**Structural and Functional Properties of a Truncated Hemoglobin from a Food-borne Pathogen**

**Campylobacter jejuni**

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**Campylobacter jejuni** contains two hemoglobins, Cgb and Ctb. Cgb has been suggested to perform an NO detoxification reaction to protect the bacterium against NO attack. On the other hand, the physiological function of Ctb, a class III truncated hemoglobin, remains unclear. By using CO as a structural probe, resonance Raman data show that the distal heme pocket of Ctb exhibits a positive electrostatic potential. In addition, two ligand-related vibrational modes, $v_{\text{Fe-O}}$ and $v_{\text{O-O}}$, were identified in the oxy derivative, with frequencies at 542 and 1132 cm$^{-1}$, respectively, suggesting the presence of an intertwined H-bonding network surrounding the heme-bound ligand, which accounts for its unusually high oxygen affinity (222 $\mu$M$^{-1}$). Mutagenesis studies of various distal mutants suggest that the heme-bound dioxygen is stabilized by H-bonds donated from the Tyr(B10) and Trp(G8) residues, which are highly conserved in the class III truncated hemoglobins; furthermore, an additional H-bond donated from the His(E7) to the Tyr(B10) further regulates these H-bonding interactions by restricting the conformational freedom of the phenolic side chain of the Tyr(B10). Taken together, the data suggest that it is the intricate balance of the H-bonding interactions that determines the unique ligand binding properties of Ctb. The extremely high oxygen affinity of Ctb makes it unlikely to function as an oxygen transporter; on the other hand, the distal heme environment of Ctb is surprisingly similar to that of cytochrome $c$ peroxidase, suggesting a role of Ctb in performing a peroxidase or P450-type of oxygen chemistry.

Benefiting from the numerous genome projects, a wide array of new Hbs was recently discovered in various microorganisms (1–6). Among them, three groups of Hbs have been identified. The group one Hbs consist of flavohemoglobins (flavoHb) from bacteria and fungi (7–13). They contain a globin domain with a classical three-over-three $\alpha$-helical sandwich motif and a flavin-containing reductase domain that is covalently associated with the globin domain. The group two Hbs consist of single domain Hbs that comprise high sequence homology and structural similarity to the globin domain of flavohb (14–22). The group three Hbs consist of Hbs with 110–140 amino acid residues and a novel two-over-two $\alpha$-helical sandwich motif, which have been termed truncated Hbs (trHb) (23–31). trHbs are characterized by the absence of the A helix and the presence of an extended loop substituting for most of the F helix (32). On the basis of phylogenetic analysis, trHbs can be further divided into three subgroups as follows: trHbl, trHbII, and trHbIII (32). trHbl and trHbII have been studied extensively (see Refs. 1 and 33 and references therein). In contrast, little is known about trHbIII.

**Campylobacter jejuni** is a microaerophilic bacterium present in the gut of many food-supply animals and birds. It is one of the most important causes of bacterial gastroenteritis worldwide. Like several other pathogenic bacteria, C. jejuni contains more than one Hb, Cgb and Ctb, which belong to the group two and trHbIII class of Hbs, respectively. Neither of them is essential for growth under laboratory conditions, but both are up-regulated as components of a regulon responsive to NO and other nitrosative stresses (34). When cgb is knocked out in C. jejuni, the bacterium becomes hypersensitive to reactive nitrogen species. As such, Cgb has been proposed to protect the bacterium against NO attack (35), perhaps via an NO dioxygenase reaction. On the other hand, when ctb is knocked out in C. jejuni, the bacterium does not show extra sensitivity to nitrosative stress, but it is disadvantaged and has lowered respiration rates when grown under conditions of high aeration, suggesting a role of Ctb in moderating intracellular $O_2$ flux (35).

The proximal heme ligand of Ctb is a His at the F8 position (36, 81), which is perfectly conserved in the Hb superfamily. The amino acid residues at the B10, E7, E11, and G8 positions, which have been recognized as critical residues in stabilizing heme ligands in other Hbs, are occupied by Tyr, His, Ile, and Trp, respectively. In previous resonance Raman studies (36), the proximal iron-histidine stretching mode ($v_{\text{Fe-His}}$) of Ctb was identified at 226 cm$^{-1}$, indicating a structurally non-constrained proximal His ligand that was confirmed by the crystal structure (81). In the CO derivative, the $v_{\text{Fe-CO}}$ was found at 514 cm$^{-1}$, 7 cm$^{-1}$ higher than that of the sperm whale myoglobin (swMb), suggesting the presence of H-bonding interactions between the CO and the distal polar residue(s), Tyr(B10), His(E7), and/or Trp(G8) (36).
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The Trp(G8) is highly conserved in the trHbII and trHbIII classes of proteins. In the crystal structure of the trHbIII from either *Mycobacterium tuberculosis* (PDB code 1NGK) or *Bacillus subtilis* (PDB code 1UX8), the Trp(G8) side chain lies parallel and in contact with the heme plane; in addition, it forms an H-bond with the heme-bound ligand (31, 37). On the other hand, the His(E7)–Tyr(B10) pair is rarely observed in Hbs, because of possible steric interference of the two bulky side chains in the typically crowded distal heme pocket, as suggested by the studies of the B10Y mutant of swMb (38). Noticeably, in addition to the Tyr(B10)–His(E7) pair and Trp(G8), the E4, B9, and CD1 positions in Ctb are also occupied by bulky aromatic residues (Trp, Phe, and Phe, respectively), which are all highly conserved in trHbIII.

As a first step to investigate this peculiar distal heme environment of Ctb and how it may impact its function, we have systematically studied the structural properties of the CO and O2 derivatives of Ctb as a function of distal mutations, including B10_YF, E7_HL, and B10_YF/E7_HL, with resonance Raman spectroscopy. The functional implications were further evaluated by examining the oxygen dissociation and association kinetics of the mutants with respect to the wild type protein.

