Bengis-Garber, C., & Gromet-Elhanan, Z. (1979) *Biochemistry 18*, 3577–3581.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Carmeli, C. (1970) *FEBS Lett. 7*, 297–300.
Casadio, R. (1988) in *Ion Pumps: Structure, Function, and Regulation* (Stein, W. D., Ed.) pp 201–206, Alan R. Liss, Inc., New York.
Clayton, R. K. (1963) in *Bacterial Photosynthesis* (Gest, H., San Pietro, A., & Vernon, L. P., Eds.) pp 495–500, Antioch Press, Yellow Springs, OH.
Edwards, P. A., & Jackson, J. B. (1976) *Eur. J. Biochem. 62*, 7–14.
Falk, G., Hampe, A., & Walker, J. E. (1985) *Biochem. J. 228*, 391–407.
Ferguson, S. J. (1985) *Biochim. Biophys. Acta 811*, 47–95.
Gromet-Elhanan, Z. (1974) *J. Biol. Chem. 249*, 2522–2527.
Gromet-Elhanan, Z. (1977) *Trends Biochem. Sci. 2*, 274–277.
Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028.
Gromet-Elhanan, Z., & Khananshvili, D. (1986) *Methods Enzymol. 126*, 528–538.
Gromet-Elhanan, Z., Weiss, S., & Eshel, I. (1985a) *Physiol. Veg. 23*, 705–715.
Gromet-Elhanan, Z., Khananshvili, D., Weiss, S., Kanazawa, H., & Futai, M. (1985b) *J. Biol. Chem. 260*, 12635–12640.
Hackney, D. D., Stempel, K. E., & Boyer, P. D. (1980) *Methods Enzymol. 64*, 60–83.
Hiller, R., & Carmeli, C. (1988) in *Ion Pumps: Structure, Function and Regulation* (Stein, W. D., Ed.) pp 307–314, Alan R. Liss, Inc., New York.
Hochman, Y., Lanir, A., & Carmeli, C. (1976) *FEBS Lett. 61*, 255–259.
Johansson, Bo. C., Baltscheffsky, M., & Baltscheffsky, H. (1973) *Eur. J. Biochem. 40*, 109–117.
Khananshvili, D., & Gromet-Elhanan, Z. (1982) *J. Biol. Chem. 257*, 11377–11383.
Khananshvili, D., & Gromet-Elhanan, Z. (1984) *FEBS Lett. 178*, 10–14.
Khananshvili, D., & Gromet-Elhanan, Z. (1986) *Biochemistry 25*, 6139–6144.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Merchant, S., & Selman, B. R. (1985) *Photosynth. Res. 6*, 3–31.
Mitchell, P. (1981) in *Mitochondria and Microsomes* (Lee, C. P., et al., Eds.) pp 427–452, Addison Wesley, Reading, MA.
Mitchell, P. (1985) *FEBS Lett. 182*, 1–7.
Oren, R., & Gromet-Elhanan, Z. (1977) *FEBS Lett. 79*, 147–150.
Oren, R., & Gromet-Elhanan, Z. (1979) *Biochim. Biophys. Acta 548*, 106–118.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Petrack, B., Craston, A., Sheppy, F., & Farron, F. (1965) *J. Biol. Chem. 240*, 906–914.
Philosoph, S., Binder, A., & Gromet-Elhanan, Z. (1977) *J. Biol. Chem. 252*, 8747–8752.
Pick, U., & Weiss, M. (1988) *Eur. J. Biochem. 173*, 623–628.
Richter, M. L., Gromet-Elhanan, Z., & McCarty, R. E. (1986) *J. Biol. Chem. 261*, 12109–12113.
Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 103–124.
Taussky, H., & Shorr, E. (1953) *J. Biol. Chem. 202*, 675–685.
Whatley, F. R., Allen, M. B., & Arnon, D. I. (1959) *Biochim. Biophys. Acta 32*, 32–46.

Identification of the Membrane Anchor of Microsomal Rat Liver Cytochrome P-450†

Guy Vergères, Kaspar H. Winterhalter, and Christoph Richter*
Laboratorium für Biochemie, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland
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ABSTRACT: Cytochrome P450IIB1 isolated from rat liver microsomes was incorporated into phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (10:5:1 w/w) liposomes. Trypsinolysis of proteoliposomes and sequencing of the membrane-bound domains revealed that only one peptide, comprising amino acid residues 1–21, spans the membrane. Modification of the N-terminal methionine by membrane-impermeable fluorescein isothiocyanate occurred with the protein in solution but not in proteoliposomes. We conclude that in proteoliposomes cytochrome P-450 spans the membrane only with amino acid residues 1–21, the N-terminal methionine facing the lumen.

