Structural and Functional Properties of a Single Domain Hemoglobin from the Food-borne Pathogen Campylobactor jejuni

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Campylobacter jejuni contains two globins, a truncated hemoglobin, Ctb, and a single domain hemoglobin, Cgb. The physiological function of Ctb remains unclear, whereas Cgb has been linked to NO detoxification. With resonance Raman scattering, the iron-histidine stretching mode of Cgb was identified at 251 cm\(^{-1}\). This frequency is unusually high, suggesting an imidazolate character of the proximal histidine as a result of the H-bonding network linking the catalytic triad involving the F8His, H23Glu, and G5Tyr residues. In the CO-complex, two conformers were identified with the \(v_{\text{C}-\text{O}}/v_{\text{C}-\text{O}}\) at 529/1914 cm\(^{-1}\) and 492/1963 cm\(^{-1}\). The former is assigned to a “closed” conformation, in which the heme-bound CO is stabilized by the H-bond(s) donated from the B10Tyr-E7Gln residues, whereas the latter is assigned to an “open” conformer, in which the H-bonding interaction is absent. The presence of the two alternative conformations demonstrates the plasticity of the protein matrix. In the \(O_{2}\)-complex, the iron-O\(_2\) stretching frequency was identified at 554 cm\(^{-1}\), which is unusually low, indicating that the heme-bound \(O_{2}\) is stabilized by strong H-bond(s) donated by the B10Tyr-E7Gln residues. This scenario is consistent with its low \(O_{2}\) off-rate (0.87 s\(^{-1}\)). Taken together the data suggest that the NO-detoxifying activity of Cgb is facilitated by the imidazolate character of the proximal F8His and the distal positive polar environment provided by the B10Tyr-E7Gln. They may offer electronic “push” and “pull,” respectively, for the O-O bond cleavage reaction required for the isomerization of the presumed peroxynitrite intermediate to the product, nitrate.

Three groups of hemoglobins (Hbs)\(^2\) have been identified in microorganisms: flavohemoglobins (FHbs), single domain Hbs (sdHbs), and truncated Hbs (trHbs) as shown in Scheme 1 (1). The FHbs contain a globin domain with a classical three-over-three \(\alpha\)-helical structure and an additional flavin-containing reductase domain covalently attached to it (2–6). The sdHbs share high sequence and structure homology with the globin domain of the FHbs. The trHbs, on the other hand, are much smaller; they contain \(-110\)–\(-140\) amino acid residues and exhibit a two-over-two \(\alpha\)-helical structure, which is characterized by the absence of the A-helix and the presence of an extended loop substituting for the F-helix (7, 8). On the basis of phylogenetic analysis, the trHbs can be further divided into three subgroups, trHb-I, trHb-II, and trHb-III (1).

The various classes of microbial Hbs may coexist in the same organism. For example, Mycobacterium tuberculosis contains a trHb-I (trHbN) and a trHb-II (trHbO) (8), Mycobacterium avium contains three trHbs, one from each subgroup (1), whereas Campylobacter jejuni contains a trHb-III (Ctb) and a single domain Hb (Cgb) (9, 10). These findings suggest distinct functions for each class of Hb. Despite their functional diversity, all microbial Hbs discovered to date contain a highly conserved tyrosine residue at the B10 position and a histidine residue at the F8 position (8). The distal histidine at the E7 position, which is important in stabilizing heme-bound dioxygen in mammalian globins, may be replaced by a variety of different polar or non-polar residues. Spectroscopic studies have shown that H-bonding interactions involving the distal polar residues at the B10, E7, E11, CD1, and/or G8 positions play an important role in regulating the ligand binding and functional properties of microbial Hbs (1, 6, 8, 10).

C. jejuni is a Gram-negative, obligatory microaerophilic bacterium. It is present in the gut of many food-supply animals and birds and is one of the leading causes of bacterial gastroenteritis worldwide. Neither Ctb nor Cgb are required for the survival of the bacterium in air (11, 12), but the \(O_{2}\) consumption rate of ctb knock-out mutant cells showed a 50% reduction as compared with the wild-type cells, suggesting the involvement of Ctb in regulating the flux of \(O_{2}\) into and within the cell (13). Nonetheless, the role of Ctb as a direct oxygen transporter has been

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2 The abbreviations used are: Hb, hemoglobin; CCP, cytochrome c peroxidase; Cgb, single domain hemoglobin from C. jejuni; Ctb, truncated hemoglobin III from C. jejuni; mCPBA, m-chloroperbenzoic acid; FHb, flavohemoglobin; Hmp, flavohemoglobin of E. coli; Mb, myoglobin; NOD, nitric-oxide dioxygenase; sHb, single domain hemoglobin; trHb, truncated hemoglobin; trHbC, truncated hemoglobin from green algae C. eugametos; trHbN, truncated hemoglobin I from M. tuberculosis; trHbO, truncated hemoglobin II from M. tuberculosis; trHBP, truncated hemoglobin from protozoan P. caudatum; trHbs, truncated hemoglobin from cyanobacterium Synechocystis sp. PCC 6803; Vgb, single domain hemoglobin from Vitreoscilla sp; HRP, horseradish peroxidase.
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Microbial Hb

FHb

sdHb

trHb

trHb-I

trHb-II

trHb-III

SCHEME 1

excluded due to its unusually slow O2 association and dissociation rates (9.1 \times 10^9 \text{M}^{-1} \text{s}^{-1} and 0.0041 \text{s}^{-1}, respectively) (10). The expression of Ctb can be induced by NO donors, although the Ctb knock-out mutant of C. jejuni does not display any sensitivity to nitrosative stress (12). On the other hand, resonance Raman studies show that the distal heme pocket of Ctb resembles that of cytochrome c peroxidase (CCP), suggesting a role for Ctb in performing oxygen chemistry (10). In contrast to Ctb, the Cgb knock-out mutant of C. jejuni is hypersensitive to reactive nitrogen species; furthermore, the expression of Cgb is strongly and specifically induced by nitrosative stress (9). Thus, Cgb, like the FHb from Escherichia coli (Hmp), is believed to function as a NO dioxygenase (NOD) to protect the bacterium against the toxic effects of NO (9).

