Cytochrome P450

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Calculated and Experimental Spin State of Seleno Cytochrome P450**

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The cysteine thiolate ligand coordinated to the heme iron atom in cytochrome P450 is thought to be responsible for the unique spectroscopic and catalytic properties of these enzymes. To explore the role of the proximal ligand in these proteins, the cysteine has been replaced by a variety of other ligands by site-specific mutagenesis, including a histidine,[1a–c] methionine,[1d] and serine.[1e] None of these ligand substitutions resulted in a protein with the spectroscopic or catalytic properties of a cytochrome P450 monooxygenase. However, recent computational studies suggested that a P450-like species might result from substitution of the thiolate ligand by a selenolate, and furthermore, that this substitution might accelerate the rate of formation and decelerate the rate of decay of the catalytic ferryl species, possibly making it observable.[2]

No selenolate-coordinated heme protein was known until our recent demonstration that such a protein is generated by the binding of PhSeH to a heme oxygenase cavity mutant in which the proximal histidine ligand had been replaced by an alanine.[3] Furthermore, binding of PhSeH to cytochrome P450cam yielded a hyperporphyrin spectrum that is analogous to that observed when a distal thiolate ligand is coordinated to the iron along with the proximal cysteine thiolate.[3] Nevertheless, only a brief meeting abstract exists describing a P450 enzyme in which the proximal cysteine has been replaced by a selenocysteine.[4]

Herein we report the expression and characterization of a seleno cytochrome P450 in which the cysteine thiolate iron ligand is replaced by a selenocysteine. CYP119 was used for this substitution because the proximal ligand is the only cysteine in its sequence. The seleno protein was expressed in a cysteine auxotroph BL21(DE3)CysE strain of *Escherichia coli* that cannot synthesize cysteine owing to a mutation in the *CysE* gene.[5] A pCWori vector containing the CYP119 gene encoding a 6-His tag at the C-terminus was transformed into the auxotrophic BL21(DE3)Cys cells, and the seleno protein was expressed in minimal media containing L-selenocystine (see the Supporting Information for the detailed procedure). The protein yield was 2.6 mg L^{-1} of culture after affinity purification, which is approximately 8–10 times lower than that of the normal thiolate-ligated protein. This approach results in over 70% replacement of the cysteine by a selenocysteine as judged by the relative peak intensities of the Cys and SeCys proteins by LC/ESI-MS.

The UV/Vis spectrum of ferric SeCYP119, the selenocysteine-substituted enzyme, has a Soret maximum at 417 nm, which is very similar to the 416 nm maximum of wild-type (WT) CYP119.[6] Furthermore, the Q bands of the SeCYP119 protein correlate well with those of WT CYP119 (Figure 1a).

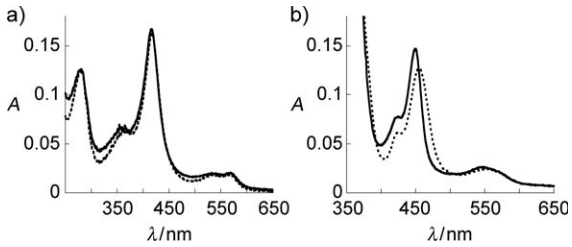

Figure 1. Comparison of the UV/Vis spectra of circa 1.5 μM WT CYP119 (——) and SeCYP119 (••••) proteins in 100 mM potassium phosphate buffer, pH 7.4. a) Ferric resting state, and b) ferrous CO complex.

The similarities in the UV/Vis spectra of ferric SeCYP119 and WT CYP119 are consistent with the presence in both proteins of a six-coordinate low-spin heme iron with a distal water ligand.

