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Transient Ligand Docking Sites in *Cerebratulus lacteus* Mini-Hemoglobin

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Abstract

The monomeric hemoglobin of the nemertean worm *Cerebratulus lacteus* functions as an oxygen storage protein to maintain neural activity under hypoxic conditions. It shares a large, apolar matrix tunnel with other small hemoglobins, which has been implicated as a potential ligand migration pathway. Here we explore ligand migration and binding within the distal heme pocket, to which the tunnel provides access to ligands from the outside. FTIR/TDS experiments performed at cryogenic temperatures reveal the presence of three transient ligand docking sites within the distal pocket, the primary docking site B on top of pyrrole C and secondary sites C and D. Site C is assigned to a cavity adjacent to the distal portion of the heme pocket, surrounded by the B and E helices. It has an opening to the apolar tunnel and is expected to be on the pathway for ligand entry and exit, whereas site D, circumscribed by TyrB10, GlnE7, and the CD corner, most likely is located on a side pathway of ligand migration. Flash photolysis experiments at ambient temperatures indicate that the rate-limiting step for ligand binding to CerHb is migration through the apolar channel to site C. Movement from C to B and iron-ligand bond formation involve low energy barriers and thus are very rapid processes in the wt protein.

Keywords

Fourier Transform Infrared Spectroscopy; photolysis; carbon monoxide; ligand migration

1. INTRODUCTION

The monomeric nerve hemoglobin (Hb) of the Nemertean worm *Cerebratulus lacteus* (CerHb) is a very small mini-hemoglobin with only 109 amino acids (Vandergon et al., 1998; Pesce et al., 2001; Pesce et al., 2002). The protein is expressed at a concentration of 2 – 3 mM in cells surrounding the axonal bundles and thus believed to function as an oxygen storage protein to maintain neural activity under hypoxic conditions. Compared to the classical 3-on-3 α -helix

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sandwich structure of conventional full-length globins, the CerHb structure is strikingly modified, due to extensive residue deletions (Pesce et al., 2000; Milani et al., 2001; Wittenberg et al., 2002). The N-terminal helix A of CerHb is almost completely deleted, and the H helix is trimmed to eight residues. Helix F is shortened to a single turn containing the proximal histidine F8, which covalently binds the heme iron. The metal center coordinates dioxygen (O_2), which may access the active site via an elongated tunnel in the protein matrix that extends from the distal cavity to the protein surface, where it emerges between the end of the E-helix and the beginning of the H-helix (Pesce et al., 2002). Tunnels with inner diameters of 5 – 7 Å have also been observed in several other small Hbs (Pesce et al., 2000; Milani et al., 2001; Milani et al., 2002; Milani et al., 2004; Milani et al., 2005), and crystallographic studies have revealed xenon atoms at well-defined sites along the protein matrix tunnel in crystals subjected to xenon gas at 25 bar (Milani et al., 2004). CerHb appears as an excellent model system to explore ligand binding in truncated mini hemoglobins. Conceptually, this process can be subdivided into three steps, (1) ligand migration between the solvent and transient docking sites near the active site of the protein, possibly by means of the tunnel, (2) equilibration between the docking sites, and finally, (3) bond formation at the heme iron. In this work, we have focused on steps 2 and 3, and the influence of the active-site structure on ligand dynamics between docking sites and their effects on the overall rates of ligand binding.

Carbon monoxide (CO) can serve as an internal probe to measure ligand movements in the protein and its rebinding to the iron atom due to its strong stretching absorption in the infrared ($\sim 5 \mu m$) that responds sensitively to the local electrostatic environment, both in the heme-bound and photodissociated state (Li et al., 1994; Ray et al., 1994; Vogel et al., 1999; Kriegl et al., 2003; Lehle et al., 2005). These effects have been most thoroughly studied for myoglobin (Mb) (Li et al., 1994; Ray et al., 1994; Nienhaus et al., 2003b; Nienhaus et al., 2003a). In the COligated form of sperm whale Mb (MbCO), three stretching bands A_0 ($\sim 1965 \text{ cm}^{-1}$), A_1 ($\sim 1945 \text{ cm}^{-1}$) and A_3 ($\sim 1930 \text{ cm}^{-1}$) of heme-bound CO are observed (Alben et al., 1982), corresponding to three discrete active-site structures with different ligand binding properties (Johnson et al., 1996). The interaction with the distal histidine governs the stretching frequency of the so-called A or bound substates. In A_1 and A_3 , the neutral HisE7 imidazole side chain resides within the distal pocket in two slightly different positions (Tian et al., 1993; Vojtechovsky et al., 1999). Upon protonation, it rotates towards the solvent (Yang and Phillips, 1996), and removal of the electrostatic interaction gives rise to the A_0 band at $\sim 1965 \text{ cm}^{-1}$ (Müller et al., 1999). Similar frequencies are also observed when HisE7 is replaced with apolar amino acids incapable of hydrogen bonding with the bound ligand (Li et al., 1994; Park et al., 1999). Upon photodissociation of MbCO at cryogenic temperatures, ligands cannot escape from the protein and migrate to internal cavities in the protein that serve as specific docking sites (Chu et al., 2000; Ostermann et al., 2000; Bourgeois et al., 2003; Schotte et al., 2003; Schmidt et al., 2005), as inferred from the appearance of sharp bands of the photodissociated CO between 2100 and 2200 cm^{-1} (Alben et al., 1982; Braunstein et al., 1988; Braunstein et al., 1993; Mourant et al., 1993). Three docking sites labeled B, C, and D can be distinguished by their characteristic CO stretching spectra (Nienhaus et al., 2003b; Nienhaus et al., 2003a; Nienhaus et al., 2003c). For an individual site, a specific doublet of IR bands is frequently observed due to the vibrational Stark effect, the effect of an electric field on a vibrational transition, and the doublet indicates that the CO molecules are trapped in opposite orientations (Kriegl et al., 2003; Lehle et al., 2005; Nienhaus et al., 2005). For example, in MbCO, the Stark splitting between bands B_1 (2131 cm^{-1}) and B_2 (2119 cm^{-1}) is caused by the electrostatic interaction between the CO ligand and the HisE7 $N_{\epsilon 2}-H$ (Nienhaus et al., 2005; Bredenbeck et al., 2006). Sophisticated illumination protocols have been developed to selectively populate photoproduct states B, C, and D to characterize them individually and to observe ligand migration and binding at cryogenic temperatures.

In CerHb, the active site residues TyrB10, GlnE7 and ThrE11 are involved in a tight-knit hydrogen bonding network that includes strong electrostatic interactions with the heme-bound ligand (Fig. 1a). Therefore, mutations of these amino acids are expected to markedly affect the ligand binding properties of CerHb and the spectral characteristics of bound and photodissociated CO. Fourier transform infrared (FTIR) spectroscopy at 3 K reveals only a single A substate band at 1982 cm^{-1} for wild-type (wt) CerHbCO (Pesce et al., 2002;Pesce et al., 2004), which indicates a rather rigid active-site structure in the native protein. Replacing ThrE11 by the isosteric Val changes the hydrogen bonding pattern, which has dramatic effects on the oxygen affinity and the spectral characteristics of both bound and photodissociated CO (Pesce et al., 2004). Here we have explored the migration of ligands between cavities near the active site using a set of mutants designed to alter selectively the electrostatic fields and sizes of the putative docking sites. These mutations allow a tentative structural interpretation of the B, C, and D photoproduct states in CerHb. Room temperature kinetics monitored with flash photolysis and stopped flow techniques on both the CO and O₂-ligated species complement the low temperature data and connect these results to overall ligand binding under physiological conditions.

