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Structural and Functional Studies of the Membrane Binding Domain of NADPH -Cytochrome P450 Oxidoreductase

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Abstract

NADPH-cytochrome P450 oxidoreductase (CYPOR), the essential flavoprotein of the microsomal cytochrome P450 monooxygenase system, is anchored in the phospholipid bilayer by its amino-terminal membrane-binding domain (MBD), which is necessary for efficient electron transfer to cytochromes P450. Although crystallographic and kinetic studies have established the structure of the soluble catalytic domain and the role of conformational motions in the control of electron transfer, the role of the MBD is largely unknown. We examined the role of the MBD in P450 catalysis through studies of amino-terminal deletion mutants and site-directed spin-labeling. We show that the MBD spans the membrane and present a model for orientation of CYPOR on the membrane capable of forming a complex with cytochrome P450. EPR power saturation measurements of CYPOR mutants in liposomes containing a lipid-Ni(II)chelate identified a region of the soluble domain interacting with the membrane. Deletion of more than 29 residues from the N-terminus of CYPOR decreases cytochrome P450 activity concomitant with alterations in electrophoretic mobility and an increased resistance to protease digestion. The altered kinetic properties of these mutants are consistent with electron transfer through random collisions rather than formation of a stable CYPOR:P450 complex. Purified MBD binds weakly to cytochrome P450, suggesting that other interactions are required for CYPOR-P450 complex formation. We propose that the MBD and flexible tether region of CYPOR, residues 51–63, play an important role in facilitating movement of the soluble domain relative to the membrane and promoting multiple orientations that permit specific interactions of CYPOR with its varied partners.

Introduction

The diverse family of microsomal cytochromes P450 catalyzes oxidation of a large number of substrates using molecular oxygen and electrons derived from NADPH via a common

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electron transfer partner, NADPH cytochrome P450 oxidoreductase (CYPOR, reductase)^{1, 2}. The amino-terminal membrane binding domain (MBD), also referred to as the “tail”, of CYPOR anchors the reductase to the microsomal membrane and is, with few exceptions³⁴, necessary for efficient electron transfer to cytochrome P450. Inspection of CYPOR sequences reveals that the first 27 residues of rat CYPOR are relatively hydrophilic while residues 28 to 44 comprise a hydrophobic region followed by four positively-charged residues and a flexible tether region extending from residues 44 to 63 (Figure 1)⁵.

The tether region is uniquely susceptible to protease digestion; limited trypsin proteolysis of mammalian CYPOR at Lys56/Ile57, produces the 56 amino acid amino-terminal fragment known as the MBD or tail, with a molecular weight of 6.3 kDa, and the 70.6 kDa “soluble” or “catalytic” domain, which contains structural domains for binding of FMN, FAD, and NADPH⁶⁻⁹. The catalytic domain is capable of supporting the activities of cytochrome *c* and other artificial electron acceptors but not cytochrome P450^{1, 10, 11}.

Although it has been known for 50 years that both full-length CYPOR and lipid are required for P450-mediated oxidations, the functions of the reductase MBD and the lipid bilayer remain an unanswered question. It has been proposed that the MBD contains a specific binding site for P450¹², or that it serves as a nonspecific membrane anchor¹³. Studies demonstrating the importance of cytochrome P450 and CYPOR conformational changes during turnover¹⁴⁻²⁰ and the influence of microsomal lipids on P450 function and CYPOR flavin redox potential²¹⁻²³ raise the possibility that the MBD facilitates one or more of these processes.

Here we report kinetic analyses and biochemical characterization of CYPOR mutants whose N-terminal tail has been systematically cleaved. Using site-directed spin labeling (SDSL) EPR methods, we present the structure of the tail in the membrane. We have mapped the location of specific residues relative to the membrane surface and determined the orientation of the soluble domain with respect to the membrane; this orientation is consistent with formation of a productive CYPOR-P450 electron transfer complex.

Materials and Methods:

Plasmid construction and mutagenesis:

The present studies utilize the expression plasmids, pET-OR and pIN-OR, both of which produce wildtype CYPOR proteins starting at the authentic initiating ATG of the rat protein, in contrast to pOR263⁷, which contained 8 additional amino acids at the amino terminus. pET-OR was constructed by introducing an NdeI site at the position of the amino-terminal methionine of the reductase coding sequence by PCR using the following oligonucleotides:

Nde-OR1 5'-GGCGACCCATATGGGGGACTCTCACGAAG-3'

1350R 5'-GGTTTCTGGTTCTCGTAGCT-3'

Following digestion of the PCR product with NdeI and SacI, the 735 -bp NdeI/SacI fragment and a 1.7-Kbp SacI/HindIII fragment of pOR263 containing the remainder of the reductase coding sequence were cloned into pET-21b to produce pET-OR. pIN-OR was

produced by overlap extension PCR using pOR263 as the template and the following oligonucleotides:

NEWPIN1 5'-TAGCGCAGGCCGGAGACTCTCACG-3'

NEWPIN2 5'-TTCGTGAGAGTCTCCGGCCTGCGCTA-3'

followed by PCR with the primers 311 – 5'-TAGAGAGGCTTTACACTTTATGCT-3' and 1350R. The Xba1/Sac1 fragment from this PCR product was cloned into pOR263.