The structural properties of Cgb, in contrast to its physiological role, are relatively unexplored. Here, we have overexpressed and purified Cgb from E. coli. We have examined the structural properties of Cgb by using resonance Raman spectroscopy and investigated its chemical reactivity toward peroxides and its ligand binding properties by using a stopped-flow system. We conclude that the structural and functional characteristics of Cgb are distinct from Ctb, consistent with the hypothesis that the two Hbs perform distinct physiological functions. On the other hand, albeit subtle differences, the properties of Cgb are comparable to those of Hmp from E. coli, in line with its potential function as a NO dioxygenase.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The cloning, expression, and purification of Cgb are described in detail elsewhere (9). Briefly, the plasmid containing the cgb gene was transformed into the BL21 (DE3) strain of E. coli. The starter cultures were grown overnight in LB medium supplemented with ampicillin (100 \mu g/ml). Starter cultures were then used to inoculate (at 1% v/v) 2-liter baffled flasks containing 500 ml of 2xYT (or LB) medium supplemented with 100 \mu M ampicillin, and 1 mM aminolevulinic acid/3 \mu M FeCl3, or 8.0 \mu M hemin. When the culture reached ~100 Klett units, 0.5 mM isopropyl-1-thio-

β-δ-galactopyranoside was added to induce the protein expression. After overnight growth at 37 °C, the cells were harvested and stored at −70 °C until used.

For purification, frozen cells were thawed, suspended in 50 mM Tris-HCl buffer (pH 8.0) and disrupted by a sonicator four times (20 s each) in an icy water bath with a 1-min chill in between. The cell debris was removed by centrifugation at 44,000 \times g for 20 min at 4 °C. The supernatant was passed through a 30-ml DEAE Sepharose Fastflow column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 8.0, followed by a 10-ml Butyl-Toyopearl 6505 column equilibrated with 1.5 mM ammonium sulfate in 50 mM Tris-HCl, pH 8.0. The protein thus collected was concentrated and further purified with a Superdex-200 column (Amersham Biosciences) equilibrated with 0.1 M NaCl in 50 mM Tris-HCl (pH 8.0). Pure Cgb was stored at −80 °C until used.

To avoid cyanide binding to Cgb (vide infra), we used an alternative purification method, starting with E. coli cells that had been disrupted by a French pressure cell, instead of a sonicator. This was accompanied by small modification in the subsequent purification procedures as follows. The cell debris was removed by centrifugation at 16,000 rpm for 30 min at 4 °C. The crude cell extract was purified with ammonium sulfate fractionation, followed by dialysis overnight against 10 mM Tris-HCl buffer with 50 \mu M EDTA, pH 8.0. The protein was then passed through a 400-ml anion exchange column (Whatman DE 52) equilibrated with 10 mM Tris-HCl buffer with 50 \mu M EDTA (pH 8.0). It was washed with 0.02 M NaCl in 10 mM Tris-HCl buffer and eluted with 0.05 M NaCl in 10 mM Tris-HCl buffer. The protein thus collected was concentrated with a Centricon and stored at −80 °C until used.

Preparation of the Various Derivatives of Cgb—For spectroscopic studies, Cgb purified after cell disruption by the French pressure cell was treated with 10-fold excess of potassium ferricyanide to ensure that the protein was in a homogeneous ferric state. The excess of ferricyanide and its reduction product thus generated were removed by a G25 column. The deoxy sample was prepared by first flushing the sample with Ar and then reduced with 5-fold molar excess of sodium dithionite. To prepare the CO-bound complexes, 12\text{C}18O or 13\text{C}18O was injected into the reduced Cgb under anaerobic conditions. The 16O2-bound derivative was prepared by passing the dithionite- or NADH-reduced protein through a G-25 column under aerobic conditions to remove the excess of dithionite or NADH and to allow atmospheric oxygen to bind to the protein. The 18O2-bound sample was prepared by injecting 18O2 into 16O2-bound protein solution, freshly purged with argon, and allowing the spontaneous exchange of the heme-bound 16O2 with 18O2. The cyanide complexes were formed by addition of ~20-fold excess of KCN or its various isotopic derivatives.

CO (Matheson purity) and Ar (99.999%) were obtained from Tech Air (New York). K13\text{C}14N, K12\text{C}15N, K13\text{C}15N, 18O2, and 13\text{C}18O were purchased from Icon Isotopes, Summit, NJ). All solutions were prepared with deionized (Millipore) water. Unless otherwise indicated, the Cgb protein samples were buffered with 50 mM Tris-HCl and 50 \mu M EDTA at pH 7.4.

Optical Absorption Spectroscopy—The optical absorption spectra were recorded by using a Shimazu spectrophotometer (Shimazu Co., Kyoto, Japan). All spectra were recorded at room temperature (~25 °C). The protein concentrations were ~1–10 \mu M.

Resonance Raman Spectroscopy—The resonance Raman measurements were carried out with previously described instrumentation (8). Briefly, the output at 413.1 nm from a krypton ion laser (Spectra Physics) was used as the excitation source, unless otherwise indicated. The laser line was focused on a quartz cuvette, constantly rotating at ~6000 rpm to avoid
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**RESULTS AND DISCUSSIONS**

Sequence Alignment—Phylogenetic analysis of Cgb showed that it shares 42% amino acid identity with Vgb (a sdHb from *Vitreoscilla* sp) and 33% identity with the globin domain of Hmp (the FHb from *E. coli*) (9), in contrast to the only ~10% identity with sperm whale myoglobin (swMb). As shown in Fig. 1, the proximal F8 residue in Cgb is a histidine and the distal B10, E7, E11, and CD1 residues, which have been shown to be critical for ligand-protein interactions in microbial Hbs, are Tyr, Gln, Leu, and Phe, respectively. These residues are fully conserved in Cgb, Vgb, and Hmp.