2. MATERIALS AND METHODS

2.1. Sample Preparation

Wild type (wt) and mutant recombinant CerHbs were expressed and purified by using a synthetic gene with codon usage optimized for expression in *E. coli*, as described previously (Pesce et al., 2002). For the FTIR measurements, lyophilized protein was dissolved at a concentration of ~20 mM in cryosolvent (75%/25% (v/v) glycerol/1.0 M potassium phosphate buffer, pH 9.2 (final sample pH 7.3 – 7.5). Samples were stirred under a CO atmosphere for one hour and reduced by adding sodium dithionite solution at two-fold molar excess. Prior to the experiment, samples were centrifuged for 15 min (2800 g) to remove any undissolved protein.

2.2. Fourier Transform Infrared (FTIR) Spectroscopy

About 2 μl of the sample solution were sandwiched between two CaF₂ windows (diameter 25.4 mm) inside an oxygen-free high-conductivity copper block, separated by a 75 μm thick mylar washer. The assembly was mounted on the cold finger of a closed-cycle helium cryostat (SRDK-205AW, Sumitomo, Tokyo, Japan). The sample temperature was measured by a silicon temperature sensor diode and adjusted between 3 and 325 K with a Lake Shore Cryotronics digital temperature controller (model 330, Lake Shore Cryotronics, Westerville, OH, USA). For photodissociation, the beam of a continuous-wave frequency-doubled Nd: YAG laser of 300 mW output power at 532 nm (Forte 530-300, Laser Quantum, Manchester, UK) was split and focused on the sample from both sides. Transmission spectra in the mid-IR ($1700\text{--}2350\text{ cm}^{-1}$, resolution: 2 cm^{-1}) were collected with a FTIR spectrometer (IFS 66v/S, Bruker, Karlsruhe, Germany).

2.3. Temperature Derivative Spectroscopy

Temperature derivative spectroscopy (TDS) is an experimental protocol to investigate thermally activated rate processes (Berendzen and Braunstein, 1990; Mourant et al., 1993; Nienhaus et al., 1994). Prior to the experiment, the sample is cooled to a temperature at which the reaction under study is slow (preferentially arrested) on the experimental time scale. Photolysis creates a non-equilibrium state, and the recovery of the bound state is measured while the temperature is slowly increased. In the work reported here, the sample temperature was ramped at a rate of 5 mK/s, and one FTIR transmission spectrum, $I(\nu, T)$, was taken every 1 K. Subsequently, absorbance difference spectra, $\Delta A(\nu, T)$, were calculated from spectra at successive temperatures. They reveal the spectral changes that occurred within 200 s or 1 K,

arising either due to population changes within the heme-bound or photoproduct states or due to the intrinsic temperature dependence of the bands. In some cases, the intrinsic temperature dependence of the CO stretching bands is weak and thus negligible. If the ligand undergoes librational dynamics, however, the temperature dependence can be significant (Kriegel et al., 2003; Nienhaus et al., 2004; Lehle et al., 2005). We present FTIR-TDS data as two-dimensional contour plots on a surface spanned by the temperature and wavenumber axes, with black lines indicating an absorbance increase and red lines indicating a decrease. The contour lines are spaced logarithmically.

Two different illumination protocols were employed to create non-equilibrium states prior to the start of the temperature ramp. In one set of experiments, samples were cooled to 3 K and then photolyzed for 1 s, which ensures that every CerHbCO molecule has been photodissociated at least once. For MbCO, brief illumination results in CO migration to only the primary docking site B (Alben et al., 1982; Nienhaus et al., 2003b; Nienhaus et al., 2005), from which rebinding is observed as the temperature of the sample is increased during subsequent TDS data acquisition (Nienhaus et al., 1994; Nienhaus et al., 2003b). To examine secondary sites, which can only be populated at higher temperatures, the sample is continuously exposed to laser illumination while being cooled slowly (5 mK/s) from 160 to 3 K. Then, the photolysis laser is switched off, and the TDS temperature ramp is started to monitor rebinding from these more remote sites (Nienhaus et al., 1994; Chu et al., 1995; Nienhaus et al., 2003b).

2.4. Ligand Association and Dissociation at Ambient Temperature

All ligand association and dissociation experiments were carried out using dilute (5 to 100 μ M) CerHbO₂ or CerHbCO samples in 0.1 M phosphate buffer, pH 7.0, 2.0 mM EDTA, 20°C. The sample solutions were inserted into sealed cuvettes (1 mm path length) that were previously equilibrated with the desired partial pressures of O₂ or/and CO. Association time courses were measured after complete laser photolysis of the samples using a 300 ns excitation pulse from a Phase-R model 2100B dye laser (Rohlfs et al., 1990). In most cases, simple exponential rebinding time courses were observed, and the association rate coefficients k'_{O_2} were determined from the slope of plots of the observed pseudo-first order bimolecular rates, k_{obs} , vs. [O₂] or [CO]. For samples containing ratios of O₂:CO (1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75, and 0:1), k_{obs} was fitted to the expression $k'_{O_2}[O_2] + k_{O_2} + k'_{CO}[CO]$ (Olson et al., 2003).

O₂ dissociation rate coefficients were determined from time courses for ligand replacement, either obtained from the slow phases in the photolysis experiments with O₂:CO mixtures (Olson et al., 2003) or recorded in stopped-flow spectrometer experiments. Here, 5 – 10 μ M CerHbO₂ samples in buffer containing various concentrations of O₂ were mixed with buffer equilibrated with 1 atm CO. In both cases, the O₂ dissociation rate coefficients were calculated from $k_{O_2} = r_{obs}(1 + k'_{O_2}[O_2]/k'_{CO}[CO])$, where r_{obs} is the observed first-order replacement rate coefficient. CO dissociation rate coefficients were determined by analyzing stopped-flow time courses, in which the heme-bound CO was displaced with high concentrations of NO (Olson, 1981; Rohlfs et al., 1990). Equilibrium coefficients for O₂ and CO binding were calculated from the ratios of association and dissociation rate coefficients.

3. RESULTS

3.1. Photolysis Difference Spectra at 3 K

CerHbCO samples were cooled to 3 K in the dark and illuminated for 1 s. Absorbance difference spectra, $\Delta A = \log(I_{dark}/I_{light})$, were calculated from transmission spectra, $I(\lambda, T)$, taken before and after photolysis. Peak frequencies and relative populations of CO stretching bands

corresponding to heme-bound and photodissociated ligands in different docking sites are compiled for all variants in Table 1.

In Fig. 2a and b, we have plotted the light–dark difference spectra of heme-bound CO, normalized to equal total area for better comparison, for wt and select mutant CerHbCO samples. We note that the rebinding barrier at the heme iron is rather small; its distribution peaks at 2.2 kJ/mol at low temperatures, which indicates a highly reactive heme iron (Pesce et al., 2004). Therefore, only ~70% of all CO molecules are kept photodissociated on average during the time it takes to collect a spectrum (~200 s). This fraction is further reduced to ~28% in mutants ValB6Ala and ValB6Trp.

Wt CerHbCO has a single A substate band at 1982 cm^{-1} (black line Fig. 2a). For GlnE7Phe and GlnE7Leu, we observe dominant bands at 1987 and 1993 cm^{-1} , respectively. For the mutant GlnE7Phe, an additional broad feature most likely consisting of several minor bands is seen between 1930 to 1975 cm^{-1} . In some active site mutants (Fig. 2a and b), multiple small bands of the heme-bound CO clearly indicate the coexistence of several discrete active-site conformations. The most prominent band is shifted to different extents with respect to that of wt CerHbCO, indicating changes in the electric field surrounding the bound ligand. The most dramatic shift is observed for the ThrE11Ala and ThrE11Val mutants (data not shown), which show large A substate bands at 1932 and 1937 cm^{-1} , respectively, indicating a strong hydrogen bond donation from the TyrB10 side chain (Pesce et al., 2004).