Amino-terminal deletion mutants were constructed by PCR using the designated mutant oligonucleotides, each containing an NdeI site, as the forward primers and 1350R as the reverse primer, followed by digestion with NdeI and SacI, and cloning of the mutant fragment into pET-OR. Oligonucleotides used for mutagenesis are as follows:

9 5'-GAAGACCATATGGCCACCATGCCTGAGGCCG-3'

19 5'-GTGGCTCATATGGTGTCTCTATTTCAGCACG-3'

28 5'-ACGACGCATATGGTTCTGTTTTCTCTC-3'

29 5'-GACGGACCATATGCTGTTTTCTC-3'

31 5'-CATGGTTCATATGTCTCTCATCG-3'

34 5'-TGTTTTCTCATATGGTGGGGGTCCT-3'

37 5'-ATCGTGCATATGCTGACCTACTGGTTCATC-3'

40 5'-GTCCTGCATATGTGGTTCATCT-3'

43 5'-CTACTGGCATATGTTTAGAAAGA-3'

46 5'-ATCTTTCATATGAAGAAAGAAG-3'

49 5'-AAGAAGCATATGGAGATACCGGA-3'

52 5'-AGAAGAGCATATGGAGTTCAGCA-3'

56 5'-GAGCTCCATATGATCCAAACAACGGCCCCA-3'

The Cys-less NADPH-cytochrome P450 reductase expression plasmid, designated CYPOR*, is based on pIN-OR and was used as the template for subsequent preparation of single-cysteine mutants. All seven native cysteine residues were mutated to alanine, leucine or threonine using the Stratagene QuickChange Mutagenesis Kit to produce the CYPOR* (C136A, C228A, C363T, C445L, C472T, C566A and C630A) construct in the pIN-OR backbone.

Protein expression, purification and site-specific spin labeling:

E. coli JM109 (pIN-OR-based plasmids) or BL21(DE3) (pET-based plasmids) cells were transformed with the appropriate reductase expression plasmid. Typically, a 10 ml culture grown overnight at 37°C in modified Terrific broth supplemented with ampicillin (100 µg/ml) and riboflavin (1 µg/ml) was subcultured into six flasks containing 500 ml each modified terrific broth with ampicillin (100 µg/ml) and riboflavin (1 µg/ml) and grown for about 4 hours at 25°C before induction with 0.5 mM isopropyl 1-thio-β-D-

galactopyranoside. After induction, the culture was incubated overnight at 16°C. Cells were harvested by centrifugation at 3,000 x g for 30 min and lysed by sonication in 20 mM potassium phosphate (KPi) buffer pH 7.7, 10% glycerol (Buffer A) containing 2 µg/ml aprotinin and 1 mM PMSF. The lysate was ultracentrifuged at 100,000 g for 1 hour and the membrane pellet was then extracted with 0.1% Triton X-100 in buffer A, followed by a second ultracentrifugation. The cleared supernatant was loaded onto a 2'-5'-ADP Sepharose column, as previously described⁷.

Spin labeling of single-Cys mutants was accomplished by adding a 10-fold molar excess of methanethiosulfonate spin label (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline- 3-methyl methanethiosulfonate, MTSL) to an approximately 20 µM solution of purified protein in buffer A and incubating at 4°C for overnight. Excess spin label was removed by loading the solution onto a Bio-Rad Mini CHT-I Ceramic Hydroxyapatite Cartridge followed by extensive washing with 10 mM KPi pH 7.4, containing 10% glycerol. The spin-labeled reductase was then eluted with 250 mM KPi, pH 7.4, 10% glycerol. Yellow fractions containing spin-labelled reductase were pooled, diluted 5-fold with distilled water containing 10% glycerol and concentrated to 0.5 mM protein in order to reduce the phosphate concentration to 50 mM.

Membrane reconstitution of NADPH-cytochrome P450 oxidoreductase:

For most experiments, membrane reconstitution was achieved by directly mixing spin-labeled protein and sonicated small unilamellar dilauroyl phosphatidylcholine (DLPC) vesicles in the presence of sodium cholate (200 mM DLPC, 10 mM Na cholate, 50 mM KPi, pH 7.4) at a DLPC:protein molar ratio of 500:1. This method of membrane reconstitution has been used widely and gives good P450 activities.

For experiments utilizing Ni²⁺-nitrilotriacetic acid-modified dioleoyl lipid, 4 µmole egg yolk PC and 1 µmole 1,2-dioleoyl-sn-glycero-3- $\{[N-(5\text{-amino-1-carboxypentyl})\text{iminodiacetic acid}]\text{succinyl}\}$ nickel salt (DOGS-NTA-Ni(II), Avanti Polar Lipids) in chloroform were mixed and dried under nitrogen gas at room temperature followed by at least one hour under vacuum. Egg yolk PC was used, so that the bilayers would remain in the liquid crystalline phase at room temperature. The resulting lipid film was hydrated in 1 ml buffer containing 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 20 mM sodium cholate, and the suspension was clarified by sonication. Approximately 10 nmoles of spin-labeled protein was added to the lipid solution and the sample was dialyzed at 4°C for 24 hours against 50 mM TrisCl buffer pH 7.4, 50 mM NaCl (three changes, 1 liter each). After dialysis, the large unilamellar vesicles containing CYPOR protein were pelleted by ultracentrifugation and resuspended in 50 mM TrisCl buffer pH 7.4, 50 mM NaCl. For control samples lacking Ni(II), 20 mM EDTA was added to the reconstituted vesicle solution followed by ultracentrifugation.

