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Hydrogen Bonding of Tyrosine B10 to Heme-bound Oxygen in Ascaris Hemoglobin

DIRECT EVIDENCE FROM UV RESONANCE RAMAN SPECTROSCOPY

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Ascaris has a parieric hemoglobin with an oxygen affinity up to $10^4$ times higher than that of vertebrate globins ($P_{50} = 0.004$ mm Hg in contrast to $0.5–30$ mm Hg for other Hbs or Ms). The affinity of the protein for carbon monoxide is at least as high as that of the intact octamer, demonstrating that the affinity is intrinsic and at least relatively independent of subunit interaction. Alignment of the individual domains with vertebrate globins typically shows about 15% homology, yet numerous invariant residues have been found.

One important difference between Ascaris Hb and vertebrate oxygen carriers is that the distal histidine E7 of vertebrate globins is replaced with a glutamine in both Ascaris hemoglobin domains. The distal histidine is a well studied residue known to stabilize the heme-bound oxygen through a hydrogen bond (7–9). Glutamine, the most common naturally occurring replacement for histidine, can also serve as a hydrogen bond donor to the bound oxygen in Ascaris Hb. Indeed, conversion of this residue to leucine or alanine produces a hemoglobin variant with oxygen off rates 5- and 60-fold higher, respectively, than that of unaltered D1 (10).

There is indirect evidence for an additional hydrogen bond to the bound oxygen originating from the tyrosine residue at position B10. Mutation of this residue to either phenylalanine or leucine greatly increases the off rate of oxygen, resulting in more than a 100-fold decrease in oxygen affinity (10, 11). The above studies suggest that glutamine and tyrosine side chains in the heme pocket stabilize the heme-bound oxygen, with hydrogen bonding being the likely mechanism. Surprisingly, the sperm whale myoglobin mutant where the native B10 leucine is replaced by tyrosine has a lowered oxygen affinity, with an off rate 50 times higher than that of the wild type (12), illustrating the fundamental limitations of identifying the structural or functional role of residues based solely on the site-directed mutagenesis approach. Recent x-ray crystallographic results (13) indicate that the tyrosine in the Ascaris Hb is situated close enough to the oxygen to form a hydrogen bond.

The present study reports on the use of UV resonance Raman to probe changes in the environments of the tryptophan and tyrosine residues for the D1 domain as a function of ligand binding and ligand. The D1 domain contains three tryptophans and seven tyrosines. Both the native and E7 Gln to Leu mutant forms of the D1 fragment are studied. The deoxy, carbonmonoxo, and oxy derivatives have been compared. Our results provide direct spectroscopic evidence for the existence of a
hydrogen bond between tyrosine B10 and heme-bound oxygen, both in the native D1 domain and in the QE7L mutant.

MATERIALS AND METHODS

UV Resonance Raman Spectroscopy—UV resonance Raman spectroscopy is a powerful molecular probe technique that allows for the generation of structure sensitive vibrational spectra containing resonantly enhanced Raman bands associated with aromatic amino acids within proteins (14–19). The effective utilization of this technique has been limited by the availability of appropriate laser sources and detectors. For this study, a new system was used that was designed and constructed by Dr. D. Eads for the Laser Spectroscopy Facility at the Albert Einstein College of Medicine. A key feature is that it contains a CW (continuous wave) tunable UV laser source that does not cause the higher levels of photodamage that typically occurs with the pulsed sources that are more commonly utilized.