Multiple photoproduct bands appear between 2100 and 2160 cm^{-1} , representing photodissociated CO ligands at intermediate sites after 1-s illumination at 3 K (Fig. 2c - d) and slow cooling under constant illumination from 160 to 3 K (Fig. 2e - f). From the difference spectra alone we cannot decide if the bands are associated with one or several discrete docking sites. Hence, TDS spectra were collected (*vide infra*) to help resolve this ambiguity. Point mutations in the distal pocket near the active site result in significant shifts and marked splitting of the photoproduct bands (Fig. 2c and e, Table 1), suggesting that transient ligand docking sites are within or very close to the distal pocket.

3.2. Ligand Docking Sites in wt CerHbCO

The occurrence of multiple photoproduct bands in wt and mutant CerHbCO suggests that several intermediate docking sites exist (Pesce et al., 2004). In the simplest case, we would expect two photoproduct bands for each intermediate site, representing opposite orientations of the ligand in each particular site. The active site electrostatic field will have differential effects on the bond order and stretching frequency of these orientational conformers, causing Stark splitting of the IR absorption of photodissociated CO. To discriminate between different orientations at one site and occupancy of various sites, we continuously recorded transmission spectra while illuminating the sample at a constant temperature. The spectra were referenced against a transmission spectrum collected in the dark to calculate the photolysis difference spectra. In Fig. 3, we present the photoproduct spectra obtained after ~2, 10, 10^2 , 10^3 and 10^4 s of illumination for two different laser powers and four different temperatures.

Upon photolysis with reduced laser power (0.3 mW) at 3 K, two photoproduct bands appeared at 2121.5 and 2126 cm^{-1} (Fig. 3a). Note that the band at 2121.5 cm^{-1} is larger than the one at 2126 cm^{-1} after two seconds; at longer times, it is smaller. This effect can be explained by an exchange between two bands due to rotation of the CO between two orientations at a single docking site, as has been observed earlier for MbCO (Lim et al., 1997; Nienhaus et al., 2003b; Nienhaus et al., 2003c; Nienhaus et al., 2005). We assign the two bands to the primary photoproduct site B, which is closest to the heme iron (Fig. 1c and d). The low frequency band most likely represents the conformer with the C atom of the CO adjacent to the iron. In this conformation, the negative partial charge of the Tyr hydroxyl O atom would be adjacent to the

ligand C atom, decreasing the CO bond order and stretching frequency. When the ligand flips, the ligand O atom would be pointing toward the negative partial charge of the Tyr side chain, stabilizing the $^+:\text{O}\equiv\text{C}:^-$ resonance structure, increasing the C-O bond order $\nu_{\text{C-O}}$. This conformer with the peak at 2126 cm^{-1} would also be the more stable one because of favorable electrostatic interactions.

Upon illumination at 3 K with full laser power (300 mW), these B state bands are largest after ~ 10 s of illumination; subsequently, they decrease again with concomitant appearance of two bands at 2131 and 2134 cm^{-1} (Fig. 3b). An isosbestic point at 2128 cm^{-1} suggests an interconversion between two discrete states. We associate the two new bands with a second docking site, denoted as C, which can only be populated efficiently at 3 K by exciting the protein molecules many times.

Upon photolysis at 30 K, the spectrum initially consists of a band at 2121.5 cm^{-1} , and only after prolonged illumination, the two bands associated with docking site C become populated (Fig. 3c). At the same time, the photoproduct band at 2121.5 cm^{-1} shifts to a slightly higher frequency. By observing rebinding in the dark after the extended illumination phase, we have observed very little rebinding in the low frequency side of the spectrum over the time course of 50,000 s. As we will show below in the TDS data, the majority of the B population should rebound within less than 100 s at 30 K, and therefore, only a small fraction of B substates from the high-barrier tail of the distribution still contributes at 30 K. The majority of photodissociated CO population at 2122 cm^{-1} must be associated with a new photoproduct site, D. The shift in this band during extended illumination can be explained by CO moving from B to C and D, so that less CO resides at site B at long times. The presence of a new photoproduct population is further supported by experiments with sample illumination at 50 and 70 K, where the dominant photoproduct band appears at 2123 cm^{-1} . Whereas a significant fraction of photodissociated ligands is still observable in site C at 50 K, essentially all photolyzed ligands are trapped in D at 70 K (Fig. 3e). We note here that the shift of the band associated with photoproduct site D with temperature may be a consequence of the heterogeneity of the band. Illumination at different temperatures causes different subpopulations to contribute to the overall photoproduct species (compare also the TDS data, *vide infra*). In addition, there will also be an intrinsic temperature shift of the CO absorption.

3.3. TDS Experiments after 1-s Illumination at 3 K

To disperse the recombination processes according to their rebinding enthalpy barriers, TDS experiments were started at 3 K immediately after photodissociation for 1 s. The contour plots in the left and right columns of Figs. 4 and 5, respectively, show the absorbance changes in the bands of heme-bound and photolyzed CO.

A cursory look at Figs. 4 and 5 confirms that the mutation of amino acids close to the active site markedly affects ligand recombination. In the A substate map of wt CerHbCO, the main contours are at 1982 cm^{-1} (Fig. 4a); the peak at 1938 cm^{-1} is due to a small fraction of the $^{13}\text{C}^{16}\text{O}$ isotope. Recombination is maximal at the lowest temperatures measured and extends to $>60\text{ K}$. A “wiggle” appears at $\sim 50\text{ K}$. In the photoproduct map (Fig. 4b), we see recombination from the photoproduct state B represented by the bands centered at 2126 cm^{-1} at very low temperature. In contrast, the contour lines at 2132 cm^{-1} and 50 K correspond to the “wiggle” in the A substate map and represent recombination from intermediate state C with a significantly higher enthalpic barrier against rebinding.

The contour maps of GlnE7Leu are similar to those of wt CerHb (Fig. 4c, d). Recombination from site B is also pronounced at the lowest temperatures and exhibits a maximum at 18 K. A small fraction of ligands has escaped to site C and rebinds at $\sim 38\text{ K}$. In the A substate of GlnE7Phe, recombination is represented by an increase of the band at 1987 cm^{-1} and 15 K. It

is accompanied by a loss in population from photoproduct site B with bands at 2123 and 2117 cm^{-1} (Fig. 4e, f). Compared to GlnE7Leu, more ligands have escaped to site C, as indicated by the more pronounced contours at 2132 cm^{-1} . Remarkably, rebinding from site C already starts at temperatures <20 K.

Dramatic changes with respect to wt CerHbCO are visible in the TDS maps of mutant ThrE11Ala. Rebinding in the main A substate peak at 1932 cm^{-1} has a maximum at 35 K and extends to >100 K (Fig. 2a, Fig. 4g). Besides the tiny population at 1982 cm^{-1} that is barely discernable above the detection threshold, there is a small fraction represented by the peak at 1967 cm^{-1} , which increases from 3 to 30 K, but then surprisingly decreases again above 30 K. We emphasize that this species contributes with mere 11% to the overall A substate spectrum and is only so clearly visible in the map because of the logarithmic level spacing. Four photoproduct bands at 2110, 2123, 2136 and 2144 cm^{-1} appear below 10 K (Fig. 4h). The bands at 2127 and 2133 cm^{-1} are associated with site C; they gain intensity at 10 and 15 K due to migration of CO into this site, as seen from the black contours. Rebinding from site C sets in at temperatures >20 K. Pesce *et al.* (Pesce *et al.*, 2004) described similar changes in the TDS spectra of the ThrE11Val mutant, indicating that the loss of hydrogen bonding between TyrB10 and the ThrE11 hydroxyl group markedly changes the electric field at the docking site and the enthalpy barriers for interconversion and ligand recombination at the iron atom.

