Multiple Active Site Conformers in the Carbon Monoxide Complexes of Trematode Hemoglobins

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Sequence alignment of hemoglobins of the trematodes Paramphistomum epiclitum and Gastrothylax crumenifer with myoglobin suggests the presence of an unusual active site structure in which two tyrosine residues occupy the E7 and B10 helical positions. In the crystal structure of P. epiclitum hemoglobin, such an E7-B10 tyrosine pair at the putative helical positions has been observed, although the E7 Tyr is displaced toward CD region of the polypeptide. Resonance Raman data on both P. epiclitum and G. crumenifer hemoglobins show that interactions of heme-bound ligands with neighboring amino acid residues are unusual. Multiple conformers in the CO complex, termed the C, O, and N conformers, are observed. The conformers are separated by a large difference (~60 cm⁻¹) in the frequencies of their Fe-CO stretching modes. In the C conformer the Fe-CO stretching frequency is very high, 539 and 535 cm⁻¹, for the P. epiclitum and G. crumenifer hemoglobins, respectively. The Fe-CO stretching of the N conformer appears at an unusually low frequency, 479 and 476 cm⁻¹, respectively, for the two globins. A population of an O conformer is seen in both hemoglobins, at 496 and 492 cm⁻¹, respectively. The C conformer is stabilized by a strong polar interaction of the CO with the distal B10 tyrosine residue. The O conformer is similar to the ones typically seen in mutant myoglobins in which there are no strong interactions between the CO and residues in the distal pocket. The N conformer possesses an unusual conformation in which a negatively charged group, assigned as the oxygen atom of the B10 Tyr side chain, interacts with the CO. In this conformer, the B10 Tyr assumes an alternative conformation consistent with one of the conformers seen the crystal structure. Implications of the multiple configurations on the ligand kinetics are discussed.

It is now well recognized that hemoglobins (Hbs) are widespread in all phyla. There has been a renewed interest in understanding the structure and function of Hbs in recent years (1–5), because these Hbs are located in diverse cellular compartments and possess atypical physicochemical properties. Hbs have been discovered in about 33% of the animal species, and in addition to being present in vertebrates, they are found in the phyla that include nematode, mollusc, and annelid, to name but a few. Single subunit globins have been discovered lately in algae, nonsymbiotic plants, and a number of prokaryotes ranging from bacteria to cyanobacteria (see Refs. 2–5). Additionally, flavoprotein-bound chimeric Hbs have been found in bacteria (6) and yeast (7). In most cases, the nonvertebrate Hbs are proposed to have dissimilar functions from those in vertebrate Hbs; the latter class is established as consisting of O₂ carrier proteins. However, additional cellular activities have been proposed recently for vertebrate Hbs as well (8).

Although the nonvertebrate Hbs bind O₂ and other ligands just as the vertebrate Hbs, the kinetic and structural properties of the O₂ complexes (see for example Refs. 3–5 and 9–15) differ significantly, suggesting functional diversity. However, identification of the physiological functions remains elusive. Diversity in ligand binding properties, despite sharing a common prosthetic group, can be attributed to subtle changes in the arrangement of the amino acid residues that either coordinate to the heme or form its immediate surroundings. Understanding of the interactions between heme-bound ligands in hemeproteins and the residues in its distal pocket has received a great deal of attention in recent years because of the feature of catalytic mechanisms in some proteins and the rationalization of ligand kinetic behaviors in others. Sequence alignment of hemoglobins of the trematodes Paramphistomum epiclitum and Gastrothylax crumenifer with myoglobin suggests the presence of an unusual active site structure in which two tyrosine residues occupy the E7 and B10 helical positions (Fig. 1) (16–17). In the crystal structure of P. epiclitum hemoglobin, such an E7-B10 tyrosine pair at the putative helical positions has been observed, although the E7 Tyr is seen as displaced toward the globin CD region (18). In the present study, we report resonance Raman data on two closely related Hbs of the trematodes, P. epiclitum and G. crumenifer, for elucidation of the active site structure.

Resonance Raman spectroscopy is a very powerful method to study the nature of the interactions between the heme-bound ligands and their distal environment owing to the presence of Fe-ligand modes in the spectrum. The CO derivatives of heme proteins are especially useful in this regard because the Fe-CO (νFe-C≡O) and C-O (νC≡O) stretching vibrations are sensitive to the polarity of the heme pocket, and hence can be used to assess the electrostatic properties of the distal environment that play a crucial role in ligand association/dissociation events. We report here a combination of multiple protein active site conformers in the CO complex of the two trematode hemoglobin. We also discuss the implications of such protein conformers in modulating the ligand binding kinetics.

