Formation of a Five-Coordinate Hydroxide-Bound Heme in the His93Gly Mutant of Sperm Whale Myoglobin

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The heme prosthetic group serves as the ligand-binding site, the catalytic site, or the redox site in hemeproteins. For many years, there has been interest in understanding the role of the proximal ligand in stabilizing the heme and controlling its reactivity. The role of the proximal histidine in myoglobin can be assessed by replacing it with a noncoordinating residue to create a cavity where exogenously added ligands can bind and coordinate to the heme iron.[1−3] By employing a chemical rescue approach, i.e., by addition of exogenous imidazole to the proximal histidine mutant, the properties of the native protein may be restored.[1,2,4] To evaluate the success of the chemical rescue approach, it is crucial to understand the ligation state of the mutant protein which is free of exogenous ligand. It is also important to determine whether the structure of the heme cavity is collapsed in the proximal histidine mutants.

The crystal structure of the proximal histidine mutant of ferric sperm whale myoglobin (H93G Mb) has been solved[1] for a form in which the heme is coordinated by an exogenous imidazole. A distal water molecule is present although the iron−water bond distance was 0.6 Å longer than in the wild-type protein. However, no spectroscopic data or structural information on the heme cavity in H93G Mb are available in the absence of an imidazole ligand. In the present study, we have examined the solution structure of H93G Mb in both the presence and absence of exogenous imidazole by resonance Raman spectroscopy. At high pH in the absence of imidazole the heme is five-coordinated with hydroxide as its axial ligand.

The high-frequency region (1300−1700 cm^{-1}) of the resonance Raman spectra of hemeproteins is comprised of porphyrin in-plane vibrational modes that are sensitive to the electron density in the porphyrin macrocycle and also to the coordination and spin state of the central iron atom.[5−6] Figure 1 shows the resonance Raman[7] spectra of ferric H93G Mb[8] in the high-frequency region as a function of pH in the presence and in the absence of exogenous imidazole. The electron density marker band, ν_4, appears at ∼1372 cm^{-1} in all spectra and is typical of ferric hemeproteins. At the highest pH (10.5) examined, the spin and coordination sensitive line, ν_3, shows the presence of a five-coordinate high-spin (5CHS) population (ν_3 = 1490 cm^{-1}) (spectrum a). When the pH is lowered to 7.4 a minor contribution from a 6-coordinate low-spin (6CLS) population is observed (ν_3 = 1504 cm^{-1}) in addition to the 5CHS species (spectrum b). Also, the intensities of the bands due to the 5CHS form are reduced (spectra normalized to ν_4). Thus, at the highest pH the heme is five-coordinate and as the pH is lowered, a 6CLS population appears. The 6CLS species is likely the one in which the heme is coordinated by the distal histidine in addition to the hydroxide. Upon addition of small amount of exogenous imidazole (0.4 mM) (spectrum c), a mixture of a 6CHS species (ν_3 = 1480 cm^{-1}) and a 6CLS species (ν_3 = shoulder at ∼1504 cm^{-1}) is formed, with a residual population of 5CHS species (1490 cm^{-1}). Addition of excess imidazole produced a 6CLS species (ν_3 = 1504 cm^{-1}, ν_{10} = 1639 cm^{-1}) (spectrum d) at both neutral (neutral pH data not shown) and alkaline pHs, which we assign as a bis-imidazole heme.

