Origin of the Anomalous Fe−CO Stretching Mode in the CO Complex of *Ascaris* Hemoglobin†

Tapan Kanti Das,‡ Joel M. Friedman,‡ Andrew P. Kloek,§ Daniel E. Goldberg,§ and Denis L. Rousseau*,‡

Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, and Howard Hughes Medical Institute and Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

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ABSTRACT: We report an unusually high frequency (543 cm^{-1}) for an Fe−CO stretching mode in the CO complex of *Ascaris suum* hemoglobin as compared to vertebrate hemoglobins in which the frequency of the Fe−CO mode is much lower. A second Fe−CO stretching mode in *Ascaris* hemoglobin is observed at 515 cm^{-1}. We propose that these two Fe−CO stretching modes arise from two protein conformers corresponding to interactions of the heme-bound CO with the B10-tyrosine or the E7-glutamine residues. This postulate is supported by spectra from the B10-Tyr → Phe mutant in which the 543 cm^{-1} line is absent. Thus, a strong polar interaction, such as hydrogen bonding, of the CO with the distal B10 tyrosine residue is the dominant factor that causes this anomalously high frequency. Strong hydrogen bonding between O_2 and distal residues in the oxy complex of *Ascaris* hemoglobin has been shown to result in a rigid structure, rendering an extremely low oxygen off rate [Gibson, Q. H., and Smith, M. H. (1965) *Proc. R. Soc. London B 163*, 206−214]. In contrast, the CO off rate in *Ascaris* hemoglobin is very similar to that in sperm whale myoglobin. The high CO off rate relative to that of O_2 in *Ascaris* hemoglobin is attributed to a rapid equilibrium between the two conformations of the protein in the CO adduct, with the off rate being determined by the conformer with the higher rate.

The understanding of the interactions between heme-bound ligands in hemeproteins and the residues in their distal environments has received a great deal of attention in recent years as it allows for the clarification of catalytic mechanisms in some proteins and the rationalization of ligand kinetic behaviors in others. This is especially important in hemoglobins and myoglobins, which have now been found to be expressed in many species ranging from bacteria to algae to plants (see ref *1* for a review), with unknown functions in most cases. However, the functions of some of these globins are beginning to be understood (*1*−*10*) and in a number of cases they involve NO−O_2 reactions (*2*−*5*). In the case of *Ascaris*, the hemoglobin has recently been shown to function as an NO-primed deoxygenase, utilizing a thiol−heme−tyrosyl redox triad to detoxify molecular oxygen (*5*).

Resonance Raman spectroscopy is a very powerful method to study the nature of the interactions between the 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 (ν_{Fe-CO}) and C−O (ν_{C-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 (*10*−*20*). We report here an unusually high ν_{Fe-CO} frequency (543 cm^{-1}) in the CO complex of *Ascaris* hemoglobin as compared to vertebrate hemoglobins in which the ν_{Fe-CO} frequency is in the 500−515 cm^{-1} range (*11*−*20*). We provide evidence from mutagenesis studies that hydrogen bonding of the CO with the distal B10 tyrosine residue is the dominant factor that causes this anomalously high frequency. These results suggest roles for tyrosine residues in the distal pocket of hemoglobins for stabilizing exogenous ligands.

Ascaris hemoglobin (AscHb), found in the perienteric fluid of the parasitic nematode *Ascaris suum*, contains eight identical subunits, and shows very high O_2 affinity (K_D = 2.7 nM in AscHb compared to 857 nM in myoglobin), due to an extremely slow dissociation rate (k_{off} = 0.0041 s^{-1}) (*21*−*25*). AscHb has a distal glutamine residue at the E7 position (E7-Gln) and a tyrosine residue at the B10 position (B10-Tyr) (Figure 1) (*26*). The corresponding residues in sperm whale Mb are histidine and leucine, respectively, both of which are highly conserved among vertebrate globins. The high O_2 affinity in AscHb, in which the off rate is over 1000 times lower than that in myoglobin, has been proposed to originate from the stabilization of the bound O_2 by hydrogen bonds from both E7-Gln and B10-Tyr (*26*−*31*).

Even though AscHb shows a high O_2 affinity, its affinity for CO (K_D = 1.1 nM in AscHb compared to 37 nM in myoglobin) is not remarkably different from that in mammalian Hbs and Mbs owing to an off rate (0.018 s^{-1}) that is very similar to that in myoglobin (*21*−*25*). On the basis of the idea that the Fe−CO bond is less polar than the Fe−O_2

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* Address correspondence to this author: Tel (718) 430-4264; Fax (718) 430-4230; E-mail rousseau@aecom.yu.edu .

