A Model of the Membrane-bound Cytochrome \(b_5\)-Cytochrome P450 Complex from NMR and Mutagenesis Data

Shivani Ahuja, Nicole Jahr, Sang-Choul Im, Subramanian Vivekanandan, Nataliya Popovych, Stéphanie V. Le Clair, Rui Huang, Ronald Soong, Jiadi Xu, Kazutoshi Yamamoto, Ravi P. Nanga, Angela Bridges, Lucy Waskell, and Ayyalusamy Ramamoorthy

Background: cytb5 modulates catalysis performed by cytP450, in vivo and in vitro. Results: The structure of full-length cytb5 was solved by NMR, and the cytP450-binding site on cytb5 was identified by mutagenesis and NMR. Conclusion: A model of the cytb5-cytP450 complex is presented. Addition of a substrate strengthens the cytb5-cytP450 interaction. Significance: The cytb5-cytP450 complex structure will help unravel the mechanism by which cytb5 regulates catalysis by cytP450.

Microsomal cytochrome \(b_5\) (cytb5) is a membrane-bound protein that modulates the catalytic activity of its redox partner, cytochrome P4502B4 (cytP450). Here, we report the first structure of full-length rabbit ferric microsomal cytb5 (16 kDa), incorporated in two different membrane mimetics (detergent micelles and lipid bicelles). Differential line broadening of the cytb5 NMR resonances and site-directed mutagenesis data were used to characterize the cytb5 interaction epitope recognized by ferric microsomal cytP450 (56 kDa). Subsequently, a data-driven docking algorithm, HADDOCK (high ambiguity driven biomolecular docking), was used to generate the structure of the complex between cytP4502B4 and cytb5, using experimentally derived restraints from NMR, mutagenesis, and the double mutant cycle data obtained on the full-length proteins. Our docking and experimental results point to the formation of a dynamic electron transfer complex between the acidic convex surface of cytb5 and the concave basic proximal surface of cytP4502B4. The majority of the binding energy for the complex is provided by interactions between residues on the C-helix and \(\beta\)-bulge of cytP450 and residues at the end of helix \(\alpha\) of cytb5. The structure of the complex allows us to propose an interprotein electron transfer pathway involving the highly conserved Arg-125 on cytP450 serving as a salt bridge between the heme propionates of cytP450 and cytb5. We have also shown that the addition of a substrate to cytP450 likely strengthens the cytb5-cytP450 interaction. This study paves the way to obtaining valuable structural, functional, and dynamic information on membrane-bound complexes.

Cytochromes P450 (cytP450)\(^{3}\) are a ubiquitous superfamily of mixed-function oxygenases, which are found in all kingdoms of life but are especially abundant in eukaryotes. Humans possess 57 different membrane-bound cytP450 (1). They are found in all tissues of the body and are responsible for influencing a dazzling array of biochemical and physiological processes, including embryonic development, blood coagulation, and the metabolism of carcinogens, environmental toxins, over 50% of drugs in use, vitamin D, and other exogenous and endogenous compounds (2, 3). Selected human cytP450 (cytP45017A1 and cytP45019A1) are targets for the treatment of prostate and breast cancer, respectively (4, 5).

CytP450 catalyzes the insertion of one atom of “activated” molecular oxygen into the substrate, using two electrons from NAD(P)H and two protons from water. Electrons destined for cytP450 are first delivered to its redox partners, cytP450-reductase (CPR) and cytb5, which then transfer the electrons to cytP450 (6). CPR is capable of transferring both electrons to cytP450; however, cytb5 is capable of donating only the second

\(^{3}\) The abbreviations used are: cytP450, cytochromes P450; 1-CPI, 1-(4-chlorophenyl) imidazole; two-dimensional HSQC, two-dimensional heteronuclear single quantum coherence; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; cytb5, cytochrome \(b_5\); cytP450, cytochrome P450; CPR, cytochrome P450 reductase; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPC, docosylphosphocholine; SLF, separated local field; TROSY, transverse relaxation optimized spectroscopy; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.
electron due to its high redox potential as compared with ferric cytP450 (7–10). cyt_b5 plays a key role in the oxidation of a variety of exogenous and endogenous compounds, including drugs, fatty acids, cholesterol, and sex hormones. The influence of cyt_b5 on cytP450 activity has been shown to depend on the cytP450 isozyme and the substrate involved. Remarkably, cyt_b5 enhances some catalytic reactions of cytP450 but does not affect or even inhibit others (6, 8–13). At low concentrations, cyt_b5 may enhance the rate of catalysis by up to 100-fold, whereas at high concentrations it inhibits catalysis by competing with CPR for a binding site on cytP450, thereby preventing the transfer of the first electron and the reduction of ferric cytP450 to the ferrous form (6, 7, 14). cyt_b5 and CPR are both required for the transfer of the first electron and the reduction of ferric cytP450 to the ferrous form (6, 7, 14). cyt_b5 and CPR are both negatively charged proteins, which are known to have overlapping binding sites on cytP450 (15). When the stimulatory and inhibitory effects of cyt_b5 are equal, cyt_b5 will appear to have no effect on the catalytic activity of cytP450.

To obtain an in-depth understanding of the molecular basis of the effects of cyt_b5 on cytP450 activity, it is necessary to determine the structure of the complex between the full-length forms of cyt_b5 and cytP450. Although all reported x-ray and NMR structural data pertain to cytosolic heme binding domains of truncated, microsomal cytP450 and cyt_b5, in which their membrane anchors have been removed (16–19), neither structures nor dynamics of the full-length protein (containing the transmembrane domain) are currently available. Additionally, although the interaction of cyt_b5 with various membranes has been previously studied (20–22), the structure of membrane-bound cyt_b5 is lacking. However, only the full-length membrane-binding form of microsomal cyt_b5 influences the enzymatic activity of cytP450 (23, 24); truncated cyt_b5 is only capable of electron transfer to water-soluble oxidative enzymes (e.g. cytochrome c and metmyoglobin) (25).

Despite recent advances in NMR methodology and isotopic labeling schemes, the structure determination of large membrane-bound protein–protein (~70 kDa) complexes remains a monumental task. The large size of the membrane-bound complex presents considerable challenges in terms of sample stability, spectral sensitivity, and resolution. In this study, we present the first full-length tertiary structure of rabbit ferric cyt_b5 solved in a membrane mimetic using a combination of high resolution solution and solid-state NMR spectroscopy. Subsequently, experimentally derived restraints from NMR, site-directed mutagenesis, and double mutant cycle data, obtained on the full-length proteins, were then used to generate the structure of the complex between ferric microsomal rabbit cytP4502B4 (56 kDa) and cyt_b5 (16 kDa), using a data-driven docking algorithm, HADDOCK (high ambiguity driven biomolecular docking) (26). The extensive structural knowledge of the cyt_b5–cytP450 complex interface provided here will prove to be essential in unraveling the molecular mechanism by which cyt_b5 regulates the rate of catalysis of cytP450 (14).

**EXPERIMENTAL PROCEDURES**

**Materials**—The QuikChange II XL site-directed mutagenesis kit was purchased from Stratagene. 1,2-Dilauroyl-snglycerol-3-phosphocholine (DLPC) was purchased from Doosan Serdary Research Laboratories (Toronto, Canada), and methoxyflurane was obtained from Abbott. 1,2-Dihexanoyl-snglycerol-3-phosphocholine (DHPC) and 1,2-dimyristoyl-snglycerol-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL). C41 cells were purchased from Lucigen (Middleton, WI). U-13C, U-15N, and U-15N-labeled CELTONE rich media, U-15N-CELTONE rich media, U-13C,U-15N-CELTONE-rich media, [1H]dodecylphosphocholine (DPC-D38), [13C]glucose, [15N]ammonium sulfate, 2,2-dimethyl-2-silapentane-5-sulfonate, and D2O were purchased from Cambridge Isotope Laboratories (Andover, MA). Resins, buffer components, and all chemicals, including BHT, benzphetamine and 1-CPI, were purchased from Sigma. Glycerol for NMR experiments was purchased from Sigma and Roche Applied Science. The NMR samples were placed into 5-mm symmetrical D2O-matched Shigemi NMR microtubes (Shigemi Inc., Aliso Park, PA).

**Generation of Mutants of cytP4502B4 and cyt_b5**—Mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The oligonucleotides were synthesized by Integrated DNA Technologies. Following mutagenesis, the sequence of the entire mutated gene was determined at the University of Michigan DNA Sequence Core Facility to confirm the mutation and correct sequence.

**Expression and Purification of Full-length Wild-type Rabbit cytP4502B4, Rat CPR, and Rabbit cyt_b5**—The cytP4502B4 and cyt_b5 cDNA in pLW01 and CPR cDNA in pSC-CPR plasmids were expressed in Escherichia coli C41 cells and purified as described previously (15, 27, 28). [U-15N]cyt_b5, [U-13C,U-15N]cyt_b5, and [U-13C,U-15N,U-15N]cyt_b5 were expressed using Cetalone-N, Cetalone-CN, and Cetalone-DCN complete media, respectively, with additional supplements as described previously (29). Prior to expressing [U-13C,U-2H,U-15N]cyt_b5, C41 cells containing pLW01-cyt_b5 plasmid were adapted to grow in 100% Lysogeny Broth (LB) media by gradually increasing the D2O concentration in the LB medium from 10, 30, 60, and 80 to 100% D2O. After each successive cycle, an LB culture was inoculated with three colonies that had been grown on a plate containing a lower amount of D2O. The liquid culture with the higher amount of D2O was incubated for up to ~8 h at 37 °C with shaking at 250 rpm. 50 µl of the resulting culture was plated on an LB agar plate containing 0.24 mm carbenicillin and the higher D2O concentration. This process was repeated until the cells were able to grow on LB plates containing 100% D2O. The protocols for the expression of [U-13C,U-2H,U-15N]cyt_b5, [U-13C,U-15N]cyt_b5, and [U-15N]cyt_b5 were identical; however, the cells were harvested at different times as follows: for [U-15N]cyt_b5, and [U-13C,U-15N]cyt_b5, the cells were harvested after 20 h of incubation at 35 °C with shaking at 200 rpm, and for [U-13C,U-2H,U-15N]cyt_b5, cells were harvested after 48 h. Purification of cyt_b5, cytP450, and CPR was performed as described elsewhere (15, 28). Each purified protein

