformer, and the high $K_{\text{O}_2}$ value would correspond to the outward conformer. Using this steric hindrance model, we assigned the high $K_{\text{O}_2}$ value to the inward conformer, and the low $K'_{\text{O}_2}$ value to the outward conformer. This assignment is compelling because it accounts for the observed kinetic parameters in the absence of a crystal structure of the Trp-44(E7) mutant and further investigation.

### RESULTS

#### Effects of Position 44 (E7) Mutations on Overall O$_2$ and CO Binding—Bimolecular association ($k$) and unimolecular dissociation ($k$) rate coefficients for ligand binding to Gln-44(E7) mutants of CerHb are listed in Table 1. Some of these rate parameters were reported in previous publications (21, 32). We constructed the Val-44 mutant to complete the series of size variants at the E7 gate position and re-measured all the O$_2$ and CO binding parameters for direct comparison with the same replacements in sperm whale Mb, which uses the E7 gate for ligand entry and exit (Fig. 2).

In general, replacement of His-64(E7) with smaller apolar amino acids (Ala, Val, and Leu) in Mb results in dramatic increases in both $k'_{\text{O}_2}$ and $k_{\text{O}_2}$. In the case of ligand association, the 5–10-fold increases are due to loss of noncovalently bound water and opening of the E7 channel, both of which increase the rate of ligand entry into Mb. In the case of dissociation, the large 100–1000-fold increases in $k_{\text{O}_2}$ are because of loss of hydrogen bonding to bound O$_2$, causing greater rates of thermal Fe–O$_2$ bond breakage. The increases in $k_{\text{O}_2}$ dominate, resulting in large ~100-fold decreases in O$_2$ affinity for the apolar E7 mutants of Mb ($K_{\text{O}_2}$ values Fig. 2C).

In contrast to Mb, replacing Gln-44(E7) with small apolar amino acids in CerHb results in decreases in the rates of both O$_2$ association and dissociation and small increases in O$_2$ affinity (Ala, Val, and Leu, solid bars in Fig. 2). The decreases in $K'_{\text{O}_2}$ and $k_{\text{O}_2}$ with decreasing size and polarity at the E7 position

| Mutants     | $k'_{\text{O}_2}$ | $k_{\text{O}_2}$ | $K_{\text{O}_2}$ | $K'_{\text{CO}}$ | $k_{\text{CO}}$ | $K_{\text{CO}}$ | $M(K'_{\text{CO}}/K_{\text{O}_2})$ |
|-------------|------------------|------------------|------------------|------------------|------------------|------------------|----------------------------------|
| Ala-44      | 160 (180)*       | 33 (30)*         | 4.9 (6.0)*       | 41 (40)*         | 0.05, 0.0004     | 820, 10000       | 170, 2100                        |
| Val-44      | 130              | 84               | 1.5             | 22               | 0.032            | 780              | 510                              |
| Leu-44      | 184 (200)*       | 96 (96)*         | 1.9 (2.1)*      | 36 (42)*         | 0.016            | 2200             | 1200                             |
| Gln-44(E7) WT | 230 ± 17         | 190 ± 25         | 1.2 ± 0.2       | 32 ± 4.7         | 0.054 ± 0.005    | 590 ± 100        | 490 ± 110                        |
| His-44      | 85 (130)*        | 81 (65)*         | 1.0 (2.0)*      | 4.0 (5.3)*       | 0.010            | 400              | 380                              |
| Phe-44      | 20 (23)*         | 170 (170)*       | 0.11 (0.14)*    | 1.3 (1.6)*       | 0.054            | 24               | 210                              |
| Trp-44      | 10, 170          | 330, 333         | 0.035, 5.2      | 5                | 0.035, 0.011     | 140, 460          | 4600, 89                         |

* The rate constants given in parentheses were measured previously with different preparations of position 44 mutants and were described in Refs. 21, 32. The standard deviations for the WT values for $k_{\text{O}_2}$, $k_{\text{CO}}$, and $K_{\text{CO}}$ were obtained from the analysis of 12 completely independent sets of experiments starting with at least 7 different expressions and purifications of CerHb. The standard deviation for $k_{\text{CO}}$ came from the analysis of 16 different determinations. The values of $K_{\text{O}_2}$ and $K_{\text{CO}}$ were calculated from $k'_{\text{O}_2}/k_{\text{O}_2}$ and $k_{\text{CO}}/K_{\text{CO}}$, and their standard deviations were calculated from the standard propagation of error formula.

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**TABLE 1**

Rate and equilibrium constants for O$_2$ and CO binding to position 44(E7) mutants of CerHb at pH 7.0, 20 °C.