‡ Albert Einstein College of Medicine.

§ Washington University School of Medicine.

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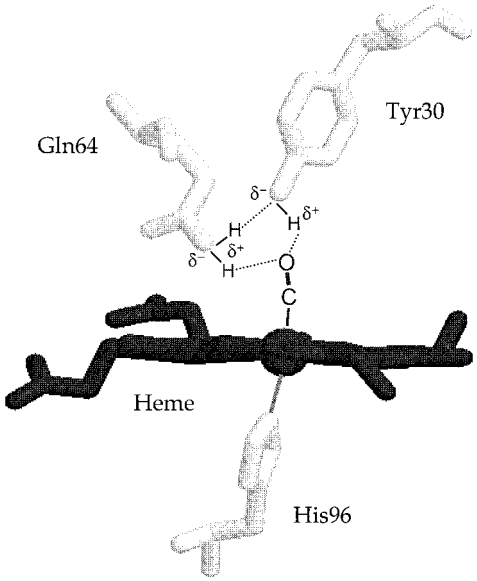

FIGURE 1: Structural model of the active site in the CO complex of *Ascaris* hemoglobin (from PDB file 1ash). Selective residues are shown: heme, His96 at F8 (proximal), Tyr30 at B10, and Gln64 at E7 helical positions. The proposed interactions of CO with Gln64 and Tyr30 are shown by dotted lines. In the oxy complex (not shown), the proximal oxygen atom (bonded directly to Fe) and the terminal oxygen atom interact with E7-Gln and B10-Tyr, respectively.

bond, it was proposed that the iron-bound O_2, and not CO, accepts a hydrogen bond from the distal residues (*28*). However, in other hemoglobins and myoglobins there is clear evidence for the interaction of the CO with the distal residues. For example, the ν_{Fe-CO} mode in Mb decreases from ~509 cm^{-1} to ~495 cm^{-1} upon replacement of the distal histidine by noninteracting residues (*11*−*20*). Furthermore, spectroscopic evidence has been presented directly supporting a hydrogen bond between the heme-bound CO and the distal histidine in myoglobin (*32*). In this study, we present evidence that in AscHb the two distinct conformations of the CO adduct result from different interactions of the iron-bound CO with the distal E7-Gln and the B10-Tyr residues. The different degree of stabilization of the CO in these two conformers is expected to give rise to very different CO off rates. From consideration of the measured CO off rate we infer that there is a rapid equilibrium between these two conformations of the protein.

MATERIALS AND METHODS

The wild-type and the B10-Tyr mutant of *Ascaris* hemoglobin were purified as described elsewhere (*27*).

The Raman experiments were carried out with 413.1 nm excitation from a CW Kr ion laser (Spectra Physics, Mountain View, CA). The sample cell (quartz, 2 mm path length, sample volume ~150 μL), into which a laser beam was focused, was spun at 3000−6000 rpm to minimize local heating. The sample cells are custom designed for strict anaerobic measurements and can be used for recording both the resonance Raman spectra and the optical absorption spectra (UV-2100U spectrophotometer, Shimadzu, Kyoto, Japan). The Raman scattered light was focused onto the entrance slit (100 μm) of a polychromator (Spex, Metuchen, NJ), dispersed by a 1200 grooves/mm grating and detected by a liquid nitrogen-cooled charged-couple device (CCD) (Princeton Instruments, Trenton, NJ). A holographic notch filter (Kaiser, Ann Arbor, MI) was used to eliminate Rayleigh scattering. Typically, several 30 s spectra were recorded and averaged. Frequency shifts in the Raman spectra were calibrated with acetone−CCl_4 (for the 100−1000 cm^{-1} region), indene (for the 100−1700 cm^{-1} region), and acetone−ferrocyanide (for the 1700−2400 cm^{-1} region) as references. The accuracy of the Raman shifts is about ±2 cm^{-1} for absolute shifts and about ±0.5 cm^{-1} for relative shifts. The Raman data were processed by using GRAMS (Galactic Industries Corporation) software. The cosmic ray spikes were removed by using CSMA subroutines (Princeton Instruments).