---

4 Unless otherwise stated, all the NMR data were collected on the oxidized (ferric; Fe(III)) form of full-length cytP450 and cyt_b5.
bicelles were prepared such that the final lipid concentration 5-sulfonate as an internal reference. The $^{13}$C and $^{15}$N chemical shifts were referenced indirectly to 2,2-dimethyl-2-silapentane-5-sulfonate (31). All NMR spectra were processed using TALOS (34), provided as part of the NMRPipe package (32), and NOE patterns. The upper bound for all NOE distance restraints was initially set to 5 Å and adjusted for nonstereospecific assignment of methylene and methyl protons using the method described originally for DYANA (35). The structure calculations were performed with CYANA 2.1 (36), which uses simulated annealing in combination with molecular dynamics in torsion angle space. Chemical shift tolerances of 0.02, 0.3, and 0.45 ppm were used for $^1$H, $^{13}$C, and $^{15}$N, respectively. For initial structure calculations, only unambiguous peak assignments, obtained from three-dimensional NOEY, were used. More than 90% of these peaks were assigned manually, and the remaining peaks were assigned using CYANA 2.1 (36). Using these assignments and TAOS-derived torsion angle restraints, an initial ensemble of NMR structures was generated. Initial violated restraints were identified and eliminated in subsequent rounds of structure calculations until a consistent set of restraints was obtained for $^1$H, $^2$H, and $^3$H $^1$H, $^{15}$N, $^{13}$C backbone resonances was achieved using a set of TROSY-based three-dimensional HNCO, HN(CA)CO, HNCA, HN(CO)CA, and HNCA experiment. Intra-residue, inter-residue, and sequential NOEs were obtained from three-dimensional $^{14}$N HSQC-NOESY and $^{13}$C HSQC-NOESY spectra on fully protonated and uniformly $^{15}$N- and $^{13}$C-labeled cytb$_5$ in DPC-D38 micelles; these experiments were conducted at optimal mixing times of 80 and 100 ms, respectively. $^{15}$N HSQC-TOCSY spectrum was also collected to assist in the assignment of intra-residue NOEs. All aromatic side chain protons and carbon atoms were assigned using two- and three-dimensional NOEY. The proton chemical shift was referenced to the methyl signal of 2,2-dimethyl-2-silapentane-5-sulfonate (31). All NMR spectra were processed using either NMRPipe (32) or TopSpin 2.0 (Bruker). Sequential and NOE assignments were made using Sparky (33).

**Structure Determination of Heme Domain of cytb$_5$—**Initial restraints for the backbone torsion angles $\phi$ and $\psi$ were obtained from chemical shifts using TALOS (34), provided as part of the NMRPipe package (32), and NOE patterns. The upper bound for all NOE distance restraints was initially set to 5 Å and adjusted for nonstereospecific assignment of methylene and methyl protons using the method described originally for DYANA (35). The structure calculations were performed with CYANA 2.1 (36), which uses simulated annealing in combination with molecular dynamics in torsion angle space. Chemical shift tolerances of 0.02, 0.3, and 0.45 ppm were used for $^1$H, $^{13}$C, and $^{15}$N, respectively. For initial structure calculations, only unambiguous peak assignments, obtained from three-dimensional NOEY, were used. More than 90% of these peaks were assigned manually, and the remaining peaks were assigned using CYANA 2.1 (36). Using these assignments and TAOS-derived torsion angle restraints, an initial ensemble of NMR structures was generated. Initial violated restraints were identified and eliminated in subsequent rounds of structure calculations until a consistent set of restraints was obtained with no violations greater than 0.2 Å in the ensemble (Table 1).

Starting **ab initio**, 100 conformers were calculated in 10,000 annealing steps each. PyMOL (Version 1.1, Schrödinger, LLC) was used to visualize the resulting ensemble of energy-minimized conformers.

**Solid-state NMR of cytb$_5$—**Magnetically aligned DMPC/DHPC bicelles, with a molar ratio of DMPC to DHPC of 3.5:1 and a weight percentage of lipid of 30% (w/v), containing uniformly $^{15}$N-labeled cytb$_5$ were prepared as described elsewhere (29). A two-dimensional separated local field (SLF) NMR experiment using the HIMSELF (heteronuclear isotropic mixing leading to spin exchange via the local field) (37) pulse sequence, which is based on the PIWIMz (polarization inversion by windowless isotropic mixing) pulse scheme, was obtained at 310 K using a ramped cross-polarization contact time of 0.8 ms, 2 s recycle delay and 32 $t_1$ increments. Radio frequency field strengths of 50 and 35 kHz were used for the WIM (windowless mixing) (38) sequence during $t_1$ and SPINAL-64 (39) decoupling during acquisition, respectively.

**Docking of Heme into cytb$_5$ NMR Structure—**HADDOCK 2.1 (26, 40) was used to incorporate the heme molecule (type B) into the 20 NMR-derived low energy structures of cytb$_5$ presented here. Unambiguous restraints were compiled based on distances (between the heme and certain cytb$_5$ residues) measured from the available crystal structure of cytb$_5$ (PDB code 1DO9) (18). As a first step, rigid body energy minimization was used for docking 2000 structures. The second step included semi-rigid simulated annealing, and the best 500 structures were selected for refinement. The best 200 structures were then selected for final refinement with explicit solvent in an 8.0-Å shell of TIP3P water molecules. The 50 lowest energy structures were then subjected to a detailed analysis.

**Solution NMR of cytb$_5$-cyp450 Complex—**Samples containing a 1:1 molar complex of 0.1 mM isopropically $^{15}$N-labeled ferric cytb$_5$ and unlabeled ferric cyp450 in NMR buffer were prepared. For solution NMR measurements, isotropic bicelles were added to the complex sample just before transferring to a Shigemi tube. For experiments in the presence of the substrate and inhibitor (BHT and 1-CP), the small molecule was added to cyp450 to a final concentration of 0.2 mM (1:2 molar ratio for

---

**Table 1**

NMR resonance assignments, constraints and refinement statistics for the cytb$_5$ structure

| NMR distance and dihedral constraints | Distance constraints | Total NOE | 1787 |
|---------------------------------------|----------------------|----------|------|
|                                       | Intra-residue         | 377      |      |
|                                       | Sequential (\(i-j=1\)) | 513      |      |
|                                       | Medium range (\(i-j<4\)) | 412      |      |
|                                       | Long range (\(i-j>5\))  | 485      |      |
|                                       | Hydrogen bonds       | 39       |      |
|                                       | Total dihedral angle restraints | 56 | |
|                                       | \(\phi\)             | 56       |      |
|                                       | \(\psi\)             | 56       |      |

**Violations**

- Max. dihedral angle violation (>2°) | 0 |
- Max. distance constraints violation (>0.2 Å) | 0 |

**Ramachandran analysis**

- Most favored | 71.70% |
- Allowed | 25.90% |
- Generously | 2.30% |
- Disallowed | 0.00% |

**Average r.m.s.d. (Å)**

- Backbone (Lys-7–Arg-89) | 0.32 ± 0.10 |
- Heavy (Lys-7–Arg-89) | 0.82 ± 0.10 |
cytP450/substrate) before the addition of isotropic bicelles. To assess the formation of the cyt_b5-cytP450 complex, three cytP450 titration points were performed. All titrations points were carried out by adding aliquots of unlabeled cytP450 solution to a final concentration of 0.03, 0.06, and 0.10 mM into a sample, containing 0.1 mM 15N-labeled cyt_b5 and 0.2 mM BHT in isotropic bicelles.

SDS-PAGE of the cyt_b5-cytP450 complex incorporated in bicelles was performed before and after NMR experiments to ensure that proteolysis, especially of the membrane anchor of cyt_b5, had not occurred during the experiments (data not shown). The proteins were full-length both before and after NMR measurements. In addition, carbon monoxide (CO) difference spectra of cytP4502B4, and activity assays of cyt_b5 and cytP4502B4 (supplemental Table S1), confirmed the presence of the functional form of cytP450 and the cyt_b5-cytP450 complex in the NMR samples (data not shown).

**Activity of cytP4502B4 in Solution and Bicelles, Methoxyflurane**—The metabolism of the anesthetic, methoxyflurane (C3H4Cl2F2O), by cytP4502B4 was measured in a reconstituted aqueous solution with and without cyt_b5. The activity of cytP450 was also measured in bicelles in the presence and absence of cyt_b5. The activity was quantified by recording the amount of fluoride ion produced. The components were mixed together in the following sequence: cytP4502B4, CPR, and DLPC. The mixture was then incubated for 5 min at room temperature, and either cyt_b5 or buffer was added to the mixture, which was then incubated for an additional 1 h at room temperature. Following the 1-h incubation, 50 mM potassium phosphate buffer of pH 7.4 saturated with methoxyflurane was added, and the solution was further incubated at 37 °C for 5 min. NADPH was added to start the reaction. The final volume of the reaction mixture was 500 μl, and it contained 50 mM potassium phosphate buffer, 1 μM cytP4502B4, 1 μM CPR, 120 μM DLPC, 1 μM methoxyflurane, 300 μM NADPH, 5 mM glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were added, and the solution was further incubated at 37 °C for 5 min.

The assay for the metabolism of methoxyflurane in the presence of bicelles was conducted as described above except that the cytP450-CPR-cyt_b5 complex was added to DMPC/DHPC (3.5/1) bicelles at 4 °C. Bicelles were prepared and precooled as described previously (29). The mixture was incubated at 4 °C for 30 min. The potassium phosphate buffer saturated with methoxyflurane was added to the protein mixture. The resulting mixture was incubated at 37 °C for 5 min at which time NADPH was added to a final concentration of 300 μM to start the reaction. The final concentration of DMPC was 110 mM. The results of the methoxyflurane assay are presented in supplemental Table S1.