EXPERIMENTAL PROCEDURES

Biological Materials—P. epiclitum and G. crumenifer (Platyhelminthes, Trematoda, Paramphistomidae) parasitic in the rumen of the common

[Further text beyond the last line of the abstract is not provided in this sample.]
Indian water buffalo, *Bubalis bubalis*, was obtained from a local slaughterhouse in Aligarh, India. Trematodes were washed thoroughly with normal saline and incubated for 1 h at 37 °C in 0.15 M NaCl, 8 mM glucose to make them shed their eggs and gut contents. Trematodes were stored at −80 °C until use.

**Purification of Trematode Hemoglobins and Sequence Determination**—Purification and primary structure determination of the *P. epiclitum* Hb is described in Rashid et al. (17). Purification and protein sequencing of the *G. crumenifer* Hb was performed as described for *P. epiclitum*. Briefly, a single isoform was isolated by semipreparative isoelectrofocusing (IEF) under denaturing conditions. Peptides, generated by digestion with trypsin, endoproteinase Asp-N, CNBr cleavage, and N-terminal deblocking were purified by RP-HPLC on a Vydac C4 column (2.1 mm) developed with a 0.1% trifluoroacetic acid/acetonitrile gradient. Peptide sequencing was performed on an ABI 471-B sequencer operated in the pulsed liquid mode as recommended by the manufacturer (20–21).

**Raman Measurements**—The Raman experiments were carried out with 413.1 nm excitation from a cw Kr-ion laser (Spectra Physics, Mountain View, CA). The instrumentation and measurement procedures have been described elsewhere (9). Deoxy (reduced) hemoglobin was prepared by the addition of a small aliquot of dithionite solution under anaerobic conditions. The CO complexes were prepared by the addition of CO (12C16O or 13C18O) to anaerobic solutions of dithionite-reduced protein (~70 µM) in 100 mM sodium phosphate buffer, pH 7.4 in tightly sealed Raman cells. All spectral measurements were made at pH 7.4 unless otherwise noted. The 13C18O gas was obtained from ICON (Mt. Marion, NY) and 12C16O was purchased from Matheson (Rutherford, NJ). The laser power was kept low at the sample (~0.5 milliwatt) to minimize CO dissociation. Optical spectra were recorded before and after Raman measurements to ensure sample integrity.

**RESULTS**

**Primary Structure of G. crumenifer Hemoglobin**—When analyzed by IEF, under native and denaturing conditions, *G. crumenifer* Hb consists, as do *P. epiclitum* and *I. hypselobagri* Hb, of different isoforms (17). It is unclear whether this heterogeneity is caused by the existence of genetically different isoforms or to post-translational modifications of a single Hb chain. A single isoform (glb1) was purified by semipreparative IEF and RP-HPLC (17).

The *G. crumenifer* globin primary structure was reconstructed from the sequence of relevant peptides generated by tryptic, endoproteinase Asp-N and CNBr cleavage, N-terminal deblocking, and trypsin as described under "Experimental Procedures." *, unidentified residues in the Dd Hb sequence. Highlighted positions are B10, CD1, E7, E10, E11, and F8 in reference to sperm whale myoglobin structure.

FIGURE 1. Alignment of trematode globin sequences compared with that of sperm whale myoglobin and a few selected nonvertebrate Hbs and reconstruction of the *G. crumenifer* hemoglobin. Abbreviations used: Phy, Physeter catodon; Mtu, M. tuberculosis; Syn, Synechocystis; Nco, Nostoc commune; Ceu, Chlamydomonas eugametos; Pca, Paramaecium caudatum; Tpy, Tetrahymena pyriformis; DDd, Dicrocoelium dendriticum; Ihy, I. hypselobagri; Pep, P. epiclitum; Gcr, G. crumenifer; an, cnbr, debloc, and trp, peptides generated by respectively digestion with protease AspN, CNBr cleavage, N-terminal deblocking, and trypsin as described under "Experimental Procedures." *+, unidentified residues in the Dd Hb sequence. Highlighted positions are B10, CD1, E7, E10, E11, and F8 in reference to sperm whale myoglobin structure.