**EXPERIMENTAL PROCEDURES**

Ctb was expressed and purified as described previously (36). The E7_HL and B10_YF single mutants and B10_YF/E7_HL double mutant were prepared by using the Stratagene QuikChange site-directed mutagenesis kit. Primers were designed with the codon for the mutated amino acid in the center. They were as follows: B10_YF forward primer (5’-GCATAGCAAAACTCATGGAAATATTGTTTGGAAAAGTGAAGATAAGGATTAG-3’) and reverse primer (5’-CTAATCTCCTATCTTTTCTAATTTTTTC-AAATTTTCCATGAGTTTTGCTATGC-3’); E7_HL forward primer (5’-CAAGCGGTAGAG-AATGGAAGAGGATACCAACGAAAATAGGAAATTTTG-GGG-3’) and reverse primer (5’-CCCAAAATTTTCTATT-TGCTTATTGCTTCTTTCCATGCTTGTG-3’). A PCR was carried out on pBAD/His (Invitrogen) carrying the ctb gene using primers appropriate for the desired mutation. The PCR was then incubated with DpnI, an endonuclease specific for methylated DNA. The remaining nonparental mutated DNA was used to transform *Escherichia coli* XL1-Blue super-competent cells. The constructs were checked by sequencing. The mutants were purified in the same way as for wild type Ctb (36).

To prepare pure ferric derivatives, the protein samples were first treated with 3–5 eq of potassium ferricyanide (Aldrich) and then purified by G-25 column chromatography. To form 18O2 into an 16O2-bound protein solution, which was then purified by G-25 column chromatography. To form the CO-bound ferrous complexes, 12C18O (Tech Air, NY) or 13C18O (Icon Isotopes, Summit, NJ) was injected into sodium dithionite-reduced Ctb under anaerobic conditions. The 18O2-bound derivative was prepared by passing the CO-bound protein through a G-25 column to allow the exchange of CO with atmospheric 16O2 and to remove the excess of dithionite. The 18O2-bound sample, on the other hand, was prepared by injecting 18O2 into an 16O2-bound protein solution, which was freshly purged with argon, followed by spontaneous exchange of heme-bound 16O2 with 18O2. The concentration of the protein samples used for the Raman measurements was ~30 μM in pH 7.0 sodium phosphate buffer (100 mM).

Optical absorption spectra were taken on a spectrophotometer (UV2100U from Shimadzu Scientific Instruments, Inc., Columbia, MD) with a spectral slit width of 1 nm. The resonance Raman spectra were taken on instruments described elsewhere (1). Briefly, 413.1 nm excitation from a Kr ion laser (Spectra Physics, Mountain View, CA) was focused to an ~30 μm spot on the spinning sample cell. The scattered light was collected by a camera lens, dispersed through a polychromator (Spex, Metuchen, NJ), which is equipped with a 1200 grooves/mm grating, and detected by a liquid nitrogen-cooled CCD camera (Princeton Instruments, Princeton, NJ). A holographic notch filter (Kaiser, Ann Arbor, MI) was used to remove the laser scattering. Typically the laser power was kept at ~1 milliwatt, and the spectral acquisition time was 60 min. The cosmic ray spikes were removed by a standard software routine (CCD spectroscopic multichannel analysis, Princeton Instruments, NJ).

The Raman shift was calibrated by using indene (Sigma) and an acetone/ferrocyanide (Sigma) mixture as the references for the 200–1700 and 1600–2200 cm−1 spectral window, respectively.

For the O2 dissociation kinetic measurements, the reaction was initiated by injecting a small volume (~50–60 μl) of concentrated O2-bound protein into a sealed quartz cuvette containing ~850–900 μl of pH 7.4 buffer (50 mM Tris and 50 mM EDTA) with 500 or 1000 μM CO. The final concentrations of O2-bound protein and free oxygen were ~4–10 and 60 μM, respectively. The spontaneous replacement of the heme-bound O2 with CO, which was rate-limited by the dissociation of the O2, was monitored as a function of time at 422 and 405 nm. The reaction rates thus obtained were independent of CO concentration applied or wavelength monitored.

The O2 association kinetics were measured with a nanosecond laser flash photolysis system (LKS.60 from Applied Photo-Physics Ltd., Leatherhead, UK). In this system, the 532 nm output (~5 ns, 110 mJ) from a Nd:YAG laser (Brilliant B from Big Sky Laser Technologies, Inc.) was employed as the photoysis beam. The output from a 150-watt xenon arc lamp, at right angles to the photolysis beam, was used as the probe beam. The probe beam passed through a monochromator prior to reaching the quartz cuvette (4 × 10 mm with a 10-mm optical path) containing the sample. The light transmitted through the sample entered a second monochromator, which was synchronized with the first monochromator and was detected by a photomultiplier tube (1P28 from Hamamatsu Corp.). The signal from the photomultiplier tube was transferred to a digital oscilloscope (Infinium from Agilent Technologies) and then to a personal computer for subsequent analysis. Typically, five or six kinetic traces were averaged to obtain a satisfactory signal-to-noise ratio.

The O2 association reactions were initiated by flashing off CO with the photolysis beam in a freshly prepared mixture of a dithionite-free CO-bound complex with ~0.2 mM CO in the presence of various concentration of O2. The dithionite-free CO-bound complex was prepared by spontaneous replacement of the O2 in the oxy derivatives with CO by extensive purging.
with CO gas. Under the conditions applied, the rebinding of the CO to the heme iron was much slower than the O₂ association reactions, which was monitored at 440 nm following the photolysis.