The UV resonance Raman apparatus is composed of a laser source, a 1.5 m single spectrograph equipped with a 3600 grooves/mm holographic grating and an liquid N2 cooled CCD detector. The CCD detector (Princeton Instruments, Princeton, N J) has a UV metaphosphate coating on its chip. An intracavity frequency-doubled ring dye laser (Coherent 899) utilizing stilbene 420 is pumped by multi-UV line output of Argon ion laser (Coherent Innova 400). To maximize the UV output with acceptable bandwidth, a single thin etalon is used instead of the usual two etalons (thick and thin). This laser system generates CW UV output from 218 to 240 nm. The maximum output of this system (at 226 nm) is 6 milliwatts. In this experiment, the average UV power used is ~1.0 milliwatts, which has proven to be sufficient for acquiring high quality Raman spectra. The samples (~1 ml) were held in a quartz tube for data collection. Typical samples were ~0.1 ml in heme in phosphate buffer (30 mM, pH 7.3). During the period of data acquisition, the tube was maintained at 10 °C and spun at 5 Hz to minimize local heating and potential photodegradation. The scattered photons were collected with a triplet lens (fused suprasil, CVI Laser Corp.) and focused onto the entrance slit (270 μm) of the spectrograph (Sopra, Inc., Bois-Colombes, France). The wavenumber shift was calibrated with the use of the Raman spectra of cyclohexane and 1,4-dioxane. For the UV Raman spectra presented here, spectral acquisitions were carried out as a series of 5-min accumulations. Each final spectrum was the sum of three to five of these 5-min spectra. Before summing the spectra, spectral spikes due to cosmic rays were removed from each spectrum using SpectralCal software (Galactic Industries Corp.). Also, each spectrum was subtracted from the first one taken from a given sample. If difference features were observed, the spectrum was discarded. In order to accurately compare different spectra from different samples, 0.2 M perchlorate was added to the stock buffer solutions from which the samples were prepared. The perchlorate Raman band at 934 cm⁻¹ serves as a protein invariant intensity reference. When comparing two spectra, the spectral intensities from the protein are normalized by matching the heights of the perchlorate internal reference bands at 934 cm⁻¹. This technique is used for accurate comparisons only for those situations where it is possible to maintain the identical concentrations of both perchlorate and protein, as in comparisons between the deoxy and ligand bound forms of a given protein where all the derivatives are made from the same stock solution.

Protein Samples—The native and E7 Gln to Leu mutant D1 domain proteins were prepared and characterized according to previously described procedures (5).

RESULTS AND DISCUSSION

Fig. 1 shows a comparison of the UV resonance Raman spectra of the deoxy and carbonmonoxy derivatives of the native D1 fragment of HbAscaris). The difference spectra between the deoxy and carbonmonoxy derivatives are shown for the QE7L mutant of the D1 fragment. It can be seen that the differences between the deoxy and carbonmonoxy derivatives are more extensive in the QE7L mutant than in the native D1 fragment.

Fig. 2 shows a similar comparison between the deoxy and oxygen derivatives of the native D1 fragment. Also shown are the oxygen and CO derivatives and, for comparative purposes, a repeat of the difference between the CO and deoxy derivatives of this protein. Except for the feature associated with the tyrosine Y8a and the lower frequency Y8b peaks at ~1616 and 1600 cm⁻¹, respectively, the changes induced in the spectrum of the deoxy derivative upon binding either ligand are the essentially the same. That point is further illustrated by virtue of the absence of prominent difference features in the oxygen versus carbonmonoxy difference (with the exception of the Y8a and Y8b peaks). It is clear that the major difference between CO and O₂ with respect to ligand binding induced changes in the deoxy spectrum, is the substantial increase in intensity associated with the Y8a and Y8b bands of the oxygen derivative. Other ligand binding induced features in the difference spectra, most noticeably the one associated with W3 band at ~1555 cm⁻¹, do appear slightly more exaggerated for the oxygen derivative relative to the CO derivative.

Fig. 3 shows a blow up of the high frequency region of the UV resonance Raman spectra of the O₂ and CO derivatives of the QE7L mutant of the D1 fragment. It can be seen that the

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2 J. M. Friedman and S. Huang, unpublished data.
oxygen binding induced increase in the intensity of the tyrosine Y8a and Y8b bands also occurs in this mutant although the fractional increase appears to be less than for the native fragment. In clear contrast to the native D1 fragment, the mutant displays distinct changes in several other Raman bands in going from CO to O2. These changes are shown in Fig. 4 which displays a blow up of the mid-frequency regime of the UV resonance spectrum. Of particular interest is the change that occurs for the W7 Fermi doublet at $1350 \text{ cm}^{-1}$. The ratio of these two bands reflects the degree of hydrophobicity in the environment of the contributing tryptophans (14–21). At this time it is not clear what the changes in Y9a are due to.

An attempt was made to study the Tyr(B10) to Phe mutant, but it proved to be too unstable to allow for spectral acquisitions.

The Raman spectra indicate that for the native protein, there are conformational or environmental changes induced in the globin upon binding of CO. These changes involve both tryptophans and tyrosines. Two of the three tryptophans in the D1 domain are situated in the G helix. The third is in the H helix. The proximity of at least the G helix tryptophans to the heme raises the possibility that the ligand binding induced changes are the results of very local changes due to the conformational and electronic changes in the heme upon ligand binding. Thus small change in W16 could be due to either the response of the tryptophans to the local electronic changes at the heme upon

switching from a five coordinate high spin ferrous heme to a six coordinate low spin ferrous heme or to small tertiary structure changes induces by ligand binding.