EPR data collection and accessibility measurements:

EPR data were collected on a Varian E 102 Century series X-band spectrometer equipped with a two-loop one-gap resonator (XP-0201, Molecular Specialties) using a 100 kHz field modulation of 1.0 G. Continuous Wave (CW) power saturation experiments were carried out on the liposome samples in a gas-permeable TPX EPR tube. Power saturation curves were

obtained from the peak to peak central line amplitude as a function of incident microwave power after equilibration with (i) N₂, (ii) air, and (iii) for samples containing 10 mM Nickel(II)-EDDA (NiEDDA) equilibrated with N₂. The half-saturation microwave power ($P_{1/2}$) was obtained by fitting the data to the equation:

$$A = IP^{1/2} \left[1 + (2^{1/\epsilon} - 1)P/P_{1/2} \right]^{-\epsilon} \quad \text{Equation 1}$$

where P is the incident microwave power, ϵ is a factor related to the homogeneity of the line, A is the amplitude of the center line, I is a scaling factor, and $P_{1/2}$ is the half-saturation parameter corresponding to the power at which A is one-half of its unsaturated value. The quantity $P_{1/2}$ is the difference between $P_{1/2}$ values in the presence and absence of a given paramagnetic relaxation agent (i.e., O₂ or NiEDDA). $P_{1/2}$ is proportional to the collision frequency of the nitroxide with the paramagnetic reagent, and therefore reflects the accessibility of the spin labeled site²⁴. For spin labels exposed to the hydrophobic phase of the lipid bilayer, the depth of the site can be calculated according to the $P_{1/2}$ values in the presence of O₂ and NiEDDA by the relationship,

$$\Phi = \ln[\Delta P_{1/2}(\text{O}_2)/\Delta P_{1/2}(\text{NiEDDA})] \quad \text{Equation 2}$$

and by calibrating the dependence of Φ on bilayer depth using lipid analog spin labels²⁵.

In experiments utilizing DOGS-NTA-Ni(II), as the relaxation agent, the paramagnetic Ni(II)-NTA headgroup is confined to a small region extending a maximum of ~14 Å from the membrane-aqueous interface²⁶. Therefore, the value of $P_{1/2}(\text{NiNTA})$ indicates the proximity of the spin labeled site to the membrane surface.

Enzyme activity assays

Protein concentration and cytochrome *c* reductase and benzphetamine N-demethylase activities were determined as described previously²⁷. Briefly, assays for benzphetamine *N*-demethylation contained 0.19 μM CYPOR, 0.04–0.72 μM cytochrome P450, 20 μg/ml sonicated dilauroylphosphatidylcholine, 1 mM benzphetamine, 1 mM NADPH, and 54 mM HEPES, pH 7.4. CYPOR, cytochrome

P450, and lipid were incubated at 37 °C for 5 min in covered microtiter plates in a volume of 20 microliters. Reactions were then initiated by addition of 180 μl of HEPES buffer containing NADPH and benzphetamine. After 4 min, reactions were terminated by addition of 50 microliters of 30% trichloroacetic acid. After centrifugation to remove protein, formaldehyde in the supernatants was assayed by the Nash method²⁸. Cytochrome P450 was purified from phenobarbital-induced rat liver microsomes, as described by Guengerich and Martin²⁹; the major forms present have been reported to be from the CYP2B family³⁰. K_m^{P450} was determined with reductase concentrations of 0.08 μM and P450 concentrations from 0.03 to 0.72 μM. Reactions for MBD inhibition studies contained 0.03 μM P450 and 0.02 to 0.3 μM reductase.

Trypsin digestions were carried out at 20° C and contained 50 mM Tris, pH 8.1, and 0.2 μg/μl wild-type or mutant reductase. Reactions were initiated by addition of trypsin and

terminated after 10 minutes by addition of soybean trypsin inhibitor (2 $\mu\text{g}/\mu\text{g}$ trypsin). For isolation of the membrane-binding peptide, samples were loaded onto a Beckman Ultrasphere ODS high-pressure liquid chromatography column equilibrated with 0.1 % trifluoroacetic acid, flow rate 1 ml/min. Peptides were eluted with gradient of acetonitrile in 0.1% trifluoroacetic acid: 0.1% trifluoroacetic acid with no acetonitrile was pumped for 5 minutes, followed by a 0–42% acetonitrile gradient over 15 minutes, then a 42%–80% gradient over 26 minutes. The soluble domain eluted at 29 minutes and the membrane-binding peptide at 43 minutes. Samples were collected and evaporated to dryness. Residual Triton X100 was removed by binding samples to a Microcon-SCX filter (Millipore) according to the manufacturer's directions and washing twice with 20 mM ammonium acetate, pH 5.5. The peptide was eluted with 1.5 N ammonium hydroxide, 50% acetonitrile, neutralized by addition of 1.7 N acetic acid, 50% acetonitrile, dried down and resuspended in double-distilled water. The molecular weight of the peptide was determined by MALDI-TOF mass spectrometry on a Bruker Reflex II machine located at the Chemistry Instrumentation Center, University of Wisconsin. Quantitation was performed by SDS-PAGE of the purified peptide in parallel with known amounts of trypsin-digested wild-type reductase. Gels were stained with SYPRO Red (Molecular Probes) according to the manufacturer's instructions and fluorescence quantitated using a Storm 860 imager and ImageQuant software (Molecular Dynamics).

Results

Amino-terminal deletions of the MBD decrease P450-dependent benzphetamine demethylase activity.

A series of N-terminal deletion mutants were prepared and assayed for ability to transfer electrons to cytochrome *c* and cytochrome P450. The amino-terminal sequences of these mutants are shown in Fig. 1. Amino-terminal deletions of the MBD had no effect on electron transfer to cytochrome *c* (Table 1). Steady-state kinetic analysis of benzphetamine N-demethylation indicates that the wild-type reductase was able to support cytochrome P450 activity with a k_{cat} of $75 \pm 5 \text{ min}^{-1}$ and $K_{\text{m}}^{\text{P450}}$ of $0.20 \pm 0.02 \mu\text{M}$ (Table 1), similar to that reported previously²⁷. The dependence of benzphetamine demethylase activity on CYPOR (not shown) yielded a similar $K_{\text{m}}^{\text{CYPOR}}$ ($0.19 \pm 0.03 \mu\text{M}$), as expected for formation of a 1:1 complex of reductase and cytochrome P450 during catalysis^{31, 32}.

