Materials and Methods

Expression and purification of CYP101A1. Competent *Escherichia coli* NCM533 cells were transformed by electroporation with a plasmid construct encoding the C334A CYP101A1mutant. The C334A mutant has been found to be spectroscopically and enzymatically identical to the wild type, but it does not form a dimer in solution.[13] C334A is ideally suited for NMR studies as a monomer, and for convenience we refer to it as wild type (WT) enzyme. Uniformly ^{15}N-labeled CYP101A1was expressed in M9 minimal media containing $^{15}NH_4Cl$ along with trace metals. The heme precursor δ-aminolevulinic acid was introduced prior to induction of CYP101A1 expression. Protein purification included a protamine sulfate cut to precipitate DNA, and an ammonium sulfate cut to concentrate CYP101A1. After desalting by dialysis, the enzyme was passed through two gravity chromatography columns, an ion exchange column and a size exclusion column. Expression and purification details have been published previously.[14] All buffers contained 1 mM (1*R*)-(+)-camphor, 98% (Sigma Aldrich). Purity of NMR samples was assessed spectroscopically and determined to be at least 90% pure with a A_{391}/A_{280} ratio of at least 1.4.

Site-directed mutagenesis. Two CYP101A1 mutants were constructed using the four-primer method described previously.[15] The first mutation substituted Gly326 with alanine (G326A). The second mutation introduced an extra glycine residue between G326 and L327 (G327'). Two side primers introduced the appropriate restriction sites on the 5'- and 3'- ends, and two middle primers introduced the desired mutation(s). The mutated gene was ligated into the vector using engineered restriction sites NdeI and HindIII.

Side primers used were:

CYP101A1 forward: 5'-TTTCACACAGGAAACAGACCATATGACGAC-3'

CYP101A1 reverse: 5'-CCAAAACAGCCAAGCTTTCAGCTACTTATAC-3'.

Mutagenic primers were:

G326A forward: 5'-GATGCTGTCTGCCCTGGATGAGC-3'

G326A reverse: 5'-GCTCATCCAGGGCAGACAGCATC-3'

G327' forward: 5'-GATGCTGTCTGGCGGACTGGATGAGC-3'

G327' reverse: 5'-GCTCATCCAGTCCGCCAGACAGCATC-3'

Custom oligonucleotides were obtained from Eurofins MWG Operon (Huntsville, AL). The new constructs were miniprepped (Qiagen, Valencia, CA) and sequenced to ensure the presence of the desired mutations.

Substrate exchange and sample preparation for NMR experiments. Two substrate analogs, norcamphor 2 and adamantanone 3, were exchanged for camphor by 24 h of dialysis against buffer saturated with the appropriate substrate prior to concentration and NMR spectroscopy. Samples were then reduced with microliter aliquots of freshly prepared sodium dithionite under an atmosphere of carbon monoxide. Typically, a total of ~8 μL of 250 mM $Na_2S_2O_4$ solution is sufficient to completely reduce 250 μL of protein sample (nominal 30-fold excess). CYP101A1 concentrations were typically ~250 μM. The reduced protein was then anaerobically transferred into a susceptibility-matched NMR tube (Shigemi, Inc., Allison Park, PA).

NADH consumption assays. A 10 μM NADH solution was freshly prepared using substrate-free buffer. Putidaredoxin reductase (PdR) and putidaredoxin (Pdx) had been expressed and purified as described previously.[14] A typical assay was as follows. To a 1

mL Eppendorf tube was added sufficient protein to reach a final concentration of 0.5 μM CYP101A1 (WT or mutant), 0.5 μM PdR and 5 μM Pdx in a buffer volume of 770 μL. The reaction mixture was allowed to incubate at room temperature for 10 min. NADH was then added (330 μM, final concentration), the reaction was mixed then transferred into a quartz cuvette to be followed by UV/visible spectroscopy. The absorbance of NADH at 340 nm was monitored as a function of time (over 1 min). The initial rate was taken over the first 15 seconds of the run. Two series were run for each CYP-substrate combination.

Detection of hydroxylation product by gas chromatography (GC). The same reaction mixtures as described for NADH standard assays were prepared in 10-mL glass GC vials, but with doubled quantities. After 10 min of incubation at room temperature, NADH was added to a final concentration of 330 μM. The vials were covered with Parafilm® and three holes were made with a needle to allow oxygen from the atmosphere to pass through. After being left overnight at room temperature, the reactions were quenched with dichloromethane, and the vials were tightly sealed with Parafilm® prior to product extraction. The bottom layer in the reaction, corresponding to the CH_2Cl_2 layer, was collected and passed through $MgSO_4$. Additional CH_2Cl_2 was added to the reaction in the 10-mL GC vial, and the bottom layer was filtered through $MgSO_4$. The dried solution was collected in a 50-mL round-bottom flask. The solution was concentrated under reduced pressure (rotary evaporator). The ~200 μL of remaining mixture was transferred into a 1.5-mL screw cap vial.

For each run, 2 μL of the mixture were manually injected into the back inlet of a HP 6890 Series GC System, operated using the Agilent ChemStation software (Agilent

Technologies). The oven temperature was increased from 60 ^{o}C to 240 ^{o}C (rate 10.00 ^{o}C/min). The back inlet was set on split mode with a split ratio of 0.283 to 1, and a split flow of 0.5 mL/min. The initial back inlet temperature was 220 ^{o}C, and the pressure was at 9.52 psi. The run was performed with a total flow rate of 6.5 mL/min with helium as a carrier. The capillary column was HP-5 5% Phenyl Methyl Siloxane (Agilent 19091J-413). The front detector was set at 230 ^{o}C, with 40.0 mL/min for hydrogen, 450.0 mL/min for air, and 1.0 mL/min for helium. Two runs were performed for each CYP-S combination.

NMR experiments. All NMR spectra were acquired at 298 K on an 18.8 T (800 MHz, ^{1}H) Bruker Avance NMR spectrometer. ^{1}H, ^{15}N correlation experiments (TROSY) were acquired as 2048 (^{1}H) x 512 (^{15}N) complex point datasets with spectral widths of 14 367 Hz and 3 243.4 Hz for ^{1}H and ^{15}N respectively. Data acquisition, processing and analysis were performed using the Topspin software package (Bruker Biospin, Inc.).