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Cysteine Thiolate Coordination in the Ferrous CO Complex of an Engineered Cytochrome b_{562}

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Cytochrome P450 is a generic name for heme proteins showing absorption maxima at about 450 nm in the ferrous CO-bound state.[1] This unique spectral characteristic, and the monooxygenase activity of P450,[2,3] have been attributed primarily to the presence of an unusual proximal heme ligand, a thiolate from a cysteine residue.[4,5] Strong electron donation from the thiolate to the iron is proposed to be responsible for heterolytic cleavage of the O−O bond[3,5,6] and for the succeeding chemical reactions including hydroxylations, epoxidations, and dealkylations.[2] Introduction of a thiolate axial ligand into heme proteins that do not naturally possess this type of ligation would offer insight into the unique chemistry of cytochrome P450s. This has been attempted in myoglobin (Mb), one strategy being simply to replace the axial ligand His93 with Cys.[7] A second approach is to substitute His93 by Gly to create a proximal cavity which can be filled with exogenous thiolate ligands such as thiophenol.[8]

In these experiments, the oxygenation reactions were performed on the ferric forms of the proteins through a shunt path which utilizes peroxides and peroxyacids to bypass the requirement for an oxygen-bound ferrous species in the P450 mimics. This is because it has so far proved impossible to engineer ferrous iron−thiolate bonding in Mb. The engineered proteins also fail to reproduce the 450-nm absorption band in the ferrous CO-bound state, the defining characteristic of P450. Here, we have succeeded in engineering cysteine thiolate ligation in the ferric, ferrous, and even the ferrous-CO ligated states of a heme protein for the first time. We utilized the four-helix bundle heme protein, cytochrome b_{562} from *Escherichia coli* as a scaffold. This protein is six-coordinate in the ferric and ferrous states with Met7 and His102 providing the axial ligands.[9] We substituted Met7 by a non-coordinating Gly to create a cavity in which CO can bind, and His102 by Cys to alter the axial ligand. In addition, two Ser replacements were made at positions Glu4 and Glu8 to stabilize the heme[10] resulting in a quadruple mutant (SGSC).

Table 1. Absorption Maxima (nm) for Thiolate-Ligated Heme Proteins

protein	Soret[a]	visible		ref
Ferric				
cytochrome P450cam (+ camphor)	391 (91)	509	646	11a
H93C myoglobin	391 (115)	509	629	7c
H93G myoglobin (+ ethanethiol)	391 (74)	508	620	8b
SGSC cytochrome b_{562}	392 (101)	513	640	this work
Ferric-Imidazole				
cytochrome P450cam (+ camphor)	425	541	578	11b
SGSC cytochrome b_{562}	425	544	578	this work
Ferrous				
cytochrome P450cam (+ camphor)	408		540	11a
SGSC cytochrome b_{562}	417		560	this work
Ferrous-CO				
cytochrome P450cam (+ camphor)	447		550	11a
SGSC cytochrome b_{562}	442		551	this work

[a] Extinction coefficients are given in parentheses (mM^{-1} cm^{-1}).

In Table 1, the wavelengths of the absorption maxima for cytochrome P450cam[11] and related model proteins are summarized. The ferric forms, including the SGSC mutant, share Soret maxima at about 390 nm. In the ferric-imidazole-bound, ferrous, and ferrous-CO states, the absorption spectra of SGSC are also quite similar to those of P450cam. Remarkably, the Soret maximum of SGSC in the ferrous-CO state is at 442 nm, which strongly suggests that thiolate coordination persists even in this state. To confirm this coordination, the active site of the SGSC mutant was investigated by resonance Raman spectroscopy, which is a powerful tool for probing the heme structure.[12]

In Figure 1A, the resonance Raman spectrum of the ferric SGSC mutant is compared with that of cytochrome P450cam. The Raman bands in the 1300−1700 cm^{-1} region are a sensitive probe of the oxidation state, the spin state, and the coordination number of the heme iron.[12] The frequencies and intensities of ν_4 (1370 cm^{-1}), ν_3 (1488 cm^{-1}), and ν_2 (1564 cm^{-1}) in SGSC are similar to those of P450cam, indicating that the mutant has a five-coordinate ferric high-spin heme. In Figure 1B, ferrous spectra of the SGSC mutant and P450cam are shown. The ν_4 and ν_3 lines in P450cam (1344 and 1466 cm^{-1}) are similar to those in SGSC but lower than those in other heme proteins which have a His ligand like Mb (1356 and 1472 cm^{-1}), and this is a signature of the anionic thiolate coordination.[13] Strong lines in the 1550−1600 cm^{-1} region are also apparent in both proteins. Therefore, the SGSC mutant faithfully traces the spectral profiles of cytochrome P450cam in both the ferric and ferrous states, consistent with the presence of a thiolate ligand.