N-terminal deletions of the hydrophilic region of the MBD region, up to 28 amino acids, did not decrease k_{cat} (Table 1). Deletions extending into the hydrophobic region, 28 or 29, produced modest decreases in activity, while deletion of two additional residues, Leu-30 and Phe-31, produced a 45% drop in k_{cat} . Activity of the 37 mutant was 20% of wildtype and activities of the 40 through 56 mutants were approximately 10% of wild-type. $K_{\text{m}}^{\text{P450}}$ was not appreciably affected by deletion of up to 30 amino acids; however further deletions produced significant decreases in $K_{\text{m}}^{\text{P450}}$ (Table 1). Activities of the 40 through 56 mutants appeared to be saturated at all concentrations of P450 tested. Activities of the 56 mutant ranged from 8.27 ± 2.6 to $10.2 \pm 2.4 \text{ nmol/min/nmol}$ reductase as the P450 concentration was varied from 0.03 to 0.72 μM and $K_{\text{m}}^{\text{P450}}$ could not be determined.

The MBD is a weak inhibitor of P450-dependent activity.

Trypsin digestion of CYPOR followed by HPLC purification of the MBD produced a single peptide which migrated on SDS-PAGE with an apparent molecular weight of 12 kDa, similar to that reported previously^{12, 13}. Mass spectroscopy of the HPLC-purified material indicated a single peptide with a molecular mass of 6260 Da, consistent with the predicted sequence of the MBD lacking the amino-terminal methionine. The purified MBD was a weak competitive inhibitor of P450-dependent benzphetamine N-demethylase activity, with a K_i of $1.0 \pm 0.3 \mu\text{M}$. Binding constants for P450-reductase complex formation have been determined to be in the nanomolar range^{32–37}, suggesting that multiple interactions in addition to the MBD contribute to complex formation. This is similar to the results of solid-state NMR studies of P450 2B4 and cytochrome *b5*^{38, 39}. However, in the complex, between 2B4 and *b5*, the contribution of interactions between the MBDs of 2B4 and *b5* is much weaker compared to those of the two heme domains.

Residues influencing conformation of the MBD and tether.

The “tether” region of CYPOR, extends from the end of the stop-transfer sequence into the soluble domain. The disordered structure of residues Ileu57 through Val64 in various rat and human CYPOR crystal structures^{9, 40} and unique sensitivity of Lys56-Ile57 to cleavage by proteases such as trypsin suggest that this region adopts a highly flexible conformation. Deletions of the MBD domain influence the conformation of the MBD and tether, leading to altered electrophoretic mobility and altered trypsin sensitivity of the Lys56-Ile57 peptide bond.

The complete CYPOR protein migrates on SDS polyacrylamide gels with the expected molecular weight of 77.2 kDa, while the soluble domain lacking the MBD migrates with an apparent molecular weight of approximately 71 kDa, consistent with removal of the 6.6 kDa MBD. Progressive deletion of amino acid residues from the amino-terminus produced anomalies in the electrophoretic behavior of the mutant proteins. Figure 2A shows the relative electrophoretic migrations of the wild-type and mutant proteins, with relative mobilities plotted in Fig. 2B.

Taking the relative mobilities of the wild-type and 56 proteins as reference points, comparison of the mobilities of the N-terminal deletion mutants showed that the relative migrations of the 9 and 19 proteins also correlated well with their molecular weights. However, the 28 and 29 proteins migrated more rapidly than expected, while further deletions had the opposite effect, producing proteins with a slower than expected mobility. Amino-terminal sequencing of the 37 protein confirmed the correct protein sequence. Trypsin cleavage produced the expected 71 kDa soluble domain (Fig. 3A), which migrated in an identical fashion for each mutant, indicating that the altered mobility was due to the presence of the MBD.

Deletions of the MBD also affected protease sensitivity of the Lys56/Ile57 peptide bond suggestive of decreased conformational flexibility in the tether region. Figure 3B shows cleavage of the Lys56/Ile57 bond of wildtype CYPOR by treatment with as little as 0.2 ng trypsin/ μg reductase and essentially complete cleavage with 3 ng trypsin/ μg reductase, with

negligible cleavage of other sites in the protein. Treatment of deletion mutants up to 29 with 3 ng trypsin/ μ g reductase also produces essentially complete cleavage of the protein; however, deletion of two additional amino acids produces a protein which is only 50% cleaved (Fig. 3A). The 37 protein was most resistant to trypsin cleavage, requiring more than 50 ng trypsin/ μ g reductase for complete cleavage (Fig. 3C). Deletion of additional amino acids (40 through 49) resulted in a gradual restoration of trypsin sensitivity (Fig. 3A).

Construction and analysis of a cysteine-free mutant of CYPOR

The dynamics of membrane-bound CYPOR and its interaction with the lipid bilayer were further investigated by site-directed spin labelling⁴¹. As a preliminary step to our site-directed spin-labelling studies, we first prepared a cysteine-free mutant, designated CYPOR*, in which all seven native cysteines were replaced. This mutant was expressed and purified under the same conditions as the wild type enzyme, and SDS-polyacrylamide gel electrophoresis showed that it was expressed in full length with an apparent molecular weight of ca. 77 kDa. Upon limited trypsin digestion, the 71 kDa soluble catalytic domain was formed, as reported previously for the wild-type enzyme. The visible absorption spectra of the oxidized and semiquinone forms of CYPOR* are similar to that of the wild-type enzyme, with broad peaks at approximately 380 and 456 nm (Supplementary Figure 1), indicating no gross effect of the seven cysteine substitutions on FMN and FAD binding and electron transfer from NADPH. Therefore, this Cys-less mutant retains co-factor binding capabilities similar to the wild-type enzyme. CYPOR* activities for cytochrome *c* reduction and P450-dependent benzphetamine N-demethylation were 2% and 19%, respectively, of wildtype. The cytochrome *c* reductase activity is similar to that previously reported for a single-cysteine mutant, C630A⁴², suggesting that the decrease in CYPOR* activity is mostly due to the mutation of Cys630, which is crucial in the hydride transfer from NADPH to FAD, and not to structural changes caused by additional cysteine mutations. This was further confirmed by the crystallographic analysis of CYPOR* (pdb code: 6NJR), showing that the overall structure of the MTSL-labeled double mutant T177C/A637C of CYPOR* is virtually identical to that of the wild type enzyme, and the cofactors NADP⁺, FAD, and FMN are all bound in their active sites with the same conformations as those found in the wild type enzyme⁹.