Multiple Fe-CO Conformers in Trematode Hbs

![Multiple Fe-CO Conformers in Trematode Hbs](image_url)

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The *G. crumenifer* globin primary structure was reconstructed from the sequence of relevant peptides generated by tryptic, endoproteinase Asp-N and CNBr digestion (Fig. 1). The N terminus was inaccessible for Edman degradation suggesting that it is blocked. Debloking by the Takara method resulted in the 6 terminal residues and proves that the N terminus is acetylated (22). All overlaps are presented, and the majority of the residues were sequenced twice. The total sequence contains 147 amino acids. The alignment with other trematode sequences is unam-
bighugous and confirms that no peptides are missing (Fig. 1). All four trematode sequences display an identity of 38 out of 147 residues (26.5%). Key positions of the globin structure at NA2, B6, B14, C2, CD1, F8, G5, and H8 are conserved. Deviations of the classical globin pattern are: Leu-A12, Tyr-B10, Tyr-C4, Tyr-E7, Tyr-F4, and Leu-H8. The presence of a Tyr at positions B10 and E7, a striking characteristic of the trematode Hbs, allows for the possible formation of two hydrogen bonds to the bound oxygen and therefore may be responsible for the high oxygen affinity because of a high k(CH2) and a low k(HB) value (16, 17, 23). We therefore suggest that, to achieve a high oxygen affinity, all trematode Hbs may have a significant difference in their heme peripheral modes widely separated in frequency and coexisting in a hemeprotein is uncommon, although two νFe-CO have been observed recently in a number of nonvertebrate hemoglobins (5, 10, 11, 32–35). Recently, in human cytoglobin three closely lying Fe-CO modes (518, 510, and 492 cm⁻¹) have been reported (36). Additionally, although a very low νFe-CO

| Presence of B10 Tyr and E7 Tyr in a total of 1040 globins from different species |
|-----------------------------------------------|---|---|
| **Globin** | **B10 Tyr** | **E7 Tyr** |
| All (1040) | 165 | 5 |
| Eukaryota (942) | 80 | 1 |
| Metazoa (859) | 29 | 1 |
| Chordata (693) | 0 | 1 |
| Arthropoda (74) | 0 | 0 |
| Platyhelminthes (5) | 5 | 4 |
| Echinodermata (3) | 0 | 0 |
| Nemertea (2) | 2 | 0 |
| Annelida (27) | 0 | 0 |
| Echiura (2) | 0 | 0 |
| mollusca (32) | 4 | 0 |
| Nematoda (19) | 18 | 0 |
| Vestimentifera (2) | 0 | 0 |
| Viridiplantae (69) | 38 | 0 |
| Mycetozoa (2) | 2 | 0 |
| Fungi (5) | 5 | 0 |
| Alveolata (7) | 6 | 0 |
| Bacteria (98) | 85 | 0 |

* Alignment and database available from M. Marden, unpublished data.
Multiple Fe-CO Conformers in Trematode Hbs

P. epiclitum HbCO

![Graph showing Raman spectra for P. epiclitum HbCO](image)

G. crumenifer HbCO

![Graph showing Raman spectra for G. crumenifer HbCO](image)

The frequencies of the Fe-CO (ν(CO)) and C-O (ν(C-O)) stretching modes are listed.

| Hemoglobin     | ν(Fe-CO) | ν(C-O) | δ(Fe-C-O) |
|----------------|----------|--------|-----------|
| P. epiclitum   | 539      | 1927   | 586       |
|                | 496      | 1956   | 578       |
| G. crumenifer  | 535      | 1929   | 582       |
|                | 492      |        | 575       |
|                | 476      |        |           |

The second bending mode (678 cm⁻¹) has not been reported in a hemoglobin, such low frequencies have been detected in other types of heme proteins, mutants, and model complexes (37–39).

Two Fe-C-O bending modes (δ(Fe-C-O)) are assigned at 586 and 578 cm⁻¹ for the ¹²C₁₆O complex that shift to 564 and 555 cm⁻¹, respectively with ¹³C₁₈O (Fig. 3). It may be noted that the width of the difference bands in the δ(Fe-C-O) frequency region of the isotope difference spectrum suggests the presence of more than one mode in this region. Therefore, two closely lying bending modes are assigned in the spectra. The δ(Fe-C-O) mode at 586 cm⁻¹ most likely corresponds to the FeCO conformer (C) with ν(Fe-CO) at 539 cm⁻¹, in analogy to the frequencies in Ascaris suum Hb (ν(Fe-CO) at 543 cm⁻¹, and δ(Fe-C-O) at 588 cm⁻¹) (10). The second bending mode (578 cm⁻¹) may correspond to the FeCO conformer (O) with ν(Fe-CO) at 496 cm⁻¹ on the basis of the empirical relation that the stretching and the bending frequencies bear a linear relationship as will be discussed in the next section. It may be noted though that the second bending mode observed here at 578 cm⁻¹ is more intense than the analogous bending modes (for a FeCO conformer with ν(Fe-CO) in the 490–500 cm⁻¹ region) in other heme proteins.