To establish thiolate coordination in the ferrous-CO complex of the SGSC mutant, resonance Raman spectra were measured with 457.9 nm excitation (Figure 2), where CO vibrations are expected to be greatly enhanced.[14] A very strong and sharp Raman line is apparent at 477 cm^{-1}, which downshifted by 12 cm^{-1} upon

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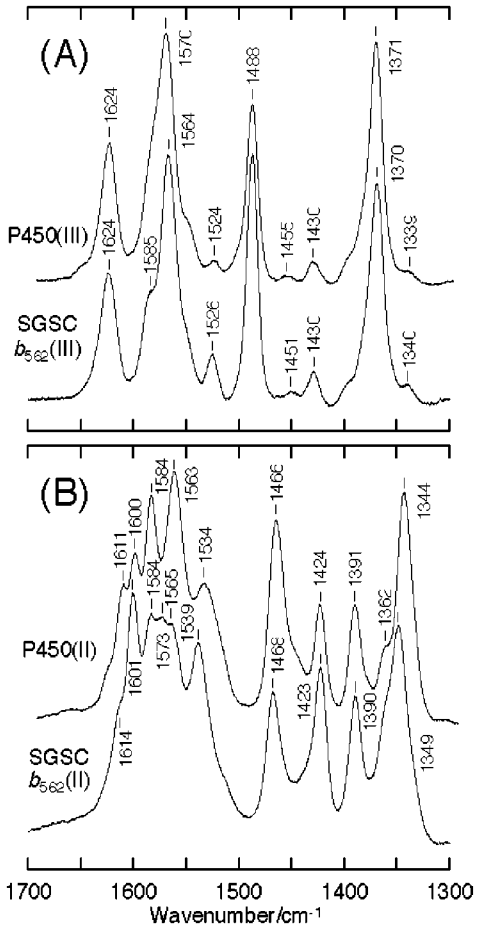

Figure 1. Resonance Raman spectra of cytochrome P450cam and the SGSC mutant of cytochrome b_{562} in the ferric (A) and ferrous (B) states. The samples were excited by the 406.7-nm line of a krypton ion laser (Coherent Innova 302C) with laser power of 50 mW at the sample. A spinning Raman cell was used throughout the measurements. The spectra were recorded using a double monochromator (Jasco R-800) with a slit width of 6 cm^{-1}. The samples contained 100 μM protein in 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl for SGSC, and 50 mM potassium phosphate (pH 7.4) and 1 mM camphor for P450cam. The ferrous samples were prepared by the addition of sodium dithionite.

CO isotope substitution of $^{12}C^{16}O$ by $^{13}C^{18}O$. The frequency shift indicates unequivocally that this line is associated with the ν(Fe−CO) stretching mode. In addition, Raman lines at 1957 and 562 cm^{-1} were also sensitive to CO isotopes, and can be assigned to the ν(C−O) stretching and δ(Fe−C−O) bending modes, respectively. The frequencies are somewhat different from those of CO−P450cam (481, 558, 1940 cm^{-1})[14] but rather close to substrate-free CO−P450scc (477, 1953 cm^{-1})[15] where the CO is located in an apolar pocket. It has been established that ν(Fe−CO) and ν(C−O) stretching frequencies are linearly correlated, and that the frequency pair reveals the identity of the axial ligand and the polarity of the environment around the bound CO.[16] The observed Fe−CO vibrations in the SGSC mutant indicate (i) that the axial ligand *trans* to CO is the Cys thiolate and (ii) that the CO is located in a hydrophobic pocket as might be anticipated from analysis of the cytochrome b_{562} structure.[9]

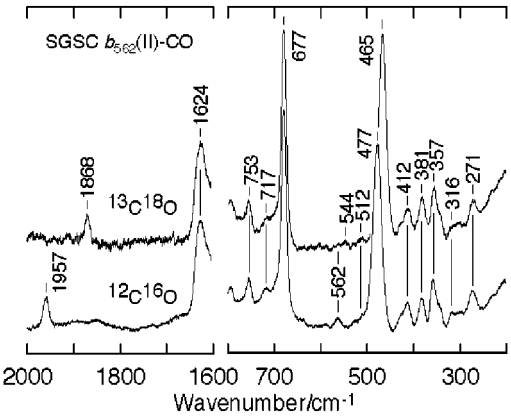

Figure 2. Resonance Raman spectra of the SGSC mutant in the ferrous CO-bound state. The spectral conditions are as in Figure 1 except that the spectra were excited by the 457.9-nm line of an argon ion laser (NEC GLS3300) with laser power of 30 mW at the sample and with a defocused laser beam. The samples contained 200 μM SGSC in 100 mM Tris-HCl (pH 9.0). Upper, $^{13}C^{18}O$; lower, $^{12}C^{16}O$.

There have been many unsuccessful attempts[17] at mimicking the ferrous-CO state of cytochrome P450. Recently, an aliphatic Leu residue was introduced into cytochrome *c* peroxidase to mimic the hydrophobic proximal environment of cytochrome P450.[17a] Exogenous cyanide coordinates the ferric protein but CO is unable to combine with the ferrous heme,[17a] even though $Fe^{3+}-CN^-$ and $Fe^{2+}-CO$ have the same charge and similar geometry. An interesting feature of the cytochrome b_{562} structure is the pair of arginines, Arg98 and Arg106, which form an integral component of the heme binding surface. These arginines flank the Fe-coordinating side chain of His102, each residing one α-helical turn away.[9] In the SGSC mutant these cationic Arg residues may form interactions with the anionic thiolate of Cys102/heme system to balance the decreasing charge in the ferrous state. In the case of cytochrome P450, the thiolate ligand is surrounded by hydrophobic residues,[4] with no nearby positively charged residues in the proximal heme pocket. This cysteine is, however, situated at the amino-terminus of an α-helix so that the anionic thiolate may be stabilized by the positive component of the helix dipole.

In summary, we have engineered a four-helix bundle protein cytochrome b_{562} into a cytochrome P450 and succeeded in achieving thiolate coordination in the ferrous CO state. The coordination is evidenced by the Fe−CO vibrational frequencies in the resonance Raman spectra.

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