The hydrophobic domain of CYPOR undergoes reorganization upon membrane reconstitution

In order to study the structure and conformation of the full-length enzyme upon membrane reconstitution, we first introduced Cys residues at four sites in CYPOR*. The cysteine mutants S9C, S32C, and S55C are spread along the MBD, while T668C is located on the surface of the FAD binding domain⁹.

Figure 4 shows the EPR spectra of these mutants, labeled with the sulfhydryl-specific spin label MTSL, in the absence and presence of DLPC liposomes. The EPR spectra of the three mutants located in the hydrophobic domain all report substantial conformational changes upon membrane reconstitution, while liposome insertion has little effect on the T668C-MTSL EPR spectrum. Subsequent site-specific labelling of 19 sites in the hydrophilic

domain of CYPOR* gave similar results, i.e., membrane reconstitution has little or no effect on local conformation throughout the hydrophilic domain (discussed below). These results suggest that the conformational changes in CYPOR* upon membrane reconstitution are restricted to the hydrophobic N-terminal tail, consistent with a membrane anchor role for the MBD domain and NMR studies suggesting that movements of the membrane binding and soluble domains are independent⁴³.

Comparison of the spectra of mutant proteins in solution with those in liposomes show that the EPR peaks of each of the three mutants located in the MBD (S9C, S32C and S55C) become much sharper following reconstitution, indicating increased mobility of the nitroxide side chain at each of these sites. Although all three residues exhibit spectral changes indicative of increased mobility upon membrane reconstitution, the change in the S32C spectrum is particularly informative. In the absence of liposomes, a broad peak is observed (Figure 4), indicating that the spin label is strongly immobilized. Upon reconstitution, this broadened peak has completely disappeared and a spectrum indicative of significantly more rapid motion is observed. These results suggest that, in aqueous solution, the hydrophobic region of the MBD is packed against a non-polar surface of the soluble domain, causing immobilization of the spin labeled residues. Reconstitution into liposomes provides a suitable environment that “solubilizes” the N-terminal tail, allowing it to extend into its native, mobile transmembrane conformation.

Residue Ser 32 is located inside the hydrophobic part of membrane while Ser 9 and Ser 55 are exposed to bulk aqueous solution

To determine the localization of S9C, S32C, and S55C relative to the membrane bilayer, we probed these residues using a series of paramagnetic relaxation agents. Broadening in the presence of chromium oxalate (CROX) is taken as a measure of accessibility to the aqueous phase while NiEDDA and O₂, which have inverse concentration gradients across the bilayer, were used to determine the depth of spin-labeled sites within the membrane²⁵.

For CYPOR* in solution, the spectra of MTSL-labelled S9C, S32C, S55C, and T668C were all broadened in the presence of CROX (spectra not shown), indicating accessibility to the aqueous phase. However, when these mutants were reconstituted into DLPC liposomes only the spectra of S9C, S55C and T668C were broadened by CROX, while this reagent had no effect on the spectrum of S32C (Figure 5). These results indicate that, upon reconstitution of CYPOR* into liposomes, S32C is buried in the hydrophobic phase of the lipid bilayer, while S9C, S55C and T668C remain accessible to aqueous phase.

The results of power saturation experiments for the membrane-reconstituted MTSL-labeled mutants in the presence of O₂ and NiEDDA are summarized in Table 2. The large $P_{1/2}(\text{O}_2)$ and small $P_{1/2}(\text{NiEDDA})$ for S32C clearly indicate that this site is buried deeply in the hydrophobic phase of the membrane, consistent with the results of the CROX accessibility experiments described above. Based on comparison to lipid-analog spin label standards⁴⁵, S32C is located about 17 Å below the membrane surface, near the center of the lipid bilayer. In contrast, the low $P_{1/2}(\text{O}_2)$ values and strong interaction with NiEDDA observed for T668C, S9C, S55C (Table 2) indicate that these sites remain exposed to the aqueous phase

following reconstitution of CYPOR* into the membrane bilayer, again consistent with the CROX results.

The MBD spans the membrane

To probe the distance of the labeled residues from the surface of the bilayer, we employed DOGS-NTA-Ni(II), a phospholipid analog that contains a NTA-Ni(II) head group which can act as a paramagnetic broadening center. When DOGS-NTA-Ni(II) molecules are incorporated into liposomes, paramagnetic relaxation due to the Ni(II) is confined to a region of about 14 Å on the hydrophilic side of the membrane-aqueous interface²⁶. This probe has previously proven useful for mapping sites of an integral membrane protein that are close to the membrane surface⁴⁶.