Two phases of roughly equal amplitudes were observed for O$_2$ association with and dissociation from Trp-44 CerHb, and rate parameters for each phase are reported.
under “Discussion” and Equation 3). Thus, it is clear that changes in the size of the position 44(E7) amino acid have little effect on ligand escape. In contrast, Scott et al. (3) observed systematic increases in the fraction of O2 geminate rebinding with increasing size of the E7 position in Mb, from ~0.12 for Ala-64 to 0.72 for Trp-64.

O2 and CO Binding to Position 55(E18) Mutants—As shown in Fig. 1, Ala-55(E18) is located at the exit and entry point of the apolar channel between the E and H helices. To see if this entrance could be blocked, Ala-55(E18) was replaced with Val, Leu, Phe, and Trp, with the premise that the barrier to ligand uptake would increase with increasing size of the amino acid side chain. As shown in Fig. 4 and Table 2, there are strong inverse correlations between amino acid size and the rates of O2 association and dissociation, with both $k_{O2}$ and $k_{O2}$ decreasing by >3-4-fold for the Ala-55(E18) to Leu, Phe, and Trp mutations and little change in $k_{O2}$. The concomitant decreases in $k_{O2}$ and $k_{O2}$ suggest strongly that the mutations are increasing the free energy barrier for entering the protein and only affecting this transition state. This pattern of effects is in contrast to the more complex behavior seen when the size of the amino acid at position 44(E7) is increased (Fig. 4C versus Fig. 2).

As shown in Table 2, the affinity of CerHb for O2 shows almost no dependence on the size of the amino acid at position 55(E18). This observation argues strongly that the mutations only affect movement into the apolar channel, and this conclusion is supported by the much smaller effects observed for CO association and dissociation. In most globins, CO binding is limited by the rate of internal bond formation or bond breakage and not movement into and out of the protein (48, 49). As a result, the overall CO association and dissociation rate coefficients are little affected by mutations that only affect entry and exit into the protein, as is observed for the position 55(E18) mutants (Table 2, part B).

Effects of Position 55 Mutants on Geminate Recombination—Replacing Ala-55(E18) with larger amino acids increases the fraction of geminate recombination, $F_{gem}$, for both O2 and CO but slows the rate, $k_{gem}$, markedly (Fig. 3, B and C, and Table 2). In the case of CO geminate rebinding, $F_{gem}$ increases almost 10-fold, from 0.05 to 0.44, whereas $k_{gem}$ decreases ~10-fold from ~80 to ~9 μs$^{-1}$. Similar increases in $F_{gem}$ and decreases in $k_{gem}$ occur for internal O2 rebinding, but in this case the values are more difficult to assign because extensive geminate recombination is occurring during excitation and at speeds approaching the decay rate of the laser pulse. However, the trends are identical to those seen for CO rebinding. The key observation is that the rate of geminate recombination decreases, whereas the fraction of rebinding increases as the channel is blocked.

These trends demonstrate that the increase in geminate rebinding is because of slowing of escape and not an increase in the rate of internal bond formation. The observed decay rate of the nanosecond intermediate is equal to the sum of the rate of internal Fe(II)-ligand bond formation and the rate of ligand escape. The fraction of geminate rebinding is given by the ratio of the rate of bond formation divided by the sum of the rates of bond formation and escape, $F_{gem}$ for WT CerHb is very small, indicating the rate of ligand escape must be much larger than

widely with increasing size, from Ala to Trp-44, supporting the idea that the amino acid at the E7 position is not part of the gate for ligand movement into the distal pocket of CerHb.

The largest fraction of geminate recombination is 0.19 (Leu-44), and the smallest is ~0.02 (Phe-44), with WT CerHb (Gln-44) being 0.05. The rates of geminate recombination ($k_{gem}$) do not vary greatly, 60 to ~100 μs$^{-1}$, and because the extent of geminate recombination is small (~20%), the values of $k_{gem}$ represent the rates of escape from the CerHb mutants (see
the rate of internal bond formation. Consequently, the observed rate of the small geminate phase is roughly equal to the speed of escape, which then decreases as the larger mutations inhibit exit from the apolar channel. Eventually, the rate of escape becomes roughly equal to the rate of internal bond formation, and then the extent of internal rebinding is ~50%. This interpretation is described quantitatively under “Discussion” (see Equations 3 and 4).
Association Rate Coefficients for NO Binding—The bimolecular rate of NO binding to globins is determined solely by the rate of entry into the protein because, once captured in the distal pocket, virtually all the internal ligands bind to the iron atom before escaping because of the highly reactive, free radical nature of NO. This high reactivity accounts for the large extent of picosecond geminate rebinding of NO in photolysis experiments, the very low overall quantum yield of NO complexes (≤0.01), and the large bimolecular association rate coefficients for NO binding, \( k'_{\text{NO}} \) (48). Scott et al. (3) have shown that there is a 1:1 correlation between the bimolecular rate of ligand entry, \( k'_{\text{entry}} \), calculated from a combined analysis of geminate and overall \( \text{O}_2 \) rate coefficients and the values of \( k'_{\text{NO}} \) determined for over 90 different sperm whale Mb mutants. Thus, rates of NO binding to the position 55(E18) mutants of CerHb were measured to examine more directly if the larger amino acids are slowing the rate of ligand entry. As shown in Fig. 5 and Table 3, the rate of NO binding decreases over 5-fold when Ala-55 is replaced with Leu, Phe, and Trp, and the values of \( k'_{\text{NO}} \) are similar to those for \( k'_{\text{O}_2} \) implying that the rate-limiting step for \( \text{O}_2 \) binding is also movement into the protein and through the channel (see also Table 3 and under “Discussion”).