CO complexes of the wild-type and the mutant hemoglobin were prepared by the addition of CO ($^{12}C^{16}O$ or $^{13}C^{18}O$) to anaerobic solutions of dithionite-reduced protein (~60 μM) in 100 mM sodium phosphate buffer, pH 7.4, in tightly sealed Raman cells. $^{13}C^{18}O$ gas was a product of Icon (Mt. Marion, NY) and $^{12}C^{16}O$ was obtained from Matheson (Rutherford, NJ). The laser power was kept low at the sample (~0.5 mW) to minimize carbon monoxide dissociation. Absorption spectra were recorded before and after the Raman measurements to ensure the stability of the species studied. For hydrogen/deuterium isotope studies, solutions of the CO complex were prepared as 40 μM protein (40 mM sodium phosphate, pH 7.4) in either 100% H_2O or in 80% D_2O + 20% H_2O (from 99.9% D_2O, Aldrich Chemical Co. Inc., Milwaukee, WI).

RESULTS

In the low-frequency region of the resonance Raman spectrum of the CO complex of AscHb, two frequencies for ν_{Fe-CO} are detected at 543 and 515 cm^{-1} (Figure 2A). Assignment of these frequencies as Fe−CO stretching modes was confirmed by isotope ($^{13}C^{18}O$) replacement experiments, where the corresponding lines appeared at 531 and 500 cm^{-1}, close to the values expected for a two-body harmonic oscillator between iron and CO. Although the presence of a porphyrin mode at ~493 cm^{-1} partially obscures the 500 cm^{-1} band with $^{13}C^{18}O$, curve-fitting analyses and the difference spectrum confirmed the above assignment. The Fe−C−O bending mode (δ_{Fe-C-O}) is detected at 588 cm^{-1} for the $^{12}C^{16}O$ complex and shifts to 567 cm^{-1} with $^{13}C^{18}O$. No hemoglobin or myoglobin has ever been reported previously to have a ν_{Fe-CO} frequency as high as 543 cm^{-1} and a bending mode as high as 588 cm^{-1}.

We measured the resonance Raman spectrum of the B10-Tyr → Phe mutant (Figure 2B). In the mutant, the 543 cm^{-1} line is absent in the resonance Raman spectrum of the CO complex as shown in Figure 2B, and instead a single ν_{Fe-CO} frequency is observed at 520 cm^{-1} (504 cm^{-1} with $^{13}C^{18}O$). This difference in ν_{Fe-CO} (520 vs 515 cm^{-1}) is postulated to arise from additional interactions with the B10-Phe residue or a slight restructuring of the distal pocket. Consistent with this, a small increase in the ν_{Fe-CO} frequency was reported in the double mutant B10-Leu → Phe/E7-His → Gln (513 cm^{-1}) relative to the E7-His → Gln mutant (507 cm^{-1}) of sperm whale myoglobin (*18*). The absence of the line at 543 cm^{-1} in the mutant of AscHb confirms the influence of the B10-Tyr on this form of the protein. To determine if there