The catalytic activity of the enzymes was examined using a hydrogen peroxide mediated shunt pathway in the presence of the substrate dodecanoic (lauric) acid. The specific activity of SeCYP119 was estimated to be 90 ± 20 pmol min^{-1} per nmol of enzyme, which is approximately half that for the WT CYP119 (170 ± 18 pmol min^{-1} per nmol of enzyme).[7] Oxidation of lauric acid resulted in hydroxylation at the ω, ω-1 and ω-2 positions, as determined by GC-MS. Interestingly, the regiospecificity of hydroxylation showed the same trend for both the WT and SeCYP119 proteins, with ω-1 being the predominant product, followed by ω-2 and ω (see the

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Supporting Information). Overall, our results suggest that the novel seleno mutant is indeed catalytically active.

The EPR spectrum of ferric SeCYP119 is characteristic of a rhombic $S = 1/2$ low-spin heme iron(III) with g values that are distinct from those of the WT protein (Supporting Information, Figure S4). Interpreting the changes in g values in electronic or structural terms is not straightforward, because the selenium substitution may affect tetragonal and rhombic splittings of the low-spin iron(III) d orbitals and also the spin-orbit coupling constant.[8] The EPR spectra also support the presence of a WT CYP119 contaminant that varies from preparation to preparation but remains below 30%. The resonance Raman spectrum of ferric SeCYP119 also supports a six-coordinate low-spin configuration[9] with porphyrin skeletal modes ν_3, ν_2, and ν_{10} at 1502, 1583, and 1636 cm^{-1}, respectively (Figure 2). These frequencies are within 1 cm^{-1} of those observed in WT CYP119. The ν_4 mode shows a 1 cm^{-1} downshift upon substitution of sulfur by selenium, which reflects an increased electron density on the antibonding porphyrin π^* orbitals.[10]

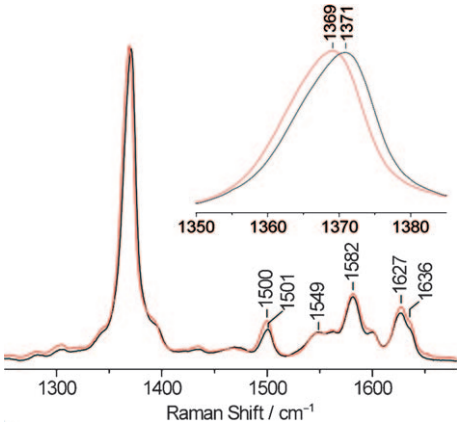

Figure 2. Resonance Raman spectra of ferric wild-type (WT) CYP119 (black) and SeCYP119 (red) at room temperature. Excitation wavelength = 413 nm.

The impact of the Cys317SeCys substitution in SeCYP119 was investigated further by characterizing the ferric nitrosyl complexes using resonance Raman spectroscopy and isotopic labeling of the nitrosyl group. In SeCYP119, the ν(Fe-NO) and ν(N-O) modes are observed at 526 ($\Delta^{15}N = 3$ cm^{-1}) and 1838 cm^{-1} ($\Delta^{15}N = 37$ cm^{-1}), respectively (Supporting Information, Figure S5). These frequencies are 4 and 14 cm^{-1} lower than in the WT protein. These decreases in stretching frequencies can be interpreted in terms of a weakening of the Fe-NO and N-O bond strengths as the σ-trans effect of the coordinating selenolate ligand on the Fe-N-O unit increases compared to that of the thiolate in the WT protein. Recent DFT calculations have examined in detail the trans effect of thiolate ligation in ferric heme nitrosyl complexes.[11]

The UV/Vis spectra of the ferrous carbon monoxide complexes show a pronounced shift in the Soret maximum from 449 nm in WT CYP119 to 454 nm in SeCYP119 (Figure 1b), but the resonance Raman characterizations of these carbonyl complexes show nearly identical ν(Fe-CO) and ν(C-O) frequencies (Supporting Information, Figure S6). Therefore, in the ferrous carbon monoxide complexes, and in contrast with the ferric-NO complexes, the trans effect of the selenolate and thiolate ligands on the Fe-C-O unit are equivalent.