Purification of Cgb—On the basis of the reduced-minus-oxidized pyridine-hemochromogen spectrum (14), the isolated Cgb protein contains a noncovalently bound B-type heme (data not shown). The as-isolated protein prepared with the sonication method exhibits a Soret maximum at 417 nm and a broad visible band at ~542 nm (Fig. 2). To further characterize this
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FIGURE 3. Resonance Raman spectra of the as-isolated Cgb prepared with the sonication method as a function of time following the treatment with sodium dithionite under anaerobic conditions (A), and the comparison between the cyanide-bound protein with and without acid-treatment (B). In A, the top three traces are the spectra of the as-isolated protein in the absence and presence of K\(^{13}C^{15}N\) and their difference spectrum; the bottom trace is the \(^{12}C^{14}N-^{13}C^{15}N\) difference spectrum of the as-isolated protein pre-treated with acid as described in the text.

species, we measured its resonance Raman spectrum with Soret excitation at 413.1 nm.

Resonance Raman spectroscopy is a powerful method for the studies of hemeproteins, because excitation in resonance with the porphyrin \(\pi-\pi^*\) electronic transition selectively enhances vibrational modes of the heme and its bound ligands, without interference from the modes associated with the protein matrix (8). The spectrum in the high frequency region (1100–1700 cm\(^{-1}\)) consists of porphyrin in-plane vibrational modes, which are markers for the oxidation-state, coordination-state and spin-state of the heme iron (15). On the other hand, the spectrum in the low frequency region (200–1200 cm\(^{-1}\)) contains in-plane and out-of-plane vibrational modes of the porphyrin macrocycle, as well as modes associated with the ligands and the peripheral groups of the heme, including propionate and vinyl groups (16).

As shown in Fig. 3A, the \(\nu_2\), \(\nu_3\), and \(\nu_4\) modes of the as-isolated form of Cgb are identified at 1584 and 1505 and 1374 cm\(^{-1}\), respectively, characteristic of a six-coordinate low-spin (6CLS) heme. No changes in optical and Raman spectra were observed upon the treatment of the as-isolated protein with oxidants, such as ammonium persulfate or potassium ferricyanide, suggesting that the heme iron was in the ferric state. In contrast, treatment with sodium dithionite led to slow reduction of the heme to a five-coordinate high-spin (5CHS) derivative, as indicated by the appearance of the \(\nu_3\) and \(\nu_4\) modes at 1469 and 1353 cm\(^{-1}\), respectively, at the expense of the modes associated with the 6CLS species (Fig. 3A), consistent with the proposal that the as-isolated protein was in the ferric state. It is noted that the reduction did not reach completion even after a 5-day incubation under anaerobic conditions (Fig. 3A).

A number of Hbs, such as neuroglobin, cytoglobin, and synechocystis Hb, have been found in a 6CLS state, in which the distal histidine residue coordinates to the heme iron in the absence of exogenous ligand (17–20). To test if Cgb, like these Hbs, is coordinated by an intrinsic amino acid in the ferric state and if an exogenous cyanide ligand can compete with the presumed intrinsic ligand for heme iron binding, we treated the protein with 2 mM potassium cyanide overnight. Interestingly, no spectral changes were observed. On the other hand, we found that treatment with pH 3.6 acetate buffer (50 mM), followed by a G25 column chromatography and dialysis against pH 8.0 Tris buffer, caused the Soret and visible bands to shift to 398 nm and 501/641 nm, respectively, consistent with a 5CHS ferric heme as reported for Hmp and Vgb (3, 21). Moreover, it was found that the original spectrum of the as-isolated protein (Fig. 2) can be fully restored by exposing the acid-treated protein to potassium cyanide. These data strongly suggest that the as-isolated protein prepared by the sonication method is a cyanide-bound ferric derivative of Cgb. To confirm this hypothesis, the low frequency Raman spectrum of the \(^{13}C^{15}N\)-bound protein, generated by incubating the as-isolated protein with excess of \(^{13}C^{15}N\) (to allow for the free exchange of the presumed natural abundant \(^{12}C^{14}N\) pre-bound to the heme iron with \(^{13}C^{15}N\)), was obtained and subtracted from that of the as-isolated protein without the treatment. The resulting difference spectrum is almost identical to the \(^{12}C^{14}N-^{13}C^{15}N\) difference spectrum of the cyanide-bound protein prepared from the 5CHS ferric protein pre-treated with acid as described above (Fig. 3B), confirming that the as-isolated protein is indeed in a cyanide-bound ferric state.

Because the crude cell lysate from E. coli exhibits the same spectrum as that of the as-isolated protein, contamination due to the chromatographic procedures was ruled out. On the other hand, the presence of cyanide ion has been found in the cell extract from the alga Chlorella vulgaris Beijerinck, when it is disrupted by sonication but not by the French pressure cell (22). To test if the cyanide found in Cgb is of similar origin, we repeated the protein preparation by disrupting the E. coli cells with a French pressure cell, instead of a sonicator. As shown in Fig. 2, with this new method, the Cgb protein was isolated in a form with a Soret maximum at 405 nm and visible bands at \(-538, 576, and 638 nm\). Further spectral analysis indicates that the protein is in a mixture of the oxygen-bound ferrous and exogenous ligand-free ferric states, because the spectrum can be perfectly simulated by a linear combination of their corresponding spectra shown in Fig. 4B. Subsequent treatment of the Cgb sample with an oxidant, potassium ferricyanide, converted the protein mixture to a homogeneous ferric 5CHS species with Soret and visible bands at 397 nm and 501/641 nm, respectively (Fig. 4B), identical to the spectrum of the acid-treated protein sample prepared with the sonication method. The data confirm that the exogenous cyanide ligand is an artifact derived from the sonication procedure.
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described under “Experimental Procedures.” In addition, all the purified protein samples were oxidized by potassium ferricyanide and further purified with a G25 column prior to use to ensure the homogeneity of the samples. To reveal the structural properties of Cgb, the exogenous ligand-free ferric and ferrous derivatives as well as their cyanide and CO/O2 complexes, respectively, were studied with resonance Raman spectroscopy.

