Membrane Topology of Cytochrome P450 2B4 in Langmuir-Blodgett Monolayers

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Abstract:

Using Langmuir–Blodgett monolayers of both phosphatidylethanolamines and phosphatidylcholines as membrane mimics, we have examined the topology of cytochrome P450 2B4 anchoring. The interaction of wild-type P450 2B4 with phosphatidylethanolamine monolayers can be characterized as a biphasic reaction, with the initial fast phase explained by the specific insertion of membrane-spanning segments of the protein into the monolayer. Injection of cytochrome b_5 (b_5) beneath dipalmitoyl-phosphatidylcholine monolayers also resulted in biphasic kinetics. Regardless of the nature of the lipid employed, neither a truncated cytochrome P450 2B4 (P450 2B4 Δ2–27) lacking the amino-terminal hydrophobic residues widely believed to be the major transmembrane segment nor a soluble b_5 fragment (Δb_5) lacking its carboxy terminus anchor exhibit the fast-phase behavior characteristic of specific insertion. To further characterize the membrane topology of P450 2B4, its insertion area in DPPE monolayers was measured and analyzed with use of the Gibbs equation for adsorption at an interface. The mean molecular insertion area derived from isotherms of P450 2B4 in a DPPE monolayer at a pressure of 19 mN/m, 680 ± 95 Å^2 is large enough to accommodate two to four transmembrane helices. The large insertion area and the fact that the truncated cytochrome retains as much as 30% of its membrane localization when expressed in Escherichia coli (Pernecky, S. J., Larson, J. R., Philpot, R.M., and Coon, M. J. (1993) Proc. Natl. Acad. Sci. USA 90, 2651–2655) suggest that this cytochrome is not deeply embedded but that other regions, in addition to the amino-terminal 26 residues, may be involved in the interaction of cytochrome P450 with the membrane.

Key Words:

cytochrome P450; Langmuir monolayers; membrane topology.

Article:

The role of cytochromes P450 in mediating the cellular response to pharmaceuticals and environmental toxins, as well as to physiologically occurring compounds such as retinoids, steroids, and prostaglandins, has made this class of heme–monoxygenases the target of a wide range of structure–function investigations. Unfortunately, the vast majority of cytochromes P450 are membrane proteins that are not easily addressed by standard structural techniques such as NMR and X-ray crystallography. Many groups have attempted to circumvent the difficulties inherent in studying membrane proteins by using indirect techniques to study the associated membrane topology of these cytochromes (1–3), often with conflicting results.

In the experiments described here, the Langmuir–Blodgett monolayer technique (LB)[*] has been used to directly probe the interaction of both wild type P450 2B4 and P450 2B4 Δ2–27, a truncated cytochrome lacking amino-

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[*] Abbreviations used: DLPC, dilauroyl-phosphatidylcholine; DLPE, dilauroyl-phosphatidylethanolamine; DMPE, dim yristoyl-phosphatidylethanolamine; DPPC, dipalmitoyl-phosphatidylcholine; DPPE, dipalmitoyl-phosphatidylethanolamine; DPPS, dipalmitoyl-phosphatidylserine; LB, Langmuir–Blodgett; P450$_{cam}$, cytochrome P450$_{cam}$; P450 2B4, cytochrome P450 2B4; P450 2B4 Δ2–27, truncated cytochrome P450 2B4 lacking residues 2–27; Mb, myoglobin; b_5, wild-type cytochrome b_5 from rabbit; Δb_5, soluble cytochrome b_5 from rat

terminal residues 2–27, with phospholipid monolayer membrane mimics. The LB technique allows one to form highly ordered amphiphilic lipid monolayers at the air–water interface which can be used to study the interaction of proteins and phospholipids without the need forfluorescent tags or synthetic phospholipid probes that can disrupt the structure of the protein or the membrane (4–7). Additionally, by measuring the changes in the two-dimensional pressure caused by an adsorbate, one can determine the mean molecular area any adsorbate occupies (8, 9), which can be useful in discriminating among theoretical models of protein structure. By determining the mean molecular area, one can discriminate between a deeply embedded protein that occupies a large area in the membrane and a protein that contains a single transmembrane helix and therefore occupies a much smaller area.

