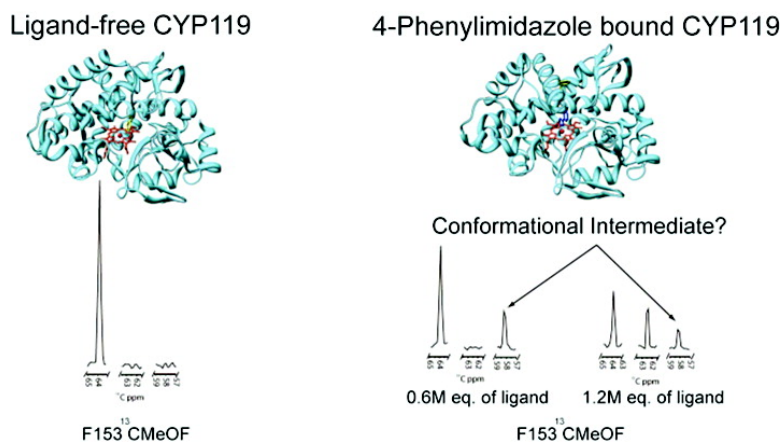


Ligand-Induced Conformational Heterogeneity of Cytochrome P450 CYP119 Identified by 2D NMR Spectroscopy with the Unnatural Amino Acid C-*p*-Methoxyphenylalanine

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Ligand-Induced Conformational Heterogeneity of Cytochrome P450 CYP119 Identified by 2D NMR Spectroscopy with the Unnatural Amino Acid ¹³C-*p*-Methoxyphenylalanine

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It has become increasingly clear that protein dynamics are required for enzymatic function.^{1–3} In fact, the native state of an enzyme can be described as a statistical ensemble of different conformational substates with similar free energies.⁴ Modern NMR techniques have proved successful at connecting conformational substates and their respective free energies with their individual contributions to catalysis.⁵ The cytochrome P450 (CYP) enzyme superfamily is a ubiquitous set of heme-containing monooxygenases that typically catalyze the incorporation of one atom of molecular oxygen into a hydrocarbon substrate.⁶ P450 enzymes are known to exhibit a wide range of dynamic enzymatic behavior, including homotropic and heterotropic cooperativity, sequential oxidation, and substrate inhibition.⁷ Due to their importance in mammalian drug metabolism and their potential utility as model systems for the study of complex protein dynamics, we report here the application of a unique high-resolution 2D NMR technique for the examination of ligand binding and its contribution to conformational dynamics in this major enzyme family. CYP119 from *Sulfolobus solfataricus* is a thermophilic P450 that is monomeric and stable at temperatures up to 90 °C.⁸ Ligand-free and ligand-bound crystal structures of this protein suggest that dramatic conformational changes occur upon ligand binding, particularly in the F/G loop region, that result in an overall “closing off” of the active site.⁸ To better understand the structural and thermodynamic contributions of specific residues in this dynamic region of the protein, we have introduced a ¹³C-labeled *p*-methoxyphenylalanine (¹³C-MeOF) unnatural amino acid site-specifically into two unique positions in the F/G loop, F153 and F162, to monitor the effects of ligand binding using 2D ¹H, ¹³C-HSQC NMR.⁹ The advantage of this technique over global labeling of the protein backbone with ¹⁵N is that it allows for site-selective incorporation of a single, specific amino acid *in vivo*, which results in the production of milligram quantities of protein and circumvents the need to make spectral residue assignments. SDS-PAGE analysis of the CYP119-F162-¹³C-MeOF mutant indicated the presence of a single band of the correct molecular weight (Supporting Information, Figure S1). Whole-protein LC-MS yielded the predicted mass shift of +31 Da, demonstrating the incorporation of a single ¹³C-MeOF residue (Figures S2 and S3). To assess the integrity of the labeled protein, a variety of ligands were examined for their ability to bind and induce a spin-state shift in the heme Soret bands (Figure S4). All ligands examined exhibited *K*_{d(app)} values similar to those of the wild-type enzyme (Table S1). Surprisingly, initial examination of the ¹H, ¹³C-HSQC spectra for both mutants revealed the presence of two symmetrical resonances of equal intensity, separated by ~0.12 ppm in the ¹H dimension (Figures 1, S5, and S6).