**Exogenous Ligand-free Derivatives**—The ν4, ν5, and ν4 modes of the ferric Cgb were identified at 1570, 1493, and 1371 cm\(^{-1}\), respectively, typical for a 5CHS heme (Fig. 4A). The ferric protein can be easily reduced to a 5CHS deoxy form with dithionite or NADH, as indicated by the shift of the Soret and visible bands from 397 and 501/641 nm to 432 and 555 nm, respectively (Fig. 4B). The resonance Raman spectrum of the deoxy form thus obtained shows ν4 and ν4 modes at 1470 and 1353 cm\(^{-1}\), respectively (Fig. 4A). The 1604 cm\(^{-1}\) band is assigned to the ν15 mode, a core size marker involving the Cα-Cα stretch, whereas the 1620 and 1627 cm\(^{-1}\) modes are assigned to the two νC=C stretching modes of the 2- and 4-vinyl groups (26). In the low frequency region (Fig. 5), the 361/372 cm\(^{-1}\) and 417 cm\(^{-1}\) modes are assigned to the out-of-plane propionate bending modes, δ(C\(_\beta\)C\(_\alpha\)C\(_\delta\)), and an in-plane vinyl bending mode, δ(C\(_\beta\)C\(_\alpha\)C\(_\delta\)), respectively. The strong band at 251 cm\(^{-1}\) is assigned to the proximal iron-His stretching mode (νFe-His), which is also present in the spectrum of the photoproduct of the CO-complex, when CO is photolyzed from the heme iron.

The frequency of the νFe-His mode of Cgb, like that of Hmp (244 cm\(^{-1}\)) (6), is much higher than that of most globins (typically 200–220 cm\(^{-1}\)) (8). The high frequency of the νFe-His mode in Hmp has been attributed to the imidazolate character of the proximal histidine due to the presence of a strong H-bonding network connecting the proximal F8His-H23Glu-G5Tyr residues making up the catalytic triad (6), as shown in Fig. 6A (27). The same H-bonding network is present in Vgb (Fig. 6B) (28), which also exhibits relatively high νFe-His frequency (252 cm\(^{-1}\)).4 Because the H23Glu and G5Tyr residues are conserved in Cgb (Fig. 1), the high νFe-His frequency found in Cgb is attributed to the same origin.

The ferric Cgb, like Hmp and Vgb, exhibits a 5CHS configuration, indicating that the distal ligand binding site of the heme is unoccupied, in contrast to the more commonly observed water-bound ferric heme in other globins (8). Like that proposed for Hmp (6), the absence of a water molecule bound to the heme iron of Cgb can be ascribed to the strong proximal Fe-His bond (as reflected by the high νFe-His frequency), which

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In addition to the alga *C. vulgaris* Beijerinck, several bacteria and plants have been described as being cyanogenic, *i.e.* to form hydrocyanic acid (HCN) (23, 24), although the source of cyanide remains unclear. Nonetheless, it has been suggested that cyanide is produced by oxidative reactions of amino acids (such as glycine or histidine) and, more interestingly, it was found that cyanide production is stimulated by the addition of peroxidases (22, 24). Similarly, it has been reported that endogenous cyanide can be generated in white blood cells and neuronal tissues, via a mechanism involving peroxidase activity (25). The fact that Cgb exhibits a peroxidase-like heme active site (*vide infra*) implies that the overexpression of Cgb in *E. coli* may facilitate cyanide generation, accounting for the presence of cyanide in the Cgb samples.

For the ensuing studies, the Cgb samples were prepared with the French pressure cell protocol with minor modifications as

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**FIGURE 4.** Resonance Raman spectra (A) and electronic absorbance spectra (B) of the various derivatives of Cgb at pH 7.4. The peak maxima in B are as follows: Fe\(^{2+}\), 432 and 555 nm; Fe\(^{3+}\), 397, 501, and 641 nm; Fe\(^{2+}\)-CO, 419, 538, and 565 nm; and Fe\(^{2+}\)-O\(_2\), 412, 541, and 576 nm.

**FIGURE 5.** Resonance Raman spectra of the deoxy derivative of Cgb in the low-frequency region under differing pH conditions. Inset, a plot of the percentage of the area under the 251 cm\(^{-1}\) peak versus the total area under the 229 and 251 cm\(^{-1}\) peaks as a function of pH.
of the H-bonding network linking the proximal catalytic triad. In addition to the modification in the $v_{Fe-His}$ mode, two modes at 377 and 392 cm$^{-1}$ are significantly enhanced at low pH, which are associated with small changes in several other heme modes in the 330–340 and 700–850 cm$^{-1}$ spectral regions, reflecting the pH-induced structural perturbations in the porphyrin ring as well as the two propionate groups of the heme. On the other hand, disturbance of the proximal catalytic triad by the mutation of the G5Tyr to Phe caused the protein to convert to a 6CLS form due to the coordination of an intrinsic amino acid to the heme iron (data not shown). Taken together the data indicate that H23Glu and G5Tyr in Cgb are important in maintaining the integrity of the protein matrix, as well as in modulating the electron donating capability of the proximal histidine heme ligand, which is important in providing electronic push for promoting the isomerization of the peroxynitrite intermediate to nitrate as suggested for Hmp (6).

**CO-bound Ferrous Complex**—The CO-derivative of Cgb displays a narrow Soret band at 419 nm and $\alpha/\beta$ bands at 565/538 nm (Fig. 4B). In the resonance Raman spectrum, the $\nu_2$, $\nu_3$, and $\nu_4$ modes are identified at 1584, 1498, and 1372 cm$^{-1}$, respectively (Fig. 4A), characteristic for a 6CLS heme. In the low frequency region of the resonance Raman spectrum (Fig. 7), the two peaks at 492 and 529 cm$^{-1}$ shift to 482 and 515 cm$^{-1}$, respectively, upon the substitution of $^{12}$C$^{16}$O with $^{13}$C$^{18}$O. They are assigned to two Fe-CO stretching modes ($v_{Fe-CO}$). Similarly, the two peaks at 1914 and 1963 cm$^{-1}$ in the high frequency region are assigned to two C-O stretching modes ($\nu_{C-O}$), as they shift to 1828 and 1873 cm$^{-1}$, respectively, upon the substitution of $^{12}$C$^{16}$O with $^{13}$C$^{18}$O.

