Solution and Crystal Structures of the H175G Mutant of Cytochrome *c* Peroxidase: A Resonance Raman Study

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Site-directed mutagenesis offers a powerful probe of heme proteins. Recent attention has focused on mutants whose proximal iron ligand is replaced by smaller, noncoordinating amino acids. These mutants form artificial cavities where the native proximal ligand resided and which are capable of binding exogenous ligands such as imidazoles. Such reconstituted systems permit detailed studies of the electronic and stereochemical influences of the proximal ligand on catalysis and activity.[1−6] The paradigm of these mutants is H175G cytochrome *c* peroxidase (CCP). Its X-ray crystal structure revealed that the iron is close to two water molecules.[1] In heme oxygenase, the H25A mutant has been proposed to contain a single water ligand on the heme iron.[4] The iron in the H170A mutant of horseradish peroxidase is probably bound by a water molecule at pH 4 and an additional sixth ligand (possibly a distal histidine) at pH 5−7.[6] The crystal structure of the imidazole adduct of H93G myoglobin has been reported,[2] but structural and spectroscopic data for the H93G mutant in the absence of imidazole are not yet available. Interestingly, UV−vis spectra of wild-type metmyoglobin and ferric *Coprinus cinereus* peroxidase in acidic buffer[7,8] bear resemblance to those of the proximal ligand-deficient heme proteins; the native proximal ligation may well be disrupted under such conditions. Among these proximal-ligand mutants, H175G CCP is unique in that its crystal structure indicates two water molecules axial to the heme. However, it is not certain whether one or both of these waters is strongly coordinated, what the water protonation states are, and whether the axial coordination of two water molecules can be maintained in solution. In this study, we have examined the crystal and solution structures of the H175G mutant by UV−vis, resonance Raman (RR), and EPR spectroscopy.

Ferric H175G CCP[9] undergoes a transition from a "red form" near pH 6 to a "green form" near pH 7 (Figure 1). The absorbance at 408 nm (λ_{max} of the red form) changes with pH to give an apparent pK_a of ∼6.5 (inset, Figure 1). A fit to these data indicates that the transition involves a cooperative two-proton process. One proton is proposed to be from the iron-bound water molecule, but the source of the second proton is unknown. The UV−vis and RR spectra of H175G at pH 10.0 are almost identical to those at pH 7.2 (not shown). Fe^{III}(TMP)s [TMP = 5,10,15,20-tetrakis(1-methylpyridinium)porphines] exhibit similar ligand ionization in aqueous solution where the five-coordinate iron is ligated by a water at pH < 6 and a hydroxide at pH < 11 and only forms a six-coordinate bishydroxo adduct at pH > 11.[10,11]

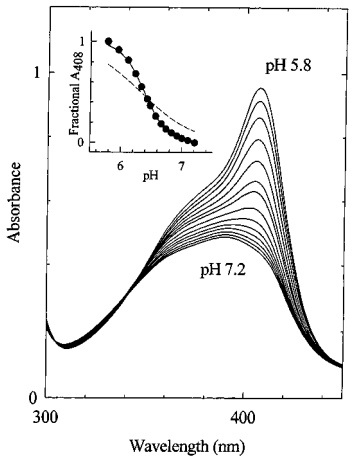

Figure 1. Electronic absorption spectra of H175G CCP (10 mM) between pH 5.8 and 7.2. The absorbance at 408 nm as a function of pH is shown in the inset together with a fit to either a single proton (pK_a ∼6.5; - - -) or a cooperative two-proton ionization (pK_a ∼ 14.8, n = 2.3; —).