Since the three protons connected to the ¹³C-labeled methoxy moiety are chemically equivalent, these two resonances can only

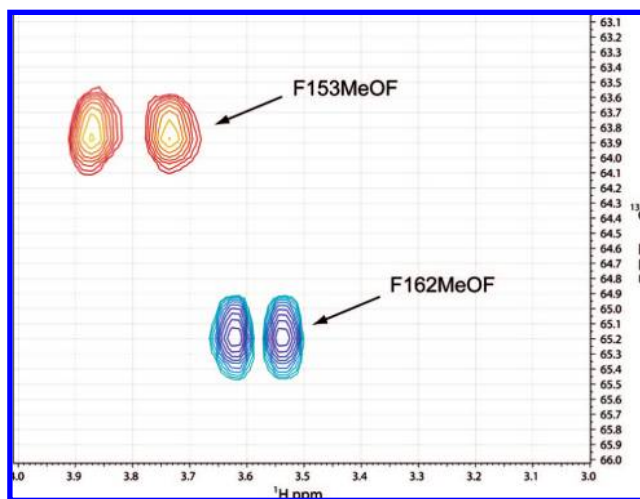


Figure 1. ¹H, ¹³C-HSQC spectra of CYP119-F153MeOF (red) superimposed upon the ¹H, ¹³C-HSQC spectra of CYP119-F162MeOF (blue).

be explained by the existence of two distinct conformers in slow chemical exchange, either conformational substates of the protein itself or specific ring-flip rotomers of the ¹³C-labeled *p*-methoxyphenylalanine residue.¹⁰ This is further illustrated by coalescence of the two resonances with increasing temperature (Figure S7). Ring-flip rotomers of tyrosine and phenylalanine have previously been shown to be distinguishable on the NMR time scale used in these experiments (i.e., ns to ms).^{10,11} Regardless of the origin of the doublet, the signal occurred in all CYP119-¹³CMeOF mutants examined. To monitor the dynamic structural changes that occur upon ligand binding, HSQC experiments were performed with a model ligand at a variety of concentrations. The ligand 4-phenylimidazole (4-PI) was chosen due to the existence of a crystal structure and the potential utility of imidazole derivatives as scaffolds for isoform-selective P450 inhibitors.¹² Upon addition of the 4-PI ligand to the F162-¹³C-MeOF labeled mutant, a single new resonance appeared in the spectrum with a chemical shift of 3.67 ppm in the ¹H dimension and 57.3 ppm in the ¹³C dimension (Figures 2b–d and S5). The intensity of the resonance increased with increasing concentration of ligand until reaching saturation at a 1:1 molar stoichiometry. 4-PI is known to form a 1:1 complex with CYP119 with a *K*_{d(app)} of ~120 nM (Table S1).⁸ In the absence of labeled protein, no resonances are detected in this region of the spectrum, further indicating that this resonance is due to ligand-bound protein (data not shown). It is interesting to note that, even at saturation, the resonances representing the ligand-free species are still present. This phenomenon has been seen previously in a similar study monitoring the binding of a model compound to the human fatty acid synthase.⁹ This may indicate that the “ligand free”