Replacement of ValB6 by alanine or tryptophan has little effect on ligand rebinding as compared to wt CerHb (Fig. 5a, b). The A state maps of both mutants display rebinding up to ~ 50 K (Fig. 5c, d). The photoproduct maps indicate recombination mainly from site B. In contrast, the contour plots of the PheB9Ala mutant reveal extensive ligand migration to site C already after brief illumination (Fig. 5e, f), suggesting that the PheB9 side chain is part of the barrier against migration from B to C. Ligand rebinding to form the dominant A substate at 1984 cm^{-1} peaks at 55 K and extends to >100 K. Additional bands at ~ 1961 and ~ 1980 cm^{-1} show a rebinding peak at 10 K. The contours of the main photoproduct band at 2132 cm^{-1} extend from 30 to 100 K and thus are assigned to recombination from site C (Fig. 5f). From 20 to 50 K, rebinding occurs from a doublet of bands at 2123 and 2127 cm^{-1} , and presumably from docking site B.

The TyrB10Ala mutant (Fig. 5g) displays multiple bound-state conformations. Rebinding to the dominant A state at 1977 cm^{-1} has two poorly separated maxima at 10 and 25 K. Additionally, we note a shoulder on the low-frequency side extending to 1950 cm^{-1} that contributes mostly around 20 K. A second well-separated A substate band at 1993 cm^{-1} also peaks at 20 K. Multiple photoproduct bands centered at 2125 and 2136 cm^{-1} indicate rebinding from various intermediate B and C sites at temperatures up to 50 K (Fig. 5h). This heterogeneity is almost certainly a result of the greater space available to the ligands upon replacement of the Tyr side chain with the methyl group of alanine.

The A state map of TyrB10Phe is similar to the one of wt CerHb, but the main absorption band is shifted down by 13 cm^{-1} and is broader (Fig. 5i) due to loss of the hydroxyl group on the benzyl side chain. The photoproduct map shows rebinding from both the B and C sites associated with bands at 2123 and 2134 and 2130 and 2136 cm^{-1} (Fig. 5j).

3.4. TDS Experiments after Slow-cool Illumination

To populate transient docking sites C and D to a large extent, all samples were cooled from 160 to 3 K under steady illumination. The photolysis difference spectra in the spectral range of heme-bound CO reveal only changes in amplitude, the photolysis yields under these conditions are close to 100% (except for ValB6Ala and ValB6Trp, with photolysis yields of 78 and 35%, respectively). The photoproduct spectra after slow cooling (Fig. 2e - f) vary less than after 1-s illumination (Fig. 2c - d). The photoproduct bands separate into two subgroups,

with frequencies of $2120 - 2125 \text{ cm}^{-1}$ and $2130 - 2135 \text{ cm}^{-1}$. The high-frequency group is associated with CO trapped at site C, the low frequency side belongs to CO in site D. The exact stretching frequencies are included in Table 1.

For an unambiguous assignment, we examined ligand recombination from these various photoproduct states after slow-cool illumination using FTIR-TDS experiments (Figs. 6 and 7). In general, the contours extend to higher temperatures, $\sim 150 \text{ K}$ instead of $\sim 80 \text{ K}$. The presence of more than one maximum for a given A substate band indicates that secondary sites have indeed been populated. Due to their very different enthalpy barriers, rebinding occurs at markedly different temperatures. All TDS contour maps are plotted in Figs. 6 and 7.

In wt CerHbCO (Fig. 6a), recombination is complete by $\sim 140 \text{ K}$. The A substate contours suggest minor recombination from site B at $\sim 10 \text{ K}$; more ligands rebind from site C at 40 K , and most rebind from intermediate D, peaking at 80 K . In the photoproduct map (Fig. 6b), rebinding from site B is too weak to be visible. The negative contours of the doublet of bands at 2131 and 2134 cm^{-1} appear from the lowest temperature up to $\sim 70 \text{ K}$, indicating ligand recombination from C. A tiny positive peak at 2130 cm^{-1} and 10 K signals rotation of the CO in site C, leading to a gain in this band and a concomitant loss of the 2134-cm^{-1} band of the Stark-split doublet. The feature at $\sim 2123 \text{ cm}^{-1}$, visible between 30 and 130 K , is associated with rebinding from site D, with a maximum at 80 K . Black contours at 2128 cm^{-1} and 50 K indicate a gain of population in photoproduct site D, presumably due to migration of CO from C to D.

Instead of discussing the contour maps of the mutant samples individually, we have focused on the integrated absorption differences, $\int \Delta \text{Adv}$, calculated from the A state TDS maps after extended illumination as a function of temperature (Fig. 8). The spectral information in the concomitant photoproduct maps enables us to assign the different features in the integrated absorption to recombination from sites B, C and D, indicated by black, blue and red arrows, respectively.

As discussed above in detail, rebinding in wt CerHb occurs mainly from site D at 80 K , and only minor signals are related to recombination from sites B and C (*upper panel* Fig. 8). In contrast, in ValB6Trp, recombination from site B clearly dominates (*lower panel* Fig. 8). The A substate map in Fig. 7c for this mutant shows additional rebinding above 50 K , which amounts to $\sim 30\%$ of the entire photoproduct population. There are two peaks at 70 and 100 K , yet the photoproduct map suggests that both should be assigned to recombination from docking site D, whereas site C is not occupied in the ValB6Trp mutant, presumably because the large indole side chain fills the site associated with the C state (Fig. 1). By contrast, a significant fraction of ligands is trapped in site C in ValB6Ala, and also in PheB9Ala. The GlnE7Phe and TyrB10Ala mutants do not show rebinding from site D at all. In the latter mutant, the rebinding process from site C peaks already at 20 K . In GlnE7Leu, there is very minor rebinding from site D, as indicated from the weak signal at 2126 cm^{-1} and 70 K in Fig. 6d and the shoulder in Fig. 8. The TyrB10Phe mutant has a lower peak temperature for recombination from site D, 65 K as compared to 80 K .

3.5. Ligand Association and Dissociation at Ambient Temperature

Nerve and body wall CerHbs have O_2 affinities similar to mammalian Mbs (Pesce et al., 2004). However, their bimolecular association and dissociation rate coefficients are both 10-fold larger than those of muscle Mbs (Table 2). The rate coefficients for O_2 dissociation from wt nerve and body wall CerHbs are also remarkably large ($k_{\text{O}_2} = 200 - 500 \text{ s}^{-1}$) compared with most other O_2 binding proteins containing polar groups in their distal pockets (Pesce et al., 2004). To elucidate the structural determinants of these specific ligand binding properties and to complement the low temperature rebinding experiments, we measured the O_2 and CO

association, dissociation and equilibrium coefficients for all of the CerHb mutants included in Table 1. The results are listed in Table 2.

As the heme-bound ligand resides in the center of an active-site hydrogen bonding network involving amino acid residues B10, E7 and E11, mutations at these locations are expected to affect ligand association and dissociation by changing the accessibility of the iron atom, stabilizing or destabilizing electrostatic interactions, and altering the size and shape of the ligand docking sites B, C, and D.