The Fe-C-O moiety in hemeproteins normally exists in the following two extreme resonance structures due to the $\pi$ back-bonding from the iron to the CO, which donates electron density from the $d_p$ orbital of the iron back to the $\pi^*$ orbital of Equation 3.

$$L^{-}Fe^{2+}C=O^{a+} (I) \leftrightarrow L^{-}Fe=CO (I) \quad (Eq. 3)$$

Here, L represents the proximal ligand of the heme iron, which is a histidine in the case of globins. In general, a positive polar environment destabilizes form (I) and facilitates the $\pi$ back-bonding interaction, leading to a stronger Fe-CO bond and a weaker C-O bond. On this basis, the frequency of the $v_{Fe-CO}$ mode is typically inversely correlated with that of the $\nu_{C-O}$ mode in a linear fashion (8, 29, 30). The inverse correlation line is sensitive to the proximal ligand, such that the line for histidine coordinated species is distinct from that of thiolate-coordinated species and five-coordinate CO adducts. Because of its
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FIGURE 9. A plot of the ratio of the peak area of the ν_{Fe-CO} mode at 492 cm⁻¹ with respect to the sum of the peak areas of the ν_{Fe-CO} mode at 492 and 529 cm⁻¹ as a function of pH (A), and the "push-pull" model for the activation of the peroxynitrite-bound intermediate of Cgb during the NO dioxygenase reaction. The Hmp data in A are taken from Ref. 6.

Here the heme-bound peroxynitrite (Fe³⁺-OONO⁻) is a postulated intermediate, which isomerizes to form the product, nitrate (33). The ability of Cgb, Hmp, and trHbN to adopt a closed conformer with high electrostatic potential surrounding the heme ligand presumably is important for providing an electronic pull, in addition to the electronic push offered by the proximal imidazolate ligand, for facilitating the isomerization of the peroxynitrite intermediate to nitrate as illustrated in Fig. 9B. On the other hand, the conformational flexibility of these Hbs, allowing them to adopt an alternative open conformer, in which no direct electrostatic interaction can take place between the ligand and the protein matrix surrounding it, may be crucial for the efficient release of the nitrate product during the multiple turnover of the NOD reaction.

It is noticeable that the relative population of the open conformer with respect to the closed conformer in the CO derivative of Cgb is pH-insensitive, in contrast to that observed in Hmp which exhibits a pKₐ of ~8.3 as shown in Fig. 9A, suggesting that, despite their similarities, the specific intramolecular interactions distinguishing the open conformer from the closed conformer in these two Hbs are distinct. The presence of the Pro residue at the E8 position in Cgb, but not in Hmp (Fig. 1), may account for the absence of the pH dependence in the former, because the Pro ring could exhibit steric effects that alter the H-bonding interaction between the heme-bound CO and the B10Tyr-E7Gln pair.

In addition to the ν_{Fe-CO} and ν_{C-O} modes, a bending mode (δ_{Fe-C-O}) is identified at 583 cm⁻¹, which shifts to 560 cm⁻¹ upon the substitution of ¹³C¹⁶O with ¹⁵C¹⁸O (Fig. 7). Recently, Das et al. (34) recognized inverse correlations between δ_{Fe-C-O} and ν_{Fe-CO (ν_{C-O})} in hemoproteins with a histidine or cysteine as the proximal heme ligand. Because the δ_{Fe-C-O} mode is typically enhanced when the Fe-C-O is in a bent form (35), the bending mode of Cgb at 583 cm⁻¹ is assigned to the closed conformer with ν_{C-O} at 1914 cm⁻¹, in which the Fe-C-O moiety is presumably forced to be bent due to the distal H-bonding interactions exerted on it. This scenario is consistent with the fact that the Cgb data with δ_{Fe-C-O}/ν_{C-O} at 583/1914 cm⁻¹ lies from nitrosative stress (6, 31, 32). The NOD reaction is believed to proceed via the following mechanism in Equation 4.

\[
\text{Fe}^{3+} - O - O + \text{N} = \text{O} \rightarrow \left[\text{Fe}^{3+} - O - O - \text{NO}_3^-\right] \rightarrow \text{Fe}^{3+} + \text{NO}_3^- \\
\text{(Eq. 4)}
\]

sensitivity to the identity of the proximal heme ligand, as well as the distal environment, CO has been widely used as a structural probe for hemeproteins.

On the basis of the inverse correlation, the ν_{C-O} and ν_{Fe-CO} of Cgb at 529 and 1914 cm⁻¹ are assigned to a "closed" conformer, in which the heme-bound CO is stabilized by H-bond(s) donated from the B10Tyr-E7Gln pair, whereas those at 492 and 1963 cm⁻¹ are assigned to an "open" conformer, in which the H-bonding interaction is absent. The data points associated with the two conformers fall on the histidine correlation curve (Fig. 8), confirming the assignment of the proximal ligand as a histidine.

In general, the CO-derivatives of microbial Hbs can be divided into two groups: (I) those adopting two conformers with ν_{Fe-CO} at ~490 and ~530 cm⁻¹ for the open and closed conformers, respectively, and (II) those displaying a single fixed conformation with ν_{Fe-CO} varying from ~490 to 525 cm⁻¹ (8, 10). Remarkably, all the Hbs belonging to the first group, such as Cgb, Hmp, and trHbN, have been implicated in performing NOD reaction in vivo to protect the corresponding organisms...
on the $\delta_{\text{Fe-C-O}}$ vs $\nu_{\text{Fe-O}}$ inverse correlation line for heme proteins with a histidine as the proximal ligand (Fig. 8B).