Measurement of the insertion area of a microsomal P450 is particularly interesting as three distinct models for the three-dimensional structure currently exist: a deeply embedded protein with 6 to 10 transmembrane segments (10–12), a cytosolic heme domain anchored to the membrane by a single transmembrane helix (13–18), or a cytosolic heme domain anchored to the membrane by both a transmembrane helix and an additional non-membrane-spanning binding site. Additionally, a comparison of the interaction of the wild- type protein and the truncated cytochrome with the monolayers can be used to determine if the amino- terminal segment is the primary membrane binding segment as proposed by several groups (13–18).

In addition to answering questions about the membrane topology, the LB technique can also provide information about the role of phosphoplipids in the monoxygenase system and the effect of head group on kinetic reaction rates. In catalytic studies, phosphatidylcholine has been found to be more effective than phosphatidylethanolamine in stimulating activity in the reconstituted monoxygenase system (19). However, the type of reaction studied and the order of addition have also been shown to effect the reaction rates. Kinetic experiments with P450scc in vesicles indicate that the nonbilayer phase propensity of phospholipids is important for catalysis in the mitochondrial monoxygenase system (20). Schwarz and co-workers found that the side chain cleavage activity increases with the fraction of nonbilayer phospholipids in the vesicles. The results indicate that the presence of phosphatidylethanolamine in the microsomal membrane may be important for in vivo catalysis.

The experiments described here indicate that P450 2B4 is not deeply embedded but is primarily anchored to the membrane by a segment containing the amino- terminal residues 2–27. The insertion kinetics of P450 2B4 have been compared to those of cytochrome b_5 (b_5), a membrane protein of known topology. Studies on the effect of headgroup and monolayer fluidity on the insertion kinetics using monolayers of dilauroyl-phosphatidylethanolamine (DLPE), dilauroyl-phosphatidylcholine (DLPC), dimyristoyl-phosphatidylcholine (DMPC), dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylserine (DPPS), dipalmitoyl-phosphatidylcholine (DPPC), and dipalmitoyl-phosphatidylethanolamine (DPPE) indicate that the protein exhibits a headgroup specificity for phosphatidylethanolamine and does not bind to phosphatidylcholine monolayers, whereas b_5 binds only to phosphatidylcholine monolayers.

EXPERIMENTAL PROCEDURES

Protein purification.
Wild-type P450 2B4 was isolated from rabbit liver microsomes as previously described (21), Rabbit b_5, which was kindly provided by Drs, K, P, Vatsis and S, J, Pernecky, was purified as previously described (22), Myoglobin (Mb) (23), P450$_{cam}$ (24), the recombinant soluble fragment of rat Δb_5 (25), and P450 2B4 $\Delta 2$–27 (2) were expressed in Escherichia coli and purified as previously described.

Sequencing of the P450 2B4 $\Delta 2$–27 has revealed an extra mutation in the protein in addition to the deleted segment, Proline 221 was mutated to serine (26), but the protein is catalytically competent and exhibits the characteristic spectroscopic features of P450 2B4.

Preparation of lipid monolayers.

All phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Monomolecular films were made on a KSV 5000 Langmuir–Blodgett trough (KSV Instruments; Helsinki, Finland) with dimensions 57 cm X 10 cm X 3 mm. Prior to each experiment, the trough and barrier were cleaned with chloroform and ethanol. Lipid stock solutions were made by dissolving powdered lipid in HPLC grade chloroform/methanol, or alternatively chloroform/toluene. Monolayers were formed by spreading 30 µl of 0.9 mg/mL DPPC, 55 µl of 0.6 mg/ML DPPE, 300 µl of 0.2 mg/ml solution of DMPE, or 80 µl of 0.6 mg/ml DLPC, DMPC, DMPE, DLPE, DPPS, or EPC stock solution. The trough was then filled with 5 mM potassium phosphate buffer (pH 7.4). To spread the lipid, drops of the stock solutions were formed on the end of a Hamilton syringe and carefully touched to the air–water interface. The solvent was allowed to evaporate for at least 20 min. After evaporation of the solvent, the interface was compressed at 10 mN/m/min until the surface pressure reached 1 mN/m, and at 1–2 mN/m/min to the final pressure of either 19 or 30 mN/m. The interface was allowed to equilibrate for a minimum of 1 h, at which point no further barrier movement was required to maintain constant pressure. The subphase was stirred with a 3-mm magnetic-Te on stir bar and a home-built motor. The pressure change kinetics were independent of the rate of stirring.