Crystal Structures of Phe-55 and Trp-55 CerHb—The crystal structures of the Trp-55 and Phe-55 mutants were determined to visualize whether the large aromatic side chains do block the entrance to the apolar channel and to examine whether perturbations of the distal pocket occur because of changes at the EF corner of the CerHb tertiary structure. The three-dimensional structures of Phe-55 and Trp-55 CerHbO\(_2\) were refined to final \( R \)-factor/\( R \)-free values of 18.8/23.0% and 16.7/21.3%, respectively, at 1.8 and 1.6 Å resolution, with good overall stereochemistry (Table 4).

The overall tertiary structures of the mutant proteins are unaffected by the mutations at position 55(E18) (Fig. 6A). The r.m.s.d. calculated between the C-α atoms of the WT and mutated proteins is 0.15 Å for both mutants, and only marginal readjustments can be detected at the end of the E helix and along the adjacent H helix (residues 97–104) because of the larger aromatic side chains at position 55(E18). When the two mutant structures are superimposed (overall r.m.s.d. of 0.13 Å), the phenyl ring of Phe-55 sits approximately in the center of mass of the indole ring of Trp-55 (Fig. 6A). The Phe and Trp side chains make 19 and 26 favorable van der Waals contacts, respectively, with adjacent regions of the protein, and the Trp-55(E18) indole N-81 atom appears to form a weak hydrogen bond with the O-62 atom of

![Figure 5. NO binding to 55(E18) mutants of CerHb at pH 7, 20 °C. CerHbCO (−50 μM) was rapidly mixed with buffer equilibrated with 1 atm of NO, and photolysis was initiated immediately afterward to generate deoxy-CerHb in −0.5 μs. NO binding was followed at 436 nm. The offset is because of the difference in absorbance between the initial CerHbCO and final CerHbNO complexes. This experiment was repeated with four different NO concentrations, and \( k'_{\text{NO}} \) was determined from the slope of plots of \( k_{\text{obs}} \) versus \([\text{NO}]\). Inset, correlations between the calculated value of \( k'_{\text{entry}} \) and the measured value of \( k'_{\text{NO}} \) for WT and the four 55(E18) mutants.]

### Table 3

| CerHb        | \( k'_{\text{NO}} \) | \( k'_{\text{entry}} \) | \( k_{\text{escape}} \) | \( K_{\text{entry}} \) | \( k_{\text{bond}} \) |
|--------------|----------------------|-------------------------|-------------------------|-------------------------|-------------------------|
|              | \( \mu\text{M}^{-1}\text{s}^{-1} \) | \( \mu\text{M}^{-1}\text{s}^{-1} \) | \( \mu\text{M}^{-1}\text{s}^{-1} \) | \( \mu\text{M} \) | \( \mu\text{M} \) |
| A. Kinetic parameters derived from NO and CO binding at pH 7.0, 20 °C | | | | | |
| Ala-55 (WT)  | 240                  | 560                     | 76                      | 7                       | 4.0                     |
| Val-55       | 140                  | 260                     | 20                      | 13                      | 3.8                     |
| Leu-55       | 47                   | 37                      | 4                       | 9                       | 3.5                     |
| Phe-55       | 48                   | 39                      | 5                       | 8                       | 4.0                     |
| Trp-55       | 45                   | 32                      | 5                       | 6                       | 4.1                     |
| B. Kinetic parameters derived from \( \text{O}_2 \) binding at pH 7.0, 20 °C | | | | | |
| Ala-55 (WT)  | 240                  | 620                     | 79                      | 8                       | 51                      |
| Val-55       | 140                  | 310                     | 27                      | 12                      | 37                      |
| Leu-55       | 47                   | 87                      | 9                       | 9                       | 45                      |
| Phe-55       | 48                   | 71                      | 8                       | 9                       | 47                      |
| Trp-55       | 45                   | 73                      | 8                       | 10                      | 47                      |
Asp-104(H15). The Phe-55 and Trp-55 side chains fit into a cleft, located between the end of the E helix and the beginning of the H helix, bordered by surface residues His-100(H11), Asp-104(H15), and by main chain carbonyl groups of the 55–62 region (EF helical corner, Figs. 1 and 6). In WT CerHb this cleft is “open” and provides access to the elongated channel, lined by the hydrophobic residues Val-7(B6), Phe-10(B9), Ile-52(E15), Ala-55(E18), Leu-86(G12), Leu-98(H9), Ala-101(H12), Ile-102(H13), and Ile-105(H16), by the hydrophilic residues Tyr-11(B10), Thr-48(E11), and Tyr-51(E14), and by the heme B-methyl and B-vinyl groups (Fig. 1A). The surface aperture of the cleft is bordered by residues Ala-55(E18), Ala-60(F1), Ala-62(F2), and Asp-104(H15) and has a diameter of between 6.8 and 7.7 Å. Thus, the major structural effect of the Ala-55(E18) to Phe and Trp mutations is blocking access to the channel from the solvent phase (Fig. 6C).