CW power saturation curves for MTSL-labeled S9C, S32C, S55C, and T668C reconstituted into liposomes containing 20% molar ratio of DOGS-NTA-Ni(II) are shown in Figure 6. As a control, Ni²⁺ was removed from the lipid head group by treatment with EDTA²⁶. The power necessary to saturate the spin-label side chains attached at S9C and S55C is greatly increased by the presence of DOGS-NTA-Ni(II), with observed $P_{1/2}(\text{NiNTA})$ values of 18.1mW and 17.6mW, respectively, indicating that these sites reside within 14 Å of the membrane surface and that they are not physically restricted from interacting with the lipid head groups. In contrast, DOGS-NTA-Ni(II) had little effect on the power saturation of either T668C or S32C (Figure 6), consistent with a structural model in which T668C is > 14 Å from the membrane surface and S32C is buried in the hydrophobic core of the bilayer. These results, combined with the location of S32 in the center of the lipid bilayer as indicated by the O₂/NiEDDA results, suggest that the MBD spans the membrane, with S9 and S55 located on opposite sides of the lipid bilayer and within 14 Å of their respective membrane surfaces. This is consistent with the results of NMR studies by Huang et al, showing that the MBD has a transmembrane helix⁴¹.

Orientation of the Soluble Domain relative to the membrane surface

In order to determine the orientation and possible interaction of the CYPOR soluble domain with the membrane surface, we created nineteen additional single-cysteine mutants in the soluble domain of CYPOR*, reconstituted each mutant into liposomes and examined the interactions of the purified, spin labeled, reconstituted proteins with DOGS-NTA-Ni(II). EPR spectra for the MTSL-labeled soluble-domain mutants in the presence of lipid are shown in Figure 7. No differences were observed between spectra in the presence or absence of lipid for any of these spin label constructs, indicating that membrane insertion did not affect the mobility of the spin label side chain at these sites. Several of the sites, including E71C, D121C, T177C, N211C, T218C, V233C, D254C, E354, L417C, and A637C have spectra with a predominant contribution from strongly-immobilized nitroxides, indicating that the spin label side chains at these locations experience a considerable degree of contact. The remaining nine sites (K75C, E127C, Q157C, R108C, V161C, Q198C, L205C, N271C, and N642C) displayed relatively higher mobility, indicative of surface exposure.

Changes in the saturation parameter in the presence of DOGS-NTA-Ni(II) [$P_{1/2}(\text{NiNTA})$], a measure of proximity to the lipid bilayer, are depicted graphically in Figure 8. None of the

spin-labeled sites in the soluble domain interacted with the lipid head group as strongly as S9C and S55C, which had $P_{1/2}(\text{NiNTA})$ values of 18.1 mW and 17.6 mW, respectively. This suggests that either the soluble domain, on a time-averaged basis, is situated $\sim 14 \text{ \AA}$ above the membrane surface, or that individual spin label side chains are inaccessible to the NiNTA head group due to physical constraints imposed by the structure of the protein. Nonetheless, many of the sites did show measurable changes in $P_{1/2}(\text{NiNTA})$, ranging up to $4.5 \text{ mW}^{1/2}$ for E71. These values likely reflect time-averaged excursions of soluble domain into positions that approach the lipid bilayer. A comparison of these values with the crystal structure of the soluble domain of CYPOR is instructive.

Figure 9A shows the structure of the soluble domain of CYPOR with the α carbons of the spin-labeled residues represented as spheres and color-coded according to their degree of interaction with the DOGS-NTA-Ni(II) head group. Except Asp254, all sites with

$P_{1/2}(\text{NiNTA})$ values greater than 2.5 mW (colored red), are located in the FMN domain, and clearly cluster along one face of the protein, suggesting that these residues are facing the bilayer surface. The most probable orientation of the CYPOR soluble, catalytic domain on the ER membrane is shown in Figures 9A and 9B, with the soluble domain of CYPOR sitting on helix A (composed of E71-K75; for helix naming convention, see⁹), which is facing the membrane. With helix A as a pivot point, swiveling and tilting motions of the soluble domain are possible, which can reposition individual residues closer to the lipid bilayer. Thus, a leftward tilt would bring V161 (located in the loop between helix D and strand 4), and both D121 and E127 of helix C closer to the bilayer; a rightward tilt would allow closer approach of E354 and D254; and a forward tilt would bring R108 into closer proximity to the membrane. Figure 9C shows the mapping of the spin-labelled residues onto the structure of CYPOR in an open conformation complexed with cytochrome P450¹⁵. It can be seen that the FMN domain is in the same orientation relative to the membrane as that shown in Figure 9B, indicating that CYPOR in this orientation can form a complex with cytochrome P450.

Discussion

Crystallographic and kinetic studies have established the structure of the soluble catalytic domain of CYPOR and the mechanism of electron transfer from NADPH to FAD to FMN to nonphysiological electron acceptors such as cytochrome *c* and have highlighted the importance of conformational motions in the control of electron transfer^{9, 14–17}. However, because the majority of these studies have been carried out in the absence of the MBD, many details of the mechanism of electron transfer from CYPOR to cytochrome P450 remain unresolved. Importantly, the structure of the membrane binding domain and the orientation of the soluble domain with respect to the lipid bilayer and cytochrome P450 are largely unknown.

With few exceptions, the MBD has been regarded as essential for efficient transfer of electrons from CYPOR to cytochrome P450. We demonstrate that the hydrophobic segment of the MBD is necessary for formation of a CYPOR-reductase complex, and that the hydrophilic amino terminal amino acids of the MBD are not required for P450 activity. Although a low level of activity is observed in the absence of the hydrophobic residues, it

does not appear to proceed through formation of a stable complex and may proceed through random collisions³⁵ or via peroxide formation³¹⁶. Previous studies have provided conflicting evidence regarding specific binding of the MBD to cytochrome P450^{12, 13, 47}. We find a relatively weak interaction between the MBD and P450, suggesting that electrostatic interactions between P450 and the soluble domain of CYPOR are the primary determinants of complex formation.