The absence of substantial tertiary changes upon ligand binding is supported by the observation$^3$ that the visible resonance Raman spectra of the deoxy form and the 10-ns photoproduct of the CO derivative are identical. The visible resonance Raman spectrum is highly sensitive to the influence of protein tertiary structure upon different degrees of freedom associated with the heme and the heme-proximal histidine linkage (25–28). An absence of a difference between the equilibrium deoxy spectrum and the 10-ns photoproduct spectrum of the CO derivative is indicative of either no difference in the tertiary structures of the deoxy and CO derivatives or no temporally persistent ($>10$ ns) ligand binding-induced tertiary changes in the photoproduct.

In general there appears to be two classes of fast tertiary relaxations in hemeprotein photoproducts. Raman studies on the photodissociated COHbA (29) and COHbI (30), the dimeric Hb from the arid clam Scapharca inaequivalvis, show that the characteristic time scale for tertiary relaxations involving significant movements of either helices, heme groups, or residues occurs from milliseconds to seconds. In HbA the time course for these relaxations are solvent- (29) and viscosity-dependent (31). In contrast to these visco-elastic type relaxations, there are also faster processes common to both Hbs and Mbs.

$^3$ S. Huang, J. Huang, A. P. Kloek, D. E. Goldberg, and J. M. Friedman, manuscript in preparation.
exposure to water for any of the tryptophan environments upon doublet.

Further comment on some of these ligand binding-induced mental dependencies for several of the Raman bands allow for changes. The heme protein does not result in large scale tertiary structure likely associated with heme changes that respond on the subnanoseconds to microseconds to relax. Instead they are most Hb are not the visco-elastic-type tertiary changes that require very much faster than the onset of the nanosecond relaxations. On the basis of these studies, it was proposed (27, 33, 34) that the fast relaxation is an elastic-like solvent-independent relaxation of the iron and proximal histidine that is driven by the repulsive interaction of the proximal imidazole and the heme nitrogens. Although the time dependence of this relaxation appears to be both protein- and solvent-independent, the heme and the heme-proximal histidine conformation at the end of this fast relaxation are strongly dependent upon the overall protein tertiary structure of the initial parent liganded protein (33). Recent femtosecond Raman studies (35) strongly support these concepts.

Based on the behavior of HbA and Mb as discussed above, it is very likely that the CO binding-induced changes in Ascaris Hb are not the visco-elastic-type tertiary changes that require nanoseconds to microseconds to relax. Instead they are most likely associated with heme changes that respond on the subnanosecond time scale upon photoligand binding in this heme protein does not result in large scale tertiary structure changes.

The assignments and known conformational and environmental dependencies for several of the Raman bands allow for further comment on some of these ligand binding-induced changes. The absence of change in the W7 tryptophan Fermi doublet at $-1350 \text{ cm}^{-1}$ indicates that there is no change in the exposure to water for any of the tryptophan environments upon ligand binding in the native protein.

It is most significant that, for the native protein, upon switching the ligand from CO to O$_2$, the only substantial change in the spectrum involves the hydrogen bond-sensitive (14, 16, 20, 24) tyrosine bands Y8a and Y8b. This doublet shows an increase in intensity of approximately 15% and a slight shift to lower frequencies. The presence of seven tyrosines makes an accurate determination of shifts difficult; nonetheless, we attempted to spectrally deconvolute the shifted peak(s) from the unshifted peaks using Lab Calc spectral deconvolution programs. The results, which are clearly fraught with uncertainty, suggest that upon oxygenation, there are spectral components appearing in the Y8a and Y8b bands that are shifted to lower frequencies from the central peaks by approximately 2 and 0.3 cm$^{-1}$, respectively. Overall these results indicate that the switch in ligand from CO to O$_2$ does not induce, to an appreciable degree, any further conformational changes in the protein other than those reflected in the intensity and frequency changes in the hydrogen bond-sensitive Y8a and Y8b tyrosine bands.