The blockage caused by the Ala-55(E18) mutations is shown in Fig. 6B, where protein cavities were calculated using a 1.4-Å radius probe (50) and is depicted as brown mesh networks. In WT CerHb, the apolar channel connects directly to the aperture between the E and H helices. In contrast, the entrance is almost fully blocked by the aromatic rings of the Phe-55 and Trp-55 side chains. The interior channel in the mutants appears as an isolated cavity of roughly the same size (57 Å³) as that found in the internal channel of the WT protein (59 Å³). In both mutants and WT CerHb, the lower portion of the channel entrance hosts two water molecules hydrogen-bonded to each other and the Ala-101(H12) carbonyl O atom, the Tyr-51(E14) hydroxyl, and the O-82 atom of Asp-104(H15). The aromatic substitutions cause only minor alterations in the

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**TABLE 4**

| X-ray data collection, refinement statistics, and model quality | Phe-55 (E18) CerHbO₂ | Trp-55 (E18) CerHbO₂ |
|---------------------------------------------------------------|---------------------|---------------------|
| **Data collection**                                          | P₂₁2₁2₁            | P₂₁2₁2₁             |
| **Cell dimensions**                                         | a = 42.88 Å, b = 43.35 Å, c = 59.88 Å | a = 42.79 Å, b = 43.32 Å, c = 60.04 Å |
| **Resolution**                                               | 43.36–1.80 Å (1.90–1.80)¹   | 43.31–1.60 Å (1.69–1.60)¹   |
| **Observations**                                             | 41,523              | 52,593              |
| **Unique reflections**                                       | 10,801              | 15,251              |
| **Completeness**                                             | 99.6 (99.7)%        | 99.7 (99.9)%        |
| **R-merge**                                                  | 12.0 (28.2)%        | 6.4 (31.5)%         |
| **I/ð(I)**                                                   | 9.5 (4.1)           | 12.7 (4.1)          |
| **Multiplicity**                                             | 3.8 (3.9)           | 3.4 (3.4)           |
| **Refinement**                                               |                     |                     |
| **R-factor/R-free**                                          | 18.8/23.0%          | 16.7/21.3%          |
| **Protein atoms in the asymmetric unit**                     | 829 (residue 0–109) | 839 (residue 0–109) |
| **Side chains with alternative conformations**               | Ser-34, Glu-44      | Lys-16, Ser-34, Glu-44, Ser-76 |
| **Dioxygen molecules**                                       | 1                   | 1                   |
| **Water molecules**                                          | 145                 | 160                 |
| **Sulfate ions**                                             | 1                   | 2                   |
| **Acetate ions**                                             | 1                   | 0                   |
| **Glycerol**                                                 | 2                   | 0                   |
| **Model quality**                                            |                     |                     |
| **Overall B-factor**                                         | 13.7 Å²             | 13.7 Å²             |
| **r.m.s.d. from ideal values**                               |                     |                     |
| **Bond lengths (Å)**                                         | 0.013 Å             | 0.010 Å             |
| **Bond angles (°)**                                          | 1.245°              | 1.080°              |
| **Ramachandran plot**                                        |                     |                     |
| **Most favored regions**                                     | 94.7                | 96.8                |
| **Additional allowed regions**                               | 5.3                 | 3.2                 |

¹ Outer shell statistics are shown within parentheses.