**Benzphetamine**—The metabolism of benzphetamine was measured by determining the amount of formaldehyde produced by the N-demethylation of benzphetamine using Nash’s reagent, as described previously (15). The final concentration of the reactants in the mixture was 50 mM potassium phosphate buffer (pH 7.4), 0.2 μM cytP4502B4, 0.2 μM CPR, 24 μM DLPC, 1 mM benzphetamine, 300 μM NADPH, or 50 mM DMPC/DHPC bicelles, and cyt_b5 (0 or 0.2 μM). The results of the benzphetamine assays are presented in supplemental Table S1.

**Determination of cyt_b5-cytP450 Equilibrium Dissociation Constant (K_d) in the Presence of a Substrate/Ligand**—The K_d value of the binding between ferric cytP4502B4 and ferric cyt_b5 was determined as described previously (15) by measuring the type I spectral change (a decrease at 420 nm and an increase at 385 nm in absorption) occurring when cyt_b5 is added to an aqueous solution of cytP4502B4, in the presence of the substrate, methoxyflurane, or BHT. The K_d value was used to calculate the free energy of binding, ΔG, using the formula ΔG = −RTln(K_d), where R is the gas constant and T is temperature. The K_d value for the complex formation of all possible pairs of wild type and mutants combinations of cytP4502B4 and cyt_b5 was determined as described previously (15) and are presented in Tables 3 and 4. The K_d value could not be calculated in the presence of either micelles or bicelles.

The second step included semi-rigid simulated annealing from the best 500 structures were selected for refinement. The second step included semi-rigid simulated annealing from the 50 lowest energy structures were selected for the final analysis and grouped into two main clusters based on the backbone r.m.s.d. The interface of the complex was analyzed by PISA (polarity index slant angle) (43) and Collaborative Computational Project Number 4 (44), and molecular graphics were prepared using PyMOL (Version 1.1).

## RESULTS

Rabbit cyt_b5 is composed of a large cytosolic heme domain and a C-terminal transmembrane domain, connected by a 15-residue linker. To identify cyt_b5 residues involved in complex formation with cytP4502B4, we first solved the three-dimensional structure of ferric cyt_b5 in DPC micelles, using a combination of high resolution solution and solid-state NMR experiments (Fig. 1).
Three-dimensional Structure Determination of Full-length Mammalian cytb$_5$—Isotopically labeled full-length wild-type ferric microsomal rabbit cytb$_5$ was reconstituted in detergent (DPC) micelles. A number of standard TROSY-based multidimensional solution NMR experiments, in combination with isotopic labeling schemes, including perdeuteration, were employed to assist in resonance assignment and structure determination of cytb$_5$. Fig. 2 presents a $^1$H-$^15$N TROSY-HSQC spectrum of uniformly $^{13}$C-, $^2$H-, and $^{15}$N-labeled cytb$_5$ in micelles exhibiting well resolved peaks. The spectrum exhibits well resolved and dispersed NH correlations from cytb$_5$ residues, suggesting that the protein is well folded and monodispersed in DPC micelles.

Using standard three-dimensional solution NMR experiments, NMR resonance assignment was achieved for 88.5% of the backbone and side chain atoms of residues from the soluble domain of full-length cytb$_5$ (Table 2). The chemical shift assignments were deposited in the Biological Magnetic Resonance Bank (code 18919). An inspection of the $^1$H-$^{15}$N TROSY-HSQC spectrum of cytb$_5$ revealed two or more NMR resonances for many of the residues. These two sets of NMR resonances originate from the two isomers (major and minor) of cytb$_5$ that differ by a 180° rotation of the heme plane about the axis that cuts through the $\text{meso}$-carbon atoms η and γ (18, 45). The ratio of the populations of the two isomers can be calculated by determining the peak intensity ratio (here in the $^1$H-$^{15}$N TROSY-HSQC spectrum of cytb$_5$) for identical residues in the two isomeric forms. The major/minor isomer ratio in our study for full-length rabbit cytb$_5$ was determined to be about 6.6:1 which is similar to 5:1 ratio previously obtained for truncated rabbit cytb$_5$ (18) and nearly identical to the isomer ratio of 6.5:1 for truncated bovine cytb$_5$ (45). The ratio depends
Model Complex between Mammalian cytb<sub>5</sub> and Cytochrome P450

Flexible Linker Domain of cytb<sub>5</sub> Lacks a Defined Secondary Structure—The linker region (Ser-90–Asp-104), which connects the cytosolic heme domain of cytb<sub>5</sub> to the transmembrane anchor, was previously characterized as random coil for various truncated forms of microsomal cytb<sub>5</sub> that lacked a transmembrane domain (18, 19); in these proteins, the C terminus had been truncated either within the linker region or beyond it. Neither intra- nor inter-residue NOEs were observed for most of the linker residues (Ser-90 to Asp-104) due to the rapid solvent exchange at those amide positions. Therefore, we show here that for full-length cytb<sub>5</sub>, incorporated in DPC micelles, the linker region is unstructured, lacking any distinct secondary structural features. A cytb<sub>5</sub> linker region of at least 6–8 amino acids has been shown to be necessary to enable formation of a functional complex between cytb<sub>5</sub> and its full-length redox partner cytP450 (23). Unlike full-length cytb<sub>5</sub>, a cytb<sub>5</sub> mutant lacking eight amino acids in the linker domain was neither able to insert efficiently into a lipid membrane (47) nor able to form a fully functional complex with cytP450 (23).

on the cytb<sub>5</sub> species and has been reported to be as high as 1.5:1 for rat cytb<sub>5</sub> (46), despite the sequence similarity between rat and rabbit cytb<sub>5</sub> (supplemental Fig. S1A). Although backbone assignments were done for the resonance peaks of both the major and minor isomers, all structure analyses were performed for the major isomer of ferric cytb<sub>5</sub>.

NMR resonance assignments of the major isomer of cytb<sub>5</sub> in DPC micelles revealed that the observed 1H-15N TROSY-HSQC spectrum is dominated by resonances from the soluble heme-containing domain and the flexible linker of cytb<sub>5</sub> (Fig. 2). Residues from the transmembrane domain of cytb<sub>5</sub> could not be identified in the 1H-15N TROSY-HSQC spectrum (Table 2). To identify the origin of the lack of transmembrane domain resonances in solution NMR, and to determine the structure and topology of the transmembrane domain of cytb<sub>5</sub>, solid-state NMR was performed on full-length cytb<sub>5</sub>, incorporated in aligned DMPC/DHPC bicelles (explained below). It is important to note here that the full-length form of cytb<sub>5</sub> incorporated in a membrane mimetic (DPC micelles or lipid bicelles) was used for all solution and solid-state NMR measurements.

Three-dimensional Tertiary Structure Calculation of the Heme Domain of Full-length cytb<sub>5</sub>—A high resolution structure of the soluble domain of the major isomer of full-length ferric cytb<sub>5</sub> (~16 kDa) in DPC micelles was calculated using a total of 1787 NOE restraints (Table 1). Distances derived from NOE restraints, in conjunction with 39 hydrogen bonds and 112 (ϕ and ψ) dihedral angles, were included for structural determination into CYANA 2.1 (36). Twenty minimum energy conformers (Fig. 3A) of cytb<sub>5</sub> with backbone and heavy atom (residues Lys-7 to Arg-89) r.m.s.d. values of 0.32 ± 0.10 and 0.82 ± 0.10 Å, respectively, were selected from 100 structures calculated in 10,000 annealing steps. Distance restraints used for the structural calculation and Ramachandran statistics can be found in Table 1. HADDOCK 2.1 was used to dock heme B into the 20 NMR-derived low energy structures of cytb<sub>5</sub> (see “Experimental Procedures”) (26, 40). Fifty low energy structures were obtained with no restraint violations. The 20 lowest energy HADDOCK-generated structures of cytb<sub>5</sub> (Fig. 3) were deposited in the Protein Data Bank (code 2M33). This solution NMR structure of cytb<sub>5</sub> was used in all subsequent analyses.

The structure of cytb<sub>5</sub> contains five α-helices, five β-strands, and one 3<sub>10</sub> helix (Fig. 3C and supplemental Fig. S1). The first β-sheet is observed for residues Lys-10 to Tyr-12 with an α-helix (α1, Leu-14 to His-20) following shortly after. The heme-binding portion of cytb<sub>5</sub> consists of two helices in the lower cleft, labeled as α4 (Thr-60 to Val-66) and α5 (Thr-70 to Phe-79), two helices in the upper cleft, labeled as α2 (Lys-39 to Glu-43) and α3 (Glu-49 to Gln-54) (Fig. 3C), and three β-strands at the bottom of the heme pocket, labeled as β3 (Lys-33 to Asp-36), β2 (Trp-27 to Leu-30), and β4 (Gly-56 to Asp-58). At the end of the structured soluble domain lies a β-strand (β5, Gly-82 to Leu-84) and a 3<sub>10</sub> helix (Pro-86 to Arg-89). The overall structure of the heme domain of full-length ferric cytb<sub>5</sub> is found to be similar to the previously determined NMR structure of the heme domain of truncated ferric cytb<sub>5</sub> from rabbit (18).