All of the active site mutations produce changes in the overall ligand binding rate and equilibrium coefficients. The largest changes occur for the ThrE11Val and ThrE11Ala mutations, which result in a 1,000 fold decrease in the rate coefficient for O₂ dissociation and a 200-fold increase in O₂ affinity. These effects were previously shown to be due to rotation of the TyrB10 hydroxyl proton away from the E11 side chain and toward the bound O₂ to form a strong hydrogen bond, which markedly stabilizes the bound ligand (Pesce et al., 2004).

Surprisingly, the TyrB10Ala and TyrB10Phe mutations cause only small changes in the overall ligand binding parameters. The largest effects are a 3-fold increase in k_{O_2} and a 3-fold decrease in k_{CO} , suggesting that a small amount of the TyrB10 conformer with hydrogen bond donation to the bound ligand occurs in the O₂ complex but that the opposite conformer dominates the CO complex, thereby destabilizing the bound ligand. Thus, loss of the Tyr OH increases the rate of O₂ dissociation but decreases the rate of CO dissociation.

The GlnE7 mutations result in either decreases or no change in the oxygen dissociation rate coefficient, suggesting that there is little hydrogen bonding with bound O₂. The PheB9 mutations cause heterogeneous bimolecular CO binding and the formation of hemichrome structures, indicating that the phenyl side chain plays an important role in maintaining a stable active site. The ValB6Ala mutation has little effect on the overall rate and equilibrium coefficients, whereas the ValB6Trp mutation causes ≥ 10 -fold decreases in both the association and dissociation rate coefficients for O₂ binding, indicating that the large indole side chain hinders entry to and exit from the CerHb active site.

4. DISCUSSION

4.1. Active Site Conformations

The active site of CerHbCO is characterized by an extensive hydrogen-bonding network that surrounds the heme-bound ligand (Pesce et al., 2002; Pesce et al., 2004). A hydrogen bond donation from the TyrB10 hydroxyl to the ThrE11 hydroxyl places the negative partial charge of the free electron pair of the TyrB10 side chain hydroxyl in close vicinity to the O atom of the bound CO. This conformation favors a resonance structure with higher bond order and a partial positive charge on the O atom and gives rise to the very high stretching frequency of the heme-bound CO ($\nu \sim 1982 \text{ cm}^{-1}$) at both cryogenic and room temperatures.

A recent molecular dynamics and quantum mechanics simulation has suggested that an equilibrium exists between two substates of wt CerHb with markedly different oxygen affinities, but rather similar CO affinities (Marti et al., 2006). These substates differ in the position of the hydrogen atom of the TyrB10 hydroxyl, which is either pointed at the second atom of the heme-bound ligand or at the ThrE11 hydroxyl oxygen. When CO is bound, FTIR spectroscopy shows that only the latter conformation, with the free electron pair pointing toward the bound ligand, occurs in wt CerHb. CO forms only weak hydrogen bonds with amino acid side chain donors and, as a result, hydrogen bond donation by TyrB10 to the O atom of the ThrE11 hydroxyl dominates. In contrast, bound O₂ can compete with the ThrE11 hydroxyl O atom for hydrogen atom donation by the TyrB10 side chain. Therefore, a small fraction of

Tyr side chains does appear to hydrogen bond to the bound O₂. A loss of this interaction increases the rate of O₂ dissociation roughly 2-fold for the TyrB10Ala and TyrB10Phe mutants.

In the absence of ThrE11, the TyrB10 side chain and the hydroxyl proton are free to rotate toward the heme-bound ligand and form a strong hydrogen bond, as seen in the x-ray structure of the ThrE11Val mutant and the dramatic decrease in the O₂ dissociation rate coefficient (Pesce et al., 2004). This positive partial charge markedly increases the back-bonding donation in the FeCO complex and shifts the major ν_{C-O} peak of the ThrE11Ala and ThrE11Val mutants to 1932 (Fig. 4g) and 1937 cm⁻¹, respectively (Pesce et al., 2004). Fig. 4g also shows a tiny population barely above threshold in ThrE11Ala, with the high frequency peak of wt CerHbCO. The other minority species at ~1967 cm⁻¹ is adopted by only ~10% of the mutant molecules (Table 1). Both minor species appear to represent conformations of TyrB10 with the hydroxyl proton pointing away from the bound CO ligand. Whereas the first minority population must have an active-site structure very similar to wt CerHbCO, the second one has a frequency close to that of a heme-bound CO in the absence of additional interactions with distal residues (Braunstein et al., 1993; Li et al., 1994). Presumably, the small side chain of AlaE11 allows TyrB10 to rotate deeper into the heme pocket, away from the bound ligand.

The appearance of negative contours in the A state TDS maps of ThrE11Ala CerHbCO (Figs. 4g and 6g) is quite remarkable and deserves an extra remark. In Mb and many other heme proteins, the A bands have been interpreted to represent different conformational substates with a different overall molecular structure (Frauenfelder et al., 1991; Nienhaus and Young, 1996). Usually, there is no population exchange between A substates at temperatures below the glass transition of the cryosolvent where structural readjustments are greatly inhibited. To the best of our knowledge, the ThrE11Ala CerHbCO mutant provides a first example of an A substate exchange in the temperature regime of a solid cryosolvent enclosing the protein. The small population transfer from the band at 1967 cm⁻¹ to the band at 1932 cm⁻¹ reflected by the negative contours in Figs. 4g and 6g indicates rotation of the of the TyrB10 hydroxyl proton toward the bound CO in a small fraction of molecules, which apparently requires little or no movements of the rest of the protein and the solvent.

The replacement of either PheB9 or TyrB10 by alanine creates a more spacious distal pocket and allows for multiple orientations of the other active-site side chains, as can be inferred from the occurrence of several heme-bound CO stretching bands (Fig. 2a). Most remarkable is the presence of high-frequency bands. TyrB10Ala CerHbCO displays a band at the extremely high frequency of 1993 cm⁻¹, which indicates that a negative charge is located close to the bound ligand, perhaps due to the electron pairs of the O atom of an internal water molecule, which fills the space vacated by the large TyrB10 side and donates hydrogen atoms to the ThrE11 hydroxyl O atom and the O atom of the GlnE7 amide side chain. This explanation seems plausible because replacement of TyrB10 by phenylalanine yields a single A state band at 1969 cm⁻¹, a value that is characteristic of a hydrophobic pocket and insignificant interactions between CO and distal residues. The line width of the 1969-cm⁻¹ band of TyrB10Phe CerHbCO is increased compared to that of the 1987 cm⁻¹ band of wt CerHbCO, indicating a larger flexibility of the Phe side chain, most likely because of lack of attachment to the ThrE11 hydroxyl.

The active site of ValB6Trp CerHbCO exists in two conformations. The minor conformer is similar to wt CerHb, whereas in the dominant conformation of the ValB6Trp mutant, the TyrB10 appears to be pushed away from the heme-bound CO, reducing its stretching frequency to a value indicative of an apolar environment. No effect on the A-state was observed for the ValB6Ala mutation.

Replacement of GlnE7 with apolar amino acids causes shifts of the A bands to higher frequencies as compared to wt CerHbCO (Table 1). In both the GlnE7Leu and GlnE7Phe mutants, the negative charge from the TyrB10 hydroxyl is still pointed at the ligand, but the positive partial charge from the GlnE7 side chain is replaced by the positively charged edge of the PheE7 phenyl side chain or the totally apolar LeuE7 side chain.