RESULTS AND DISCUSSION

Fig. 1 shows the optical absorption spectra of the exogenous ligand-free ferric as well as the CO- and O₂-bound ferrous derivatives of the wild type Ctb. These spectra, which are typical for the Hb family of proteins, are not significantly affected by the mutations in the B10 and/or E7 residues (data not shown). In contrast, resonance Raman spectroscopy, which has been demonstrated to be much more sensitive to small structural perturbations in heme proteins (39–41), reveals several unique structural features of Ctb that are sensitive to the distal mutations and are distinct from other Hbs.

CO Derivatives—The top trace in Fig. 2 shows the resonance Raman spectrum of the CO derivative of the wild type Ctb. The 515 cm⁻¹ band shifted to lower frequency when ¹²C¹⁶O was substituted by ¹³C¹⁸O, as manifested by the isotope difference spectrum shown in the insert. This band was assigned to the Fe–CO stretching mode (νFe–CO) as reported previously (36).

Likewise, an isotope-sensitive band at 1936 cm⁻¹ was assigned to the C–O stretching mode (νC–O) (36).

In general, a positive polar environment destabilizes form (I) and facilitates the π back-bonding interaction, leading to a stronger Fe–CO bond and a weaker C–O bond (see Ref. 42 and references therein). On this basis, the νFe–CO and νC–O typically follow a well known inverse correlation as illustrated in Fig. 3A (43–45). Along these lines, it has been shown that the electrostatic field in the distal pocket of Mb quantitatively correlates with νFe–CO and inversely with νC–O (46).

In swMb, the heme-bound CO is stabilized by an H-bond donated from the His(E7), which leads to a νFe–CO at 507 cm⁻¹. On the other hand, the Hbs from microorganisms studied to date can be divided into two groups based on the electrostatic environment of the CO as follows: 1) those adopting two conformations with νFe–CO at ~490 and ~535 cm⁻¹, and 2) those
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displaying a single fixed conformation with $v_{Fe-CO}$ varying from ~490 to 525 cm$^{-1}$ (see Ref. 1 and references therein). Remarkably, all the Hbs belonging to the first group, including Hmp from E. coli, Vgb from Vitreoscilla, and trHbN from M. tuberculosis, have been suggested to perform NO dioxygenase reactions physiologically to protect the corresponding organisms from nitrosative stress (2, 27, 47). The two $v_{Fe-CO}$ modes in this group of Hbs are attributed to an open and a closed conformation, respectively (1). In the closed conformation, the heme-bound CO is stabilized by H-bonding interactions with a Tyr residue at the B10 position and a Gln residue at the E7 (e.g. Hmp) or E11 position (e.g. trHbN), whereas in the open conformation these H-bonding interactions are absent. We have postulated that the conformational flexibility of these microbial Hbs, as reflected by their ability to adopt the two conformations, is a structural feature that is important for the catalysis of the isomerization of the peroxynitrite intermediate to nitrate and/or the facilitation of the release of the nitrate product during the turnover of the NO dioxygenase reaction (1, 28, 78).

Evidently, Ctb belongs to the second group of Hbs with a fixed single conformation. The location of the data point in the inverse correlation line (Fig. 3A) suggests a distal environment with a medium positive electrostatic potential. Intriguingly, the mutation of the His(E7) to Leu causes the protein to convert to a wild type-like conformation with a medium positive electrostatic potential. Intriguingly, the inverse correlation line (Fig. 3) is a structural feature that is important for the catalysis of the isomerization of the peroxynitrite intermediate to nitrate and/or the facilitation of the release of the nitrate product during the turnover of the NO dioxygenase reaction (1, 28, 78).

The $v_{Fe-CO}$ of the B10YF/E7HL double mutant at 521 cm$^{-1}$ is significantly higher than that of the wild type protein (515 cm$^{-1}$), indicating that the heme-bound CO in the mutant is stabilized by an H-bonding interaction with the Trp(G8), the only polar residue remaining in the vicinity of the ligand as illustrated in Fig. 3B. On the other hand, the presence of a wild-type-like conformation in the E7$_{II}$ mutant indicates that the His(E7) in the wild type protein does not directly interact with the heme-bound CO. On the basis of these observations, we conclude that in the wild type protein the His(E7) forms an H-bond with the Tyr(B10) that limits the conformational freedom of the phenolic side chain of the Tyr(B10) and inhibits its direct interaction with the heme ligand as illustrated in Fig. 3C. The mutation of the His(E7) to Leu disrupts this H-bonding interaction and frees up the Tyr(B10) side chain, which can either stay in the native location (E7$_{II}$) or move to a new position (E7$_{II}$) to form a new H-bond with the heme-bound CO, along with the Trp(G8). The new H-bonding network in E7$_{II}$ leads to the astonishingly high electrostatic potential surrounding the CO, as indicated by the extremely high $v_{Fe-CO}$ at 557 cm$^{-1}$. To our knowledge, this frequency represents the highest $v_{Fe-CO}$ ever reported for heme proteins with His as the proximal heme ligand.

Similarly, in the B10Y$_{III}$ mutant the disruption of the H-bond between the Tyr(B10) and His(E7) to form a new H-bond with the heme-bound CO (Fig. 3C), thereby accounting for the 9 cm$^{-1}$ up-shift in the $v_{Fe-CO}$ with respect to the wild type protein. Likewise, the 6 cm$^{-1}$ up-shift in the $v_{Fe-CO}$ of the B10YF/E7HL double mutant is attributed to the rearrangement of the Trp(G8) side chain to a new stereo orientation that favors the formation of a more stable H-bond with the heme-bound CO (Fig. 3C).