The resonance Raman spectra of the C-O stretching modes (ν(C-O)) of P. epiclitum Hb (Fig. 3, top) shows one prominent ν(C-O) mode at 1927 cm⁻¹ (1839 cm⁻¹ with ¹³C₁₈O). We assign this ν(C-O) band as being associated with the FeCO conformer (C) that has a ν(Fe-C-O) at 539 cm⁻¹. A second difference feature, albeit very weak, is seen at 1956/1874 cm⁻¹, which may be a candidate for ν(C-O) corresponding to the FeCO conformer (O) with ν(Fe-C-O) at 496 cm⁻¹.

Assignment of ν(Fe-C-O), ν(C-O) and δ(Fe-C-O) in the CO Complex of G. crumenifer Hb—Three ν(Fe-C-O) frequencies are detected at 535, 492, and 476 cm⁻¹ (Fig. 4, bottom) in the low frequency region of the resonance Raman spectrum of the CO complex of G. crumenifer Hb and they are also designated as the C, O, and N conformers. Assignment of these frequencies as Fe-CO stretching modes was confirmed by isotope (¹³C₁₈O) substitution measurements, in which the corresponding modes are detected at 514, 478, and 462 cm⁻¹, close to the values expected for a two-body harmonic oscillator between iron and CO (cal-
calculated values of $\Delta \nu_{\text{Fe-CO}}$ between the two isotopes are 17.4, 16.0, and 15.5, respectively. Two Fe-C-O bending modes ($\delta_{\text{Fe-C-O}}$) are assigned at 582 and 575 cm$^{-1}$ for the $^{12}$C$^{16}$O complex, which shift to $\sim$562 and 551 cm$^{-1}$, respectively with $^{13}$C$^{18}$O (Fig. 4). Assignment of the bending modes is made on the same basis as that presented above in the case of $P. \text{epictum}$ Hb. The correspondence of the bending and stretching modes, based on the correlation described under "Discussion," are assigned as follows: the $\delta_{\text{Fe-C-O}}$ mode at 582 cm$^{-1}$ to the $\nu_{\text{Fe-CO}}$ at 535 cm$^{-1}$ (C conformer); the $\delta_{\text{Fe-C-O}}$ mode at 575 cm$^{-1}$ to the $\nu_{\text{Fe-CO}}$ at 492 cm$^{-1}$ (O conformer). The $\nu_{\text{CO}}$ frequency in $G. \text{crumenifer}$ Hb (Fig. 4, top) is assigned at 1929 cm$^{-1}$ (1846 cm$^{-1}$ with $^{13}$C$^{18}$O). We attribute this $\nu_{\text{CO}}$ to the FeCO conformer with $\nu_{\text{Fe-CO}}$ at 535 cm$^{-1}$ (C conformer).

A spectrum was measured from a sample at low pH to determine if there was any pH dependence. In Fig. 4 (bottom) the spectrum in the low frequency region of the CO complex of $G. \text{crumenifer}$ Hb at low pH (pH 5) is shown. No major changes are observed in $\nu_{\text{Fe-CO}}$ as well as in other modes. All other CO spectra reported here are of samples at pH 7.4.

The three $\nu_{\text{Fe-CO}}$ modes, the $\nu_{\text{C-O}}$ mode as well as the two $\delta_{\text{Fe-C-O}}$ modes in $G. \text{crumenifer}$ are very similar to those detected in $P. \text{epictum}$ Hb. It may also be noted that the other low frequency region modes as well as their intensity patterns in the spectrum of $G. \text{crumenifer}$ HBCO are very similar to those seen in $P. \text{epictum}$ HBCO. Although the CO complexes of the two Hbs are very similar, the deoxyHb species are markedly different in their heme peripheral modes (cf. Fig. 2). Thus, despite a difference in conformation of the deoxy forms, upon ligand binding the active site conformation in the CO complexes becomes very similar.