4.2. Ligand Docking Sites in CerHb

The FTIR-TDS experiments have provided solid evidence for ligand recombination from a primary docking site B and two more remote secondary sites C and D (Figs. 4 - 6). Structural assignments of these sites cannot be made solely on the basis of the spectroscopic data but can be inferred by also considering the effects of mutagenesis and the crystal structure of CerHb. There are two lines of evidence indicating that all three photoproduct sites are located in the distal pocket and not further away in the hydrophobic channel. (i) FTIR/TDS data on mutant samples with bulky amino acids introduced along the channel (TyrE14Trp, IleE15Trp, LeuG12Phe, LeuH9Phe) all feature CO in photoproduct site D, and no new photoproduct sites were observed for these mutants (data not shown). (ii) The kinetic data in Table 2 show that both O₂ association and dissociation rate coefficients are decreased by introducing a bulky Trp at position B6, suggesting that residue B6 blocks access to the hydrophobic channel. In addition, the ValB6Trp mutation markedly inhibits ligand escape through the channel (data not shown). Thus, our finding that photoproduct site D can be significantly populated by extended illumination in this mutant implies that this D site is within the distal cavity and not within the hydrophobic channel.

Sites B and C are more easily accessible to photodissociated CO, and thus we assume that they are close to the active site. We have probed the x-ray structure of CerHbCO (Pesce et al., 2002) for the presence of cavities by using the program VMD (Humphrey et al., 1996). A sphere of 1.5 Å radius was used to find “empty” volumes within the protein (Fig. 1c and d). Three possible cavities are shown in Fig. 1 that are large enough to host a CO molecule. In the following sections, the effects of mutagenesis on the FTIR data were used to assign the individual B, C, and D photoproduct states to these structural features.

4.3. Primary Docking Site B

In wt CerHbCO, maximum recombination from site B occurs even at the lowest temperatures examined, 3 K (Fig. 4). The average enthalpy barrier to CO recombination is very low, $\sim 2.2 \pm 0.2$ kJ/mol (Pesce et al., 2004), which together with the sensitivity of the B state bands to amino acid changes at the active site suggests that the primary docking site is located very close to the heme iron and almost certainly coincides with volume B in Fig. 1b. The doublet of CO stretching bands for state B (Fig. 3a) arises from a vibrational Stark effect of the CO dipole. The photodissociated CO appears to be present in two orientations, one with the C atom pointing back toward the iron and the TyrB10 hydroxyl O atoms and one with the O atom pointing back toward these atoms. The local electric field at the docking site causes only small differential changes in the bond order and ν_{C-O} values of these orientational conformers, increasing stability, bond order, and stretching frequency of the CO–Fe isomer and decreasing the stability, bond order, and stretching frequency of the OC–Fe isomer (Lim et al., 1997; Kriegl et al., 2003; Lehle et al., 2005).

In wt CerHbCO, the Stark splitting of 4.5 cm^{-1} is significantly smaller than in MbCO, where it is 12 cm^{-1} due to the presence of the distal histidine. In Fig. 1b, we have overlaid the active sites of Mb and CerHb and included the CO ligand at the B site of Mb (pdb code 1ABS). It is apparent that in wt CerHbCO, the TyrB10 side chain is held away from docking site B due to the hydrogen bond to ThrE11. Consequently, its effect on the Stark splitting of the CO vibration is expected to be much smaller and is only readily visible when examining the dependence on

illumination time in Fig. 3a. In MbCO, the Nε-H of HisE7 provides a positive partial charge adjacent to the bound and photodissociated ligand and causes opposite Stark effects on the CO-Fe (lower frequency) and OC-Fe (higher frequency) conformers (Nienhaus et al., 2005). In the case of wt CerHbCO, both B state peaks (2121.5 and 2126 cm⁻¹) disappear quickly upon heating indicating very small enthalpy barriers to interconversion as well as rebinding. In contrast, there is a larger barrier to the CO-Fe (B₂, 2119 cm⁻¹) to OC-Fe (B₁, 2131 cm⁻¹) interconversion in Mb, and this interconversion is required before the ligand in state B₂ can rebound to the iron atom.

In CerHbCO mutants ThrE11Ala and ThrE11Val, the splitting of the B state peaks is much larger, 34 and 19 cm⁻¹, respectively. B state splittings of comparable size have been observed in experiments with the MbCO triple mutant YQR, which has a similar active-site architecture, including TyrB10, GlnE7, and ValE11 (Lamb et al., 2002; Lamb et al., 2004). For MbCO, cryocrystallography (Schlichting et al., 1994; Teng et al., 1994; Hartmann et al., 1996) and time-resolved crystallography (Bourgeois et al., 2003; Schotte et al., 2003) have shown that CO in the primary docking site resides on top of pyrrole ring C of the heme. In MbCO YQR, the positive partial charge of the proton on the Tyr hydroxyl is almost certainly responsible for the large Stark splitting of the CO stretching band into B₁ (OC-Fe, 2142 cm⁻¹) and B₂ (CO-Fe, 2119 cm⁻¹). A comparable Stark splitting appears to occur in the initial photoproduct of aliphatic ThrE11 CerHbCO mutants, which supports our interpretation that state B represents photodissociated CO lying on pyrrole ring C, parallel to the heme plane in a location similar to that found in Mb. As in wt MbCO, the CO in the low-frequency B₂ state of the Mb mutant YQR does not rebound directly but first rotates into the B₁ orientation and then rebounds, or migrates to photoproduct site C (Brunori et al., 1999; Brunori et al., 2000; Lamb et al., 2002; Lamb et al., 2004). These observations on wt and mutant Mbs and DFT calculations were the basis of our interpretation that the CO points with the oxygen atom toward the heme iron in the B₂ substate of Mb, so that it cannot form the Fe-C bond without rotating first into the B₁ substate with the C atom adjacent to the heme iron (Nienhaus et al., 2003c; Nienhaus et al., 2005). In the ThrE11Ala CerHbCO mutant, the small B₂ population at 2110 cm⁻¹ vanishes below 20 K, whereas the decay of B₁ at 2142 cm⁻¹ is observed up to ~50 K (Fig. 4h). By analogy with the wt and YQR MbCO results, we assume that B₂ exchanges into B₁ below 20 K, but the concomitant gain of the B₁ population is overcompensated by the loss due to strong rebinding already at the lowest temperatures. In wt and YQR MbCO, rebinding is weak below 20 K so that the population gain in B₁ is nicely visible (Lamb et al., 2002; Nienhaus et al., 2005). Therefore, the CO orientations in the ThrE11 CerHbCO mutants appear to be analogous to those of wt and YQR MbCO, both in terms of ν_{C-O} values and enthalpy barriers.

4.4. Secondary Docking Site C

The FTIR-TDS data of the PheB9Ala CerHbCO mutant (Fig. 5e and f, Fig. 7e and f, Fig. 8) show that photodissociated ligands preferentially occupy the secondary C site, even after brief illumination at 3 K. The occupancy of site C is also enhanced in the CerHbCO ValB6Ala mutant compared to wt CerHb (Fig. 8). Both mutations enlarge the open volume between residues B9, E15 and G12, which we have assigned to state C in Fig. 1c and d. This cavity is similar to site C (Xe4) in MbCO (Ostermann et al., 2000). In the case of the PheB9Ala mutation, the steric barrier to migration from B to C appears to be reduced considerably, accounting for the large population in this secondary intermediate after 1-s illumination at 3 K. In mutant ValB6Trp, the bulky indole side chain should occlude this cavity, and consistent with this interpretation, the photoproduct state C is completely suppressed in this CerHbCO mutant. Thus, we propose that site C in CerHb is the open volume defined by amino acids B9, B10, E15 and G12 (Fig. 1c and d).