Oxygen-bound Complex—The oxy complex of Cgb is stable for several months at $-20^\circ\text{C}$, in contrast to that of Hmp, which exists only transiently (3, 36). It exhibits a Soret maximum at 412 nm and $\alpha/\beta$ bands at 576/541 nm (Fig. 4B). In the low frequency Raman spectrum, the mode at 554 cm$^{-1}$ shifts to 527 cm$^{-1}$ upon the substitution of $^{16}\text{O}_2$ with $^{18}\text{O}_2$ (Fig. 10). It is assigned to the Fe-O$_2$ stretching mode ($\nu_{\text{Fe-O}_2}$), because the isotopic shift is consistent with that expected for a Fe-O$_2$ diatomic oscillator. On the other hand, although the $\nu_{\text{O-O}}$ mode is expected to be in the $\sim1120-1200$ cm$^{-1}$ region (10, 37), the positive and negative peaks at $\sim1130$ cm$^{-1}$ in the $^{16}\text{O}_2$-$^{18}\text{O}_2$ isotope difference spectrum is assigned as an artifact because the frequency shift is much smaller than that expected for an O-O oscillator.

The frequency of the $\nu_{\text{Fe-O}_2}$ at 554 cm$^{-1}$ is much lower than that of mammalian globins ($\sim570$ cm$^{-1}$), but is comparable to that of most other microbial Hbs as listed in Table 1 (8, 37). In microbial Hbs, the low $\nu_{\text{Fe-O}_2}$ frequencies have been attributed to the H-bonding interactions between the distal residues and the proximal oxygen atom of the heme-bound dioxygen (10, 38). Accordingly, the low $\nu_{\text{Fe-O}_2}$ frequency of Cgb is attributed to a similar origin, i.e. the H-bonding interaction between the distal B10Tyr-E7Gln pair and the proximal oxygen atom of the heme-bound dioxygen. This unique distal H-bonding interaction is presumably critical for the isomerization of the peroxynitrite intermediate to nitrate during the NOD reaction as illustrated in Fig. 9B.

Cyanide-bound Ferric Complex—Although Fe$^{3+}$-CN and Fe$^{2+}$-CO are isoelectronic, their chemical properties are distinct. As illustrated in Equation 3, the Fe$^{2+}$-CO bond is sensitive to the $\pi$-bonding interactions; in contrast, the Fe$^{3+}$-CN bond is dominated by the $\sigma$-bond donated from the 4$s$ and 5$d$ molecular orbitals of CN$^-$ to the $d_{z^2}$ orbital of the heme iron (39). Consequently, Fe$^{3+}$-CN is more flexible than Fe$^{2+}$-CO and they respond differently to steric and electrostatic influences exerted by the distal protein matrix surrounding the ligand in heme proteins. The higher flexibility of the Fe-C-N moiety is consistent with its lower binding frequency ($\sim350-460$ cm$^{-1}$) with respect to that of the Fe-C-O moiety ($\sim560-590$ cm$^{-1}$) (34, 40). Cyanide-bound ferric complexes of heme proteins can adopt either “linear” or “bent” conforma-

![FIGURE 10. Resonance Raman spectra of the $^{16}$O$_2$ and $^{18}$O$_2$-coordinated forms of Cgb, and the difference spectrum between them.](Image)

**TABLE 1**
The Raman and kinetic data of Cgb and other related heme proteins

| Protein | $\nu_{\text{Fe-His}}$ | $\nu_{\text{Fe-CO}}$ | $\nu_{\text{Fe-O}_2}$ | $\nu_{\text{O-O}}$ | $K_{\text{m,CD}}$ | $K_{\text{cat,CD}}$ | $K_{\text{m,CO}_2}$ | $K_{\text{cat,CO}_2}$ |
|---------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| sdHb Cgb | 5CHS | 251 | 492 | 529 | 554 | ND$^a$ | 0.024 | 150$^d$ | 0.87 |
| Vgb | 5CHS | 252$^e$ | | | | 0.19 | 31$^d$ | 200$^e$ | 4.2$^e$ |
| FHB | 5CHS | 244$^e$ | 494 | 535$^e$ | | 0.66 | 22$^e$ | 0.057$^f$ | 38$^a$ | 0.44$^a$ |
| trHbs | 6CHS+LS | 226$^e$ | 500 | 534$^e$ | | | | | |
| trHbO | 6CHS+LS | 226$^e$ | 525 | | | | | | |
| HRP | 6CHS+LS | 226$^e$ | 515 | | | | | | |
| CCP | 5CHS | 244$^{mm}$ | 541 | 519$^{mm}$ | | | | | |
| swHb | 6CHS+LS | 220$^e$ | 508$^{mm}$ | | | | | | |

$^a$ The 6C, 5C, HS, and LS stand for six coordinate, five coordinate, high spin, and low spin heme iron, respectively.

$^b$ ND, not detected.

$^c$ Ref. 49.

$^d$ Ref. 46.

$^e$ Ref. 38.

$^f$ To be published.

$^g$ Ref. 6.

$^h$ Ref. 56.

$^i$ Ref. 57.

$^j$ Ref. 11.

$^k$ Ref. 10.

$^l$ Ref. 8.

$^m$ Ref. 59.

$^n$ Ref. 8.

$^o$ Ref. 60.
tions. Moreover, in the “linear” conformation, the $\nu_{\text{Fe-CN}}$ frequencies of hemeproteins with histidine as the proximal ligand are typically higher than those of hemeproteins with a cysteine or tyrosine as the proximal ligand (e.g., P450s or catalase, respectively), because cyanide competes with the proximal ligand for the $\sigma$-bonding with the $dz^2$ orbital of the heme iron.