**Resonance Raman Spectra of the Oxy Complexes**—To test for the presence of multiple conformations in the oxy complexes of these two Hbs we recorded the spectra of the $O_2$-bound proteins. In both cases the high frequency resonance Raman spectra and the optical absorption spectra confirmed that the oxy complex was formed. In the $G. \text{crumenifer}$ Hb a mode was detected at 573 cm$^{-1}$ with $^{18}$O$_2$, in the resonance Raman spectrum, which shifted to 548 cm$^{-1}$ with $^{13}$O$_2$ (see supplemental data, Fig. S1). No additional oxygen isotope-sensitive lines from alternate conformations were detected in the spectrum. This line is assigned as the Fe-O$_2$ stretching mode. In the $P. \text{epictum}$ Hb no oxygen-isotope sensitive line was found (see supplemental data, Fig. S2). Thus, for this complex the Fe-O$_2$ stretching mode is too weak to be detected. The Fe-O$_2$ stretching mode of many oxy complexes is weak so the absence any contribution from the $P. \text{epictum}$ Hb is not unprecedented.

**DISCUSSION**

**Low Frequency of Fe-His Stretching Mode**—The frequency of the Fe-His stretching modes in the $P. \text{epictum}$ and $G. \text{crumenifer}$ Hbs (206 and 207 cm$^{-1}$, respectively) are both very low compared with most other Hbs in which the mode is typically in the 220–230 cm$^{-1}$ region. The frequency of the Fe-His stretching mode in globins is sensitive to both proximal steric strain and the electronic coupling between the orbitals of the histidine and those of the heme iron. Both of these factors depend on the iron out-of-plane displacement, the hydrogen bonding between the imidazole $N_4$ with proximal side residues, the tilting of the imidazole with respect to the heme and the orientation of the histidine with respect to the iron-pyrrrole nitrogen bonds (32, 40–43).

The $P. \text{epictum}$ hemoglobin crystal structure shows typical hydrogen bonding of the imidazole $N_4$ and a staggered orientation of the proximal imidazole with respect to the heme pyrrole nitrogens resulting in a planar heme macrocycle (18). In addition, the tilt of the imidazole ring, within its plane, with respect to the heme plane is typical of that in other hemoglobins. However, the plane of the imidazole ring is highly tilted with respect to the heme plane as may be seen for the two crystalline forms shown in Fig. 5. In one form (Structure A) the angle is $\sim$75° and in the other (Structure B) it is $\sim$60°. We propose that this tilted heme imidazole plane weakens the electronic coupling between the imidazole orbitals and the iron $\pi$-orbitals resulting in a weaker bond and the associated lowered Fe-His stretching frequencies for these two Hbs.

**Correlation among the Iron Carbonyl Modes**—It is well established that there is an inverse correlation between the frequencies of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ modes shown in Fig. 6A. The two sets of modes assigned to the C and O structures of $P. \text{epictum}$ lie on the inverse correlation curve and the one set of frequencies from the C conformer of $G. \text{crumenifer}$. 

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**Multiple Fe-CO Conformers in Trematode Hbs**

![Figure showing two different structures of $P. \text{epictum}$ hemoglobin](image)

**FIGURE 5.** Structure of the distal pocket of $P. \text{epictum}$ showing the two different distances of the B10 Tyr$_{32}$ to the heme iron for the two conformers. Structures are from the PDB files 1H97 (18) and 1KFR (58).

**FIGURE 6.** A, inverse correlation for the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies for various hemoproteins and model heme complexes that have histidine or imidazole as the proximal ligand. The open circles correspond to the stretching modes of globins, peroxidases, and model heme complexes. The filled symbols represent the frequencies of the stretching modes of $P. \text{epictum}$ Hb (filled triangle) and $G. \text{crumenifer}$ Hb (filled circle). The FeCO frequencies other than trematodes are taken from Refs. 10, 35, and 38 and appropriate source references cited therein. B, linear relationship of the $\nu_{\text{Fe-CO}}$ and $\delta_{\text{Fe-C-O}}$ frequencies for several heme proteins and model heme complexes that have histidine/imidazole (open square) or cysteine (open triangle) as the proximal ligand. The filled symbols represent the frequencies of $P. \text{epictum}$ Hb (filled triangle) and $G. \text{crumenifer}$ Hb (filled circle). Data are shown in Table 3.
Multiple Fe-CO Conformers in Trematode Hbs