In the B10Y$_{III}$ mutant of swMb, the simultaneous presence of the Tyr(B10) and His(E7) has been shown to introduce conformational heterogeneity to the protein because of steric interference between the side chains in the crowded distal pocket (38). In contrast, the naturally occurring Tyr(B10)–His(E7) pair in the leghemoglobin (LegHb) from the roots of leguminous plants avoids the steric problem by forming H-bonds with each other (49, 50). Similarly, the data reported here indicate that the H-bond between the His(E7) and the Tyr(B10) in Ctb (Fig. 3C) reduces the unfavorable steric interactions between the two residues and positions the Tyr(B10) in a proper stereo orientation for O$_2$ stabilization as will be discussed in more detail below.

It is important to note that although the H-bonds between the heme-bound CO and the Tyr(B10) and Trp(G8) residues are consistent with the crystallographic data of the cyano-met derivative, the H-bond between the Tyr(B10) and His(E7) residues is not observed in the structure (Fig. 3B). This difference indicates that the distal H-bonding network reported here is sensitive to the oxidation and coordination state of the heme iron, as reported for several other microbial Hbs (1).

$O_2$-bound Derivatives—Fig. 4 shows the resonance Raman spectrum of the $O_2$-bound wild type Ctb. Upon isotope substitution of $^{16}O_2$ with $^{18}O_2$, the two bands at 542 and 1133 cm$^{-1}$ shift to 506 and 1063 cm$^{-1}$, respectively (see the isotopic differ-
those in the B10YF/E7HL double mutant shift to 557/1144 cm

proximity (1).

The specific mutations are defined in Fig. 2. The 16O2-18O2 differ-
ence spectra shown in the right panel were obtained as described in Fig. 4.

FIGURE 5. Resonance Raman spectra of the O2-bound wild type (wt) and various mutants of Ctb and their associated 16O2-18O2 difference spectra. The specific mutations are defined in Fig. 2. The 16O2-18O2 difference spectra shown in the right panel were obtained as described in Fig. 4.

FIGURE 6. The νFe-O2 versus 1/νFe-O2 correlation curve of Ctb and pictorial illustrations of the O2-protein interactions in Ctb. The data in A were taken from Fig. 3. The distal residues of Ctb shown in B are as defined in Fig. 3. C and D, illustrate the overlap of the electronic orbitals of O2 and the heme iron in Ctb. The angles θ and φ define the bending angle of the Fe–O–O moiety and the dihedral angle between the Fe–O–O plane and the Fe–pyrrole bonds, respectively. The H-bonds are indicated as dotted lines. WT, wild type.

ference spectrum shown at the bottom), indicating that they are associated with the νFe–O2 and νO–O modes, respectively. The νO–O mode of heme proteins with His as the proximal ligand is typically not detected in the Raman spectrum. It was not experimentally observed until recently in several trHbI (trHbC and trHbP) and trHbII (trHbO) families of proteins (1, 28, 51). The appearance of the νO–O mode in the Raman spectra of these trHbs has been linked to an interlaced H-bonding network between the heme-bound O2 and the distal residues in its close proximity (1).

In the B10YF and E7HL mutants of Ctb, the νFe–O2/νO–O shift to 550/1139 and 552/1139 cm⁻¹, respectively (Fig. 5), whereas those in the B10YF/E7HL double mutant shift to 557/1144 cm⁻¹. It is noted that the negative peaks for the two modes associated with the 18O2 samples in the isotope difference spectra are in some cases significantly weaker, because of the difficulties in exchanging 16O2 in the oxy derivative with 18O2 (see “Experimental Procedures”). Nonetheless, all the 16O2-associated modes are clear and definitive. Markedly, all the νFe–O2 reported νFe–O2 and νO–O in fact has been reported for five coordinate (5C) O2-bound model heme complexes, although there is no clear correlation found in six coordinate (6C) O2-bound heme proteins (51, 52).

In general, the proximal oxygen atom of the heme-bound dioxygen donates the lone pair electrons in the sp² orbital to the d₅z orbital of the heme iron, constituting the σ-bond that leads to a Fe–O–O bond angle approaching 120°; in addition, the Fe–O bond is strengthened by π-back bonding from the iron d₇z orbital to the π* orbital of the dioxygen (Fig. 6C). Within protein matrices, these interactions are more intricate, because the amino acid residues lining the interior of the distal heme pocket may affect the tilting and bending of the Fe–O–O moiety as well as the relative orientation of the Fe–O–O plane with respect to the Fe–pyrrole bonds of the porphyrin.

On the basis of the structure shown in Fig. 3B, we hypothe-
size that the Trp(G8) in the wild type as well as the various mutants of Ctb forms an H-bond with the proximal oxygen atom of the dioxygen as illustrated in Fig. 6D, which, to a certain extent, restricts the position of the proximal oxygen atom, thereby minimizing the disturbance to the π-back bonding sys-
tem introduced by the distal residues. On the other hand, the stereo-orientation of the terminal oxygen of the heme-bound O2 is sensitive to its interactions with the B10 and E7 residues. Accordingly, the positive νFe–O2 versus νO–O correlation in Ctb may be attributed to the perturbation of the σ-bonding system because of the modifications of the bending angle (θ) of the Fe–O–O moiety and the dihedral angle (φ) between the Fe–O–O plane and the Fe–pyrrole bonds (Fig. 6D). This proposal is consistent with the observation that the isotopic shift of the νFe–O2 mode is sensitive to distal mutations (Fig. 5) and is relatively large with respect to that expected for a simple har-
monic diatomic oscillator (≈ 27–36 cm⁻¹ versus 24 cm⁻¹). It is noted that the latter signifies a significantly bent Fe–O–O moiety (55).