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**FIGURE 6.** Comparison of WT CerHbO₂ and 55(E18) mutant structures. A, overlay of ribbons drawings of WT (cyan), Phe-55 (green), and Trp-55 (red) CerHb. The same amino acid side chains shown in Fig. 1A are presented as sticks in this drawing, but only the E7 and E18 positions are labeled and show different positions. B, disruption of the apolar channel at the end of the E helix by the Phe-55 and Trp-55 mutations. Protein cavities were calculated with a 1.4-Å radius probe using the program SURFNET (50) and are represented as a brown network. Side chains of the mutated residues at position 55(E18) are shown in ball-and-stick representation. C, “back” space-filling view of Trp-55 showing that the indole ring (green) blocks the entrance to the apolar channel. This structure should be compared with the same view of WT CerHbO₂ in Fig. 1C.

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Asp-104(H15). The Phe-55 and Trp-55 side chains fit into a cleft, located between the end of the E helix and the beginning of the H helix, bordered by surface residues His-100(H11), Asp-104(H15), and by main chain carbonyl groups of the 55–62 region (EF helical corner, Figs. 1 and 6). In WT CerHb this cleft is “open” and provides access to the elongated channel, lined by the hydrophobic residues Val-7(B6), Phe-10(B9), Ile-52(E15), Ala-55(E18), Leu-86(G12), Leu-98(H9), Ala-101(H12), Ile-102(H13), and Ile-105(H16), by the hydrophilic residues Tyr-11(B10), Thr-48(E11), and Tyr-51(E14), and by the heme B-methyl and B-vinyl groups (Fig. 1A). The surface aperture of the cleft is bordered by residues Ala-55(E18), Ala-60(F1), Ala-62(F2), and Asp-104(H15) and has a diameter of between 6.8 and 7.7 Å. Thus, the major structural effect of the Ala-55(E18) to Phe and Trp mutations is blocking access to the channel from the solvent phase (Fig. 6C).

The blockage caused by the Ala-55(E18) mutations is shown in Fig. 6B, where protein cavities were calculated using a 1.4-Å radius probe (50) and is depicted as brown mesh networks. In WT CerHb, the apolar channel connects directly to the aperture between the E and H helices. In contrast, the entrance is almost fully blocked by the aromatic rings of the Phe-55 and Trp-55 side chains. The interior channel in the mutants appears as an isolated cavity of roughly the same size (57 Å³) as that found in the internal channel of the WT protein (59 Å³). In both mutants and WT CerHb, the lower portion of the channel entrance hosts two water molecules hydrogen-bonded to each other and the Ala-101(H12) carbonyl O atom, the Tyr-51(E14) hydroxyl, and the O-82 atom of Asp-104(H15). The aromatic substitutions cause only minor alterations in the
Multiple Conformations of Gln-44(E7)—Even though there appear to be few changes in the positions of the C-α atoms in the N-terminal region of the E helix and the CD corner (Fig. 6A), the amide side chain of Gln-44(E7) is found in two conformations in both the Phe-55 and Trp-55 mutant structures (Fig. 7). The electron density clearly defines two positions of roughly equal occupancy, which will affect electrostatic fields near the iron atom and could potentially influence the rates of O2 binding and the FTIR spectra of bound CO. This double conformation is particularly well defined in the Trp-55 CerHbO2 structure, perhaps because of the higher resolution of the x-ray diffraction data for this mutant (1.6 Å). The innermost Gln-44(E7) conformer exactly matches the rotamer found in the oxygenated WT protein and appears to form a hydrogen bond between the Gln-44(E7) N-ε2 atom and the O-1 of the bound ligand (O-1–N-ε2 distances of 2.61 and 2.66 Å in the Phe-55 and Trp-55 mutants, respectively). The Gln-44(E7) N-ε2 atom may also weakly interact with the Tyr-11(B10) hydroxyl O-ζ atom (distances of 3.26 and 3.28 Å in the Phe-55 and Trp-55 mutants, respectively) and with the Thr-48(E11) O-γ1 atom (distances of 3.36 and 3.25 Å in the Phe-55 and Trp-55 mutants, respectively).

Note that in all the structures, the Thr-48(E11) O-γ1 atom is accepting a strong hydrogen bond from the hydroxyl proton of Tyr-11(B10) (2.48 and 2.56 Å in Phe-55 and Trp-55 CerHbO2, respectively). Thus, all bound ligands are experiencing a partial negative charge from the nonbonded electrons of the O-ζ atom of Tyr-11(B10), which accounts for the moderate O2 affinity (Table 2) and high stretching frequency of bound CO (~1980 cm⁻¹, Fig. 8) (21, 32) for WT CerHb and all five position 55 mutants. A complete discussion of the key role of Thr-48(E11) in regulating O2 binding in CerHb is given in Pesce et al. (32).