Examination of the mobility of specific residues in the MBD demonstrates that in the presence of lipid, the MBD moves away from a position packed against the soluble domain and adopts a conformation extending into the lipid bilayer and exhibiting increased mobility. As expected from its location in the hydrophobic region of the MBD, Ser32 is buried in the membrane, while Ser9, in the hydrophilic amino terminus, and Ser55, in the flexible tether region, are exposed to the aqueous phase. The strong interaction of Ser55 with the paramagnetic phospholipid analog DOGS-NTA-Ni(II) suggests that it is located in close proximity to the lipid headgroup, consistent with the presence of a stop-transfer sequence at residues 44–49. The demonstration that Ser9 and Ser55 are both accessible to the aqueous phase is in accord with the membrane-spanning orientation of the MBD helix suggested by NMR studies⁴³ and studies indicating that the amino terminus is located in the lumen of the endoplasmic reticulum, with the catalytic domain located on the cytoplasmic face of the membrane.⁴⁸

Although the MBD spans the lipid bilayer, removal of up to 28 amino acids has only a modest effect on P450-dependent benzphetamine N-demethylase activity and does not affect reductase-P450 complex formation as measured by K_m^{P450} . However, deletion of additional residues severely decreases both activity and complex formation. The shortened MBD exhibits alterations in electrophoretic mobility and protease sensitivity suggestive of an altered conformation with decreased flexibility. It is likely that the shortened MBD, although still membrane-associated, is unable to assume an extended conformation that extends into the lipid bilayer and is necessary for electron transfer to P450. Since EPR spectra at soluble-domain labeling sites did not change in the presence and absence of lipid, we believe that the hydrophilic domain, as a whole, is in motion relative to the lipid bilayer, as opposed to local conformational changes which would be expected to alter spin label mobility at individual sites. Solid state NMR studies have also suggested differences in the dynamics of the soluble and membrane-binding domains⁴³.

Although the soluble domain is situated $>14\text{\AA}$ above the membrane for much of the time, there are weak interactions of residues E71C, K75C, R108C and V161C from the FMN domain and D254C from the FAD domain with DOGS-NTA-Ni(II) head group, reflecting time-averaged excursions that approach the bilayer surface. These results suggest that dynamics of the soluble domain are such that, as a unit, it is swiveling and tilting on the bilayer surface, with individual residues making transient interactions with the bilayer and presumably with P450s. From examination of the locations of these spin-labeled residues in the CYPOR crystal structure, we have proposed a model for the orientation of the CYPOR soluble domain relative to the membrane (Figure 9). In this model, helix A containing residues E71 and K75 is located in close proximity to the membrane surface. Tilting movements transiently allow other residues to approach the membrane, while swiveling

movements may permit multiple electrostatic interactions with electron transfer partners. Mapping NiNTA accessibilities of the labelled residues onto CYPOR in an open conformation known to transfer electrons to P450¹⁵ complexed with CYP2B4 shows that this FMN domain orientation is in proper position to form a complex with cytochrome P450 (Figure 9). It is likely that the highly flexible tether, extending as far as 30 Å laterally and/or vertically from Ser55 (Figure 9), mediates these movements. It would be interesting to compare these movements with those of the open form of the CYPOR *i.e.*, the four-amino acid deletion mutant (delete 236-TGEE-239, referred to as TGEE) that is capable of forming a stable complex with P450 2B4¹⁵, and whose movement we would expect to be restrained compared to uncomplexed wildtype reductase.

CYPOR is unique in that it is able to transfer electrons to a variety of enzyme acceptors, including not only various P450s but also heme oxygenase isozymes and cytochrome *b5*. The MBD and flexible tether are able to facilitate movement of the soluble domain relative to the membrane and promote multiple orientations that permit specific electrostatic interactions of CYPOR with its varied enzyme partners. Adjustments in the orientation of soluble domain may also mediate the reported lipid-induced change in FMN redox potential²¹. Finally, the MBD and flexible tether may facilitate second electron transfer to P450. Previous studies have established conformational changes and domain movements upon NADPH binding and NADP⁺ release that coordinate hydride transfer from NADPH to FAD and electron transfer from FAD to FMN^{16, 17}. The fully reduced FMNH₂ becomes FMN semiquinone after donating one electron to P450. As the FMN semiquinone is not competent to transfer the second electron to P450, there is an additional requirement for a conformational change from the P450-bound open conformation to the closed conformation necessary for interflavin electron transfer¹⁷. Weak binding of the MBD to P450 coupled with flexible positioning of the soluble domain with respect to the membrane surface may provide a mechanism for efficient second electron transfer from CYPOR to P450, wherein P450 is loosely bound to CYPOR during this second round of interflavin electron transfer. Deletions of the MBD that prevent its extension into the membrane and alter the conformation and flexibility of the tether region disrupt this process. Observations of decreased coupling and increased hydrogen peroxide formation in the absence of the MBD³ and of multiple contact points between P450 and the FMN domain^{27,49–51} are consistent with this hypothesis.

In conclusion, we have provided evidence that the membrane binding domain is essential in anchoring CYPOR in the microsomal membrane, and, together with the tether, permitting reorientation as needed for regulated electron transfer to the diverse electron transfer partners of CYPOR. Based on the interaction of specific MTSL-labeled residues with the lipid bilayer, we present a model for an orientation of the reductase relative to P450 which facilitates electron transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sequence of the amino terminal Membrane Binding Domain and tether region of CYPOR. Residues are numbered beginning from the amino-terminal methionine. The protease-sensitive Lys56/Ileu57 bond is shown in bold. STS, stop-transfer sequence.

Figure 2.

Altered electrophoretic mobilities of CYPOR N-terminal deletion mutants. A. SDS-polyacrylamide gel electropherogram. The first and last lanes, marked WT, contain a mixture of full-length and trypsin-digested wildtype protein. Arrows indicate the positions of the 77 kDa full-length protein and 71 kDa soluble domain. B. Relative electrophoretic mobilities of wildtype and N-terminal deletion mutants.