Flexible Linker Domain of cytb<sub>5</sub> Lacks a Defined Secondary Structure—The linker region (Ser-90–Asp-104), which connects the cytosolic heme domain of cytb<sub>5</sub> to the transmembrane anchor, was previously characterized as random coil for various truncated forms of microsomal cytb<sub>5</sub> that lacked a transmembrane domain (18, 19); in these proteins, the C terminus had been truncated either within the linker region or beyond it. Neither intra- nor inter-residue NOEs were observed for most of the linker residues (Ser-90 to Asp-104) due to the rapid solvent exchange at those amide positions. Therefore, we show here that for full-length cytb<sub>5</sub>, incorporated in DPC micelles, the linker region is unstructured, lacking any distinct secondary structural features. A cytb<sub>5</sub> linker region of at least 6–8 amino acids has been shown to be necessary to enable formation of a functional complex between cytb<sub>5</sub> and its full-length redox partner cytP450 (23). Unlike full-length cytb<sub>5</sub>, a cytb<sub>5</sub> mutant lacking eight amino acids in the linker domain was neither able to insert efficiently into a lipid membrane (47) nor able to form a fully functional complex with cytP450 (23). The extended form of the cytb<sub>5</sub> linker region, presented here, should provide the flexibility and orientational freedom necessary for efficient complex formation with its redox partners.
Model Complex between Mammalian cytb5 and Cytochrome P450

![Diagram of the Model Complex between Mammalian cytb5 and Cytochrome P450]

**FIGURE 4. Transmembrane domain of cytb5 is visible under magic angle spinning NMR.** A, 1H-15N-HMQC spectrum recorded on a selectively [15N]alanine-labeled sample of cytb5 incorporated in DPC micelles. This spectrum was obtained from a 600 MHz Varian solid-state NMR spectrometer under a 2.5 kHz spinning speed of the sample at 37 °C using a double-resonance magic angle spinning nanoprobe (Agilent/Varian). B, representation of cytb5 highlighting all the alanines (red sphere) in the protein.

Structure of the Soluble Domain of cytb5 Is Unaffected by Its Membrane Environment—The 1H-15N TROSY-HSQC spectrum was also obtained for full-length cytb5 in 10% (w/v) DMPC/DHPC isotropic bicelles with a q ratio of 0.25. The majority of the spectrum was nearly identical to the one obtained in DPC micelles (data not shown). The largest chemical shift changes were observed for the tryptophan indole side chain (NH) resonances of Trp-109, Trp-110, and Trp-113 residues. These tryptophan residues are predicted to be at the edge of the transmembrane domain and hence should be most affected by the change in the membrane environment when going from micelles to lipid bicelles.

Establishing the Topology and Structure of the Transmembrane Domain of Full-length cytb5 in Bicelles—As mentioned above, the resonances for residues in the transmembrane domain of cytb5, reconstituted in either isotropic bicelles (DMPC/DHPC) or DPC micelles, were not identified in the 1H-15N TROSY-HSQC solution NMR spectra. Although sequential NOE assignments could not be carried out for the residues in the transmembrane domain, ambiguous NOE assignments, without secondary structural information, were possible for the Hα and side chain protons of residues Asn-121 to Asp-134 in solution NMR. A 1H-15N-HMQC spectrum recorded under magic angle spinning (2.5 kHz) on a selectively [15N]alanine-labeled sample of cytb5 incorporated in DPC micelles displayed broad resonances for the backbone amide-NHs of the four alanines present in the transmembrane domain of cytb5, along with narrow resonances for the alanines in the soluble domain (Fig. 4). These data suggest that the restricted motion of the transmembrane domain of cytb5 incorporated in a DPC micelle, or isotropic bicelles, causes significant broadening of the transmembrane domain resonances due to fast spin-spin relaxation. To obtain the structure of the transmembrane domain of cytb5, we employed an alternative technique of static solid-state NMR spectroscopy on uniformly 15N-labeled full-length cytb5 incorporated in bicelles (48, 49) composed of DMPC and DHPC lipids in a 3:5:1 molar ratio, which were magnetically aligned in the external magnetic field.

A two-dimensional SLF NMR experiment using the HIMSELF (29) pulse sequence, which is based on the PiWIzM pulse scheme, was performed on magnetically aligned bicelles containing cytb5 (Fig. 1C). The resultant two-dimensional SLF spectrum correlates 15N chemical shifts with 1H-15N dipolar couplings. The two-dimensional spectrum in Fig. 1C exhibits a distinct circular PISA-wheel pattern of resonances between 60 and 100 ppm, which is indicative of an α-helical conformation and was assigned to the transmembrane anchor of cytb5 based on our previous work (29, 50). This is in agreement with previous circular dichroism (51) and Fourier transform infrared (52) experiments, which indicated that the transmembrane domain is at least 50% helical.

The observed PISA wheel was empirically fitted (53) to determine the average tilt angle of the transmembrane α-helix with respect to the bilayer normal. The resonance pattern was consistent with an average tilt of 15 ± 3°, in agreement with our previously published work (29). The value of the helix’s order parameter was estimated to be 0.86. Additionally, a “structure fitting algorithm” (54) was used in combination with the solid-state SLF NMR data to determine the backbone structure of the transmembrane anchor as a whole, as presented in Fig. 1A. Interestingly, the transmembrane domain of cytb5 is conserved among vertebrates (sequence similarity of 78–96% (23)) and is essential for complex formation with redox partners (55); both suggest that this α-helical domain plays an important role in the function of cytb5 and its interactions with redox partners.

cytP450B4-binding Epitope on cytb5 by NMR—Perturbations in the amide-NH chemical shifts (Fig. 5) and peak heights (Fig. 6) of 15N-labeled full-length ferric cytb5 upon complex formation with unlabeled full-length ferric cytP450B4 in isotropic bicelles composed of DMPC and DHPC lipids (DMPC/DHPC = q ratio of 0.25) were measured to identify the binding cytb5 epitope for cytP450. The addition of cytP450 to cytb5 in an equimolar ratio had two effects as follows: (a) it caused an overall reduction of the cytb5 amide signal intensities, indicating complex formation between cytP450 and cytb5, which increases the overall correlation time of cytb5 (Fig. 6A, yellow
A histogram depicting the weighted average chemical shift perturbations ($\delta$) observed for residues of cyt$b_5$ upon complex formation with cytP450 is presented in Fig. 5. The average chemical shift perturbations observed for the backbone amides of cyt$b_5$, upon addition of cytP450, are relatively small in magnitude, $<0.01$ ppm, and are spread over a large area of cyt$b_5$. As a result, no specific regions of cyt$b_5$ can be highlighted as being part of the interaction epitope, based on chemical shift perturbations. This lack of widespread changes in chemical shifts across the $^1$H-$^1$N TROSY-HSQC spectrum indicates that there is no notable change in the overall tertiary fold of cyt$b_5$ upon interaction with cytP450.

The reason for the small average chemical shift perturbations could be 2-fold. First, fast-to-intermediate (ns-$\mu$s) chemical exchange between the free observable cyt$b_5$ in isotropic bicelles and the unobservable high molecular weight bound-state of the cyt$b_5$-cytP450 complex in isotropic bicelles ($>100$ kDa) (56, 57) would explain the overall broadening of the observed cyt$b_5$ resonances. Our findings are in agreement with a recent NMR study, where they observed very modest chemical shift perturbations upon complex formation between truncated forms of cytP45017A1 and human cyt$b_5$ lacking the membrane domains (58). Second, small and widespread chemical shift perturbations could be a result of the formation of an ensemble of dynamic “encounter complexes” as have been reported previously for other metalloprotein complexes (56, 59–62) such as cyt$b_5$-myoglobin (56, 57). Encounter complexes are composed of an ensemble of protein orientations within the complex; this leads to very small chemical shift perturbations as observed for the cyt$b_5$-cytP450 complex here and for other redox partners previously (56).
A closer inspection of Fig. 6A reveals differential line broadening of cyt\textsubscript{b\textsubscript{5}} resonances upon complex formation with substrate-free cytP450 (yellow). The line broadening could be the result of the following: (a) changes in chemical shifts suggesting a conformational change in the protein or (b) a change in the transverse relaxation rate of cyt\textsubscript{b\textsubscript{5}} resonances caused by a direct interaction with cytP450 (63). The absence of significant chemical shift perturbations (as mentioned above) suggests that the differential line broadening observed is predominantly due to a direct interaction with cytP450, enabling the characterization of the interaction interface between cyt\textsubscript{b\textsubscript{5}} and cytP450 (63, 64). cyt\textsubscript{b\textsubscript{5}} residues that exhibited significant differential line broadening (with a decrease in peak height of greater than 20\% as compared with free cyt\textsubscript{b\textsubscript{5}}) were mapped onto the NMR structure of cyt\textsubscript{b\textsubscript{5}}. These residues, which include Glu-48, Glu-49, Asp-65, Val-66, and Thr-70 to Ser-76, highlight a region of cyt\textsubscript{b\textsubscript{5}} around the solvent-exposed edge of the heme that potentially forms the interaction interface with cytP450 (Fig. 6B). Broadening of His-68 and Ser-69 resonances of cyt\textsubscript{b\textsubscript{5}} was also observed and may be due to the close proximity of the paramagnetic center in cytP450 and/or due to steric interaction between His-68 and Ser-69 and residues on cytP450 in the interface. We also observed broadening of the backbone amide-NH resonances corresponding to residues Met-96 to Val-103 in the flexible linker domain of cyt\textsubscript{b\textsubscript{5}} (Fig. 5); this observed broadening is likely due to restriction of the motion of the linker upon complex formation (65).

Interestingly, extensive line broadening and disappearance of most of the cyt\textsubscript{b\textsubscript{5}} amide resonances in the \textsuperscript{1}H-\textsuperscript{15}N TROSY-HSQC spectrum were observed upon addition of an equimolar amount of substrate-bound unlabeled cytP450 (Fig. 6C). Two different compounds were tested as follows: BHT (type I substrate), and the heme iron-binding inhibitor, 1-(4-chlorophenyl)imidazole (1-CPI; type II ligand).\textsuperscript{5} The widespread broadening of the cyt\textsubscript{b\textsubscript{5}} resonances might suggest that the interaction of cyt\textsubscript{b\textsubscript{5}} with substrate-bound cytP450 has shifted from a fast-to-intermediate (nanosecond to microsecond) to an intermediate-to-slow time scale (microsecond to millisecond), which causes the disappearance of the majority of cyt\textsubscript{b\textsubscript{5}} resonances upon titration of substrate-bound cytP450. This conjecture is further supported by the measurement of a submicromolar $K_d$ value for the cyt\textsubscript{b\textsubscript{5}}-cytP450 complex in the presence of the substrates methoxyflurane ($K_d \sim 0.9 \mu M$) (Table 3), BHT ($K_d \sim 0.3 \mu M$) (data not shown), and 1-CPI ($K_d \sim 0.03 \mu M$) (data not shown) in aqueous solution. These submicromolar $K_d$ values are consistent with an intermediate-to-slow exchange on the NMR time scale leading to extensive line broadening of cyt\textsubscript{b\textsubscript{5}} amide resonances (57). A previous kinetic study has also reported a greater than 10-fold decrease in $K_d$ of the cyt\textsubscript{b\textsubscript{5}}-cytP450 complex upon addition of the substrate, benzphetamine (66). However, due to extensive line broadening and disappearance of most of the resonances of the heme domain of cyt\textsubscript{b\textsubscript{5}} in the \textsuperscript{1}H-\textsuperscript{15}N TROSY-HSQC spectrum, relaxation NMR experiments could not be performed to validate the change in the time scale of interaction.