In swMb, the residues lining the distal heme pocket are mostly hydrophobic, except the His(E7), which stabilizes the heme-bound O2 by H-bonding to it. Despite the fact that the oxygen affinity of swMb is dramatically affected by the muta-
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...tion in the His(E7) residue, the \( \nu_{\text{Fe-O}_2} \) mode, typically at ~570 cm\(^{-1}\), is insensitive to it (56). In contrast, trHbs in general exhibit much higher oxygen affinity and a significantly lower \( \nu_{\text{Fe-O}_2} \) (554–560 cm\(^{-1}\)), both of which are sensitive to the mutations in the critical distal residues H-bonding to the heme ligand (especially those that directly interact with the proximal oxygen atom of the dioxygen) (1). The extremely low \( \nu_{\text{Fe-O}_2} \) of Ctb at 542 cm\(^{-1}\) represents the lowest \( \nu_{\text{Fe-O}_2} \) ever reported for heme proteins with His as the proximal ligand, consistent with a distal environment involving multiple H-bonding interactions that are unique to Ctb. To further investigate the protein-ligand interaction in Ctb, we examined its \( \text{O}_2 \) dissociation and association kinetics and their responses to the distal mutations.

**O\(_2\) Dissociation Kinetics**—The \( \text{O}_2 \) dissociation reactions were measured by spontaneous exchange of \( \text{O}_2 \) in the \( \text{O}_2 \)-bound protein with CO. Under the conditions applied, the exchange reactions were rate-limited by the dissociation of the \( \text{O}_2 \) from the heme iron (see “Experimental Procedures”). Fig. 7A shows the comparison of the \( \text{O}_2 \) dissociation kinetics of the wild type and the various mutants of Ctb. All the kinetic traces can be fitted with single exponential functions with the rate constants, \( k_{\text{off}} \) of the following order: E7HL (0.0003 s\(^{-1}\)) < B10\(_{\text{YF}}$/E7HL (0.0028 s\(^{-1}\)) < wild type (0.0041 s\(^{-1}\)) < B10\(_{\text{YF}}\) (0.0088 s\(^{-1}\)), as listed in Table 1. The astonishingly low \( k_{\text{off}} \) values of all the Ctb derivatives reported here manifest the unique structural properties of the distal heme pocket of Ctb. Especially interesting is the low \( k_{\text{off}} \) of the B10\(_{\text{YF}}$/E7HL double mutant, in which the heme-bound dioxygen is presumably solely stabilized by the Trp(G8). The data strongly support the proposal that the Trp(G8) plays a pivotal role in ligand stabilization in Ctb as suggested by the resonance Raman data discussed above.

Oxygen binding and dissociation in heme proteins can be simplified as a two-step reaction as illustrated in Fig. 7A (right panel). For the binding reaction, the \( \text{O}_2 \) in free solution first encounters the protein via a bimolecular binding process; it enters the protein matrix and then migrates into the active site, as described by the S \( \rightarrow \) B transition. Once the ligand is in the temporary docking site (the B state or the caged state), it may form a chemical bond with the heme iron, leading to the ligand-bound state A (the B \( \rightarrow \) A transition), or escape out of the protein matrix into the free solution (the B \( \rightarrow \) S transition). Conversely, during the dissociation reaction, the ligand bound to the heme iron in the A state dissociates from the heme iron by breaking the Fe–O\(_2\) bond thermally (A \( \rightarrow \) B), followed by escaping out of the protein matrix into the free solution (B \( \rightarrow \) S) or rebinding to the heme iron (B \( \rightarrow \) A). In this framework, the ligand binding and dissociation rate constants can be approximated by Equations 1 and 2 (57, 58).

\[
\begin{align*}
\kappa_{\text{on}} & = \kappa_{SB}/(\kappa_{BA} + \kappa_{BS}) \quad (\text{Eq. 1}) \\
\kappa_{\text{off}} & = \kappa_{AB}/(\kappa_{BA} + \kappa_{BS}) \quad (\text{Eq. 2})
\end{align*}
\]

The inner and outer energy barriers for ligand binding are defined by \( \text{TS}_{\text{inner}} \) and \( \text{TS}_{\text{outer}} \), respectively.

In swMb, the free energies of the \( \text{TS}_{\text{inner}} \) and \( \text{TS}_{\text{outer}} \), as well as those of the A and B states, are sensitive to the nature of the distal residues surrounding the heme ligand. Consequently, the mutations in the distal

**TABLE 1**

The oxygen association and dissociation rate constants \( (k_{\text{on}} \) and \( k_{\text{off}} \)) of Ctb, the oxygen affinities \( (K) \) calculated from \( k_{\text{on}}/k_{\text{off}} \) and the \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{O}_2} \) modes determined by resonance Raman spectroscopy.