This structural assignment of state C in CerHb is supported by the results for the ThrE11 and TyrB10 mutants. Positive (black) contours are observed in the photoproduct maps of mutants ThrE11Ala (Fig. 4g) and ThrE11Val (Pesce et al., 2004) that can be associated with thermally activated ligand migration from B to C at ~20 K. A similar migration process from site B into the Xe4 cavity is present in Mb mutant YQR (Lamb et al., 2002).

4.5. Secondary Docking Site D

The D state is the preferred ligand docking site in wt CerHb at elevated cryogenic temperatures (80 – 160 K, but still below the glass temperature, Fig. 6a). Only tiny fractions of photolyzed ligands are still found in sites B and C after extended slow cool illumination (Fig. 8). Our initial interpretation was that the D state represented CO trapped in the first portion of the interior apolar channel between the G and E helices. Introducing a tryptophan at the ValB6 not only fills photoproduct site C but also blocks the inner entrance to the hydrophobic tunnel. However, photodissociation of the ValB6Trp CerHbCO mutant still results in substantial population of the D state, suggesting strongly that the ligand is not in the apolar channel, which is sealed by the TrpB6 side chain, but probably still near the active site in the space labeled D in Fig. 1c and d.

This structural assignment of site D is supported by the observation that rebinding from state D is particularly sensitive to modifications of GlnE7 and TyrB10, which form part of the walls of the cavity shown in Fig. 1c and d. Replacement of TyrB10 by phenylalanine reduces the peak rebinding temperature and thus the average enthalpy barrier for recombination from site D. Presumably the non-hydrogen-bonding phenylalanine is more flexible and allows easier migration of CO to the active site. No trapping of photodissociated CO in the D state occurs for the TyrB10Ala mutant, most likely because the loss of the large tyrosine side chain allows direct interconnection between the B and D sites, preventing trapping of ligands at site D. This behavior resembles the situation in Mb, where ligand trapping in the Xe4 cavity is only efficient with large aromatic amino acids at position B10 (Brunori et al., 2000; Ostermann et al., 2000; Bourgeois et al., 2003; Schotte et al., 2003). Trapping of ligands at site D is significantly reduced in the GlnE7Leu and GlnE7Phe CerHbCO mutants, presumably because the structure of the cavity is modified, becoming smaller in the case of GlnE7Phe. Our structural assignment of the D cavity is further supported by its lower occupancy in the ThrE11Val and ThrE11Ala mutants (Fig. 8). Replacement of ThrE11 by the isosteric Val allows the TyrB10 side chain to move away from the E helix and towards the heme iron, partially blocking the migration pathway to site D (Fig. 1a) (Pesce et al., 2001; Pesce et al., 2002). However, a smaller alanine at the E11 position appears to allow some movement to site D.

4.6. Secondary Sites and Pathways for Ligand Entry and Exit at Ambient Temperature

Cavities of different sizes have been found in a number of heme proteins. In Mb, two cavities, Xe4 and Xe1, have been shown to play a crucial role as transient ligand storage sites C and D for dissociated ligands, respectively, thereby influencing rates of geminate ligand recombination and in the case of site C, rates of overall association and dissociation (Scott and Gibson, 1997; Scott et al., 2001; Nienhaus et al., 2003b). Under physiological conditions, ligands can shuttle back and forth between primary and secondary docking sites B and C on 0.1 – 10 nanosecond time scales, whereas equilibration with site D occurs on longer time scales, from 100 – 1000 ns. Site B is the only site from which bond formation occurs, and therefore, fast internal equilibration with sites C and D reduces the rate of geminate rebinding. Consequently, ligands can stay inside the protein without rebinding for much longer time periods. Blocking of internal Mb cavities C and/or D leads to a higher probability for the ligand to reside at site B and thus enhances the rate of geminate rebinding.

In CerHb, photoproduct site B also plays a key role as the site from which the bond to the heme iron is formed. Photoproduct site C also appears to be directly involved in the overall binding process. It is present in all the variants studied except for ValB6Trp, where C is almost completely blocked (Figs. 1 and 8). This mutation causes dramatic increases in the fraction of geminate recombination at room temperature, from ~ 0.05 to 0.75 for CO and from ~ 0.2 to ≥ 0.9 for O₂, data not shown). Consequently, only about 25% and $\leq 10\%$ of photodissociated CO and O₂, respectively, escape into the solvent. This mutation also causes marked decreases in both the overall association and dissociation rates of O₂ and CO binding. Taken together, these geminate and overall kinetic results suggest that cavity C plays an important role as a way station for ligands entering or exiting the protein through the apolar channel.

The physiological role of photoproduct site D is less clear. It is efficiently populated in wt CerHbCO and several mutants at cryogenic temperatures. However, there are no correlations between the appearance of ligands at this site and the overall ligand binding parameters at room temperature. For example, in TyrB10Ala and TyrB10Phe CerHbCO, photoproduct D is absent and present, respectively, but the overall association and dissociation rate coefficients for both CO and O₂ binding to these B10 mutants are essentially identical. It could even be that site D is too small to host ligands at room temperature or, alternatively, it is on a side pathway as is the case for the D or Xe1 site in Mb and has no influence on the overall ligand binding parameters. We have tried to measure the competition between CO and Xe for the internal cavities in wt CerHbCO by equilibrating samples with xenon gas, which has been shown to work very well for the Xe1 cavity in Mb (Nienhaus et al., 2003b). Interestingly, it was impossible to detect an effect of Xe on the occupancy of site D by photodissociated CO in wt CerHbCO, which implies that this cavity may be too small to accommodate xenon atoms at room temperature (Deng, 2005).

4.7. Summary

Photodissociated CO can occupy one of three docking sites adjacent to the distal pocket in CerHbCO. The primary docking site (state B) appears to be structurally similar to that observed in mammalian Mbs, with the photodissociated CO lying directly above pyrrole ring C and parallel to the heme plane. This site is easily accessible to ligands even at the lowest temperatures, whereas secondary site C becomes occupied only after photodissociation at high temperatures and/or long illumination times. Based on mutagenesis studies and analyses of the crystal structure of wt CerHbO₂, this site is assigned to a cavity surrounded by the B and E helices at internal opening, which connects the distal portion of the heme pocket to the apolar channel. It appears to be part of the pathway for ligand entry and exit. Blocking this cavity enhances the rate and extent of geminate rebinding and at the same time reduces the overall rates of ligand entry (association) and exit (dissociation). The D site has been assigned to a small cavity located above the active site surrounded by TyrB10, GlnE7, and the CD corner. It appears to be on a side pathway and only affects the speed of geminate rebinding but not the overall association and dissociation rate coefficients. Perhaps the most dramatic result is the extremely low enthalpy barrier to CO rebinding from site B in wt CerHb. These results, coupled with the effect of the mutations that open or block site C, indicate that the rate limiting step for ligand binding to CerHb is migration through the apolar channel between the G and E helices to site C. Movement from C to B and iron-ligand bond formation are very rapid processes with low energy barriers in the wt protein.

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Abbreviations

FTIR, Fourier Transform Infrared Spectroscopy; CerHb, *Cerebratulus lacteus* hemoglobin; CerHbCO, CO-ligated *Cerebratulus lacteus* hemoglobin; TDS, Temperature Derivative Spectroscopy; EDTA, ethylenediaminetetraacetic acid; CO, carbon monoxide.

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FIG. 1.