Protein insertion into DPPE monolayers.
Various quantities of protein or buffer (20% glycerol, 10 mM potassium phosphate, 0.1 mM EDTA) were injected in 100 uL aliquots beneath DPPE monolayers to give subphase concentrations of 0–90 nM. The subphase concentration was measured by removing a small volume of the subphase at the end of the experiment and measuring the absorbance of the oxidized substrate-free protein in a Cary 3 UV–VIS spectrophotometer (Varian Instruments), A linear baseline correction was performed on spectra of low concentration samples, The change in pressure after injection was measured by the Wilhelmy plate method at constant interfacial area and recorded for a minimum of 20 min after injection, The P450 2B4 Δ2–27 sample contained a small quantity of thrombin used during preparation, However, in control experiments a stock solution of thrombin exhibited no surface activity.

Spectra of aspirated monolayers were also measured and exhibited the spectral features characteristic of P450, including a Soret maximum at 418 nm.

Measurement of the insertion area of P450 2B4.
Injection of P450 2B4 caused a biphasic increase in the pressure. The slow phase was fit to a single exponential using the Igor software package (Wavemetrics, Lake Oswego, OR) (Fig, 1) using data collected from 2 to 20 min after injection, The fast phase, which occurred immediately after injection of the protein, was determined to be that portion of the pressure increase that did not fit the single exponential, The fast- phase pressure change was generally completed in less than 1 min, The major source of error in these results is in the measurement of subphase protein concentration and was determined by calculating the standard error using a least-squares algorithm.
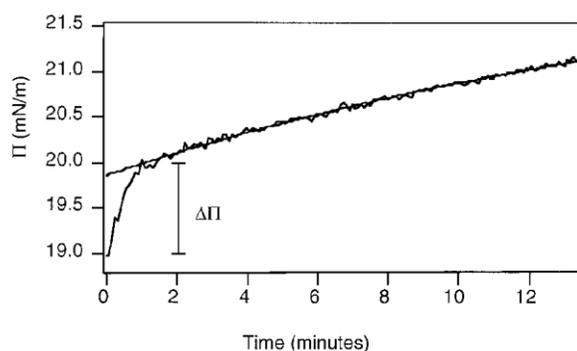
FIG. 1. The surface pressure of a DPPE monolayer is monitored as a function of time following the injection of cytochrome P450 2B4 at a concentration of 18 nM. The derived incremental pressure ΔΠ is the quantity used for the Gibbs isotherm analysis (see text).
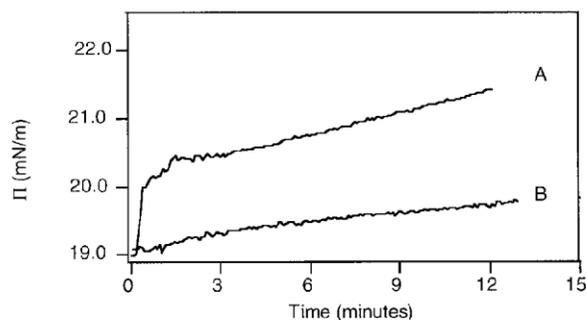
FIG. 2. Binding of P450 2B4 to DPPE monolayers. (A) Pressure changes induced by injection of P450 2B4 to a concentration of 30 nM beneath a DPPE monolayer. (B) Pressure changes induced by injection of the truncated form of the cytochrome, P450 2B4 Δ2±27, to the same concentration.