Mutagenesis Identifies Residues in Contact in the cyt\textsubscript{b\textsubscript{5}}-cytP450 Complex—To complement the NMR data collected on the cyt\textsubscript{b\textsubscript{5}}-cytP450 complex, we carried out site-directed mutagenesis of residues on both cyt\textsubscript{b\textsubscript{5}} and cytP450 (supplemental Fig. S2B and Tables 3 and 4). Residues (Glu-42, Glu-43, Pro-45, Gly-46, Glu-49, Val-50, Glu-53, Gln-54, Asn-62, Asp-65, Val-66, Asp-71, and Leu-75) on the anionic surface surrounding the solvent-exposed heme of cyt\textsubscript{b\textsubscript{5}} were mutated to alanine to explore the role of atoms distal to the $\beta$-carbon of the wild-type amino acid in binding to cytP4502B4 (supplemental Fig. S2B). After purification, the mutant proteins were assayed for their ability to bind cytP4502B4 and stimulate catalysis in an aqueous reconstituted system (Table 3). Of the 13 different single mutations of cyt\textsubscript{b\textsubscript{5}}, only two, D65A and V66A, exhibited both a significantly lower affinity for cytP4502B4 (15- and 7-fold higher $K_d$, respectively) and a decreased ability (85 and 43\%, respectively) to stimulate cytP4502B4 catalysis (Table 3).

\begin{table}[h]
\centering
\caption{Values and methoxyflurane metabolism of cyt\textsubscript{b\textsubscript{5}}-cytP450 complexes, determined with both wild-type and mutant proteins.}
\begin{tabular}{llll}
\hline
& $K_d$ cyt\textsubscript{b\textsubscript{5}}-cytP450 complex ($\mu M \pm S.D.$) & Methoxyflurane metabolism & Ratio of activity \pm cyt\textsubscript{b\textsubscript{5}}
\hline
\textbf{cyt\textsubscript{b\textsubscript{5}} mutants} & & & \\
No cyt\textsubscript{b\textsubscript{5}} (control) & 0.022 \pm 0.003 & 0.19 \pm 0.2 & 7.1 \\
Wild-type cyt\textsubscript{b\textsubscript{5}} & 0.012 \pm 0.002 & 1.10 \pm 0.1 & 5.8 \\
E42A & 0.015 \pm 0.003 & 1.41 \pm 0.2 & 7.4 \\
E43A & 0.002 \pm 0.004 & 1.17 \pm 0.2 & 6.2 \\
V50A & 0.016 \pm 0.001 & 1.08 \pm 0.2 & 5.7 \\
N62A & 0.010 \pm 0.004 & 0.92 \pm 0.1 & 4.8 \\
D65A & 0.332 \pm 0.033 & 0.22 \pm 0.02 & 1.1 \\
V66A & 0.152 \pm 0.019 & 1.77 \pm 0.1 & 4.1 \\
D71A & 0.017 \pm 0.008 & 1.66 \pm 0.2 & 8.7 \\
\hline
\textbf{cytP4502B4 mutants} & & & \\
Wild-type cytP450 & 0.022 \pm 0.003 & 1.32 \pm 0.02 & 8.3 \\
R122A & 0.221 \pm 0.006 & 0.54 \pm 0.03 & 3.4 \\
R126A & 0.453 \pm 0.006 & 1.99 \pm 0.1 & 1.2 \\
R133A & 1.502 \pm 0.18 & 0.06 \pm 0.1 & 0.29 \\
F135A & 0.205 \pm 0.005 & 0.43 \pm 0.1 & 2.7 \\
M137A & 0.379 \pm 0.007 & 0.33 \pm 0.2 & 1.7 \\
K139A & 0.611 \pm 0.13 & 0.12 \pm 0.2 & 0.75 \\
K433A & 0.458 \pm 0.004 & 0.05 \pm 0.01 & 0.31 \\
H226A & 0.032 \pm 0.009 & 1.37 \pm 0.3 & 8.5 \\
\hline
\end{tabular}
\end{table}

\footnote{Type I ligands displace the water coordinating the Fe(III) in the heme as the sixth ligand, shifting the Fe(II) spin equilibrium toward the high spin form, whereas type II ligands can replace the water by coordinating to the Fe(III) thereby stabilizing the low spin form.}
These data indicate that Asp-65 and Val-66 of cytb5 are important for both binding to cytp450 and its function as an enhancer of cytp450 catalysis. Site-directed mutants of cytb5, P45A, G46A, E53A, Q54A, D71A, and L75A were found to be indistinguishable from wild type (data not shown). Whereas E42A, E43A, E49A, V50A, and N62A exhibited a modest decrease in binding affinity to cytp450, these mutants did not show a decrease in their ability to stimulate cytp4502B4 activity (Table 3); as a result, these residues were deemed to only play a minor role in the interprotein interactions.

Previous mutagenesis studies on cytp4502B4 have shown that residues in the C-helix and C-D loop, which were mutated to alanine (Arg-122, Arg-126, Arg-133, Phe-135, Met-137, and Lys-139) and Lys-433 in the β-bulge near the axial Cys-436, are important for binding to cytb5 (Table 3) (15). The R133A mutant showed a drastic decrease in binding affinity to cytb5 in fact, the binding was too weak to obtain a robust ΔKd measurement. All other mutants showed a decrease in binding affinity of at least 10-fold.

To determine the amino acids that are in contact at the interface between cytb5 and cytp450, a “double mutant cycle” (15, 67) analysis was then performed using mutants of both cytb5 and cytp450 that are defective in binding to one another (Table 4). In such cycles, the sum of the free energy change for the two single amino acid mutant proteins is compared with that of the double mutant protein complex. When the sum of the free energy change of the single mutants is not equal to that of the double mutant, the two residues are defined as interacting and not behaving independently. This assumes that the two residues are not interacting indirectly, e.g. through a structural perturbation (67, 68). A difference of greater than 1.0 kcal/mol was considered significant (67, 68). The free energy of binding, ΔG, of all possible pairs of wild type and poorly binding alanine mutants (Table 3) of both cytp4502B4 (15) (Arg-122, Arg-126, Phe-135, Met-137, Lys-139, and Lys-433) and cytb5 (Asp-65 and Val-66) was measured. Table 4 presents the results of the double mutant cycle analysis, which indicate that Lys-433 of cytp450 interacts with both Asp-65 and Val-66 of cytb5, and that Arg-122 of cytp450 interacts with Asp-65 of cytb5. Because of the 68-fold decreased affinity of the R133A-cytp450 mutant for cytb5, a robust double mutant cycle analysis could not be performed (15); however, R133A-cytp450 mutant’s poor affinity for cytb5 indicates that Arg-133 is critical to the interprotein interaction.

**Structure of the cytb5-cytp450 Complex**—A structural model of the cytb5-cytp450 complex was generated using the data-driven docking program HADDOCK (26), governed by unambiguous and ambiguous intermolecular restraints obtained from mutagenesis and NMR data (Table 5). The solution NMR structure of the heme domain of the membrane-bound rabbit cytochrome b5 (25) has been determined (12) and is in good agreement with the X-ray crystal structure of the heme domain (26) of bovine cytb5 (27). The structure of the cytb5-cytp450 complex was modeled (28) using the cytb5 and cytp450 sequences, and the X-ray crystal structures of cytb5 (27) and cytp450 (26) as a starting point.

### Table 4

**Double mutant cycle analysis of mutants of cytp450 and cytb5**

A comparison of the difference in free energy of binding values (ΔG) between mutant and wild-type proteins to determine the residues interacting at the cytb5-cytp450 interface.