To examine the cyanide-associated vibrational modes of Cgb, the resonance Raman spectra of the ferric derivative of Cgb were obtained in the presence of $^{12}\text{C}^{14}\text{N}, ^{13}\text{C}^{14}\text{N}, ^{12}\text{C}^{15}\text{N}$ or $^{13}\text{C}^{15}\text{N}$, as shown in Fig. 11A. The various isotope difference spectra shown in Fig. 11B exhibit similar patterns as that reported for the cyanide derivatives of catalases and peroxidases (40–43). Accordingly, we assigned the bands at 440 and 403 cm$^{-1}$ to the Fe-CN stretching ($\nu_{\text{Fe-CN}}$) and bending modes ($\delta_{\text{Fe-CN}}$), respectively, of a linear Fe-C-N moiety, whereas the bands at 353 and 417 cm$^{-1}$ are assigned to the $\nu_{\text{Fe-CN}}$ and $\delta_{\text{Fe-CN}}$ modes, respectively, of a bent Fe-C-N moiety.

An additional cyanide isotope-sensitive mode found at 314 cm$^{-1}$ (Fig. 11B) is tentatively assigned to the Fe-His stretching mode ($\nu_{\text{Fe-His}}$). It is important to note that, although the $\nu_{\text{Fe-His}}$ mode is typically active only in the spectrum of a 5CHS ferrous heme with the iron located out of the heme plane, it has been identified in various 6C heme-proteins. The sensitivity of this mode to the cyanide isotope substitution is presumably due to its coupling to the distal Fe-CN stretching and/or bending modes. A similar assignment has been made for a 309 cm$^{-1}$ band in the cyanide complex of the Hb from Chironomus, which exhibits sensitivity to both CN and iron isotope substitution (44). Likewise, the bands at 315 and 311 cm$^{-1}$ in the cyanide complexes of the Chlamydomonas Hb and lactoperoxidase, respectively, were assigned to the same origin (40, 45).

The cyanide derivatives of all the globins reported to date exhibit only a linear conformer; in addition, their $\nu_{\text{Fe-CN}}$ frequencies are typically located at $\sim$452–458 cm$^{-1}$ (40), which are more than 10 cm$^{-1}$ higher than that of the linear conformer of Cgb reported here (440 cm$^{-1}$). On the other hand, most peroxidases, like Cgb, display both linear and bent conformers. These data indicate that, unlike conventional globins, Cgb has a distal pocket similar to peroxidases, in which the distal H-bonding interaction involving the B10Tyr-E7Gln residues weakens the Fe-CN bond and causes it to adopt an additional bent conformer.

Comparison with Hmp and Vgb—The resonance Raman data presented here show that Cgb shares two common structural features with Hmp and Vgb: (I) a proximal histidine with imidazolate character resulting from the conserved F8His-H23Glu-G5Tyr catalytic triad, as indicated by the unusually high proximal iron-histidine stretching frequency at 251 cm$^{-1}$, as well as the 5CHS configuration of the ferric protein, and (II) a polar distal environment with B10Tyr-E7Gln forming H-bond(s) with the heme-bound CO in the CO complex, as indicated by the presence of the closed conformer with high $\nu_{\text{Fe-CO}}$ at 529 cm$^{-1}$, and the plasticity of the protein matrix, as indicated by its ability to exist in an alternative open conformer with high $\nu_{\text{Fe-CO}}$ at 492 cm$^{-1}$.

Despite their similarities, Vgb exhibits structural signatures distinctive from Hmp on the basis of the crystal structures of their exogenous ligand-free ferric derivatives shown in Fig. 6: (I) the peptide segment connecting the B and E helices (amino acids 44–52) is disordered and unresolved; (II) the E7 to E11 region of the peptide adopts a coil-like structure, instead of a helical turn as that observed in Hmp; and (III) the side chain group of the E7Gln protrudes into the solvent, instead of pointing toward the heme propionate group as observed in Hmp. In any case, on the basis of the crystallographic data, a structural transition must take place upon ligand binding to Vgb and Hmp, perhaps Cgb as well, to enable the interaction between heme-bound ligand and the B10Tyr-E7Gln pair, as indicated by the resonance Raman data. This type of ligand-induced structural transition is plausibly facilitated by the plasticity of the protein matrix of these Hbs. Intriguingly, several properties of Cgb also set it apart from Hmp: (I) the relative population of the open conformer with respect to the closed conformer in the CO derivative of Cgb is pH-insensitive, whereas that in Hmp exhibits a $pK_a$ of $\sim$8.3 (Fig. 9A), (II) the O$_2$ complex of Cgb is stable, whereas that of Hmp spontaneously auto-oxidizes to the ferric state (3, 46). Taken together these data suggest that the specific structural elements controlling ligand protein interactions in Cgb, Vgb, and Hmp are distinct. It is noteworthy that the E7-E11 sequence, QPKAL, of Cgb (Fig. 1) is identical to that of Vgb, but is different from the QREAL sequence in Hmp. The sequence difference, especially the replacement of the E8Pro in Cgb/Vgb with the E8Arg in Hmp, may be at least partially responsible for the observed differences between Cgb/Vgb and Hmp.

Reactivity toward Peroxides—The imidazolate character of the proximal histidine ligand and the positive polar distal ligand environment of Cgb revealed by the resonance Raman data reported here are similar to those of peroxidases. In peroxidases, these structural characteristics have been shown to be important in catalyzing the O-O bond cleavage reaction of heme-bound peroxides, on the basis of the well-known “push-pull” model (47) as that illustrated in Fig. 9B. On this basis, we sought to investigate the peroxidase activity of Cgb by examining its reaction with hydrogen peroxide. For this work, the reaction was initiated by mixing Cgb with at least 5-fold excess of H$_2$O$_2$ in a stopped-flow instrument; the initial decay kinetics of the ligand-free ferric protein were monitored as a function.
of time following the mixing. Similar reactions were carried out with hhMb and Hmp as comparisons.

To our surprise, both Cgb and Hmp exhibit lower reactivity toward H₂O₂ as compared with hhMb. As shown in Fig. 12A, the apparent rate constants for all three globins vary linearly with the concentration of H₂O₂, and the bimolecular rate constants calculated based on the slopes are 95, 18, and 292 M⁻¹ s⁻¹ for Cgb, Hmp, and hhMb, respectively. Nonetheless, the 5-fold higher activity of Cgb as compared with Hmp is in line with the structural differences manifested in the resonance Raman data shown in Fig. 9A.