| Species       | Protein | \( \nu_{\text{Fe-CO}} \) \( \text{cm}^{-1} \) | \( \nu_{\text{Fe-O}_2} \) \( \text{cm}^{-1} \) | \( \nu_{\text{O}_2} \) \( \text{cm}^{-1} \) | \( k_{\text{on}} \) \( \mu \text{s}^{-1} \) | \( k_{\text{off}} \) \( \text{s}^{-1} \) | \( K \) \( \mu \text{M} \) | Ref. |
|---------------|---------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|------|
| *C. jejuni*   | Ctb (WT)| 515                                         | 542                                         | 1132                                        | 9.1 \times 10^2                              | 0.0041                                      | 222                                         | This work |
|               | Ctb (B10\(_{\text{YF}}\)) | 524                                         | 552                                         | 1139                                        | 1.2 \times 10^3                              | 0.0088                                      | 14                                          | This work |
|               | Ctb (E7HL) | 517, 557                                   | 550                                         | 1139                                        | 4.9 \times 10^3                              | 0.0005                                      | 1885                                        | This work |
|               | Ctb (B10\(_{\text{YF}}$/E7HL) | 521                                         | 557                                         | 1144                                        | 4.2 \times 10^3                              | 0.0028                                      | 15,000                                      | This work |
| *M. tuberculosis* | trHB0 (WT) | 527                                         | 559                                         | 1140                                        | 1.3 \times 10^4                              | 0.0032                                      | 40                                          | 28 |
|               | trHB0 (B10\(_{\text{YF}}\)) | 527                                         | 559                                         |                                              |                                              |                                              |                                              | 28 |
|               | trHB0 (CD1YF) | 515                                         | 556                                         |                                              |                                              |                                              |                                              | 28 |
|               | trHB0 (CD1YF) | 500, 535                                   | 560                                         |                                              | 7.1 \times 10^4                              | 0.2                                         | 357                                         | 27, 39 |
| *Chlamydomonas* | trHB0 (B10\(_{\text{YF}}\)) | 502                                         | 570                                         |                                              |                                              |                                              |                                              | 39 |
| *Synechocystis* | trHB0 (WT) | 491                                         | 554                                         | 1136                                        |                                              |                                              |                                              | 51, 75 |
| *Paramacium* | trHB0 (WT) | 493                                         | 563                                         |                                              |                                              |                                              |                                              | 66 |
| *E. coli*     | Hmp (WT) | 494, 535                                    | 569                                         |                                              | 3.8 \times 10^7                              | 0.44                                        | 86                                          | 77, 78 |
| *Squid*       | Mb (WT)  | 507                                         | 569                                         |                                              | 1.4 \times 10^7                              | 12                                          | 1.1                                         | 79, 80 |

| \( ^a \) C. Lu and S.-R. Yeh, manuscript in preparation. |
residues cause the binding and dissociation constants to vary by a factor of $10^3$ (from $10^5$ to $10^8$ M$^{-1}$ s$^{-1}$) and $10^7$ (from $10^{-3}$ to $10^4$ s$^{-1}$), respectively (59). Flash photolysis studies of the O$_2$-bound swMb show $\sim$50% geminate recombination yield (i.e. $k_{BA}/(k_{BA} + k_{BS}) \approx 0.5$), suggesting that the inner energy barrier is comparable with the outer energy barrier (60). In contrast to swMb, our photolysis data show that the photodissociated O$_2$ in Ctb almost fully rebinds to the heme iron within 5 ns (data not shown), indicating that the inner energy barrier is much smaller than the outer energy barrier as illustrated in Fig. 7A.

As shown in Fig. 7B, $\Delta G$($k_{\text{off}}$) for O$_2$ inversely correlates with $v_{\text{Fe-CO}}$, which reflects the electrostatic force surrounding the heme ligand, with the B10/Y$_7$ mutant as the only exception. It is important to note here that only the more polar conformation of the E7/M$_{10}$ mutant with $v_{\text{Fe-CO}}$ at 557 cm$^{-1}$ was considered, because this conformation is expected to be the lowest energy state for the O$_2$-bound complex because of the polar nature of the Fe–O$_2$ moiety. The deviation of the B10/Y$_7$ data from the correlation line is plausibly because of the differences in the structures of the O$_2$- versus CO-bound derivatives. Considering the fact that $k_{\text{off}}$ depends on $k_{AB}$ as well as the branching ratio for ligand escape ($k_{BS}/(k_{BA} + k_{BS})$), the mutation does not only affect the equilibrium stability of the Fe–O$_2$ moiety (the A state), but it also influences the relative energy levels of the B, TS$_{inner}$, and TS$_{outer}$ states. Taken together, the data suggest that, in general, a positive polar distal environment in globins disfavors the O$_2$-iron bond breakage and O$_2$ escape from the protein matrix.

It is important to point out that although only key distal mutations were examined, the trend shown in Fig. 7B is very clear, and the data are fully reproducible. Similar positive correlations between $\Delta G$($k_{\text{off}}$) and the electrostatic potential of the heme pocket (as reported by $v_{\text{Fe-CO}}$) have been demonstrated in swMb and soybean LegHb mutants (46, 50), giving independent support for the correlation. The absence of any correlation between $\Delta G$($k_{\text{off}}$) and $v_{\text{Fe-CO}}$ (Table 1), on the other hand, is perhaps because of the fact that O$_2$ is not a good probe for the electrostatic potential as compared with CO, as pointed out by Olson and co-workers (46).

**O$_2$ Association Kinetics**—The bimolecular oxygen association reactions of Ctb cannot be studied by monitoring the re-binding kinetics of O$_2$ following direct photolysis of the oxy complex, because of its $\sim$100% geminate rebinding yield. To overcome this problem, the O$_2$ association reactions were measured by photolyzing CO from the CO-bound complex in the presence of various concentrations of O$_2$, with a nanosecond laser pulse (532 nm). It is noted that 0.2 mM CO is also present in the solution to maintain the integrity of the CO-bound reactant, and under the conditions applied, the rebinding of the CO to the heme iron is much slower than the O$_2$ association reactions, which were monitored at 440 nm following the photolysis.

Fig. 8A shows the comparison of the O$_2$ association kinetics of the wild type and various mutants of Ctb. The rate constants obtained from single exponential fits of the data are plotted as a function of O$_2$ concentration in Fig. 8B. The bimolecular rate constant, $k_{\text{on}}$, of the wild type protein thus obtained, 9.1 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$, is 15-fold slower than that of swMb (Table 1). The single mutation in the B10 or E7 residue reduces the on-rate by a factor of 8 or 2, respectively. On the other hand, the on-rate of the B10/E7 double mutant increases by a factor of 46 to 4.2 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$.