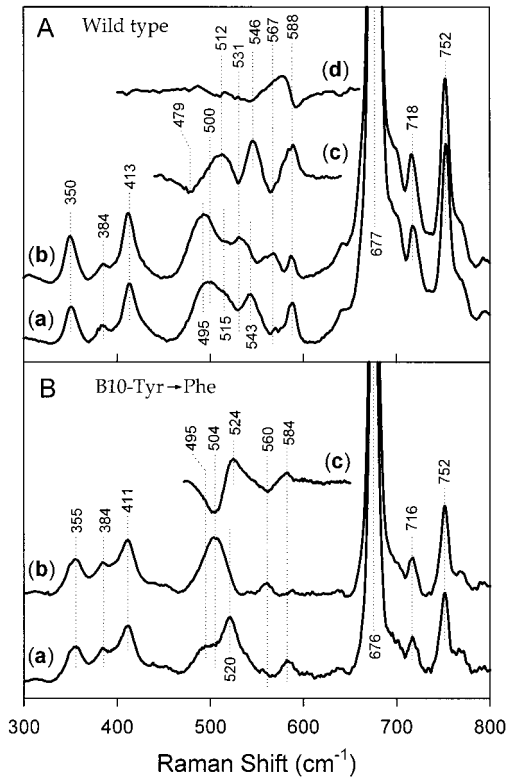

FIGURE 2: (A) Resonance Raman spectra of the CO complex of wild-type AscHb in the low-frequency region: (a) $^{12}C^{16}O$, (b) $^{13}C^{18}O$, (c) difference spectrum $^{12}C^{16}O - {}^{13}C^{18}O$, and (d) difference spectrum $H_2O - D_2O$ of the $^{12}C^{16}O$ complex. The $H_2O - D_2O$ spectrum shows a difference feature at 578/593 cm^{-1} due to an apparent upshift of the bending mode by ~3 cm^{-1} in D_2O. The trough seen at ~543 cm^{-1} is because of an increase in both width (~1 cm^{-1}) and intensity and a small upshift (~0.3 cm^{-1}) of the Fe−CO stretching mode (543 cm^{-1}) in D_2O. (B) Resonance Raman spectra of the CO complex of the B10-Tyr → Phe AscHb mutant in the low-frequency region: (a) $^{12}C^{16}O$, (b) $^{13}C^{18}O$, and (c) difference spectrum $^{12}C^{16}O - {}^{13}C^{18}O$.

is any evidence for hydrogen bonding to the CO, we measured the resonance Raman spectrum of the wild-type complex in deuterated buffer and detected a large change in the Fe−CO bending mode (Figure 2A, spectrum d). The absolute shift in the mode was estimated to be ~3 cm^{-1} to higher frequency in the deuterated buffer. This deuteration shift is consistent with a hydrogen-bonding interaction as has been seen in CO-bound myoglobin (*32*) and barley hemoglobin (*10*).

The resonance Raman spectra of the C−O stretching modes (ν_{C-O}) were measured for the wild-type and the mutant proteins of AscHb (Figure 3). The wild-type protein shows two ν_{C-O} frequencies (spectrum a) at 1909 and 1948 cm^{-1} (1823 and 1863 cm^{-1}, respectively, with $^{13}C^{18}O$). In the mutant, only one ν_{C-O} mode appears at 1928 cm^{-1} (spectrum b). It should be noted that the Fourier transform infrared (FTIR) spectra of the CO derivative (*31*) of the wild-type protein shows a distinct band at 1912 cm^{-1} as well as a broad structured band at ~1956 cm^{-1}. For the tyrosine mutant a broad band is observed at 1940 cm^{-1}. A ν_{C-O} frequency as low as 1909 cm^{-1} in AscHb is very uncommon and heretofore not observed in any hemoglobin or myoglobin. In an FTIR study by Li et al. (*17*) on a large number of myoglobin mutants, the lowest observed frequency for ν_{C-O} was at 1916 cm^{-1} for the CO complex of E11-Val → Asn, which was attributed to the effect of the positive polarity of Asn on the CO.

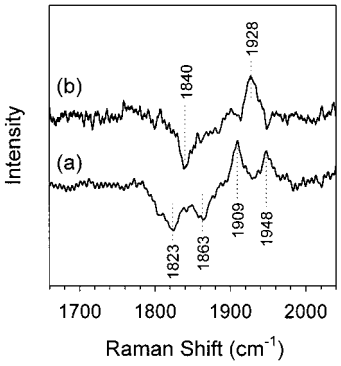

FIGURE 3: Resonance Raman difference spectra ($^{12}C^{16}O - {}^{13}C^{18}O$) of the CO complexes of (a) wild-type AscHb and (b) B10-Tyr → Phe AscHb mutant, showing the assignment of ν_{C-O} in the high-frequency region.

DISCUSSION

The two ν_{Fe-CO} modes are postulated to be associated with two different conformations of the protein that have different electronic environments around the Fe−CO moiety. The conformer with low frequency for ν_{Fe-CO} at 515 cm^{-1} (form L) is assigned as one in which the CO interacts only with the E7 Gln. This assignment is consistent with the resonance Raman spectrum of elephant myoglobin in which also the E7 position is occupied by a glutamine (*11*) and ν_{Fe-CO} is detected at 515 cm^{-1}. We attribute the conformer with the unusually high frequency for ν_{Fe-CO} at 543 cm^{-1} (form H) as one in which there is a strong positive polar interaction (including hydrogen bonding) between the oxygen atom of the CO and the B10 Tyr side chain and an additional interaction with the E7 Gln side chain (Figure 1). It is well established that positive polar interactions decrease the bond order of C−O and concomitantly increase the bond order of Fe−CO, thereby resulting in an inverse correlation between the Fe−CO and the C−O stretching frequencies (*10*−*16*, *33*). On that basis, we assign the two ν_{C-O} frequencies at 1909 and 1948 cm^{-1} in AscHb as being associated with the H and L conformers, respectively. The ν_{Fe-CO} and ν_{C-O} frequencies for both the H and L forms in the wild-type and the single form in the mutant of AscHb fall on the correlation line (*10*−*16*, *33*) that is characteristic of the heme proteins and model compounds containing histidine or imidazole as a proximal ligand (Figure 4).