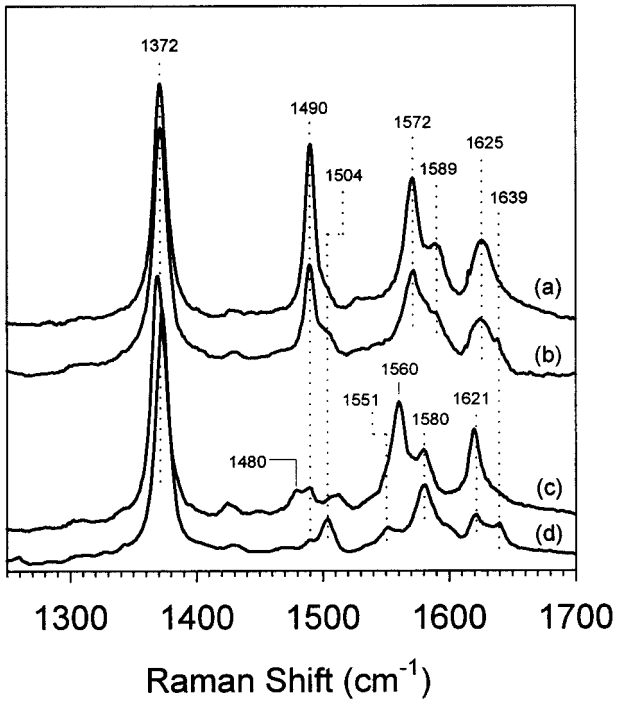

Figure 1. High-frequency resonance Raman spectra of ferric H93G Mb as functions of pH and imidazole concentration. The spectra in the absence of imidazole were measured at (a) pH 10.5 (CAPS) and (b) pH 7.4 (phosphate). The two spectra at the bottom were obtained at pH 10 (CAPS) in the presence of (c) 0.4 mM and (d) 60 mM imidazole.

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(7) The 413.1 nm laser beam (Kr-ion) was used as excitation source in the resonance Raman measurements.[5] The power at the spinning sample cell was ∼15 mW. The composition of the protein samples in isotopic water was 25 μM H93G, 20 mM buffer, 85% $H_2{}^{18}O$ (98% $H_2{}^{18}O$ from CIL, Andover, MA) and 15% $H_2{}^{16}O$.
(8) The H93G mutant was obtained by applying cassette mutagenesis to the sperm whale Mb gene in the plasmid pMb413b that was transformed into BL21 (lambda DE3) *E. coli* cells.[1, 2]

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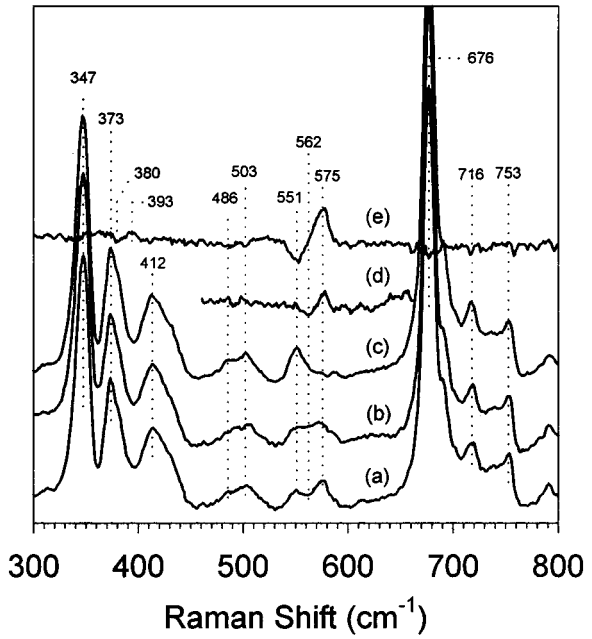

Figure 2. Low-frequency resonance Raman spectra of ferric H93G Mb at pH 7.4. The spectra shown are for samples in (a) $H_2{}^{16}O$, (b) $D_2{}^{16}O$, and (c) $H_2{}^{18}O$. Traces (d) and (e) are the $H_2{}^{16}O-D_2{}^{16}O$ and $H_2{}^{16}O-H_2{}^{18}O$ difference spectra, respectively.

The identification of an iron−axial ligand vibrational mode is extremely useful as it directly demonstrates the presence of a particular ligand and the nature of its interactions in the heme pocket.[5] As seen in Figure 2, at pH 7.4 the intense Raman line at 575 cm^{-1} in $H_2{}^{16}O$ (spectrum a) shifts to 551 cm^{-1} in $H_2{}^{18}O$ (c), and yields a very clean difference spectrum (e). This band shifts down from 575 cm^{-1} in H_2O to 562 cm^{-1} in D_2O (spectra b and d). Similar isotopic sensitivity is also observed at pH 10.5 (data not shown). We assign this line as the iron-hydroxide stretching mode (ν_{Fe-OH}) arising from the Fe−OH moiety of a five-coordinate heme in H93G Mb.