On the basis of the mutagenesis studies of swMb, Olson et al. (60) suggest that the bimolecular ligand association kinetics is controlled by two factors as follows: 1) the size of the free accessible space in the distal ligand binding site, based on entropic considerations, and 2) the stabilization of a water molecule in the distal site of the deoxy derivative, which has to be released prior to the binding of any exogenous ligand. Consistent with this argument, ligand association in swMb has been shown to be retarded by replacing apolar residues in the distal pocket with polar substitutes that stabilize the water molecule, or with bulkier substitutes that decrease the ligand-accessible space in the distal heme pocket.

In Ctb, the mutation of Tyr(B10) to Phe or His(E7) to Leu does not drastically change the size of the side chain group; however, the mutations are expected to destabilize distal water molecule(s), if there is any, thereby accelerating the association reaction. This is opposite to our experimental observations shown in Fig. 8B. As such, the mutation in either the B10 or E7 residue may cause movement of the B and/or E helix closer to the heme (as a result of the disruption of the H-bond between the B10 and E7 residues), which reduces the free accessible space in the distal pocket, thereby retarding the ligand binding. Conversely, the double mutation in the B10 and E7 residues increases the conformational freedom of the B and E helices, thereby providing larger accessible space for the ligand and facilitating ligand entry.

Collectively, the data indicate that the energy barrier for ligand association is mostly defined by steric factors, instead of electrostatic forces (as indicated by the lack of correlation between $\Delta G$($k_{\text{on}}$) and $v_{\text{Fe-CO}}$, shown in Fig. 7C). On the basis of
the free energy diagram shown in Fig. 7A, the steric energy barrier for the O₂ association is mainly associated with the S → B transition, similar to that reported for the G8 mutants of swMb (61).

**Oxygen Affinity**—The oxygen affinities (Kₒ₂) of the wild type and mutants of Ctb were calculated based on the ratio of kₐn versus kₐf. As listed in Table 1, the Kₒ₂ values are of the following order: B10YP (14 μM⁻¹) < wild type (222 μM⁻¹) < E7HL (1885 μM⁻¹) < B10YS/E7HL (15000 μM⁻¹). The much higher oxygen affinities of the wild type as well as the mutants of Ctb with respect to those of swMb derivatives (0.02 to 15 μM⁻¹) are conceivably a result of the unique stabilizing effect of the Trp(G8). Furthermore, the more than 3 orders of magnitude variation in oxygen affinity demonstrates the importance of the H-bonding interactions involving the Tyr(B10) and His(E7) residues.

As depicted in Fig. 6B, the data suggest that the heme-bound O₂ in the wild type Ctb is stabilized by H-bonding interactions with the Trp(G8) and Tyr(B10); furthermore, the Tyr(B10) is positioned in a constrained stereo orientation by an H-bond donated from the His(E7). The mutation of the His(E7) frees up the Tyr side chain, which rearranges to a new position to form a more favorable H-bond with the heme-bound O₂, leading to the 8-fold increase in the Kₒ₂. Likewise, the mutation in both the E7 and B10 residues allows the Trp(G8) to reposition itself to a new stereo orientation to form a more favorable H-bond with the O₂, causing the 68-fold increase in the oxygen affinity. On the other hand, the oxygen affinity of the B10YP mutant decreases by a factor of ~20, suggesting that in the absence of the Tyr(B10), the new H-bond between the His(E7) and the terminal oxygen atom of the dioxygen destabilizes the H-bonding interaction between the Trp(G8) and the heme-bound dioxygen.

**Ligand Discrimination**—For most Hbs studied to date, the affinity of O₂ is significantly lower than that of CO, although O₂ is the most abundant diatomic gas molecule in the atmosphere and is the most important physiological ligand. Recently, trHbO was recognized as the first naturally occurring Hb that exhibits much higher oxygen affinity with respect to CO, mainly because of its extremely slow O₂ dissociation rate as shown in Table 1 (62). The higher oxygen affinity with respect to CO has been attributed to the distal heme-pocket network involving Trp(G8) and CD1Tyr. Intriguingly, the trHbII from *B. subtilis*, which possesses Trp at the G8 position, but a Phe at the Tyr(B10) position, has also been demonstrated to display unusually high oxygen affinity (48), suggesting that the relatively high oxygen affinities of the trHbII family of proteins are primarily a result of the stabilization offered by the Trp(G8). Along these lines, our data show that Ctb exhibits higher affinity for O₂ as compared with CO (~17-fold), indicating that this peculiar oxygen stabilization and discrimination characteristic linked to the Trp(G8) is a general property for the trHbII and trHbIII class of proteins.

**Conclusion**—Our data indicate that there is no direct H-bond between the His(E7) and heme-bound ligand because if there were any H-bonds between the His(E7) and heme ligand, an increased oxygen off-rate would be expected for the E7HL mutant with respect to the wild type protein because of the loss of the H-bonding energy. Instead, we found that the oxygen dissociation rate constant is reduced by a factor of ~10 upon the mutation, indicating that the His(E7) must indirectly regulate the ligand affinity. On the basis of the crystal structure shown in Fig. 3B as well as the mutagenesis/Raman data shown in Figs. 2 and 5, it is most reasonable to assume an H-bond between the His(E7) and Tyr(B10).

We propose that the unusually high oxygen affinity of Ctb is a result of the strong H-bonding interactions between the heme-bound dioxygen and the Trp(G8) and Tyr(B10), which are consistent with those found in the crystallographic structure of the cyanide-bound ferric derivative shown in Fig. 3A. We hypothesize that these interactions are modulated by the additional H-bond between the His(E7) and the Tyr(B10), by positioning the phenolic side chain of the Tyr(B10) to a less favorable stereo orientation for H-bonding with the ligand (Fig. 6B). The unique H-bonding interaction between the His(E7) and Tyr(B10) offers a conduit for Ctb to fine-tune its oxygen affinity to a level that is optimum for the execution of its physiological function, which remains to be determined. It is important to note that this type of distal control mechanism is not unprecedented. A similar H-bonding interaction between the His(E7) and Tyr(B10) in LegHb, like Ctb, has been proposed to prevent overstabilization of heme-bound oxygen (50).