The adsorption of an amphiphilic molecule to either a liquid–liquid or an air–water interface can be described by the Gibbs equation

$$c_i = \frac{1}{RT}\left(\frac{\partial\Pi}{\partial\ln c_b}\right)$$

where Π is the surface pressure, c_i is the interfacial excess concentration, c_b is the subphase protein concentration, R is the universal gas constant, and T is temperature (22°C) (8, 9), The area per molecule is equal to the reciprocal of the excess surface concentration. A plot of the change in pressure as a function of the natural log of the subphase concentration was fit to a straight line using a least-squares linear regression.

RESULTS

Effect of P450 2B4 and cytochrome b_5 on the interfacial pressure of phospholipid monolayers.
Injection of P450 2B4 beneath a DPPE monolayer caused a dramatic increase in the interfacial pressure within 60 s. A similar rapid pressure increase was seen when b_5 was injected beneath DPPC monolayers. The pressure increased by as much as 1.5 mN in the first 20 s after injection at high subphase concentrations (Fig. 1). The pressure continued to rise for the next several hours, but at a much slower rate (approximately 0.5–2 mN/h). This slow pressure rise is described well by a single exponential but did not correlate well to the subphase protein concentration. Conversely, the change in surface pressure during the fast phase was found to be dependent only on the subphase concentration.

Injection of P450 2B4 Δ2–27 or Δb_5 into the subphase of either DPPE or DPPC monolayers (Fig. 2) caused only a slow-phase pressure increase. No fast phase was observed with either truncated protein, suggesting that the fast phase is due to the specific interaction of the deleted segments of the proteins with the monolayers.

Interaction of Mb and $P450_{cam}$ with DPPC and DPPE monolayers.
To rule out the possibility that nonspecific interactions induce the fast-phase pressure change, two soluble proteins were injected into the subphase under preformed DPPE and DPPC monolayers. Injection of either $P450_{cam}$ or Mb, soluble heme proteins not known to exhibit any membrane or surface activity, resulted in a slow increase in the surface pressure that was comparable to the slow phase observed when P450 2B4 or b_5 was injected (Fig. 3). No fast phase was observed. Injection of 100 μL of buffer had no effect on the interfacial pressure, but small decreases of interfacial pressure were occasionally observed immediately after injection when small quantities of lipid adhered to the syringe and were subsequently removed from the interface.

Effect of lipid fluidity and chain length on P450 2B4 insertion into lipid monolayers.
Injection of P450 2B4 beneath a DPPE monolayer held at 19 mN/m caused a dramatic increase in the interfacial pressure. However, P450 2B4 does not insert into DPPE monolayers maintained at a physiological surface pressure of 30–35 mN/m. To determine if the lack of insertion at physiological pressure is due to the nonfluid

state of dipalmitoyl-phospholipids, the insertion of the protein into more fluid short-chain monolayers was examined. The
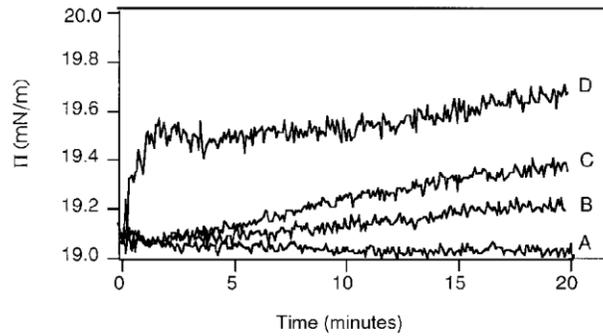
FIG. 3. Effect of soluble heme proteins on interfacial pressure. Pressure changes induced in a DPPE monolayer by injection of: (A) 5.0 mM (pH 7.4) potassium phosphate, (B) Mb (17 nM), (C) $P450_{cam}$ (30 nM), (D) P450 2B4 (11 nM).
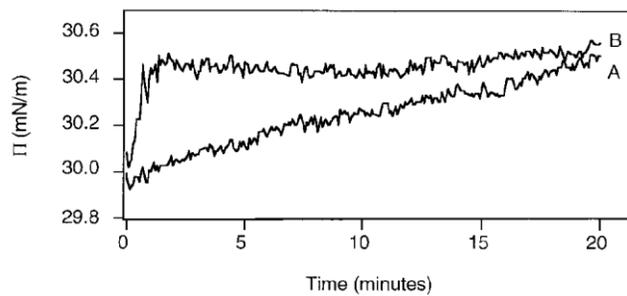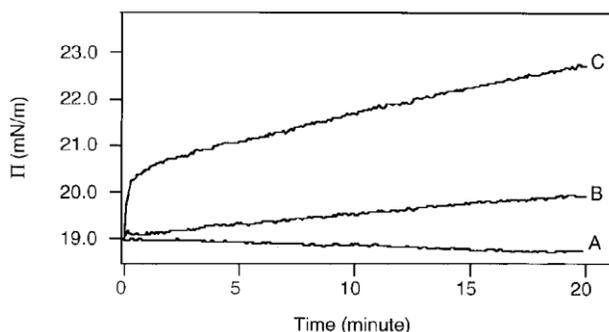