| cytp450    | cytb5    | ΔKd (μM) | Free energy of binding (kcal/mol) | ΔΔG interaction of double mutants |
|-----------|----------|----------|-----------------------------------|---------------------------------|
| Wild type | Wild type| 0.022±0.003 | -10.43 | 1.36 |
| R122A     | Wild type| 0.221±0.010 | -9.07  | 1.61 |
| Wild type | D65A     | 0.332±0.032 | -8.82  | 1.91 |
| R122A     | D65A     | 0.558±0.065 | -8.52  | 1.14 |
| Wild type | V66A     | 0.152±0.019 | -9.29  | 2.14 |
| R122A     | V66A     | 0.814±0.033 | -8.29  | 1.79 |
| Wild type | D65A     | 0.454±0.042 | -8.64  | 1.61 |
| R126A     | D65A     | 18.33±1.500 | -6.45  | 3.98 |
| Wild type | V66A     | 0.152±0.010 | -9.29  | -0.58 |
| R126A     | V66A     | 5.149±0.930 | -7.20  | 3.23 |
| Wild type | V66A     | 0.205±0.021 | -9.11  | 1.32 |
| F135A     | Wild type| 0.332±0.042 | -8.82  | 1.61 |
| Wild type | D65A     | 1.420±0.110 | -7.96  | 2.47 |
| F135A     | D65A     | 0.152±0.010 | -9.29  | 1.14 |
| Wild type | V66A     | 0.959±0.130 | -8.20  | 2.23 |
| Wild type | D65A     | 0.379±0.040 | -8.75  | 1.68 |
| M137A     | Wild type| 0.342±0.012 | -8.82  | 1.61 |
| Wild type | D65A     | 1.951±0.440 | -7.36  | 3.07 |
| M137A     | D65A     | 0.152±0.010 | -9.29  | 1.14 |
| Wild type | V66A     | 1.583±0.142 | -7.90  | 2.53 |
| K139A     | Wild type| 0.611±0.050 | -8.46  | 1.97 |
| Wild type | D65A     | 0.332±0.012 | -8.82  | 1.61 |
| K139A     | D65A     | 4.831±0.042 | -7.24  | 3.19 |
| Wild type | V66A     | 0.152±0.010 | -9.29  | 1.14 |
| K139A     | V66A     | 1.683±0.212 | -7.86  | 2.57 |
| K433A     | Wild type| 0.458±0.053 | -8.63  | 1.80 |
| Wild type | D65A     | 0.332±0.012 | -8.82  | 1.61 |
| K433A     | D65A     | 0.869±0.071 | -8.26  | 2.17 |
| Wild type | V66A     | 0.152±0.010 | -9.29  | 1.14 |
| K433A     | V66A     | 0.487±0.052 | -8.60  | 1.83 |
cytb5 with a backbone r.m.s.d. of 0.32 ± 0.10 Å (Fig. 1A) and a 1.9 Å resolution crystal structure of the heme-containing domain of cytP4502B4 (PDB code 1SUO (41)) were used in HADDOCK calculations. Docking was performed in the absence of a membrane environment, and therefore our structure of the complex reveals the interactions between the structured heme domains of cytb5 and cytP450. However, it is important to note that all NMR and mutagenesis data were collected on membrane-bound full-length proteins, and the enzymatic function of the complex under these conditions was confirmed by activity assays (supplemental Table S1).

As described above, the double mutant cycle analysis revealed that Lys-433 of cytP450 interacts with both Asp-65 and Val-66 of cytb5 and that Arg-122 of cytP450 interacts with Asp-65 of cytb5; these interactions were incorporated as ambiguous intermolecular restraints in HADDOCK. The ambiguous intermolecular restraints were generated using active and passive residues for cytb5 and cytP450 (all >40% solvent-accessible). As active residues, eight cytb5 residues exhibiting significant differential line broadening upon complex formation with cytP450, and seven cytP450 residues deemed essential for binding to cytb5, based on site-directed mutagenesis, were selected. As passive residues, solvent-accessible (>40% solvent-accessible) amino acids flanking the active residues were selected for cytb5 and for cytP450, all residues on the proximal side where the heme is closest to the surface were selected (Table 5).

The docking simulations reveal not a single specific complex but rather an ensemble of low energy complex orientations (supplemental Fig. S3A), where the acidic convex surface of cytb5 is sampling an extended surface area on the concave basic proximal side of cytP450 (data not shown). The two dominant subpopulations of low energy complexes (Table 6 and supplemental Fig. S3), titled clusters I and II, include two unique but overlapping clusters of residues on cytb5 and cytP4502B4 (Figs. 7 and 8, supplemental Figs S2 and S3, and supplemental Table S2). The residues of cytb5 that are common between the majority of the low energy complex structures (supplemental Fig. S3) are found mostly on the α4 and α5 helices. The cytP450 and cytb5 hemes are nearly perpendicular to one another in both clusters, and the shortest distance between the two heme edges is 9.0 and 7.4 Å, respectively, in clusters I and II, which is well within the 14.0 Å limit predicted for efficient electron transfer (Fig. 7B) (69). The Fe-Fe distance is 20.9 and 19.3 Å in clusters I and II, respectively.

**DISCUSSION**

The structure of the heme domain of full-length rabbit cytb5, incorporated in DPC micelles, was found to be similar but not identical to the previously reported structure of truncated rabbit cytb5 (18). Our NMR structure has additional β-strands (β1 and β4) and longer (α1, β2, α2, and α3) and shorter (β5 and 310 helix) segments for some of the secondary structure elements (Figs. 1 and 3 and supplemental Fig. S1A). The linker region (Ser-90 to Asp-104) in our NMR structure of cytb5, which connects the cytosolic heme domain to the α-helical transmembrane anchor, was found to be random coil. As mentioned under “Results,” the extended form of the cytb5 linker region likely allows for proper interaction of the cytb5 soluble domain with its redox partners. Solid-state SLF NMR data, in combination with a structure fitting algorithm (54), were used to determine the α-helical structure of the transmembrane domain of cytb5 (Fig. 1C). Our NMR structure of full-length microsomal ferric cytb5 was used subsequently to establish the interaction interface between microsomal, rabbit, ferric cytb5, and cytP450.

**cytb5-cytP450 Interaction Interface**—As mentioned above, the modest average chemical shift perturbations (Δδ <0.01 ppm) observed for the heme domain of full-length cytb5 upon complex formation with substrate-free cytP450 could be a result of the combination of interaction on the fast-to-interme-
mediate NMR exchange time scale and formation of an ensemble of dynamic encounter complexes, as has been reported previously for other metalloprotein complexes (59, 62). Complex formation between electron transfer proteins, like cytP450 and cyt_b, has been shown to proceed via the formation of dynamic encounter complexes driven by the oppositely charged surfaces of the proteins (supplemental Fig. S2) (62, 70). In our experiment, we hypothesize that the different complex orientations, within the encounter complexes, are interchanging among themselves at a fast-to-intermediate time scale, because we do not see any significant chemical shift perturbations. These encounter complexes formed by cyt_b and cytP450 in the absence of a substrate are most likely in equilibrium with a well defined complex orientation, known as the stereospecific complex (70). The stereospecific complex is characterized by a tighter affinity of the two proteins for one another and more hydrophobic interactions, whereas the weaker encounter complexes are stabilized predominantly by long range electrostatic interactions. The lifetime and the populations of the individual orientations govern the effect of the encounter and the stereospecific complex on the NMR data.

The extensive line broadening of cyt_b resonances upon complex formation with substrate-bound cytP450 (Fig. 6) suggests that substrate binding modulates the affinity of the proteins for each other. Our result is in agreement with two recent studies where binding of the substrate modulates the interaction between cytP450 and cyt_b (58, 65). Addition of the substrate could be shifting the equilibrium from the weaker encounter complexes toward the stereospecific complex.

The two lowest energy cyt_b-cytP450 complex orientations (clusters I and II), calculated from HADDOCK driven by the NMR and mutagenesis data presented here, likely represent “productive” cyt_b-cytP450 complex orientations, because the heme-edge to heme-edge distances would allow for efficient electron transfer (69). Both complex structures are typical of other redox complexes in that, although there is a large interfacial area of contact (∼937 Å² in cluster I and ∼903 Å² in cluster II, see Table 6), the bulk of the binding energy can be attributed to a small number of complementary residues (71). Our result is in agreement with a recent chemical cross-linking study on cytP4502B4 where the authors observed evidence for the existence of two mutual orientations of the cyt_b-cytP450 complex (72).

The cyt_b-cytP450 complex orientations reveal that the acidic convex surface of cyt_b docks, like a ball in a socket, into the entire concavity on the proximal surface of cytP450, with the C-helix residues contributing the vast majority of the binding energy as indicated by the mutagenesis data (Table 3). A closer look at the complex structures from clusters I and II (Fig. 8, supplemental Fig. S2, C and D, and supplemental Table S2) highlights that the interactions at the complex interface occur between 14 residues and the heme-D-propionate of cyt_b and 14 residues of cytP450. Based on the double mutant cycle analysis (Table 4), we identified that the interactions contributing...
the most binding energy to the complex formation were between the cytP450 C-helix residue Arg-122 and Lys-433 in the /H9252-bulge (near the axial Cys-436) and Asp-65 and Val-66 at the C terminus of helix /H9251 of cytb5. These interactions were shown to be critical for both complex formation and function. From the HADDOCK structures, we see that Asp-65 of cytb5 is able to form hydrogen bonds and/or salt bridges with Arg-122 and Lys-66 of cytP450 and that Val-66 of cytb5 is in van der Waals contact with Lys-433 (and Arg-125) of cytP450. Arg-133 of cytP450, which was found to be very important for binding to cytb5 in our studies, is hydrogen-bonded with the heme propionate group on cytb5 in both clusters (additional interactions were also found for Arg-133 in cluster II, see supplemental Table S2). The function of other cytP4502B4 residues previously mutated to alanine (Table 3) (15) can also be discussed. Arg-126 of cytP450 was found to form hydrogen bonds and salt bridges with Glu-64 of cytb5 in both clusters and hydrogen bonds with His-68 (supplemental Table S2). Surprisingly, the C-D loop residues of cytP450, Met-137, and Lys-139, which mutagenesis data revealed were important for cytb5 binding (Table 3), were not in the complex interface (Fig. 8 and supplemental Fig. S2, C and D). We hypothesize that their mutation might induce a structural perturbation in the flexible C-helix, which in turn destabilizes the interaction with cytb5. The K139A mutation was previously shown to disrupt a hydrogen bond network between the Lys-139 amino group and Pro-261 and Asn-260 in the G-H loop (41), suggesting an allosteric interaction between the C-helix and the G-H loop. Arg-422 on cytP450, which is in contact with cytb5 in the predicted complex structure from cluster II, was previously considered to be important only for binding to CPR (15). However, review of the previously reported mutagenesis data revealed that the R422A-cytP450 mutant exhibited a 50% decreased affinity for cytb5, which at the time was considered insignificant (15). Residue Arg-443, which was shown to be important only for cytP450 reductase (15), is not found in the interaction interface of the predicted complex structures (Fig. 8).
On cytb₅, both Asp-65 and Val-66, which were shown to be in the binding site for cytochrome c (18, 73), are now shown to be in the binding site for cytP450B4 both experimentally and in our HADDOCK complex structures. A double cytb₅ mutant E48G and E49G mutant (which introduced a very flexible sequence of four glycine residues) has been shown to be deficient in its ability to stimulate the activity of cytP450c17 (74). This observation is consistent with the presence of Glu-48 and Glu-49 in the interaction interface of cluster II and is in accordance with our NMR data where we observed considerable line broadening for Glu-48 and Glu-49 upon complex formation with cytP450.