The role of H-bonding interactions in ligand binding and discrimination in Hb remains a major unresolved question in biology. In all the Hbs discovered to date, the distal heme ligand-binding site is universally made up by the B-E helix bundle and part of the G helix. In Mb, the heme-bound ligand is mainly stabilized by an H-bond donated by the His(E7) residue, as supported by the fact that the mutation of the His(E7) to Gly introduces more than a 100-fold increase in the oxygen dissociation rate (63). In nonvertebrate Hbs, more than one H-bond donor in the distal pocket often coexists, and their structural roles appear to be much more intricate. For example, a Tyr(B10)–Gln(E7) pair in AscHb from the nematode parasite *Ascaris suum* forms an interlaced H-bonding network stabilizing the heme-bound dioxygen, which leads to an extremely low oxygen dissociation rate (0.004 s⁻¹) and high oxygen affinity (64). Similarly, the Tyr(B10)–Gln(E7) pair present in several microbrial Hbs has been shown to be linked to high oxygen affinity (1).

Nonetheless, the same Tyr(B10)–Gln(E7) pair in CerHb, a Hb from *Cerebratulus lacteus* nerve tissue, does not seem to play the same stabilizing role, in view of the fact that the oxygen dissociation rate of CerHb is more than 4 orders of magnitude faster (180 s⁻¹) with respect to AscHb (65). The drastic increase in the dissociation rate has been attributed to an H-bond donated from the Tyr(B10) to the Thr(E11), which forces the phenolic oxygen of the Tyr(B10) to face the heme-bound O₂ (Fig. 9C), thereby creating a destabilizing negative electrostatic force around the heme-bound O₂ (65). This scenario is supported by the fact that the mutation of the Thr(E11) to Val in CerHb causes a 1000-fold decrease in the oxygen dissociation rate (65). Likewise, the Tyr(B10)–Gln(E7) pair is present in trHbP from the unicellular protozoan *Paramecium caudatum*; however, the oxygen dissociation rate is relatively fast (25 s⁻¹) (66). Like CerHb, the E11 position of trHbP is occupied by Thr, and as such, a modulation mechanism comparable with that of...
CerHb may account for the fast oxygen dissociation rate of trHbP. Evidently, the His(E7) in Ctb (Fig. 9A) plays a role analogous to the Thr(E11) in CerHb and trHbP and His(E7) in LegHb. In view of the fact that almost all the trHbs discovered to date exhibit at least three polar residues at critical topological positions in the distal heme pocket (1), this type of regulation mechanism may be more general than has been recognized.

Although the H-bond interaction we propose is not directly detected in our data, we believe that it provides the most reasonable scenario accounting for all the data presented in this work. However, because of the uncertainty associated with a conclusion drawn from indirect evidence, this hypothesis certainly warrants further investigation. Nonetheless, our Ctb data presented here demonstrate that it is the delicate balance of the various distal H-bonding interactions that leads to the unique ligand binding/discrimination properties of Ctb. We propose that the rearrangement of the H-bonding network in response to ligand binding is an integral part of the mechanism controlling the migration of the ligands within the protein matrix. In Mb, it is well accepted that the ligand association and dissociation pathway is controlled by the so-called “His(E7) gate” as well as several hydrophobic cavities, called “Xe sites” (67–73). Recently, crystallographic data of the trHbl group of proteins suggest that, instead of cavities, long hydrophobic tunnels connecting the distal pocket directly to the solvent may guide ligand entry and exit (33). These tunnels are not observed in trHbls (31) and Ctb (81), perhaps because of the fact that these Hbs comprise distal heme pockets congested with bulky aromatic amino acids. Although an His(E7) gate has been suggested to play a role in controlling ligand entry/exit in Ctb, based on the observation that two alternative conformations of the His(E7) side chain were found in the crystal structure (81), our kinetic data do not support this hypothesis.

Finally, we would like to point out that the polar nature of the distal heme pocket of Ctb is surprisingly similar to that of cytochrome c peroxidase (CcP) as illustrated in Fig. 9, A and B. In CcP, the side chain of the Trp-51 lies parallel and in contact with the porphyrin ring and forms H-bonds with both oxygen atoms of the heme-bound dioxygen. Another polar distal residue, Arg-48, also lies parallel with the porphyrin ring and interacts indirectly with the terminal oxygen of the heme-bound dioxygen via a water molecule. In addition, the His-52 positioned in-between Trp-51 and Arg-48 also forms an H-bond with the terminal oxygen atom of the heme-bound dioxygen. These three residues have been shown to play important roles in facilitating the deprotonation of hydrogen peroxide required for its binding and the subsequent heterolytic cleavage of the O–O bond, the key step in CcP catalysis (74). As may be seen in Fig. 9, A and B, the topological positions of the Trp-51, His-52, and Arg-48 residues in CcP are quite similar to those of the Trp(G8), Tyr(B10), and His(E7), respectively, in Ctb. As such, we hypothesize that Ctb is involved in CcP or P450-like oxygen chemistry physiologically. The high oxygen affinity of Ctb suggests that it is unlikely for Ctb to function as an oxygen transporter. Our preliminary studies also show that the B10E7E11 double mutant of Ctb, although not the wild type protein, exhibits heme oxygenase activity,3 consistent with the hypothesis Ctb is involved in oxygen chemistry. The unique chemical and structural properties of Ctb revealed in this work certainly warrants further investigation.

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