In the second outermost conformation, the Gln-44(E7) side chain is rotated by about 180° toward the solvent interface (Fig. 7). As a consequence, polar interactions with the bound ligand, Tyr-11(B10), and Thr-48(E11), are lost. However, in the outer conformation of Trp-55 CerHbO2, the Gln-44(E7) N-ε2 atom appears to be part of a hydrogen bonding network, which connects the carbonyl of Ala-40(E3) to the N-ζ of Lys-47(E10) through well defined water molecules, and there may also be electrostatic interactions with the heme propionates. This network is less well defined in the Phe-55 structure.

The outward rotation of Gln-44(E7) in the 55 mutants is puzzling for four reasons. First, it is not clear how mutations at the end of the E helix cause this conformational shift of the E7 side chain without causing a significant change in the position 44 C-α atom. A simple interpretation would be that the larger Phe-55 and Trp-55 side chains sterically clash with their surroundings altering the dynamics of the MACROS BELOW ARE FOR THE VISUALE-helix that allows two conformations for the Gln-44 side chain, but this idea remains speculative, and other explanations are possible.

Second, if the E7 residue served as a gate for ligand entry and exit, then the outward movement of the Gln-44 side chain would be expected to increase the rates of O2 association and dissociation and decrease the fraction of geminate recombination by enhancing the rate of ligand escape. However, the Phe-55 and Trp-55 mutations cause the opposite effect, decreasing k'_{O2} and k_{O2} and increasing the fraction of internal rebinding. In addition, the outer Gln-44(E7) conformer does not create a significant opening to solvent, even though there is net movement away from the iron atom (Fig. 7). In WT sperm whale Mb, protonation of the imidazole side chain of His-64(E7) at low pH causes an upward and outward rotation into a truly open conformation, creating a direct channel from solvent to the iron atom (51). A similar opening is created by the Phe-46(CD4) to Val mutation in MbCO and deoxyMb (52), the binding of imidazole to metMb (53), and the binding of long chain isocyanides to deoxyMb (54–56). In contrast, access to the iron atom is still blocked in the outer Gln-44 conformer of Trp-55 CerHb (Fig. 7). In general, it is more difficult for the Gln-44 side chain to move out of the E7 channel because of the need for concerted rotations about C-α–C-β, C-β–C-γ, and C-γ–C-δ bonds and for disruption of two possible hydrogen bonding interactions for each of the N-ε and O-ε amide atoms. In contrast, the side chain of His-64 in Mb can rotate almost completely out of the E7 channel by changing only the torsion angle of the C-α–C-β bond (53), and the aromatic and secondary N atoms of the neutral imidazole side chain can only interact with one hydrogen bond donor and acceptor, respectively.

The third and fourth puzzles are that the O2 affinities of WT CerHb and the position 55(E18) mutants are virtually identical (Table 2) and that stretching frequency of bound CO is unaffected by the Phe-55 and Trp-55 substitutions (Fig. 8 and below). These results suggest that the exact conformation of Gln-44(E7) has little effect on either the rate or equilibrium coefficients for ligand binding. The latter conclusion is partially
supported by the results for the position 44(E7) mutants in Table 1. The Ala-44, Val-44, and Leu-44 mutations have little effect on the ligand association rates and, unexpectedly, cause small to moderate decreases in the rate of O₂ dissociation. However, the Phe-44 and Trp-44 mutations cause marked decreases in $k_{O2}$ with little change in $k_{H2O2}$. The simplest interpretation of the latter result is that the large aromatic E7 side chains occupy the inner conformation and sterically hinder access to the iron atom and the bound ligand.

**FTIR-TDS of Position 55 Mutants**—We have previously shown that, when photodissociated at low temperatures, CO occupies several positions (denoted as B, C, and D) within the distal pocket of CerHb before migrating into the apolar channel (21). On the basis of the effects of distal pocket mutations, the B state was assigned to a position underneath Phe-10(B9) and Tyr-11(B10), directly above pyrrole C. The C state was identified as a position located further into the protein underneath Val-7(B6) and above the vinyl group of pyrrole B, just at the beginning of the apolar channel (21) (Fig. 9). The D state was tentatively assigned to a location within the distal pocket between Tyr-10(B10) and Gln-44(E7) and $\approx$5 Å above the heme plane.