In the CO complex, the H conformer (ν_{Fe-CO} at 543 cm^{-1}, ν_{C-O} at 1909 cm^{-1}) represents a rigid (*closed*) structure in which the ligand is stabilized by hydrogen bonding. On the other hand the L conformer (ν_{Fe-CO} at 515 cm^{-1}, ν_{C-O} at 1948 cm^{-1}) represents an *open* structure that allows faster ligand escape than the H conformer. The H conformer experiences electrostatic effects from both the B10-Tyr and E7-Gln. Thus, in the L conformer, the CO is expected to move away from the tyrosine (or conversely the tyrosine moves away from the CO) so that the CO sees the potential primarily from glutamine. In the absence of the tyrosine residue (in the Tyr → Phe mutant), only one conformer is stabilized, the vibrational frequency of which may be governed by the combined effect of the glutamine and the phenylalanine or by only one of the residues.

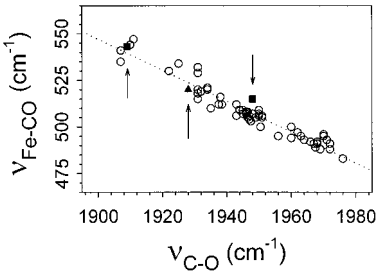

FIGURE 4: Inverse correlation curve for the ν_{Fe-CO} and ν_{C-O} frequencies for various heme proteins and model complexes that have histidine or imidazole as the proximal ligand. The open circles correspond to the stretching modes of globins, peroxidases, and heme complexes. The solid squares represent the frequencies of the modes in the two conformations of wild-type AscHb, and the solid triangle reports the frequencies of the conformation seen in the B10-Tyr → Phe mutant of AscHb.

A very strong interaction from a single residue is present in barley hemoglobin, which has a histidine at the E7 position and a phenylalanine at the B10 position. The Fe−CO frequency is observed at 534 cm^{-1} at neutral pH (*10*), which arises from the interaction of CO with E7-His. When the distal histidine swings out at pH 5, the 534 cm^{-1} conformer is converted to an open form with the Fe−CO frequency of 493 cm^{-1} (*10*). The distal histidine in barley hemoglobin lies sufficiently close to the CO to exert a strong positive potential, rendering the high frequency of the Fe−CO mode. The effect of the positive field from the phenyl multipole of the B10-Phe in barley hemoglobin is not strong enough to cause such a high frequency. For comparison, in the two B10-Leu → Phe mutants of sperm whale myoglobin the Fe−CO frequencies are observed at 513 cm^{-1} (B10-Leu → Phe/E7-His → Gln) and 525 cm^{-1} (B10-Leu → Phe), respectively (*34*). If these values are compared with the Fe−CO frequencies of the E7-His → Gln mutant (507 cm^{-1}) and the wild-type protein (509 cm^{-1}) (*34*), the contribution of Phe is clearly not additive. It is possible that the disposition of two aromatic rings (imidazole and phenyl) in the distal pocket of the B10-Leu → Phe mutant in Mb causes steric crowding and the histidine is pushed closer to CO, causing a conformation resembling the distal pocket of barley hemoglobin. It appears that the contribution of the phenyl multipole to the modulation of the Fe−CO frequency is relatively small (5−6 cm^{-1}), as seen in the myoglobin double mutant and also in the AscHb mutant. Thus, although individual residues may supply the dominant interaction for modulation of the Fe−C−O frequencies, multiple effects also exist and heme pocket reorganization is also likely.