TABLE 3

Fe-CO bending and stretching frequencies of selected hemoproteins and model complexes

| Protein | \(\nu_{\text{Fe-CO}}\) (cm\(^{-1}\)) | \(\delta_{\text{Fe-CO}}\) (cm\(^{-1}\)) | Ref. |
|---------|-----------------------------------|----------------------------------|-----|
| Imidazole axial ligands | | | |
| P. epiclitum | 539 | 586 | This work |
| G. crumenifer | 496 | 578 | |
| Ascaris B10 Tyr-Phe | 520 | 584 | 35 |
| Ascaris E7 Glu-Leu | 536 | 587 | 35 |
| Ascaris E7 Gln-Leu | 493 | 576 | 35 |
| Ascaris E7 Gln-Asn | 492 | 575 | 35 |
| Ascaris E7 Gln-His | 495 | 573 | 35 |
| Ascaris F7 Arg Ser | 543 | 587 | 35 |
| Ascaris F7 Arg-Leu | 542 | 587 | 35 |
| Ascaris F7 Arg-Ser | 515 | 575 | 35 |
| A. suum | 513 | 577 | 35 |
| Barley | 534 | 586 | 11 |
| Scapharca His\(^{\alpha-Val} \) | 517 | 582 | 64 |
| Scapharca His\(^{\alpha-Val} \) & scapharca His\(^{\alpha-Val} \) | 488 | 572 | 65 |
| Human HbA | 507 | 578 | 66 |
| C. eleganetos | 491 | 572 | 67 |
| P. caudatum | 493 | 570 | 9 |
| Synecocystis PC6803 | 492 | 576 | 68 |
| Mb His\(^{\alpha-} \)Leu | 491 | 575 | 46 |
| Mb L29F | 526 | 582 | 69 |
| Elephant Mb | 513 | 577 | 70 |
| Horseradish peroxidase C, pH 6 | 539 | 590 | 71 |
| Horseradish peroxidase C, pH 7 | 541 | 590 | 72 |
| Cytochrome c peroxidase | 537 | 587 | 73 |
| TCP-Py | 465 | 560 | 39 |
| Thiolate axial ligands | | | |
| eNOS | 512 | 567 | 74 |
| iNOS | 487 | 560 | 74 |
| nNOS | 491 | 562 | 75 |
| nNOS+1-Arg | 503 | 566 | 75 |
| Wild-type P450cam | 484 | 562 | 29 |
| Chloroperoxidase | 486 | 560 | 75 |
| TCP-TG/THF | 458 | 547 | 39 |
| TCP-TB/THF | 429 | 539 | 39 |

Note: The Fe-CO bending and stretching frequencies of selected hemoproteins and model complexes are listed in Table 3. The frequencies are listed in Table 3. A linear correlation is seen between the bending and stretching modes. Resolving and assigning the Fe-C-O bending mode is not straightforward when there are multiple CO conformers present (35, 45) or when there are spectroscopic complexities such as Fermi resonance coupling of the bending mode (e.g., wild-type myoglobin shows a doublet at 576 and 585 cm\(^{-1} \); Ref. 46). Finally, linking a \(\delta_{\text{Fe-CO}}\) mode to the associated \(\nu_{\text{Fe-CO}}\) mode (i.e., of the same CO conformer) requires assumptions in a multiconformer system. Fig. 6B provides a framework that will facilitate assignment of Fe-C-O bending modes in a complex system provided the bending modes are sufficiently resolved in the spectra. If the linear correlation holds for very low Fe-CO stretching frequency ranges (such as below 480 cm\(^{-1} \)) one would expect a low frequency of the Fe-C-O bending mode as well. The model complex data supports this trend (Table 3); however, more data covering the low frequency ranges would be helpful for further confirmation of the correlation. The positive correlation is likely a consequence of the strengthening of the Fe-C bond. As the bending mode is primarily a motion of the carbon, when the Fe-C bond order increases, the carbon motion is more restricted so the bending frequency also increases.

The C Conformer—The active site conformer with the highest frequency of \(\nu_{\text{Fe-CO}}\) (and low \(\delta_{\text{Fe-CO}}\)) is designated as the C conformer. This conformer represents a rigid (closed) structure in which we attribute strong ligand stabilization by hydrogen bonding to the B10 Tyr. Such conformers with strong hydrogen bonding stabilization to the heme-bound ligand have also been observed in other nonvertebrate Hbs. For example, in A. suum Hb, the C conformer is stabilized by the B10 Tyr as well as E7 His (11). In barley Hb such conformers are stabilized primarily by the E7 His (11). In the two Hbs from Mycobacterium tuberculosis the conformer is stabilized by the B10 Tyr and the CD-1 Tyr (5, 12, 14, 15, 34). Corresponding to the high \(\nu_{\text{Fe-CO}}\) frequencies in P. epiclitum and G. crumenifer Hbs, the \(\nu_{\text{Fe-CO}}\) modes are assigned at 1927 and 1929 cm\(^{-1} \), respectively, for the C conformer and lie on the inverse correlation curve (Fig. 6A).