At room temperature, photodissociated CO appears to equilibrate rapidly between those sites and the channel, and only a single, exponential geminate rebinding phase is observed on nanosecond time scales for the position 55 mutants and WT CerHb (Fig. 3). To examine the effects of blocking the channel entrance on ligand migration and rebinding from near the heme group in more detail, we measured low temperature FTIR photolysis difference spectra of WT, Phe-55, and Trp-55 CerHbCO (Fig. 8). These data serve to connect the initial dissociation events to the rates of ligand escape, entry, and bond formation measured at room temperature and to test more
directly whether or not the replacements of residue 55 have an effect on the reactivity of the heme iron atom and the innermost barrier to bond formation. Moreover, the low temperature FTIR data also allow assessing the functional consequences of the two conformers of Gln-44(E7) observed in the crystal structures of Phe-55 and Trp-55 CerHbO₂.

Two sets of similar experiments for WT, Phe-55, and Trp-55 CerHbCO are shown in Fig. 8. In the first protocol, the CerHbCO samples were cooled to 3 K in the dark and then illuminated for 1 s. Under these conditions, the photolyzed ligands are trapped mainly at the primary docking site B. In the second protocol, the CerHbCO samples were exposed to continuous laser illumination while being cooled slowly from 160 to 3 K. This protocol allows sampling of all internal transient ligand docking sites. In both types of experiments, absorbance difference spectra were calculated from transmission spectra measured before and after illumination (Fig. 8A). The negative features between 1900 and 2000 cm⁻¹ represent the missing absorbance of the originally bound CO (A state bands), whereas the positive features between 2000 and 2150 cm⁻¹ represent photodissociated CO trapped in different locations in or near the distal pocket.

As shown in Fig. 8A, the negative peaks representing bound CO occur at the same position, 1982 cm⁻¹, for WT CerHbCO and the two mutants. The presence of only a single CO stretching band for each sample reflects a structurally homogeneous binding site in the ligand-bound state. However, the photoproduct spectra at 3 K are different. A single B state band (B1) is observed for WT CerHbCO, suggesting that the dissociated ligand occupies a single site with a single orientation of the C-O molecule. Little or no conformational heterogeneity of the surrounding polar amino acids and the resultant electrostatic field at the CO ligand position is observed. In contrast, two peaks (B1 and B2) are observed for the photoproduct in Phe-55 CerHbCO, implying either two orientations, two distinct locations of the photolyzed CO, or two distinct conformations of the distal pocket amino acids. In the case of Trp-55 CerHbCO, three photoproduct bands (B1, B2, and C) are seen. Two are identical to the photoproduct states seen in WT (B1) and Phe-55 (B1 and B2) CerHbCO. The third, higher frequency band is associated with the secondary docking site C, which was observed previously for CerHb mutants in which Val-7(B6), Phe-10(B9), or Tyr-11(B10) were mutated to smaller amino acids (21) (Fig. 9). The appearance of this band in the Trp-55 mutant suggests that the large indole ring may cause minor perturbations along the E helix that reduce the volume of the distal pocket and site B. This reduction in volume might force the Gln-44 side chain outward and the dissociated ligand toward site C at the interior entrance to the apolar channel. However, this interpretation is speculative. After slow cooling from 160 to 3 K, all samples show still another photoproduct spectrum; the main band is associated with site D (Fig. 8A, lower right).

To examine thermally activated rebinding from the photoproduct states, the sample temperature was ramped upward at a rate of 5 mK/s, and an FTIR transmission spectrum was recorded every 1 K. Difference spectra were calculated for each successive temperature rise and then plotted as a function of both frequency and temperature as shown in Fig. 8B. The temperature at which the A state is regenerated (Fig. 8B, left column, solid contour lines) and the photoproduct states disappear (right column, dashed contours) provides a measure of the enthalpy barrier to return to the iron atom and bond formation. After a 1-s illumination (Fig. 8B), rebinding from both states B1 and B2 occurs at roughly the same low temperature, ~15–20 K, in WT CerHbCO and both position 55 mutants, indicating that these two bands are associated with ligands close to the iron atom. Thus, the Phe and Trp replacements have little effect on the inner barrier to CO rebinding from the B states. In the case of the Trp-55 mutant, the C state is partially populated, even after brief illumination at 3 K. As a result, a significant increase in the A state population occurs at ~65 K, reflecting rebinding from the C state.

DISCUSSION

The E7 Gate Versus the Apolar Channel—The effects of mutagenesis of Gln-44(E7) in CerHb are complex and markedly different from those observed for the same set of replacements at the E7 position in sperm whale Mb (Table 1 and Fig. 2). Replacement of Gln-44 with smaller apolar amino acids results in small decreases in both the association and dissociation rates for O₂ binding to CerHb. In contrast, when His-64(E7) is replaced with Ala, Val, or Leu in sperm whale Mb, 10–1000-fold increases in kₖO₂ and kₑO₂ are observed. These results argue against an E7 gate pathway for ligand movement into CerHb. The His-44 and Phe-44 CerHb mutants and one of the conformers of the Trp-44 mutant do show significant decreases in kₑO₂, which could be interpreted as due to blockage of the E7 gate. However, the dissociation rate coefficients for the Phe-44 mutant and that for one of the Trp-44 conformers are large and equal to kₑO₂ for WT CerHbO₂.
O₂ Binding to Cerebratulus Hb through an Apolar Tunnel

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 $k'_{\text{entry}}$ 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 ($k'_{\text{CO}}$ 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 O₂ and NO, respectively (48). Thus, the large ~10-fold decreases in $k'_{\text{CO}}$ 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. 3A. 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. 3A).