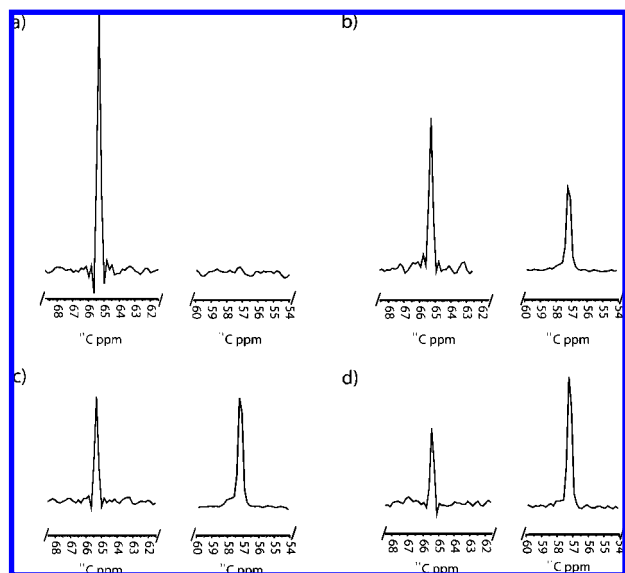


Figure 2. 1D slices from the ^{13}C axis of the ^1H , ^{13}C -HSQC spectra of CYP119-F162- ^{13}C MeOF with (a) 0 M, (b) 0.3, (c) 0.9, and (d) 1.2 molar equiv of 4-PI. Slices were taken at the midpoint of the resonance in the ^1H dimension. All figures are scaled relative to the same y -axis.

ground-state conformation of the enzyme, represented by the corresponding crystal structure, may be accessible even in the presence of saturating amounts of ligand. In comparison, upon titration with 4-phenylimidazole the F153- ^{13}C -MeOF mutant initially showed a similar pattern with a single resonance appearing at ^1H 3.65 ppm, ^{13}C 58.2 ppm, increasing in intensity with increasing ligand concentration (Figures 3 and S6). However, at higher concentrations of ligand, this resonance begins to decrease in intensity as a new resonance appears at ^1H 3.65 ppm and ^{13}C 62.3 ppm in the spectrum (Figure S6d, e), suggesting that the ^1H 3.65 ppm, ^{13}C 58.2 ppm resonance represents a conformational intermediate on the path to the ground state (crystal structure) conformer. The data suggest that this large scale conformational change proceeds through a stable intermediate that is readily detectable on the time scale of the ^1H , ^{13}C HSQC NMR experiments. In comparison, the F162- ^{13}C -MeOF residue appears to be insensitive to this conformational intermediate. It has recently been demonstrated that mammalian P450s may form an initial, nonproductive, “encounter” complex with ligands that leads to secondary conformational changes that transition the ligand into a productive binding orientation within the active site.¹³ This phenomenon may be represented in the CYP119 F153- ^{13}C -MeOF mutant by the appearance of a transient complex at low ligand occupancy.

In summary, here we have demonstrated the efficacy of a unique NMR approach utilizing isotopically labeled artificial amino acids to examine ligand-dependent conformational substates of a model cytochrome P450. In the future, this approach may also shed additional light on the role conformational dynamics plays in the

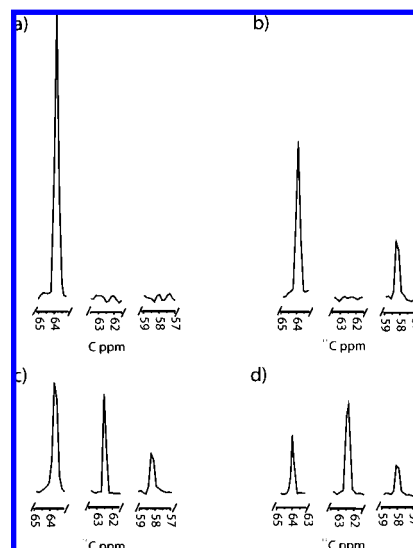


Figure 3. 1D slices from the ^{13}C axis of the ^1H , ^{13}C -HSQC spectra of CYP119-F153- ^{13}C MeOF with (a) 0, (b) 0.3, (c) 0.6, and (d) 1.2 molar equiv of 4-PI. Slices were taken at the midpoint of the resonance in the ^1H dimension. All figures are scaled relative to the same y -axis.

complex enzymatic phenomena exhibited by mammalian cytochrome P450 enzymes, such as heterotropic cooperativity and substrate promiscuity. Moreover, the approach may be universally applicable in studying complex enzymatic behavior in a variety of systems.

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Supporting Information Available: Experimental procedures, UV–vis and NMR data, and full ref 9. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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