To model the three-dimensional structure of the selenium resting state in SeCYP119, we used theoretical DFT(B3LYP)/MM calculations to probe an analogous SeCYP101 mutant.[2,12a] Three snapshots (60 ps, 120 ps, and 200 ps) from the equilibrium trajectory of the WT enzyme were mutated by replacing cysteine by selenocysteine and fully optimized by DFT(B3LYP)/MM geometry optimization for the spin states $S = 1/2$, 3/2, and 5/2.[12b] (For further details, see the Supporting Information). All three snapshots display similar properties, and Figure 3 depicts representative geometric data,

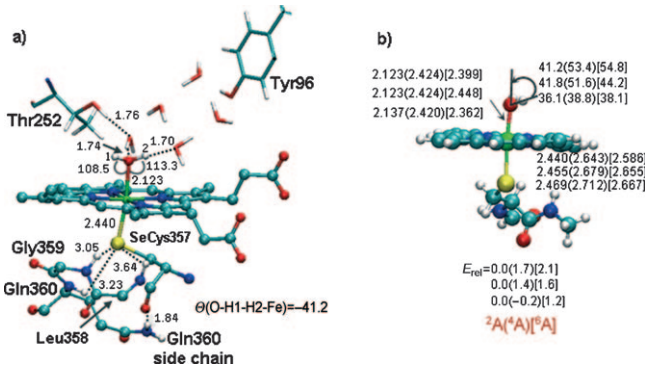

Figure 3. B3LYP/MM calculated geometric features and spin-state energies of the SeCys mutant of CYP101: a) Detailed structure of a representative snapshot (60 ps) in the doublet ^2A state. b) Relative QM/MM energies (kcal mol^{-1}) and key geometric parameters for different snapshots calculated with the largest basis set (B_L//B1 in the Supporting Information). The geometric parameters correspond to snapshots at 60, 120, and 200 ps (arranged vertically) in the $^2A(^4A)[^6A]$ spin states.

calculated at the highest basis set, of triple-zeta quality augmented by polarization and diffuse functions. The Fe–Se distance is 2.44 Å, which is within the range found in iron seleno complexes.[13] As seen from Figure 3b, the calculations predict small energy gaps between the three spin states, which are packed within about 2.1 kcal mol^{-1}. Given that B3LYP/MM slightly overestimates the stability of high spin states,[12b] along with the BLYP/MM data (Supporting Information), which strongly prefer the doublet state, we can assign the ground state to have a doublet spin. This feature is similar to the computed situation for the WT and to the experimental finding that the ground state (doublet) is in spin equilibrium with the higher spin states.[14] Comparisons to the WT geometries[12b] suggest that in both cases, the water ligand is tilted by 40–60° owing to H-bond interactions within the protein pocket (Figure 3b). The Fe–O bond length is slightly shorter by ca 0.02 Å, whilst the Fe–Se bond is longer than Fe–S by circa 0.2 Å, as previously reported for the seleno and WT Cpd I species.[2] Therefore, the DFT/MM calculations support the doublet spin-state assignment by EPR.

In conclusion, we have expressed and characterized the first selenium-incorporated P450 enzyme. Our preliminary results reveal that the spectral characteristics of this novel CYP119 are comparable to those of the corresponding WT protein, indicating the presence of a six-coordinate low-spin heme iron with water as a distal ligand. More importantly, the catalytic activity of the seleno mutant is comparable to that of the WT enzyme. Furthermore, computational calculations clearly support the experimentally assigned spin state. Future studies will focus on both examining how this substitution affects the stability of the putative Cpd I species and the development of novel catalysts.

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[1] a) K. Auclair, P. Moënne-Loccoz, P. R. Ortiz de Montellano, *J. Am. Chem. Soc.* **2001**, *123*, 4877; b) S. Yoshioka, S. Takahashi, H. Hori, K. Ishimori, I. Morishima, *Eur. J. Biochem.* **2001**, *268*, 252; c) K. P. Vatsis, H.-M. Peng, M. J. Coon, *Arch. Biochem. Biophys.* **2004**, *434*, 128; d) R. Murugan, S. Mazumdar, *ChemBioChem* **2005**, *6*, 1204; e) K. P. Vatsis, H.-M. Peng, M. J. Coon, *J. Inorg. Biochem.* **2002**, *91*, 542.