FIG. 4. Insertion of P450 2B4 into DLPE monolayers at 30 mN/m. (A) Pressure changes induced by injection of P450 2B4 $\Delta 2\pm 27$ to a concentration of 14 nM, beneath a DLPE monolayer. (B) Pressure change induced by injection of P450 2B4 to a concentration of 10 nM.

insertion kinetics of both full-length and truncated P450 2B4 into monolayers of DLPE and DMPE was measured (Figs. 4 and 5). P450 2B4 inserts into both DLPE and DMPE monolayers at 19 mN/m with the fast-phase kinetics characteristic of specific insertion as seen with DPPE. Additionally, in DLPE monolayers, P450 2B4 inserts at pressures as high as 30 mN/m. Furthermore, neither the truncated protein nor $P450_{cam}$ induce fast-phase changes in either short-chain lipid.

Headgroup specificity.
While injection of P450 2B4 beneath monolayers of DPPE resulted in a specific insertion of the protein into the monolayer, injection beneath DPPC monolayers did not give rise to a fast-phase pressure change (Fig. 6). In contrast, wild-type b_5 induces biphasic kinetics, characteristic of specific insertion, when injected beneath DPPC but not with DPPE. No conditions could be found under which either the truncated proteins or the soluble proteins would insert into monolayers regardless of the nature of the lipid headgroup or chain length. To ensure that the difference in the interactions of P450 2B4 and b_5 with ethanolamine and choline monolayers is due to a headgroup effect and not a difference in the fluidity of the monolayers, P450 2B4 was also injected beneath DLPC monolayers, which are more fluid than the DPPE monolayers. P450 2B4 did not insert into the fluid phosphatidylcholine monolayers and exhibited only slow phase kinetics, indicating a headgroup specificity for monolayer insertion.

FIG. 5. Binding of P450 2B4 to DMPE monolayers at 19 mN/m. (A) Time-dependent behavior of DMPE monolayer after injection of 5 mM potassium phosphate buffer (pH 7.4). (B) Pressure change induced by injection of P450 2B4 $\Delta2\pm27$ to a concentration of 3 nM. (C) Pressure change induced by injection of P450 2B4 to a concentration of 9 nM.
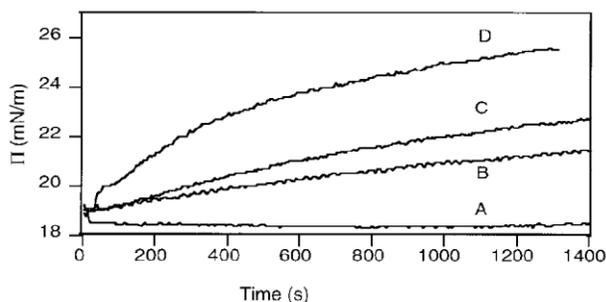
FIG. 6. Effect of headgroup on monolayer insertion. (A) Δb_5 insertion into DPPC (11 nM). (B) Wild-type cytochrome b_5 insertion into DPPE monolayers (7 nM). (C) Insertion of P450 2B4 beneath DLPC monolayers (19 nM). (D) Wild-type cytochrome b_5 insertion into DPPC monolayers (7.5 nM).