Resonance Raman spectra of heme proteins are dominated by porphyrin skeletal vibrations, i.e., totally symmetric ν_2, ν_3, and ν_4 with Soret excitation, nonsymmetric modes ν_{10}, ν_{11}, and ν_{19} with Q-band excitation, and ν_2 and ν_{10} with near-UV (∼350 nm) excitation.[12−15] The frequencies of these modes are determined by the iron coordination and spin states. The RR spectra of H175G[16] are shown in Figure 2. The well-defined ν_3 and ν_{10} frequencies at 1494 and 1630 cm^{-1}, respectively, of the red solution (pH 5.9) are clearly characteristic of a five-coordinate high-spin (5cHS) ferric heme.[15,17−20] The RR spectra of the green solution (pH 7.2) also indicate a dominant 5cHS

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(9) The construction, expression (in *Escherichia coli*), purification, and crystallization of H175G CCP mutant have been described in ref 1. While the crystals of this mutant were grown from distilled water, a crystal suspension of H175G had a pH of 5.8. For solution studies, crystals were dissolved in buffers. To prepare enzyme in ^{18}O-labeled buffers, crystals were twice washed with $H_2{}^{18}O$ water (95%, ICON, Mt. Marion, NY) and subsequently dissolved in a 250 mM potassium phosphate/$H_2{}^{18}O$ buffer (pH 5.9) or in a similar 100 mM buffer (pH 7.2). The final $H_2{}^{18}O$ percentage was ∼90% in these solutions. The RR spectra of the crystalline H175G protein were obtained from individual crystals whose surface water was removed with a Kimwipe tissue.

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(16) Resonance Raman spectra were collected on a CCD-equipped spectrograph.[6] Spectra of solution samples in glass capillaries at room temperature were collected in a 90°-scattering geometry with incident laser power of ∼12 mW at 350.1, 413.1, and 441.6 nm and ∼25 mW at 514.5 nm. For the crystals, a 145°-backscattering geometry was used. The crystal sample temperature was varied between 17 and 120 K by use of a closed-cycle helium refrigerator. The incident laser power was kept low (∼0.5 mW at 413.1 nm) because of severe photoreduction at higher power. Spectral resolution was ∼5.0 cm^{-1}.
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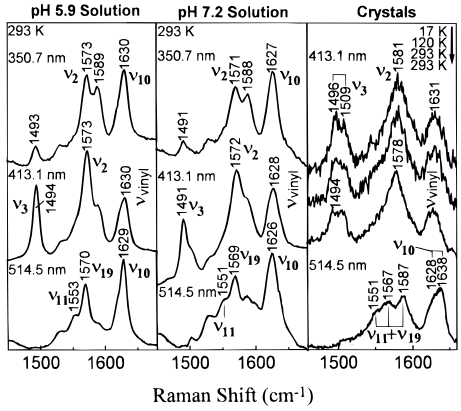

Figure 2. High-frequency, variable-wavelength resonance Raman spectra of H175G CCP solutions as a function of pH and of crystals as a function of temperature.

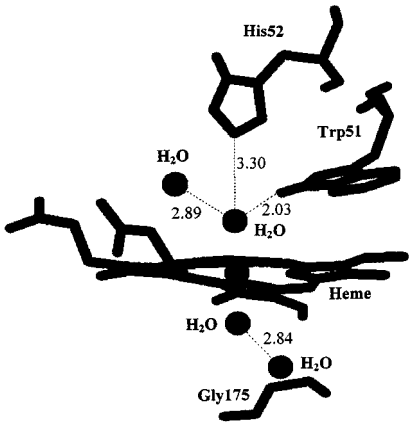

Figure 3. X-ray crystal structure of the heme site in H175G CCP. The two axial water ligands are strongly H-bonded and may be hydroxo ions (see text). A second and third species that contain only one axial water or hydroxide ligand are not shown in this diagram. The indicated distances (Å) are between heavy atoms, except for O···H(indole N of Trp51).

heme by the frequencies at 1491 (ν_3) and ∼1627 cm^{-1} (ν_{10}).[15,17−20] In contrast, the RR spectra of the H175G crystals show a mixture of 5cHS (ν_3 at ∼1495 cm^{-1}) and low- and/or intermediate-spin (ν_3 at 1509 cm^{-1} and ν_{10} at 1638 cm^{-1}) species.[18,21,22] However, EPR data (12 K; not shown) of H175G CCP crystals rule out intermediate-spin species, showing a predominant (>50%) HS heme with an axial $g = 6$ signal, a six-coordinate low-spin (6cLS) heme, and a minor (∼10%) second HS species with rhombic character. The relative populations of HS:LS do not change strongly with temperature as indicated by the relative intensities of the RR bands at 1495 and 1509 cm^{-1}.