The O Conformer—The conformer with intermediate frequency for \(\nu_{\text{Fe-CO}}\) (at 496 and 492 cm\(^{-1} \), respectively, in P. epiclitum and G. crumenifer) is designated as the O conformer. This conformer represents an open structure that should allow much faster ligand escape than the C conformer. In the O conformer, we postulate that the B10 Tyr has moved away from the CO such that the CO no longer experiences positive polarity from it or any other residue. Such an open conforma-
tion created by slight structural rearrangements in the active site is not unusual in hemeproteins. The most common examples of such open conformer are Mb mutants in which the distal E7 His is replaced by apolar residues (see Ref. 53 and references therein), and the acidic form of Mb in which the E7 His swings away from the ligand site (54–56). These open conformers in Mb have $v_{Fe-CO}$ frequencies in the 490 cm$^{-1}$ region. Open conformers also naturally occur in some nonvertebrate Hbs, such as in barley (11), M. tuberculosis (5, 12), and in human cytochrome oxidase (36), and may remain in equilibrium with other CO conformers as demonstrated by the pH dependence in barley Hb (11). Such conformational changes have also been associated with mechanisms of inter-subunit communication (15).

The N Conformer—The conformer with the lowest frequency for $v_{Fe-CO}$ in P. epiclitum and G. crumenifer (at 479 and 476 cm$^{-1}$, respectively) is designated as the N conformer. A conformer with such a low frequency is rare and has not been reported in wild-type Hbs or Ms, although such low frequencies have been detected in mutant Ms and model complexes (38, 39). The only reported example of such a low $v_{Fe-CO}$ frequency in a native hemeprotein is at 473 cm$^{-1}$ in guanoylate cyclase (37). On the basis of the hypothesis that an interaction between a negative charge (or polarity) with the heme-bound CO would increase the C-O bond order, and that the Fe-CO bond order would concomitantly decrease because of the loss of strong Fe$^{3+}$-electron back donation, the N conformer may be envisaged as one in which a negative dipole in the active site interacts with CO, and the structure is pushed toward Fe-C=O$^-$ configuration rather than a Fe=C=O$^+$ configuration resulting from a positive distal environment and strong back bonding (5, 10, 38, 39, 57). Empirical calculations using point negative charges predict that such a bond order relation would hold, and the Fe-CO bond order is expected to decrease because of the negative charge (44). This effect was confirmed in a Mb mutant (His(E7)Val/Val(E11)Thr) in which the Fe-CO stretching mode shifted from ~510 cm$^{-1}$ in the wild-type protein to 478 cm$^{-1}$ in the mutant (45, 47). This was attributed to the interaction of the CO with the hydroxyl oxygen atom of the threônine. Thus, the N conformer likely results from an interaction between the CO and a negatively charged or non-polar group.

Structural Origin of the Conformer Differences—To determine the origin of the conformer differences we first examined the original crystal structure of P. epiclitum Hb (18). In the distal pocket the only residue that could interact with the heme ligand is the B10 Tyr. However, recently Milani et al. (58) reported that there is a great deal of plasticity in the structure of P. epiclitum Hb. Of particular interest was their observation that the Fe-O distance of the B10 Tyr32 was 5.53 Å in one structure and 3.37 Å in another as shown in Fig. 5. This flexibility of the Tyr suggests that through changes in its position several different types of interaction could occur as illustrated in Fig. 7 and thus account for the three different CO forms. When B10 Tyr32 is closest to the ligand, its oxygen atom may directly interact with the oxygen atom of the CO resulting in weak back bonding and a correspondingly low Fe-CO stretching mode (the N conformer). When the distance between B10 and the CO is more distant, the interaction will depend on the orientation of the proton on the Tyr hydroxyl group. When it is rotated away from the CO an open structure (O) is formed whereas when it is pointed toward the CO the positive interaction results in the closed structure (C) and the very strong back-bonding. The three different forms then are a consequence of the two different reported structures and two different rotational conformers of the Tyr hydroxyl group.