The microsomal monooxygenase system comprises several membrane-bound proteins. NADPH–cytochrome P-450 reductase is inserted in the membrane via a 6-kilodalton (kDa)[1] N-terminal peptide, which spans the bilayer twice. A water-soluble protein can be set free with protease, the protein being then unable to interact with cytochrome P-450 (P-450) (Gum & Strobel, 1981). Cytochrome b_5 spans the membrane only once with its C-terminus, the localization of the last amino acid being unclear (Tennyson & Holloway, 1986; Arinc et al., 1987). The membrane topology of P-450, the terminal and key enzyme of the microsomal monooxygenase system, is not known with certainty. Models in which a large part of the protein is imbedded in the bilayer were proposed on the basis

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* To whom correspondence should be addressed.

[1] Abbreviations: DPH, diphenylhexatriene; FITC, fluorescein isothiocyanate; kDa, kilodalton(s); P-450, cytochrome P-450; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; r^s, steady-state fluorescence anisotropy; SDS, sodium dodecyl sulfate.

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of hydrophobicity calculations when the amino acid sequence became known (Heinemann & Ozols, 1982; Tarr et al., 1983). More recent data suggested that the main mass is extramembranous and directed toward the cytoplasm, and new models appeared with the protein spanning the membrane only once (De Lemos-Chiarandini et al., 1987; Sakaguchi et al., 1987; Monier et al., 1988) or twice (Nelson & Strobel, 1988).

We report here the use of trypsinolysis and of site-specific modification with fluorescein isothiocyanate to analyze the topology of P-450 in proteoliposomes. We conclude that only the N-terminal peptide spans the membrane, the first amino acid residue facing the liposomal interior.

Materials and Methods

Enzyme Purification. P450IIB1[2] was purified from phenobarbital-induced male Sprague-Dawley rats (150 g, Madoerin AG, Füllinsdorf, Switzerland) in an active form by the method of Waxman and Walsh (1982) except that Emulgen was removed by detergent exchange while the cytochrome was bound to the hydroxylapatite column. The protein gave a single band on a sodium dodecyl sulfate–polyacrylamide (SDS–polyacrylamide) gel. The specific content was 14 nmol of P-450 heme/mg of protein.

Preparation of Proteoliposomes. Reconstitution of P-450 in phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine (PC/PE/PS) (10:5:1) (w/w) vesicles was done by cholate dialysis (Kawato et al., 1982). The buffer was 50 mM potassium phosphate, pH 7.4, 0.1 mM ethylenediaminetetraacetic acid, and 20% glycerol (standard buffer). The lipid to protein ratio was 5:1 (w/w) unless indicated otherwise.

Characterization of Proteoliposomes. Electron microscopy was carried out as described by Müller et al. (1980). Proteoliposomes and P-450 in solution were analyzed on Sepharose 4B (43 × 2 cm) equilibrated at 4 °C with standard buffer plus 100 mM KCl. The flow rate was 12 mL/h. Chromatography was followed at 220 nm. Phospholipids were detected with diphenylhexatriene (DPH, 2 μM) fluorescence intensity (λ_{ex} 357 nm; λ_{em} 420 nm) (Kunz et al., 1985). Aliquots of 45-min fractions were analyzed on a 12% SDS–polyacrylamide gel and silver stained to follow the P-450 pattern.

Proteolytic Digestion of P-450 Proteoliposomes. Peptides facing the outside of the vesicles were cleaved by trypsin (bovine pancreas) (1 μg/50 μg of P-450 at time zero and after 2.5 h) at 37 °C overnight in standard buffer. The liberated peptides were separated from the vesicles on a Bio-Gel A 1.5-m column (29 × 2 cm) equilibrated with standard buffer plus 100 mM KCl at room temperature. The flow rate was 12 mL/h. Fifteen-minute fractions were analyzed for peptides and lipsomes (light scattering) at 220 nm.

Characterization of Digested Proteoliposomes. To check the vesicles' quality after trypsin treatment, they were analyzed on Sepharose 4B as described above. The DPH fluorescence intensity pattern was compared with that of undigested proteoliposomes and of protein-free liposomes. The fractions containing vesicles were then pooled, and the steady-state fluorescence anisotropy (r^s) was measured at 22 °C according to Kunz et al. (1985).