To further examine the peroxidase activity of the three globins, their reactions with an organic peroxide, m-chloroperbenzoic acid (mCPBA), were investigated. Intriguingly, the reactivities of Cgb and Hmp toward mCPBA are comparable, both of which are greatly enhanced as compared with their reactions with H₂O₂. The bimolecular rate constants of Cgb, Hmp, and hhMb, calculated based on the data shown in Fig. 12B are 4.1 × 10⁵, 4.9 × 10⁵, and 7.9 × 10⁴ M⁻¹ s⁻¹, respectively. The much higher reactivities of Cgb and Hmp toward mCPBA as compared with hhMb, demonstrate their potential function as organic peroxidases.

The high reactivity of Hmp toward organic peroxides has been reported for its ferrous derivative, but not the ferric derivative (48). On this basis, it was proposed that Hmp is involved in the repair of oxidatively damaged membrane lipids due to oxidative stress. Although the peroxidase activities of Cgb and Hmp discovered in this work are distinct from that reported for the ferrous derivative of Hmp, as the active species is a ferric heme, the data underscore the scenario that the active site of Cgb and Hmp are optimized to perform oxygen chemistry, rather than oxygen delivery.

**Ligand Binding Properties**—It has been reported that the O₂ association reaction of Cgb follows single exponential kinetics with a rate constant of 150 M⁻¹ s⁻¹, whereas the CO association reaction follows bi-exponential kinetics with rate constants of 45 and 0.19 M⁻¹ s⁻¹ (49). The dissociation rate constant of O₂ is determined to be 0.87 s⁻¹ in this work, as shown in Fig. 13A. It is similar to that reported in the literature (49). On the other hand the dissociation rate constant of CO is determined to be 0.024 s⁻¹ (Fig. 13B), which is 17-fold slower than that reported in the literature (49). To examine if the faster literature value is a result of a common artifact due to the photodissociation of CO by the probing light source during the stopped-flow kinetic measurements, we repeated the same measurements as a function of light intensity. We found that...
under our experimental conditions, decreasing the light intensity does not affect the rate of the reaction, whereas increasing the light intensity causes significant increase in the rate (Fig. 13B), confirming that the faster rate reported in the literature is an artifact due to the photodissociation of CO.

As listed in Table 1, the on-rates of O2 and CO in Cgb are ~10- and 90-fold faster than swMb, respectively. On the other hand, the off-rate of O2 in Cgb is more than 13-fold slower than swMb, whereas that of CO is similar to swMb (Fig. 13). In globins, heme-bound dioxygen is typically stabilized by distal polar interactions. Accordingly, a linear correlation between log(k_{on,CO}) and the electrostatic potential of the heme pocket (as reported by ν_C-O or ν_{Fe-CO}) have been recognized in swMb and soybean LegHb derivatives (50, 51). In Fig. 14A, we plot the log(k_{on,CO}) of the various microbial Hbs along with those of swMb derivatives as a function of ν_{Fe-CO}. It is important to note that for Cgb, Hmp, and trHbN only the ν_{Fe-CO} associated with the closed conformers are used for the plot, since the O2-complexes are in a closed state as indicated by the relatively low ν_{Fe-O2} as discussed earlier (Fig. 10) (10). Intriguingly, all the Hbs, which have been implicated in performing the NOD reaction in vivo, including Cgb, Hmp, and trHbN, are located in the right lower corner of the swMb correlation line; on the other hand, all the trHbs, except trHbN and trHbP, sit on a separate correlation line. The significantly slower O2 dissociation rate constants of the trHbs and its lower sensitivity to the electrostatic potential of the distal environment surrounding the heme ligand (as indicated by the smaller slope of the inverse correlation line) manifest the unique chemical nature of the Fe-O-O moiety in trHb-type of globins. On the other hand, the slower O2 dissociation rate constants of Cgb/Hmp and trHbN with respect to swMb demonstrate the importance of the distal B10Tyr-E7Gln and B10Tyr-E11Gln residues, respectively, in regulating the ligand binding properties of these three Hbs.

As shown in Fig. 14B, the O2 and CO on-rate of microbial Hbs, which span more than 4 orders of magnitude, are also inversely correlated with the frequency of the ν_{Fe-CO} mode, suggesting that ligand entry in microbial Hbs is somehow regulated by the electrostatic potential of the heme ligand binding pocket. Here only the open conformers of Cgb, Hmp, and trHbN are considered, assuming that the open and closed conformers are in a thermal equilibrium and only the open conformation can uptake ligands. It is noticeable that Cgb, like Hmp and trHbN, is located in the upper left corner of the inversely correlation lines; all have fast on-rates. Because the O2-complex is the key derivative of Cgb that detoxifies NO as illustrated in Equation 4, the fast on-rate of O2, as well as its slow off-rate, is expected to be critical for Cgb to execute the NOD reaction efficiently, by sequestering O2 under O2-limiting conditions (which might be particularly pertinent for this microaerophilic bacterium) and by avoiding the energy wasting O2 dissociation reaction.

**CONCLUSIONS**

*In vivo* studies have suggested that Cgb function as a NOD to protect *C. jejuni* against the toxic effects of NO (9). In line with this hypothesis, here we show that the architecture of Cgb is characteristic of those proteins that perform oxygen chemistry rather than oxygen transport. Our resonance Raman data show that Cgb is characterized by having a proximal histidine with strong imidazolate character, due to the presence of the H-bonding network linking the proximal catalytic triad, F8His-H23Glu-G5Tyr, and a polar distal pocket, due to the presence of the B10Tyr-E7Gln pair, which may offer electronic push and electronic pull, respectively, for facilitating the isomerization of the heme-bound peroxynitrite intermediate to nitrate during the NO dioxygenase reaction. The plasticity of the heme pocket of Cgb, on the other hand, may be important for the efficient multiple turnover of the NOD reaction by facilitating the release of the product nitrate.

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