The HADDOCK structures of the cytb₅-cytP450 complex, as well as previous experiments (7, 14), demonstrate that cytb₅ and CPR compete for an overlapping binding site on cytP450, and they rule out the existence of separate functional binding sites for cytb₅ and CPR and the formation of a ternary complex between the three proteins (75).

Electron Transfer Pathway between cytb₅ and cytP450—The structure of the cytb₅-cytP450 complex generated from HADDOCK shows that the guanidinium group of Arg-125 on the C-helix of cytP450 forms a salt bridge between the heme-𝛥-proponates of both cytb₅ and cytP450. This network was predicted, using HARLEM (76), to serve as one of the shortest electron transfer pathways between the two proteins (Fig. 7B). Arg-125 is one of the most highly conserved cytP450 residues. It is homologous to Arg-112 in cytP450 cam, which has been shown to be essential for electron transfer (77). The physiological significance of Arg-125 was also highlighted when mutation of the Arg-125 homolog in human cytP4502A1 resulted in a defect in vitamin D degradation (78). We have previously attempted to characterize the R125A mutant of cytP4502B4 (15), and we found that the mutation rendered the protein unstable. The proposed network involving the heme propionates is consistent with previous studies that have demonstrated that the heme propionate groups of cytb₅ interact with charged groups on cytochrome c (79, 80) and cytP450 isoforms (81), suggesting that other cytP450 complexes employ a similar interface. Fig. 7B presents another possible electron transfer pathway between the heme-𝛥-proponate of cytb₅ and Ile-435, the nonconserved amino acid preceding the axial ligand, Cys-436, on cytP450. However, our mutagenesis data have shown that the I435A mutant is as active as the wild-type cytP450 (data not shown), suggesting that it does not play a critical role in electron transfer between the two proteins.

Conclusion—Here, we have presented the first full-length structure of rabbit cytb₅ incorporated in a membrane mimetic (DPC micelles or lipid bicelles) obtained using a combination of solution and solid-state NMR spectroscopy. The heme domain and linker region structures were established using solution NMR, and the transmembrane domain structure and topology were identified using solid-state NMR (full-length cytb₅ was used for all NMR measurements). The highly conserved nature of the transmembrane domain of cytb₅ (with a sequence similarity of 78–96% (23) among vertebrates) suggests that these results and methodology should be applicable to other mammalian cytsb₅ as well. Subsequently, HADDOCK (26), driven by experimental constraints obtained from site-directed mutagenesis and solution NMR spectroscopy on full-length membrane-bound monosomal cytb₅ and cytP450B4, was used to generate a model of the cytb₅-cytP450 complex. The two proteins form a dynamic complex mediated by both hydrophobic and electrostatic interactions (Fig. 8 and supplemental Table S2). The electrostatic interactions between the oppositely charged residues, as well as the fact that the two proteins are anchored in the membrane, play important roles in orienting the two proteins prior to complex formation and help considerably by increasing the number of productive collisions that control and direct the flow of electrons from cytb₅ to cytP450. Addition of a small molecule substrate (BHT) or inhibitor (1-CPI) significantly increased the binding affinity between cytb₅ and cytP450, moving the dynamic interaction between the two proteins from a fast-to-intermediate to an intermediate-to-slow regime on the NMR time scale, based on the extensive line broadening of cytb₅ amide NMR resonances upon complex formation with substrate-bound cytP450. The structure of the cytb₅-cytP450 complex presented allows us to identify the interactions at the interface and to propose the pathway of interprotein electron transfer from cytb₅ to cytP450 through the highly conserved Arg-125 residue on cytP450. Our study demonstrates how a combinatorial approach, involving NMR and mutagenesis studies, can be exploited to obtain atomic level structural, functional, and dynamic information on intact, membrane-bound, and large metalloprotein redox complexes in a near-native environment. The extensive knowledge of the structure of the cytb₅-cytP450 complex provides insights into the principles governing interprotein interactions and will markedly facilitate our ability to unravel the molecular mechanism by which the rate of cytP450 catalysis is regulated by its redox partners, cytb₅ and CPR.

REFERENCES

1. Guengerich, F. P., Wu, Z. L., and Bartleson, C. J. (2005) Function of human cytochrome P450s: characterization of the orphans. Biochem. Biophys. Res. Commun. 338, 465–469
2. Shen, A. L., O’Leary, K. A., and Kasper, C. B. (2002) Association of multiple developmental defects and embryonic lethality with loss of microsomal NA-DPH-cytochrome p450 oxidoreductase. J. Biol. Chem. 277, 6536–6541
3. Nebert, D. W., and Russell, D. W. (2002) Clinical importance of the cytochromes P450. Lancet 360, 1155–1162
4. O’Donnell, A., Judson, I., Dowsett, M., Raynaud, F., Dearnaley, D., Mason, M., Harland, S., Robbins, A., Halbert, G., Nutley, B., and Jarman, M. (2004) Hormonal impact of the 17α-hydroxylase/C(17,20)-lyase inhibitor abiraterone acetate (CB7630) in patients with prostate cancer. Br. J. Cancer 90, 2317–2325
5. Orlando, L., Schiavone, P., Fedele, P., Calvani, N., Nacci, A., Rizzo, P., Marino, A., D’Amico, M., Sponziello, F., Mazzoni, E., Cinefra, M., Fazio, N., Maiello, E., Silvestris, N., Colucci, G., and Cinieri, S. (2010) Molecurly targeted endocrine therapies for breast cancer. Cancer Treat. Rev. 36, 567–571
6. Im, S. C., and Waskell, L. (2011) The interaction of microsomal cytochrome P450 2B4 with its redox partners, cytochrome P450 reductase, and cytochrome b₅. Arch. Biochem. Biophys. 507, 144–153
7. Zhang, H., Hamdane, D., Im, S. C., and Waskell, L. (2008) Cytochrome b₅ inhibits electron transfer from NAPDH-cytochrome P450 reductase to ferric cytochrome P450 2B4. J. Biol. Chem. 283, 5217–5225
8. Gruenke, L. D., Konopka, K., Ćadić, M., and Waskell, L. (1995) The stoichiometry of the cytochrome P-450-catalyzed metabolism of me-
Model Complex between Mammalian cytb\textsubscript{5} and Cytochrome P450

thoxyflurane and benzphetamine in the presence and absence of cytochrome b\textsubscript{5}. J. Biol. Chem.\textbf{270}, 24707–24718

9. Finn, R. D., McLaughlin, L. A., Ronseaux, S., Rosewell, I., Houston, J. B., Henderson, C. J., and Wolf, C. R. (2008) Defining the \textit{in vivo} role for cytochrome b\textsubscript{5} in cytochrome P450 function through the conditional hepatic deletion of micrornal cytochrome b\textsubscript{5}. \textit{J. Biol. Chem.} \textbf{283}, 31385–31393

10. Guengerich, F. P. (2006) Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS J. \textbf{8}, E101–E111

11. Shimada, T., Mernaugh, R. L., and Guengerich, F. P. (2005) Interactions of mammalian cytochrome P450, NADPH-cytochrome P450 reductase, and cytochrome b\textsubscript{5} enzymes. \textit{Arch. Biochem. Biophys.} \textbf{435}, 207–216

12. Canova-Davis, E., Chiang, J. Y., and Waskell, L. (1985) Obligatory role of cytochrome b\textsubscript{5} in the mammalian microsomal metabolism of methoxyflurane. \textit{Biochem. Pharmacol.} \textbf{34}, 1907–1912

13. Morgan, E. T., and Coon, M. J. (1984) Effects of cytochrome b\textsubscript{5} on cytochrome P-450-catalyzed reactions. Studies with mammalian-substituted cytochrome b\textsubscript{5}. \textit{Drug Metab. Dispos.} \textbf{12}, 358–364

14. Zhang, H., Im, S. C., and Waskell, L. (2007) Cytochrome b\textsubscript{5}. \textit{Biochemistry} \textbf{46}, 1149, 1224–1243

15. Halpert, J. R. (2011) Structure and function of cytochromes P450 2B: from mechanism-based inactivators to x-ray crystal structures and back. \textit{Drug Metab. Dispos.} \textbf{39}, 1113–1121

16. Scott, E. E., He, Y. A., Wester, M. R., White, M. A., Chin, C. C., Halpert, J. R., Johnson, E. F., and Stout, C. D. (2003) An open conformation of mammalian cytochrome P450 2B4 at 1.6-Å resolution. \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{100}, 13196–13201

17. Banci, L., Bertini, I., Rosato, A., and Scacchi, S. (2000) Solution structure of oxidized microsomal rabbit cytochrome b\textsubscript{5}. Factors determining the heterogeneous binding of the heme. \textit{Eur. J. Biochem.} \textbf{267}, 755–766

18. Nunez, M., Guittet, E., Pompon, D., van Heijenoort, C., and Truan, G. (2010) NMR structure of oxidized microsomal human cytochrome b\textsubscript{5}. \textit{J. Biol. Med.} \textbf{47}, 289–295

19. Basaran, N., Doebeler, R. W., Goldston, H., and Holloway, P. W. (1999) Effect of lipid unsaturation on the binding of native and a mutant form of cytochrome b\textsubscript{5} to membranes. \textit{Biochemistry} \textbf{38}, 15245–15252

20. Greenhut, S. F., Taylor, K. M., and Roseman, M. A. (1993) Tight insertion of cytochrome b\textsubscript{5} into large unilamellar vesicles. \textit{Biochim. Biophys. Acta} \textbf{1149}, 1–9

21. Fung, B. M., Khitrin, A. K., and Ermolaev, K. (2000) An improved broad-band decoupling sequence for liquid crystals and solids. \textit{J. Magn. Reson.} \textbf{142}, 97–101