Injection of P450 2B4 beneath monolayers of DPPS induced a slow-phase pressure increase of an unusually large magnitude. Optical spectra of the subphase and monolayer fractions showed no Soret peak, indicating complete protein denaturation with heme loss. Protein injected beneath all other lipids exhibited Soret peaks at the expected position and displaying the correct magnitude.

Monolayers of egg phosphatidylcholine (EPC) were also made to measure the effects of mixed lipid systems on protein insertion. Unfortunately, due to the relatively high solubility of the short-chain lipids and the requirement that the subphase be stirred, the EPC monolayers were found to be too unstable to measure insertion kinetics.

The insertion area of P450 2B4 in DPPE monolayers.
To further characterize the membrane topology of P450 2B4, the insertion area was measured in DPPE by determining the concentration dependence of the fast-phase magnitude as described previously. The change in pressure versus the natural log of the concentration is plotted in Fig. 7. Data were collected for protein from two different preparations to ensure reproducibility. A least-squares linear regression was performed and the correlation was determined to be 0.72. The line was found to have a slope of 6.0×10^{-4} mN/m•M, giving an insertion area of 680 ± 95 Å^2. Because of the temporal instability of the short-chain lipid monolayers, insertion areas could not be measured in those systems.

DISCUSSION

The goal of these experiments was to use LB mono- layers as membrane mimics to characterize the mem-
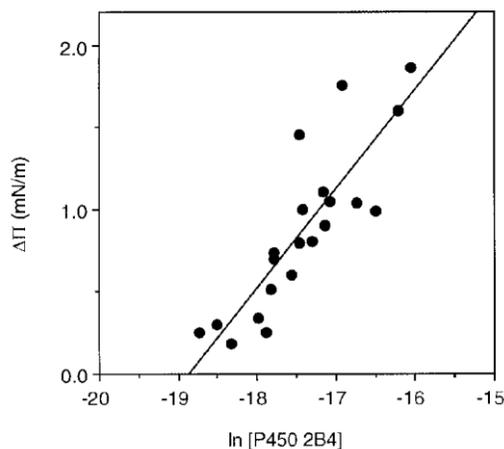
FIG. 7. Gibbs analysis of binding isotherms. Fast-phase pressure changes induced by injection of P450 2B4 beneath DPPE monolayers are plotted as a function of the natural log of protein concentration. The resulting slope yields the mean molecular surface area as described in the text.

brane topology of P450 2B4 and to explore the possibility of phospholipid headgroup affinity. Previous experiments using LB monolayers of $P450_{scc}$ and adrenodoxin to probe the topology of the mitochondrial monoxygenase system have shown that this cytochrome retains its structure at both at both the air–water and air–solid interface (27, 28).

The interaction of P450 2B4 with phospholipid monolayers of phosphatidylethanolamine is characterized by biphasic kinetics including a rapid initial phase and a much slower secondary phase. Cytochrome b_5, a membrane-bound component of the endoplasmic reticulum that is known to exist as a soluble heme domain with a single transmembrane α-helical anchor, exhibits identical biphasic kinetics when injected beneath phosphatidylcholine monolayers. The soluble cytochromes, $P450_{cam}$ and Mb, and the truncated proteins, P450 2B4 Δ2–27 and Δb_5, in contrast, induce only slow-phase pressure changes. The lack of a fast-phase pressure increase with soluble proteins indicates that the initial fast phase is due to the specific insertion of membrane-spanning segments into the monolayer.

In contrast to the fast-phase interaction, the slow-phase pressure increase is due to nonspecific interactions between proteins and the monolayers as evidenced by the fact that both soluble and membrane proteins are capable of inducing the latter phase. The cause of the slow-phase pressure increase can be ascribed to a combination of factors. First, charged groups on the surface of subphase proteins may interact with the lipid headgroups, altering the electrostatics of the monolayer and the lipid packing. Conversely, the fast-phase pressure increase cannot be explained by lipid repacking or electrostatic effects; else, the soluble proteins would induce the fast-phase behavior seen with the membrane proteins. Small quantities of denatured proteins that are naturally present in all protein preparations may also contribute to the slow phase by gradually inserting into the monolayer. The slow phase is not due to denaturation of native protein because in all but one case the subphase protein concentration, as measured optically, agreed with the expected value. The exception was seen when P450 2B4 was injected beneath highly charged DPPS monolayers which are known to denature some membrane proteins. After aspiration of the monolayer and subphase and measurement of the optical spectra, no native P450 was detected in either the phospholipid mono- layer or the subphase fractions.