The identification of three species in H175G crystals is in accord with structural studies of the mutant protein and model systems.[1,10,11,23] The X-ray structure of the CCP mutant reveals two axial water ligands (Figure 3).[1] $Fe^{III}(TPP)(H_2O)_2$ (TPP = tetraphenylporphine) and $Fe^{III}(TMP)(OH)_2$ have been determined to be in high-spin and low-spin states, respectively.[10,11,23] We propose that H175G crystals contain 5cHS aqua and hydroxo species together with a 6cLS species arising from strong

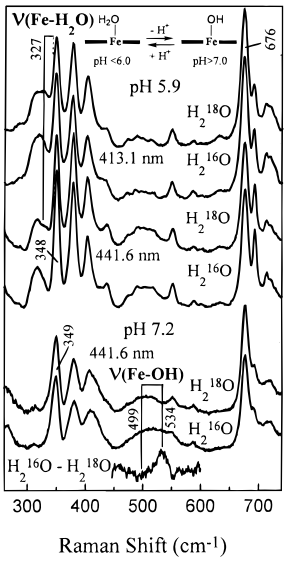

Figure 4. Low-frequency resonance Raman spectra of H175G CCP in $H_2{}^{16}O$ and $H_2{}^{18}O$ buffer solutions at room temperature.

H-bonding to axial water or hydroxide ligands, especially in the distal pocket (Figure 3).

The axial ligands of H175G in solution are concluded to be a water and a hydroxide at pH 5.9 and 7.2, respectively (Figure 4). In the pH 7.2 spectrum, a broad Raman band at ∼534 cm^{-1} is shifted to ∼499 cm^{-1} in $H_2{}^{18}O$ buffer. The frequency is in the range of ν(Fe−OH) (450−558 cm^{-1}) for heme proteins with a histidine and a hydroxo ligand.[24,25] The ν(Fe−OH) of Fe^{III}(TMP)(OH) is at 554 cm^{-1} (514 cm^{-1} in $H_2{}^{18}O$)[11] and is broad like that of H175G at pH 7.2. The preservation of the ∼534-cm^{-1} ν(Fe−OH) band at pH 10 indicates that the H175G heme iron maintains a hydroxo ligand between pH 7.0 and 10.0 (data not shown). At pH 5.9, Raman peaks observed at ∼500 cm^{-1} no longer show ^{18}O-sensitivity. Instead, a new feature is observed at ∼327 cm^{-1} in the $H_2{}^{18}O$ spectrum that is missing in the $H_2{}^{16}O$ spectrum (Figure 4). Analysis of the difference spectrum (not shown) has minima and maxima at 333 and 348 cm^{-1} that we assign to $\nu(Fe-{}^{18}OH_2)$ and $\nu(Fe-{}^{16}OH_2)$, respectively. The latter is overlapped by the very intense porphyrin mode at 348 cm^{-1} (a diatomic model estimates $\nu(Fe-{}^{16}OH_2)$ at ∼346 cm^{-1} relative to $\nu(Fe-{}^{18}OH_2)$ at 333 cm^{-1}). Although Fe−OH_2 vibrations have not been previously identified in ferric heme proteins, they have been observed in the 340−460-cm^{-1} range in non-heme Fe(III) complexes.[26−28]

It is of interest that heme protein mutants with noncoordinating proximal residues[4,6] and native ferric heme proteins in acid solution[7,8] show a broadened and, perhaps, blue-shifted Soret absorption. Their λ_{max} are similar to that of the red or the green form of H175G CCP, reflecting that the heme iron atoms in these proteins are coordinated by a single water or hydroxide ligand with different degrees of H-bonding to the protein.

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