[2] S. Cohen, D. Kumar, S. Shaik, *J. Am. Chem. Soc.* **2006**, *128*, 2649.

[3] Y. Jiang, P. R. Ortiz de Montellano, *Inorg. Chem.* **2008**, *47*, 3480.

[4] I. Gromov, I. Garcia-Rubio, C. Aldag, A. Schweiger, D. Hilvert, 6th European Federation EPR Groups Meeting, Abstract OT3, **2006**. When this work was under review, Aldag et al. published a paper on the expression and characterization of the seleno mutant of P450cam. See C. Aldag, I. A. Gromov, I. G. -Rubio, K. von Koenig, I. Schlichting, B. Jaun, D. Hilvert, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5481.

[5] S. Müller, H. Senn, B. Gsell, W. Vetter, C. Baron, A. Böck, *Biochemistry* **1994**, *33*, 3404.

[6] L. S. Koo, R. A. Tschirret-Guth, W. E. Straub, P. Moënne-Loccoz, T. M. Loehr, P. R. Ortiz de Montellano, *J. Biol. Chem.* **2000**, *275*, 14112.

[7] The catalytic activity of SeP450cam is also reported to be twofold less than the activity of the corresponding WT control (see Ref. [4]).

[8] G. Palmer in *Iron Porphyrins*, *Part II* (Eds.: A. B. P. Lever, H. B. Gray), Addison-Wesley, Reading MA, **1983**, pp. 43 – 88.

[9] T. G. Spiro, X. Y. Li in *Biological Applications of Raman Spectroscopy*, *Vol 3*, Wiley, New York, **1998**; T. G. Spiro in *Resonance Raman spectra of hemes and metalloproteins*, Wiley, New York, **1998**, pp. 1 – 37.

[10] T. G. Spiro, J. M. Burke, *J. Am. Chem. Soc.* **1976**, *98*, 5482.

[11] F. Paulat, N. Lehnert, *Inorg. Chem.* **2007**, *46*, 1547.

[12] a) It has been shown that the spectral properties and geometric features of CYP119 are very similar to those in CYP101 for a few species in the catalytic cycle; see I. G. Denisov, S. C. Hung, K. E. Weiss, M. A. McLean, Y. Shiro, S. Y. Park, P. M. Champion, S. G. Sligar, *J. Inorg. Biochem.* **2001**, *87*, 215. In view of this similarity of the two isozymes, we preferred to carry out the QM/MM calculations on CYP101 for which we already have all the necessary MM parameters, as well as QM/MM features of the active Cpd I species: b) J. C. Schöneboom, W. Thiel, *J. Phys. Chem. B* **2004**, *108*, 7468.

[13] a) R. Hauptmann, R. Kliss, G. Henkel, *Angew. Chem.* **1999**, *111*, 389; *Angew. Chem. Int. Ed.* **1999**, *38*, 377; b) L.-C. Song, Y. Sun, Q.-M. Hu, Y. J. Liu, *Organomet. Chem.* **2003**, *676*, 80; c) L.-C. Song, J. Yang, Q.-M. Hu, Q.-J. Wu, *Organometallics* **2001**, *20*, 3293; d) L.-C. Song, H.-T. Fan, Q.-M. Hu, Z.-Y. Yang, S. Yi, F. H. Gong, *Chem. Eur. J.* **2003**, *9*, 170; e) M. El-khateeb, *Inorg. Chim. Acta* **2004**, *357*, 4341.

[14] a) H. Thomann, M. Bernardo, D. Goldfarb, P. M. H. Kroneck, V. Ullrich, *J. Am. Chem. Soc.* **1995**, *117*, 8243; b) M. Sharrock, P. G. Debrunner, C. Schulz, J. D. Lipscomb, V. Marshall, I. C. Gunsalus, *Biochim. Biophys. Acta Protein Struct.* **1976**, *420*, 8.