Once the interaction between P450 2B4 and phosphatidylethanolamine monolayers was determined to be specific, wild-type P450 2B4 and P450 2B4 Δ2–27 were used to determine whether the amino-terminal 26 residues are in fact the primary transmembrane sequence. When injected beneath phospholipid monolayers of either phosphatidylethanolamine or phosphatidylcholine, this truncated cytochrome did not exhibit the fast-phase pressure increase characteristic of specific insertion regardless of the lipid chain length or the concentration of protein. This result is particularly interesting because P450 2B4 Δ2–27 is known to retain as

much as 30% of its membrane localization when expressed in E. coli and cannot be removed from the membrane fraction by exposure to high salt concentrations (21). Several groups have constructed truncated forms of other cytochromes P450 that lack the putative membrane-spanning sequence but contrary to the prediction that the truncated proteins would be located entirely in the cytosol, these shortened cytochromes also retain much of their membrane localization (29–32). However, the lack of interaction between the truncated protein and the monolayers in the absence of the NH_2-terminal 26 residues suggests that these residues are, in fact, the primary membrane-binding segment.

The difference in the interaction between the truncated and wild-type P450 2B4 rules out the possibility that the protein is deeply embedded but not the possibility of a second binding site that does not span the membrane. In addition to explaining the membrane localization of the truncated cytochromes, a secondary binding interaction could explain the slow rotation rate of P450 observed in phospholipid vesicles (33). The possibility that this secondary binding site might involve the active site of the protein has previously been explored by attempting to dislodge the protein from the membrane using pseudosubstates (21).

The presence of a second binding site was probed in the present experiments by measuring the insertion area of the protein. The measured area, 680 ± 95 Å^2, is too large to be accounted for by a single transmembrane helix because the average diameter of an α-helix ranges from 7.5 to 9 Å which would occupy an area of 170 to 250 Å^2. On the other hand, the area is also too small to accommodate the large number of transmembrane helices predicted by sequence homology-based models and X-ray diffraction studies (11, 12). The large insertion area could be explained either by a tilted α-helix or a non-membrane-spanning site. However, assuming a diameter of 10 Å and a length of 5–6 nm (the width of a phospholipid bilayer), a helix would have to lie flat at the air–water interface to occupy an area of 500–600 Å^2. If the palmitoyl fatty acid side chains provide so little support that the helix is lying flat in the interface, one would expect the area of the protein in DPPE to be the same as that in the short-chain lipids DLPE and DMPE which provide an even smaller hydrophobic area to support the transmembrane segment. While the insertion area of the protein in the short-chain lipids could not be measured, the magnitude of the fast phase was in fact larger in DLPE and DMPE than in DPPE, indicating that the same helix occupies a larger area in the shorter lipids and thus cannot be lying parallel to the interface in DPPE.

One final explanation of the large insertion area that must be considered is that P450 2B4 may interact with the monolayers as a protein micelle instead of a monomer and that the differential binding is due to differences in the oligomeric state of the protein as P450 2B4 exists as a hexamer in solution in the absence of phospholipids and detergent. Electron micrographs of the hexamer indicate that the protein exists as two trimers stacked on top of each other to form a disk-shaped structure with a diameter of approximately 11.5 nm and a height of 7 nm (34). A micelle inserted into the membrane would occupy an area of 103 nm^2/micelle, or roughly 1730 Å^2/P450 2B4 molecule, much larger than the measured insertion area.