Relation of the Conformers to Ligand Kinetics—Our data confirm the presence of multiple conformers of the distal pocket for the CO-bound complexes of these trematode Hbs. It has been shown that the nature of the residues in the distal pocket significantly affects the ligand kinetics but quite unexpectedly there is no correlation between the Fe-CO vibrational frequencies and the ligand affinity (47). On the other hand there is a rough correlation between the frequency of the Fe-CO stretching mode and the CO dissociation rate constant. The lack of a correlation between the affinity and the mode frequency has been attributed to the need, upon ligand entry, to displace a water molecule from the distal pocket that has a stabilization which depends on the electrostatic properties of the distal residues, whereas the CO dissociation rate depends only on its direct stabilization (47). In a case such as that presented here, the ligand binding properties are further dependent on the equilibrium among the three conformers.

The combination of stabilizing and destabilizing interactions in trematode Hbs is different from the multiple conformations observed in other hemoglobin, For example in A. suum Hb, E7 Gln and B10 Tyr both provide hydrogen bonding stabilization to the bound ligand (10, 32, 59–63). In trematode Hbs, while B10 Tyr provides hydrogen-bonding stabilization to the bound carbon monoxide, interaction with a negative polarity of the B10 Tyr lone pair in the alternate conformation causes a destabilizing effect. This effect may be manifested in the oxy complex as well. However, the presence of only one Fe-O$^-$ mode in the G. crumenifer Hb at a high frequency (573 cm$^{-1}$) suggests that the O$_2$ is bound in the conformation in which the Tyr is at the longer distance from the heme group. It is unlikely that the oxygen would bind to the heme in the alternate conformation, the N conformer, as in that case the negatively charged environment resulting from the nearby Tyr would destabilize the structure. There is a substantial increase of ligand off-rates in the oxy complex of trematode Hbs in comparison to A. suum Hb (see Table 4) despite the fact that the Fe-O$^-$ stretching mode in the A. suum Hb is at $570$ cm$^{-1}$ (10). However, the higher frequency (573 cm$^{-1}$) may signify a weaker interaction in the G. crumenifer Hb with the bound oxygen. Whereas in A. suum Hb a dual hydrogen bonding from both E7Gln and B10 Tyr provides strong stabilization of the oxy complex, the single B10

### TABLE 4

| Hemoglobin      | $k_{cat}$ O$_2$ | $k_{cat}$ CO | Ref. |
|-----------------|-----------------|--------------|------|
| P. epiclitum    | 0.07            | 0.1          | 18   |
| G. crumenifer   | 0.4             | 1.2          | 16   |
| A. suum         | 0.0041          | 0.018        | 59   |
| Sperm whale myoglobin | 12              | 0.019        | 19   |

FIGURE 7. Proposed structural origin of the three different conformers of the CO complexes of the trematode hemoglobins. For the N conformer the B10 Tyr is close to the CO so the hydroxyl oxygen interacts directly with the bound CO. For the O and N conformers the Tyr is further away. In the former there are no interactions with the CO and in the latter there is an H-bonding interaction.
Multiple Fe-CO Conformers in Trematode Hbs

Tyr in the trematode Hbs may not stabilize the bound oxygen as much, resulting in the higher off-rates. It is difficult to pinpoint the exact contribution of the three active site conformers toward ligand kinetics. However, a qualitative assessment can be made by assuming a kinetic equilibrium between the conformers for the CO complex. A similar mechanism was proposed in the case of A. suum Hb (10). If a rapid equilibrium between the conformers is assumed, the most destabilizing conformer (N conformer) will determine the ligand dissociation rate as shown in Reaction 1.

\[
[(\text{Hb-CO})_c \leftrightarrow (\text{Hb-CO})_o] \leftrightarrow [(\text{Hb-CO})_N] \rightarrow \text{Hb + CO}
\]

**REACTION 1**

The brackets represent the two different reported crystal structures (18, 58). The O and the C conformers, differing only by a rotation of the Tyr hydroxyl group, are both in the configuration in which the Tyr is far from the heme iron (5.53 Å). The N conformer is in the configuration in which the Tyr is close to the heme iron (3.37 Å). This model means that the most stabilized conformer (C conformer) has minimal effect. Such a proposal is consistent with the nanosecond photolysis data that show a minimal effect of the initial distribution of stabilized conformers on rebinding kinetics in mutant Ms and nonvertebrate Hbs.* Instead, a rapid relaxation of the B10 residue subsequent to photodissociation dictates rebinding kinetics. This model accounts for the high CO dissociation rates listed in Table 4.

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Multiple Fe-CO Conformers in Trematode Hbs

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