(a) Active site of wtCerHb, with amino acids GlnE7, ThrE11 and TyrB10 represented by stick models (pdb code 1KR7). The heme-bound oxygen is plotted in van der Waals representation. Residue TyrB10 of mutant ThrE11Val (pdb code 1V07) is plotted in white. The CO ligand at the Mb B site is included in panel (b). (c) Side and (d) top views of the open volumes B, C and D in wt CerHb probed by a 1.5 Å sphere.

FIG. 2.

FTIR photolysis difference spectra of CerHbCO mutants. (a, b) Absorption spectra of heme-bound CO after 1-s illumination at 3 K. (c, d) Photoproduct bands after 1-s illumination at 3 K. (e, f) Photoproduct bands after cooling from 160 to 3 K under constant illumination.

FIG. 3.

CerHbCO photoproduct spectra after 2, 10, 10², 10³ and 10⁴ s illumination at (a) 3 K (laser intensity attenuated with 3.0 OD filter), (b) 3 K, (c) 30 K, (d) 50 K, (e) 70 K. Dotted/bold line: initial/final spectrum.

FIG. 4.

TDS contour plots of wt CerHbCO and active site mutants after 1-s illumination at 3 K. Left column: Absorbance changes in the bands of heme-bound CO. Right column: Absorbance changes in the bands of photodissociated CO. Black/red contours indicate an absorbance increase/decrease, respectively. Contours are spaced logarithmically.

FIG. 5.

TDS contour plots of CerHbCO mutant samples after 1-s illumination at 3 K. Left column: Absorbance changes in the bands of heme-bound CO. Right column: Absorbance changes in the bands of photodissociated CO. Black/red contours indicate an absorbance increase/decrease, respectively. Contours are spaced logarithmically.

FIG. 6.

TDS contour plots of wt CerHbCO and active site mutants after slow cooling under light from 160 to 3 K. Left column: Absorbance changes in the bands of heme-bound CO. Right column: Absorbance changes in the bands of photodissociated CO. Black/red contours indicate an absorbance increase/decrease, respectively. Contours are spaced logarithmically.

FIG. 7.

TDS contour plots of CerHbCO mutant samples after slow cooling under light from 160 to 3 K. Left column: Absorbance changes in the bands of heme-bound CO. Right column: Absorbance changes in the bands of photodissociated CO. Black/red contours indicate an absorbance increase/decrease, respectively. Contours are spaced logarithmically.

FIG. 8.

Integrated absorbance differences $[\Delta A_{dv}]$ calculated from the A state TDS plots after extended illumination in Figs. 6 and 7. Black, blue and red arrows indicate ligand recombination from sites B, C and D, respectively.

TABLE 1
Stretching frequencies and fractions (in percent) of the absorbance bands of heme- bound and photolyzed CO in CerHb mutants, determined at 3 K.

Sample	ν (heme-bound CO) (cm^{-1})/ (%)	1-s illumination at 3 K		ν (photolyzed CO) (cm^{-1})/ (%)			
		A	B_2/B_1	C	B_2/B_1	C	D
Wild-type CerHb	1982		2122 (15) / 2126 (80)	2132 (5)		2131 (12) / 2134 (6)	2123 (81)/ 2128 (1)
Active site mutants							
ValB6Ala	1981 (100)		2122 (18) / 2126 (81)	2133 (1)	2126 (6)	2131 (18) / 2134 (26)	2123 (50)
ValB6Trp	1971 (72), 1981 (28)		2117 (5) / 2126 (95)	—	2127 (50)	—	2124 (50)
PheB9Ala	1961 (29), 1980 (40), 1984 (31)		2123 (46) / 2127 (8)	2132 (39) / 2137 (7)	2133 (48)		2124 (52)
TyrB10Ala	1962 (14), 1972 (30), 1977 (41), 1993 (15)			2136 (68) / 2142 (4) 2118 (3) / 2125 (25)	2136 (68) / 2141 (6)		2132 (20)
TyrB10Phe	1969 (100)		2122 (54) / 2135 (28)	2129 (9) / 2131 (9)	2127 (6)	2129 (6)	2124 (36) / 2134 (58)
GlnE7Leu	1993 (100)		2119 (15) / 2124 (72)	2133 (13)	2118 (5)	2131 (25)	2125 (19) / 2134 (51)
GlnE7Phe	1987 (100)		2117 (11) / 2123 (50)	2132 (35) / 2139 (4)	2118 (8) / 2124 (22)	2134 (38)	2131 (32)
Lys(E10)Trp	1983 (100)		2122 (33) / 2126 (42)	2131 (6) / 2133 (19)		2131 (11) / 2134 (11)	2123 (78)
ThrE11Ala	1932 (89), 1967 (11), 1982 (<1)		2110 (5) / 2144 (40)	2127 (29) / 2133 (26)		2134 (47)	2125 (42) / 2130 (11)
ThrE11Val	1937 (88), 1955 (6), 1963 (6)		2124 (43) / 2143 (34)	2131 (23)	2143 (8)	2131 (22) / 2134 (54)	2125 (16)

Overall rate coefficients for O₂ and CO binding at pH 7, 293 K. Association rate coefficients were measured using either rapid mixing or 300 ns laser photolysis techniques. The dissociation rate coefficients were determined from the analysis of rapid mixing, ligand displacement reactions. The errors in the rates and equilibrium coefficients are ~±20% and ~±35%, respectively, based on completely independent experiments with different starting mutant or wt samples.

TABLE 2

Sample	k'_{O_2} ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{O_2} (s^{-1})	K_{O_2} (μM^{-1})	k'_{CO} ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{CO} (s^{-1})	K_{CO} (μM^{-1})	M (K_{CO}/K_{O_2})
wt CerHb	240	180	1.3	28	0.048	580	450
ValB6Ala	250	280	0.9	35	0.033	1100	1200
ValB6Trp	35	16	2.2	12	0.0042	2900	1300
PheB9Ala	70 ^d (hexacoordination)	~40 ^b (hexacoordination)	~1.8	6.8 (55%) 2.2 (35%) 10% slower phases	0.02	~340 ^b	~190
PheB9Leu	130	~160	~0.8	12 (81%) 2.6 (13%) 6% slower phases	0.04	~300 ^b	~380
PheB9Trp	65	~8.5	~7.6	12 (89%) 2.6 (11%) No slower phases	0.008	~1500 ^b	~200
TyrB10Ala	120	440	0.3	33	0.015	2200	7300
TyrB10Phe	140	460	0.3	34	0.0081	4200	14000
GlnE7Ala	180	30	6.4	40	0.059	680	110
GlnE7Leu	200	96	2.0	42	0.017	2500	1200
GlnE7His	130	65	1.9	5.3	0.011	480	250
GlnE7Phe	23	170	0.10	1.6	0.038	42	420
LysE10	180	180	1.0	27	0.058	470	470
ThrE11Ala	41	0.26	160	2.6	0.0065	400	2.5
ThrE11Val	30	0.18	170	3.0	0.0070	430	2.5
ThrE11Trp	~4.3	~0.025	170	1.4	0.0035	400	~2.3

^aThe PheB9Ala mutant forms a very stable high spin, hexacoordinate ferric complex that reduces very slowly even in the presence of CO. The resultant ferrous protein shows markedly biphasic bimolecular CO rebinding but roughly monophasic O₂ rebinding after photolysis with a ~500 ns dye laser. The O₂ and CO displacement time courses were roughly monophasic. The equilibrium constants are approximate and were computed using the fast phase parameters for CO binding.

^bThe other B9 mutants had “normal” ferric spectra and were easily reduced. These proteins also showed more normal bimolecular CO kinetics, with a major phase (≥ 80%) showing a rate constant (~12 $\mu\text{M}^{-1}\text{s}^{-1}$) of only half that of the wild-type protein.