While the measured insertion area is strong evidence for an additional binding site, one would expect the truncated protein to exhibit some monolayer binding like the full-length protein. Experiments with the NH_2-terminal truncated P450s expressed in E. coli showed that upon removal of the putative transmembrane segment, the membrane localization decreased, indicating that the secondary site binds the membrane more weakly (2). Furthermore, in the absence of residues 2–27, any proximity effect between the transmembrane segment and the second binding site that might contribute to the strength of the secondary interaction would be lost. It is also possible that monolayer binding through the secondary site does not result in a substantial displacement of phospholipids and thereby is undetectable by the Wilhelmy plate method. Finally, despite the fact that P450 2B4 appears to exhibit an affinity for phosphatidylethanolamine, biological membranes consist of a wide variety of phospholipids, the second binding interaction may require an additional membrane component for binding.

The fact that P450 2B4 does not insert into monolayers of long-chain lipids at physiological surface pressures is not surprising. P450 2B4 inserts into DPPE and DPPC at pressures of 19 mN/m or less, but not at physiological pressure. The mammalian cell contains complex machinery responsible for the insertion of integral proteins into

the membrane, which is absent in this in vitro system. In addition, biological membranes, unlike the long-chain lipid monolayers, consist of a complex mixture of lipids, steroids, and proteins— all carefully regulated by the cell to maintainfluidity, membrane charge, and membrane phases. In a fluid short-chain monolayer like DLPE, P450 2B4 does insert at physiological pressures, thus apparently indicating that the lack of insertion is solely a function of the rigid nature of the saturated phospholipid monolayer.

Since P450 2B4 clearly displays a headgroup specificity, the role of phospholipids in catalysis must be further explored. In kinetic studies with detergent-solubilized liver microsomal fractions, DLPC was found to most effectively stimulate the reaction (19) but the monolayer data indicate that P450 2B4 preferentially binds phosphatidylethanolamine over phosphatidylcholine. The conflicting results may be due to the fact that the kinetic studies were conducted with detergent-solubilized liver microsomal fractions which may have contained other P450s in small amounts in addition to 2B4 as the major cytochrome.

Combined, the results of the kinetic studies and the monolayer experiments indicate that the headgroup affinity may be due to the physical state of the lipid and differences in phospholipid packing. When proteins insert into a monolayer, they induce local defects in the phospholipid packing (35). Thus, phospholipids with phosphatidylethanolamine headgroups may pack in such a manner that they can accommodate the trans-membrane portions of the P450 more easily than can phosphatidylcholine. The fact that b_5 only binds to phosphatidylcholine monolayers may indicate that it packs more efficiently with the larger choline headgroups. Interestingly, in the kinetic studies the phase of the lipids was found to greatly effect the kinetic rates. In fact, depending on the reaction studied and the lipids used, the optimal size of the vesicle varies greatly indicating that the nature of the lipid and its phase state are important.

The fact that P450 and b_5 do not appear to bind the same lipids suggests that the lateral distribution of phospholipids in a bilayer may be important for catalysis. Studies of biological membranes have indicated that phospholipids are not homogeneously distributed but separate into domains or regions with a higher concentrations of a particular component. In the case of protein kinase C, domain formation is required for optimal enzymatic activity and lateral domains serve to localize the reaction components on the vesicle surface, concentrating the substrates, protein components, and activators (36). Evidence for a heterogeneous localization of P450 on the endoplasmic reticulum can be found in electron microscope images of P450 2B4, which clearly show that the protein is localized in discrete clusters (37). Because P450 and b_5 must interact for the catalytic oxidations of certain substrates, it is possible that the head group affinity may serve either a regulatory function or to mediate the domain structure of the membrane so that both proteins can localize in the same region. Further experiments will be conducted to explore the role of phospholipid domains on P450 catalysis and protein–protein interactions using fluorescence spectroscopy.

In these experiments Langmuir–Blodgett monolayers were formed at an air–buffer interface, and the kinetics and equilibrium process of P450 2B4 insertion into the monolayer were quantitated. These results with the full-length cytochrome and with the genetically engineered N-terminal deletion mutant suggest that the dominant interaction of P450 2B4 with the membrane is through the N-terminal helical domain, but that a second site of interaction on the surface of the cytochrome may also contribute. In addition to providing topological information, the Langmuir–Blodgett technique should allow direct measurement of cytochrome P450 interactions with its flavoprotein reductase and with b_5.

ACKNOWLEDGMENTS

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