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 stereochemistry 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 (CCP) is probably bound by a water molecule at pH 4 and an additional sixth ligand (possibly a distal histidine) at pH 5.7 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 buffer7,8 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 CCP9 undergoes a transition from a “red form” near pH 6 to a “green form” near pH 7 (Figure 1). The absorbance at 408 nm (λmax of the red form) changes with pH to give an apparent pK1 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(Cp)(TMP) [TMP = 5,10,15,20-tetrakis(1-methylpyridinium)porphine] exhibits 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

Resonance Raman spectra of heme proteins are dominated by porphyrin skeletal vibrations, i.e., totally symmetric ν3, ν5, and ν4 with Soret excitation, nonsymmetric modes ν10, ν11, and ν14 with Q-band excitation, and ν3 and ν10 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 are shown in Figure 2. The well-defined ν3 and ν10 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.13,17−20 The RR spectra of the green solution (pH 7.2) also indicate a dominant 5cHS

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 (pK1, ∼6.5; ‒•‒) or a cooperative two-proton ionization (pK2, ∼14.8, n = 2.3; ‒`).

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heme by the frequencies at 1491 (ν 3) and ∼1627 cm −1 (ν 10). 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 5 at 1509 cm −1 and ν 10 at 1638 cm −1) species. 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. The X-ray structure of the CCP mutant reveals two axial water ligands (Figure 3). FeIII(TPP)(H2O)2 (TPP = tetraphenylporphine) and FeIII(TMP)(OH)2 have been determined to be in high-spin and low-spin states, respectively. We propose that H175G crystals contain 5cHS aqua and hydroxido species together with a 6cLS species arising from strong 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 H218O buffer. The frequency is in the range of ν(Fe–OH) (450–558 cm −1) for heme proteins with a histidine and a hydroxido ligand. The ν(Fe–OH) of FeIII(TMP)(OH) is at 554 cm −1 (514 cm −1 in H218O) 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 hydroxido 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 18O-sensitivity. Instead, a new feature is observed at ∼327 cm −1 in the H218O spectrum that is missing in the H216O spectrum (Figure 4). Analysis of the difference spectrum (not shown) has minima and maxima at 333 and 348 cm −1 that we assign to ν(Fe–18OH2) and ν(Fe–16OH2), respectively. The latter is overlapped by the very intense porphyrin mode at 348 cm −1 (a diatomic model estimates ν(Fe–16OH2) at ∼346 cm −1 relative to ν(Fe–18OH2) at 333 cm −1). Although Fe–OH2 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.

It is of interest that heme protein mutants with noncoordinating proximal residues and native ferric heme proteins in acid solution show a broadened and, perhaps, blue-shifted Soret absorption. Their A 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 hydroxido ligand with different degrees of H-bonding to the protein.

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