Figure 3.

Trypsin sensitivity of amino terminal deletion mutants. **A.** Digestion of wildtype and amino-terminal mutants. – indicates untreated and + indicates trypsin digested (3 ng trypsin/ μ g protein). Arrows indicate the positions of the 77 kDa full-length protein and 71 kDa soluble domain. **B.** Trypsin digestion of wildtype protein.

C. Trypsin digestion of 37 protein. 1 μ g of purified protein was digested with the indicated amounts of trypsin. Molecular weights are indicated at the right.

Figure 4. EPR spectra of four MTSL-labeled CYPOR* mutants in both aqueous solution (black) and in DLPC liposomes (red). All spectra were recorded with a 100 Gauss scan width and were normalized to reflect equal spin concentrations.

Figure 5.

EPR spectra of four MTSL-labeled CYPOR* mutants in DLPC liposomes in the presence (black) or absence (red) of 20 mM chromium oxalate. All spectra were recorded with a 100 Gauss scan width and were normalized to reflect equal spin concentrations.

Figure 6. EPR power saturation curves in the presence of Ni(II) of the DOGS-NTA head group (squares) and after Ni(II) removal by addition of EDTA (triangles). The solid line is a fit to Equation 1. The liposomes contained 20% DOGS-NTA-Ni(II) and 80% egg yolk PC lipid.

Figure 7.

EPR spectra of MTSL-labeled CYPOR mutants reconstituted in liposomes. The mutant proteins were reconstituted in liposomes containing 20% DOGS-NTA-Ni(II) and 80% egg yolk PC lipid. Spectra were recorded at an incident microwave power of 2 mW with a scan width of 100 Gauss.

Figure 8.

Changes in the saturation parameter in the presence of DOGS-NTA-Ni(II). The Y axis value represents the $P_{1/2}$ difference of each mutant in the presence of DOGS-NTA-Ni(II) in the liposomes and those in which the Ni(II) was removed by EDTA (see Materials and Methods).

Figure 9.

Orientation of CYPOR on the membrane. (A) Orientations of the CYPOR structure (pdb code: 5URD) on the membrane according to the DOGS-NTA-Ni(II) saturation parameters described in Figure 8 are shown. The symbol ($\curvearrowright$) represents swiveling and tilting movement of the CYPOR molecule on the ER membrane. The red balls represent mutant positions which have $P_{1/2}$ (Ni-NTA) > 2.4 mW, the pink balls between 1.5 and 2.4 mW, and the green balls < 1.5 mW, as shown in Figure 8. The most probable CYPOR model orients helix A (including residues E71 and K75⁹) towards the membrane. (B) 90° rotation of CYPOR shown in (A). (C) the same orientation of CYPOR as in (B) but in the open conformation (pdb code: 3ES9) and complexed with P450 2B4 (pdb code: 1SUO) as described previously¹⁵.

Table 1.

Catalytic activities and kinetic properties of NADPH-cytochrome P450 oxidoreductase N-terminal deletion mutants.

Protein	Cyt c Specific Activity	P450-dependent benzphetamine N-demethylase Activity	
	$\mu\text{mol/min/mg protein}$	$\text{kcat (min}^{-1}\text{)}$	$K_m^{\text{P450}} (\mu\text{M})$
WT	54.8 $\pm$ 2.1 (4)	75.0 $\pm$ 5.7 (3)	0.20 $\pm$ 0.02 (3)
9	50.3 $\pm$ 1.3 (3)	74.2 $\pm$ 8.7 (3)	0.22 $\pm$ 0.06 (3)
19	47.3 $\pm$ 6.6 (3)	77.4 $\pm$ 4.5 (3)	0.22 $\pm$ 0.03 (3)
28	53.5 $\pm$ 10.4 (3)	64.6 $\pm$ 5.5 (3)	0.18 $\pm$ 0.04 (3)
29	63.2 $\pm$ 2.1 (4)	55.6 $\pm$ 4.6 (3)	0.20 $\pm$ 0.04 (3)
31	39.8 $\pm$ 6.2 (3)	29.8 $\pm$ 7.7 (3)	0.26 $\pm$ 0.15 (3)
34	66.7 $\pm$ 5.5 (4)	28.3 $\pm$ 2.2 (3)	0.08 $\pm$ 0.02 (3)
37	35.4 $\pm$ 8.0 (3)	15.7 $\pm$ 1.0 (3)	0.03 $\pm$ 0.01 (3)
40	58.8 $\pm$ 6.0 (3)	9.4 $\pm$ 1.2 (3) *	ND
43	58.5 (1)	9.2 $\pm$ 1.9 (3) *	ND
46	58.8 (1)	7.6 $\pm$ 1.6 (3) *	ND
49	55.3 (1)	12.7 $\pm$ 1.9 (3) *	ND
52	61.5 (1)	11.1 $\pm$ 0.6 (3) *	ND
56	45.2 $\pm$ 13.4 (3)	10.4 $\pm$ 1.4 (3) *	ND

Values are expressed as mean $\pm$ SD (n).

* indicates activities determined at 0.72 μM P450.

ND, not determined.

Table 2.

Power saturation parameters of four MTSL-labeled CYPOR* mutants in DLPC liposomes in the presence of oxygen or NiEDDA.

Mutant	$P_{1/2}(N_2)$	$P_{1/2}(O_2)$	$P_{1/2}(NiEDDA)$	$P_{1/2}(O_2)$	$P_{1/2}(NiEDDA)$	ϕ
S9C	3.36	6.57	37.15	3.21	33.79	-2.35
S32C	5.65	18.89	6.36	13.24	0.71	2.93
S55C	3.73	7.54	12.65	3.81	8.92	-0.58
T668C	4.31	8.59	11.30	4.28	6.99	-0.49