In summary, our data suggest that both the E7-Gln and B10-Tyr residues interact with CO, which is consistent with the proposal that both of these residues provide hydrogen-bonding stabilization to the bound O_2 in the oxy complex of AscHb that results in a dramatically slow O_2 dissociation rate (*21*−*31*). However, the measured CO dissociation rate is the same as that in sperm whale myoglobin. This may be understood by our observation that there are two conformers for the CO adduct, H and L, whereas for the oxy complex there is only one conformer. In the oxy complex the proximal and the distal oxygen atoms are hydrogen-bonded to E7-Gln and B10-Tyr, respectively, forming one rigid structure. As shown in Figure 1, in the CO complex only the oxygen atom of CO is hydrogen-bonded to the distal residues. The H conformer is more strongly stabilized by the polar interactions than the L conformer. We infer that there is a rapid equilibrium between the H and L forms of the CO adduct, and thus the CO dissociation rate, *k*, is determined by that of the L conformer, the one with the higher dissociation rate:

$$\text{H}-\text{CO} \leftrightarrow \text{L}-\text{CO} \xrightarrow{k} \text{L} + \text{CO} \qquad (1)$$

Although in the H conformer of AscHb the CO interacts very strongly with the residues in the distal pocket, the effect of the highly stabilized structure on the CO dissociation kinetics is minimal due to the rapid equilibrium between the two forms.

The above mechanism is consistent with the observation of a large increase of the geminate yield for the CO complex of AscHb upon lowering the temperature (*31*), which suggests a temperature dependence of the equilibrium between the two distal pocket conformers. We predict that, upon lowering the temperature, the magnitude of the CO off rate in AscHb should concomitantly decrease. Furthermore, as expected, the geminate yield in the B10-Tyr → Phe mutant is significantly reduced relative to that in wild-type AscHb (*31*).

The modulations of the Fe−C−O frequencies reported here are presumed to arise from electrostatic rather than steric effects. Recently, models have been put forward in attempts to quantitate such interactions. The electrostatic calculations by Kushkuley and Stavrov (*33*) demonstrated that the vibrational frequency of ν_{C-O} (and thus ν_{Fe-CO}) in a heme−CO complex is strongly modulated by the distance of a charged group from the CO. A more recent report (*18*) described the electrostatic potential field distribution in the distal pocket of myoglobin and several of its mutants. The potential was correlated with the vibrational frequencies and ligand dissociation rates. On the basis of these correlations, an estimate of the electrostatic potential on the oxygen atom of the CO corresponding to the Fe−CO frequencies of AscHb (543 and 515 cm^{-1}) gives values of ∼33 and ∼15 kcal/mol, respectively. According to the kinetic model presented above (eq 1), the L conformer is responsible for determining the CO dissociation rate. From the empirical relation between the potential and CO dissociation rate (*18*), the predicted rate for the L conformer is ∼0.04 s^{-1}, reasonably close to the experimental value of 0.018 s^{-1} given that the data points in the correlation graph (*18*) are very scattered.

If the potential values, which were calculated on the basis of the vibrational frequencies of CO in AscHb, are used to determine the O_2 dissociation rate, a low dissociation rate of ∼0.1 s^{-1} is obtained for the H conformer, which is in qualitative, but not quantitative, agreement with the experimental rate (0.0041 s^{-1}). It demonstrates that the calculated potentials based on interactions of the distal residues with the CO cannot be transferred quantitatively to the O_2 structure. However, qualitative predictions appear to be valid.

UV resonance Raman studies (*30*, *31*), indicate that the strength of hydrogen bonding by the tyrosine is higher in the oxy complex than in the CO complex. This conclusion is substantiated by the observation of an ultrafast (<5 ps) geminate rebinding phase for O_2 in AscHb (*31*) and an extremely low O_2 off rate. Therefore, on the surface it may

seem surprising that only a single Fe−O_2 stretching mode is detected at 570 cm^{-1} (data not shown), the same as in mammalian hemoglobins and myoglobins. However, this lack of sensitivity is expected for the O_2 adduct in contrast to the CO adduct, since it has been demonstrated that the O_2 adduct in many heme proteins studied thus far is very insensitive to the surrounding electrostatic field (*35*).

In conclusion, strong polar interactions on the CO from the B10 tyrosine in the distal pocket give rise to an anomalously high frequency for the Fe−CO stretching mode of AscHb. However, the CO off rate is not as low as would be predicted from this stabilization due to an equilibrium with a less well-stabilized conformer. Effective stabilization of bound ligands depends on the absence of an equilibrium with an unstabilized structure. As shown here when such an equilibrium is present, the stabilized structure plays no role in the observed dissociation kinetics. In such cases structural inferences cannot be drawn from kinetic analyses.

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