22. Scott, E. E., White, M. A., He, Y. A., Johnson, E. F., Stout, C. D., and Halpert, J. R. (2004) Structure of mammalian cytochrome P450 2B4 complexed with 4-(4-chlorophenyl)imidazole at 1.9-Å resolution: insight into the range of P450 conformations and the coordination of redox partner binding. \textit{J. Biol. Chem.} \textbf{279}, 27294–27301

23. Schüttelkopf, A. W., and van Aalten, D. M. (2004) PRODRG: a tool for high throughput crystallography of protein-ligand complexes. \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{60}, 1355–1363

24. Potterton, E., Briggs, P., Turkenburg, M., and Dodson, E. (2003) A graphical user interface to the CCP4 program suite. \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{59}, 1131–1137

25. Zhang, Q., Cao, C., Wang, Z. Q., Wang, Y. H., Wu, H., and Huang, Z. X. (2004) The comparative study on the solution structures of the oxidized bovine microsomal cytochrome b\textsubscript{5} and mutant V45H. \textit{Protein Sci.} \textbf{13}, 2161–2169

26. Lee, K. B., La Mar, G. N., Kehres, L. A., Fujinari, E. M., Smith, K. M., Pochapsky, T. C., and Silar, S. G. (1990) 1H NMR study of the influence of hydrophobic contacts on protein-prosthetic group recognition in bovine and rat ferricytochrome b\textsubscript{5}. \textit{Biochemistry} \textbf{29}, 9623–9631

27. Santos, C. R., Hare, B. I., Howard, K. P., and Prestegard, J. H. (1994) Magnetically oriented phospholipid micelles as a tool for the study of membrane-associated molecules. \textit{Prog. Nucl. Magn. Reson. Spectrosc.} \textbf{26}, 421–444

28. Sanders, C. R., Gildenberg, M., and Ramamoorthy, A. (2012) The magic of bicelles lights up membrane protein structure. \textit{Chem. Rev.} \textbf{112}, 6054–6074

29. Soong, R., Smith, P. E., Xu, J., Yamamoto, K., Im, S. C., Waskell, L., and Ramamoorthy, A. (2010) Proton-evolved local-field solid-state NMR
studies of cytochrome \( b_5 \) embedded in bicelles, revealing both structural and dynamical information. J. Am. Chem. Soc. 132, 5779–5788.

51. Dailey, H. A., and Strittmatter, P. (1978) Structural and functional properties of membrane binding segment of cytochrome \( b_5 \). J. Biol. Chem. 253, 8203–8209.

52. Holloway, P. W., and Buchheit, C. (1990) Topography of the membrane-binding domain of cytochrome-B5 in lipids by Fourier-transform infrared-spectroscopy. Biochemistry 29, 9631–9637.

53. Denny, J. K., Wang, J., Cross, T. A., and Quine, J. R. (2001) PISEMA powder patterns and PISA wheels. J. Magn. Reson. 152, 217–226.

54. Nezvoriev, A. A., and Opella, S. J. (2003) Structural fitting of PISEMA spectra of aligned proteins. J. Magn. Reson. 160, 33–39.

55. Seygers, G., and Waskell, L. (1992) Expression of cytochrome \( b_5 \) yeast and characterization of mutants of the membrane-anchoring domain. J. Biol. Chem. 267, 12583–12591.

56. Prudêncio, M., and Ubbink, M. (2004) Transient complexes of redox proteins: structural and dynamic details from NMR studies. J. Mol. Recognit. 17, 524–539.

57. Zijderveld, E. R. (2002) Mapping protein-protein interactions in solution by NMR spectroscopy. Biochemistry 41, 1–7.

58. Estrada, D. F., Laurence, J. S., and Scott, E. E. (2013) Substrate-modulated cytochrome P450 17A1 and cytochrome \( b_5 \) interactions revealed by NMR. J. Biol. Chem. 288, 17008–17018.

59. Volkov, A. N., Ferrari, D., Worrall, J. A., Bonvin, A. M., and Ubbink, M. (2005) The orientations of cytochrome \( c \) in the highly dynamic complex with cytochrome \( b_5 \) visualized by NMR and docking using HADDOCK. Protein Sci. 14, 799–811.

60. Tang, C., Iwahara, J., and Clore, G. M. (2006) Visualization of transient encounter complexes in protein-protein association. Nature 444, 383–386.

61. Volkov, A. N., Ubbink, M., and van Nuland, N. A. (2010) Mapping the encounter state of a transient protein complex by PRE NMR spectroscopy. J. Biomol. NMR 48, 225–236.

62. Suh, J. Y., Tang, C., and Clore, G. M. (2007) Role of electrostatic interactions in transient encounter complexes in protein-protein association investigated by paramagnetic relaxation enhancement enhancement. J. Am. Chem. Soc. 129, 12954–12955.

63. Matsuo, H., Walters, K. J., Terauchi, K., Tanaka, T., Gassner, G. T., Lippard, S. J., Kyogoku, Y., and Wagner, G. (1999) Identification by NMR spectroscopy of residues at contact surfaces in large, slowly exchanging macromolecular complexes. J. Am. Chem. Soc. 121, 9903–9904.

64. Zamoon, J., Nitu, F., Karim, C., Thomas, D. D., and Veglia, G. (2005) Mapping the interaction surface of a membrane protein: unveiling the conformational switch of phospholamban in calcium pump regulation. Proc. Natl. Acad. Sci. U.S.A. 102, 4747–4752.

65. Koberova, M., Jecmen, T., Sulc, M., Cerna, V., Kizek, R., Stiborova, M., and Hodek, P. (2013) Photo-cytochrome \( b_5 \) – a new tool to study the cytochrome P450 electron-transport chain. Int. J. Electrochem. Sci. 8, 125–134.

66. Tamburini, P. P., and Gibson, G. G. (1983) Thermodynamic studies of the protein-protein interactions between cytochrome P450 and P450 spin state in the coupling of substrate and cytochrome \( b_5 \) binding to the terminal hemoprotein. J. Biol. Chem. 258, 13444–13452.

67. Frisch, C., Schreiber, G., Johnson, C. M., and Fersht, A. R. (1997) Thermodynamics of the interaction of barnase and barstar: changes in free energy versus changes in enthalpy on mutation. J. Mol. Biol. 267, 696–706.

68. Harel, M., Cohen, M., and Schreiber, G. (2007) On the dynamic nature of the transition state for protein-protein association as determined by double-mutant cycle analysis and simulation. J. Mol. Biol. 371, 180–196.

69. Page, C. C., Moser, C. C., and Dutton, P. L. (2003) Mechanism for electron transfer within and between proteins. Curr. Opin. Chem. Biol. 7, 551–556.

70. Ubink, M. (2009) The course of a transient protein complex. FEBS Lett. 583, 1060–1066.

71. Clackson, T., and Wells, J. A. (1995) A hot spot of binding energy in a hormone-receptor interface. Science 267, 383–386.

72. Suh, M., Jecmen, T., Najadrova, R., Novak, P., Martinek, V., Hodek, P., and Hucek, J. (2012) Mapping of interaction between cytochrome P450 284 and cytochrome \( b_5 \) the first evidence of two mutual orientations. Neuro Endocrinol. Lett. 33, 41–47.

73. Shao, W., Im, S. C., Zuiderweg, E. R., and Waskell, L. (2003) Mapping the binding interface of the cytochrome \( b_5 \)-cytochrome \( c \) complex by nuclear magnetic resonance. Biochemistry 42, 14774–14784.

74. Naffin-Olivos, J. L., and Auculis, R. J. (2006) Human cytochrome \( b_5 \) requires residues E48 and E49 to stimulate the 17,20-lyase activity of cytochrome P450c17. Biochemistry 45, 755–762.

75. Tamburini, P. P., and Schenkman, J. B. (1987) Purification to homogeneity and enzymological characterization of a functional covalent complex composed of cytochromes P450 isozyme 2 and \( b_5 \) from rabbit liver. Proc. Natl. Acad. Sci. U.S.A. 84, 11–15.

76. Kurnikov, I. V. (2000) HARLEM Molecular Modeling Package, Version 1.0, Department of Chemistry, University of Pittsburgh, Pittsburgh.

77. Nakamura, K., Horiuchi, T., Yasukochi, T., Sekimizu, K., Hara, T., and Sagara, Y. (1994) Significant contribution of arginine 112 and its positive charge of Pseudomonas putida cytochrome P-450cam in the electron transport from putidaredoxin. Biochem. Biophys. Acta 1207, 40–48.

78. Schlingmann, K. P., Kaufmann, M., Weber, S., Irwin, A., Goos, C., John, U., Misselwitz, J., Klaus, G., Kuwertz-Bröking, E., Fehrenbach, H., Wingen, A. M., Güran, T., Hoenderop, J. G., Bindels, R. J., Prosser, D. E., Jones, G., and Konrad, M. (2011) Mutations in CYP24A1 and idiopathic infantile hypercalcaemia. N. Engl. J. Med. 365, 410–421.

79. Rodgers, K. K., Pochapsky, T. C., and Sligar, S. G. (1988) Probing the mechanisms of macromolecular recognition-the cytochrome-\( b_5 \)-cytochrome-\( c \) complex. Science 240, 1657–1659.

80. Deep, S., Im, S. C., Zuiderweg, E. R., and Waskell, L. (2005) Characterization and calculation of a cytochrome \( c \)-cytochrome \( b_5 \) complex using NMR data. Biochemistry 44, 10654–10668.

81. Tamburini, P. P., and Schenkman, J. B. (1986) Mechanism of interaction between cytochromes P-450 RLM5 and \( b_5 \): evidence for an electrostatic mechanism involving cytochrome \( b_5 \) heme propionate groups. Arch. Biochem. Biophys. 245, 512–522.

82. Popovych, N., Sun, S., Ebright, R. H., and Kalodimos, C. G. (2006) Dynamically driven protein allostery. Nat. Struct. Mol. Biol. 13, 831–838.

83. Ahmad, S., Gromiha, M., Favareh, H., and Sarai, A. (2004) ASAView: database and tool for solvent accessibility representation in proteins. BMC Bioinformatics 5, 51.