The relationship between the hydrogen bonding status of tyrosine and its UV Raman spectroscopic behavior has been studied by examining the spectral response of p-cresol (CH$_3$C$_6$H$_4$OH) (14, 20, 21, 24) in different solvents. These studies show that the Y8a and Y8b bands exhibit an approximately linear correlation with the strength of the hydrogen bond formed in apotic solvents. In apotic solvents, an increase in hydrogen bonding involving the tyrosine phenolic hydrogen lowers the Y8a and Y8b frequency and increases the intensity. In solvents where the hydrogen bonding is to the tyrosine oxygen, the Y8a and Y8b bands both increase in frequency and decrease in intensity relative to values observed for a nonhydrogen-bonding environment. The change in intensity is due to a shift in the tyrosine UV absorption upon change in hydrogen bonding status of the hydroxyl group. A change from a non-hydrogen-bonding situation to one in which the tyrosine becomes a strong proton donor should increase the cross-section for Raman scattering by a factor of two for a 230 nm excitation (20). If one assumes that only one out of seven tyrosines switches from being non-hydrogen bonding to a proton donor, the intensity of the Y8a, Y8b doublet should increase approximately 15% as is observed.

The above analysis is consistent with CO and O$_2$ both inducing similar local tertiary changes within Ascaris Hb D1 but with oxygen, ligand binding is also associated with the formation of a hydrogen bond involving a tyrosine that acts as the proton donor. The absence of additional changes in the spectrum upon switching from CO to O$_2$ indicates that ligand binding per se creates a conformation that allows the tyrosine to form the hydrogen bond without further conformational changes in the protein. Given these points, the fact that dioxygen and carbon monoxide are nearly identical in size but differ in that the former forms hydrogen bonds much more readily when bound to the iron, and the favorable position of Tyr(B10) (13), it can be concluded that the formation of the phenolic hydrogen bond directly involves the bound oxygen.

From the results on the native form of D1 it is still unclear to what extent the E7 glutamine plays a role in the formation of the hydrogen bond discussed above. X-ray crystallographic studies (13) indicate that E7 can form a hydrogen bond with both the tyrosine and the bound dioxygen possibly creating a hydrogen bonding network. Raman spectral differences between the CO- and O$_2$-bound derivatives of the E7 Gln to Leu mutant D1 fragment indicate that the switch to dioxygen results in the formation of the above discussed tyrosine hydrogen bond. Thus the glutamine is not necessary for the formation of this hydrogen bond. The diminution of the intensity increase suggests that the B10 tyrosine is not forming as strong a
hydrogen bond as in the native fragment.

The observation that for the mutant (but not the native form of D1) there are additional spectral changes besides those involving YBa,YB upon switching ligands, indicates that the binding of CO to the mutant does not result in the same conformation as for the oxygen derivative (minus the strong hydrogen bond). In contrast to native D1, the mutant protein shows both the hydrogen bond formation and additional conformational changes upon switching from CO to O2. This finding suggests that the glutamine may play a role in maintaining the geometry of the distal pocket, possibly through its direct interaction with the B10 tyrosine. The absence of the glutamine may therefore result in a less structured pocket which requires a greater degree of reorganization in order to create the conformation that maximally stabilizes the bound oxygen.

The change in the relative intensity of the tryptophan Fermi doublet Raman band at $-1350$ cm$^{-1}$ is indicative of a more hydrophilic tryptophan environment in the oxy form of the mutant vs. the CO derivative of the mutant. No such change is observed for the native protein. A possible explanation is that water replaces the glutamine in the network of hydrogen bonding that stabilizes the bound oxygen in the E7 Gln to Leu mutant and that upon binding oxygen there is a reorganization of the relatively mobile water-tyrosine unit so as to create this stable hydrogen bonding network with the water.

Differences in the off rates between the leucine and alanine E7 mutants may reflect differences in the occupancy of the distal heme pocket for water.

**CONCLUSION**

Ligand binding to the wild type D1 fragment of Ascaris Hb with either CO or dioxygen produces identical changes in the high frequency region of the UV resonance Raman spectrum in all of the tyrosine and tryptophan bands except the tyrosine bands with known sensitivity to hydrogen bonding. This result indicates the presence of ligand-independent tertiary changes in the overall globin upon binding diatomic ligands and the presence of an oxygen-dependent strong hydrogen bond in which the proton donor is the tyrosine oxygen. Together the results directly support the claim that tyrosine B10 is hydrogen-bonded to the bound oxygen in the native protein.

The detection of a spectral signature for this key localized interaction in conjunction with the existing mutagenic capabilities open the door for studies in which this hydrogen bond can be easily monitored as a function of well-defined changes in both local and global tertiary structure.

Results obtained on the E7 Gln to Leu mutant indicate that this oxygen-dependent hydrogen bond is still formed even in the absence of the glutamine. The spectral changes observed for this mutant raise the possibility that the glutamine helps stabilize a hydrophobic distal heme pocket conformation that is “preset” for hydrogen bond formation. The Raman data suggest that water may replace the glutamine in the hydrogen bonding network of the mutant.

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