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 O₂ 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 photoprotein 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 $k'_{\text{bond}}$ and 3) escape from the intermediate state out through the channel past the Ala-55(E18) position at a rate equal to $k'_{\text{escape}}$. The fraction and rate of geminate rebinding, $F_{\text{gem}}$ and $k'_{\text{gem}}$, are given by Equation 3,

$$F_{\text{gem}} = \frac{k'_{\text{bond}}}{k'_{\text{bond}} + k'_{\text{escape}}}$$

$$k'_{\text{gem}} = k'_{\text{bond}} F_{\text{gem}}$$

(Eq. 3)

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 $k'_{\text{gem}}$ and the normalized amplitude for rebinding and the offset are equal to $F_{\text{gem}}$ and $1 - F_{\text{gem}}$, respectively (48). These parameters can then be used to compute $k'_{\text{bond}}$ and $k'_{\text{escape}}$ as shown in Equation 3. An expression for the overall bimolecular rate coefficient for ligand binding, $k'_{\text{X}}$, where $X$ is the ligand (O₂, CO, NO, etc.), can be obtained by assuming a steady-state approximation ($d[I]/dt = 0$) for the intermediate on μs and ms time scales. Under these conditions, $k'_{\text{X}}$ is given by Equation 4,

$$k'_{\text{X}} = \frac{k'_{\text{bond}}}{k'_{\text{bond}} + k'_{\text{escape}}} = k'_{\text{entry}} F_{\text{gem}}$$

(Eq. 4)

Equations 3 and 4 and the measured kinetic parameters for O₂ and CO binding in Table 2 were used to compute estimates of $k'_{\text{entry}}, k'_{\text{escape}}$, and $k'_{\text{bond}}$ 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 O₂ and CO binding. The estimated value of $k'_{\text{bond}}$ for O₂ rebinding is, as expected, roughly 10 times greater than that for CO, ~50 μs⁻¹ versus ~4 μs⁻¹. Both values of $k'_{\text{bond}}$ 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 $k'_{\text{entry}}$ and $k'_{\text{escape}}$ 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 $k'_{\text{entry}}$ and $k'_{\text{escape}}$ calculated using the O₂ and CO data is remarkably good, considering that it is difficult to determine the exact values of $k'_{\text{gem}}$ and $F_{\text{gem}}$ for O₂. 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 computational values of $k'_{\text{entry}}$. NO is expected to show a quantum yield for complete photodissociation to the solvent (data not shown). Thus $k'_{\text{NO}}$ should be approximately equal to $k'_{\text{entry}}$. As shown in Table 3 and the Fig. 5. inset, there is a strong linear correlation between $k'_{\text{NO}}$ and $k'_{\text{entry}}$. However, the calculated values of $k'_{\text{entry}}$ appear to be ~2-fold greater than $k'_{\text{NO}}$ 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 photo-dissociated 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 \( k'_{\text{entry}} \). 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 \( k'_{\text{NO}} \) and the calculated values of \( k'_{\text{entry}} \) 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, \( K_{\text{entry}} \), 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 \( -9.1 \pm 2.0 \text{ M}^{-1} \) 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 \( -5.6 \text{ Å}^3 \). The value of \( K_{\text{entry}} \) for ligand capture in WT sperm whale Mb is \( -5.5 \pm 1.8 \text{ M}^{-1} \), 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 \( K_{\text{entry}} \) for sperm whale Mb increases to \( -10 \text{ M}^{-1} \), 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 \( k'_{\text{entry}} \) or \( k_{\text{escape}} \) as the size of residue 55 is increased from Leu to Phe to Trp suggests that the small, limiting values of these parameters, \( -50 \mu \text{M}^{-1} \text{s}^{-1} \) and \( -6 \mu \text{s}^{-1} \), 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 (\( k'_{\text{entry}} \approx 34 \pm 7 \mu \text{M}^{-1} \text{s}^{-1} \) and \( 6.3 \pm 1 \mu \text{s}^{-1} \)) (3). However, our current sets of single mutants and FTIR data cannot define the location of alternative pathways.

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