In six-coordinate ferric hydroxide complexes of hemeproteins with histidine or imidazole as an axial ligand, ν_{Fe-OH} is observed in the 450−555 cm^{-1} region[9−12] at room temperature depending on the spin state of the heme iron. Alkaline metMb and metHb contain a mixture of six-coordinate high- and low-spin states of the iron−hydroxide species in solution at room temperature.[10] The ν_{Fe-OH} frequency of the high-spin hydroxide complex appears at ∼490 cm^{-1}, while in the low-spin hydroxide complex it appears at ∼550 cm^{-1}. The alkaline hydroxide complex of horseradish peroxidase is six-coordinate low-spin and the ν_{Fe-OH} frequency appears at 503 cm^{-1} at room temperature.[10] In contrast, in the five-coordinate high-spin hydroxide species (with no axial histidine/imidazole) ν_{Fe-OH} appears at a higher frequency, 541 cm^{-1}, in a model heme complex,[13] and at ∼534 cm^{-1} in the proximal histidine mutant of cytochrome *c* peroxidase.[14] In the five-coordiate hydroxy complex of H93G Mb, ν_{Fe-OH} appears at a significantly higher frequency (575 cm^{-1}). A similar frequency is seen in the 5-coordinate ferriheme hydroxide complex of native *Scapharca inaequivalvis* hemoglobin (HbI).[15] We attribute the high frequency to two origins. First, the absence of a ligand trans to the hydroxide can strengthen the Fe−OH bond in comparison to the case when the proximal histidine is present. Second, the higher frequency (>30 cm^{-1}) of the line as well as it being significantly narrower compared to some of the other 5-coordinate hydroxide complexes[13,14] indicates that there are very few polar interactions between the −OH and its neighboring residues. Thus, the −OH moiety appears to be located in a hydrophobic environment.

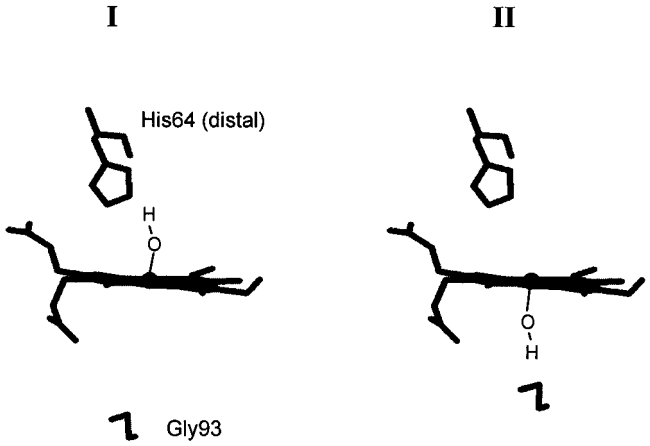

Figure 3. Schemes showing the two possible ligation states of the heme in ferric H93G Mb without the imidazole. The hydroxide group can bind either on the distal side (**I**), or on the proximal side (**II**) of the heme. In structure **II**, the proximal pocket is shown as partially collapsed. As discussed in the text, we postulate that the hydroxide binds on the proximal side of the heme (structure **II**).

It is important to note that the 24 cm^{-1} isotope shift ($^{16}O/^{18}O$) corresponds to an ideal value (23.8 cm^{-1} assuming that the two oscillating units are the Fe and the −OH) that would be expected from an "isolated" Fe−OH harmonic oscillator. The 13 cm^{-1} shift of ν_{Fe-OH} in D_2O is also nearly an ideal value. If the −OH group has strong hydrogen-bonding interactions with its neighboring groups, the expected isotope shift may not occur. In such cases, the ν_{Fe-OH} line may show a smaller isotope shift than expected, and even can move to higher frequencies in D_2O than in H_2O, as seen in alkaline horseradish peroxidase[10] and a model heme complex.[13] Thus, the observation of a nearly ideal shift to lower frequency of ν_{Fe-OH} in D_2O suggests that the hydroxide group in H93G Mb does not experience strong polar interactions with its surroundings.

From the resonance Raman data reported here a model for the coordination of the heme in H93G Mb could be formulated. First, the high frequency of the ν_{Fe-OH} mode indicates that the −OH is not interacting with any polar residue, although it may be close to some nonpolar residues. Therefore, we postulate that the −OH is coordinated to the iron on the proximal side of the heme (Figure 3). If it were on the distal side of the heme it would expectedly be connected by a hydrogen-bonding network to the distal histidine but we find no evidence for any hydrogen bonding. For the same reason, it can be argued that the proximal cavity is partially collapsed in the absence of imidazole. Should the proximal cavity in H93G Mb have retained its shape as in the wild-type protein, water molecules would be expected to be present which could hydrogen bond to the hydroxide ligand.

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