Analysis of Liposome-Associated Peptide(s). The liposomal pool obtained after exhaustive trypsin digestion and chromatography on Bio-Gel A-1.5m was analyzed in two ways:

(a) Vesicles were first dialyzed against 20% glycerol for 3 h and against water for the next 4 h and then dried on a glass filter at 50 °C with nitrogen. Phospholipids were removed by extensive washing with cyclohexane (3 × 5 mL). The efficiency was measured with proteoliposomes containing 1 μCi of [*choline-methyl*-^{14}C]PC. The peptides remaining on the filter were sequenced with a gas-phase protein sequencer.

(b) Vesicles were mixed at 0 °C with 100 μg of deoxycholate/mL and precipitated with ice-cold trichloroacetic acid (final concentration 10%). The pellet was dissolved in formic acid/ethanol (1:2.8 v/v) and applied to a Sephadex LH-60 column (30 × 1 cm) equilibrated with the same solvent. The flow rate was 3 mL/h. Aliquots of the 30-min fractions were analyzed by Tricine–SDS–polyacrylamide gel electrophoresis (16.5%) according to Schägger and von Jagow (1987). The fractions containing peptide(s) were pooled and sequenced. Since in the presence of formic acid/ethanol sequence analysis stops at serine residues (see Discussion), the peptides were treated with methanol/HCl prior to sequencing. To avoid blocking of the N-terminus by lyophilization, all fractions to be sequenced were dried with nitrogen.

To analyze partitioning of P-450 tryptic peptides between aqueous and membrane phases, P-450 was digested in solution under conditions as for proteoliposomes. The digest was mixed with protein-free liposomes and incubated at 37 °C for 2 h. The solution was then chromatographed on Bio-Gel A-1.5m and Sephadex LH-60 and analyzed for peptides on an SDS–polyacrylamide gel as above.

Modification with Fluorescein Isothiocyanate. Modification of the N-terminal methionine was done according to Bernhardt et al. (1983, 1984). P-450 (5 nmol/mL) in solution or incorporated into liposomes [lipid to protein ratio of 30:1 (w/w), 7.8 mg of phospholipids/mL] was incubated with fluorescein isothiocyanate (FITC) for 2 h at 22 °C in standard buffer. The reaction was started by the addition of a 35-fold molar excess of FITC over P-450 (50 μL of a 3.5 mM stock solution). It was stopped with mercaptoethanol and acetic acid (final concentration 1% and 1 M, respectively). In control experiments, mercaptoethanol and acetic acid were added before FITC to test the efficiency of stopping the reaction. Samples were then dialyzed overnight at room temperature against 2 L of water and lyophilized. To avoid a possible incomplete protein recovery after acid precipitation of the proteoliposomes, the sample was centrifuged, and the supernatant was dialyzed as above, mixed with the pellet, and lyophilized. The lyophilizates were dissolved in 300 μL of formic acid/ethanol (1:2.8 v/v) and sonified when necessary. To free the protein of phospholipids and unreacted FITC, the solutions were chromatographed on Sephadex LH-60 by a modification of the microfuge desalting method (Helmerhorst & Stokes, 1980). The columns were packed with a 2-mL bed volume of Sephadex LH-60 swollen in formic acid/ethanol. They were first centrifuged at 1000*g* for 15 min, 2 mL of solvent was added, and the columns were centrifuged for 30 min to free the gel's top of solvent. The applied samples were then centrifuged for 15 min. The column eluates were dried with nitrogen and dissolved, aided by sonification, in SDS sample buffer for gel electrophoresis. Aliquots of the samples were analyzed on a 12% SDS–polyacrylamide gel. FITC was visualized by excitation at 355 nm, and the protein bands were then stained with Coomassie blue.

To test whether FITC reacts with amino groups of phospholipids and/or dissolves into the lipid bilayer, liposomes were incubated with FITC as above and analyzed on Bio-Gel A-1.5m.

Materials. Trypsin (bovine pancreas) was obtained from Sigma (St. Louis, MO), fluorescein isothiocyanate and DPH

[2] The nomenclature follows the recommendation of Nebert et al. (1987).

FIGURE 1: Electron micrograph of freeze-fractured P-450 proteoliposomes. Lipid to protein ratio was 5:1 (w/w); protein concentration was 1 mg/mL. The bar represents 100 nm.

were from Fluka (Buchs, CH), Sephadex LH-60 and Sepharose 4B were from Pharmacia (Uppsala, Sweden), and Bio-Gel A-1.5m was from Bio-Rad Laboratories (Richmond, CA). Egg PC, egg PE, and bovine spinal PS were purchased from Lipid Products (Nutfield, U.K.). Emulgen 911 was a kind gift from Kao Atlas Chemicals (Tokyo, Japan). [*choline-methyl*-^{14}C]PC (50 mCi/mmol) was supplied from New England Nuclear. Sequencing was done with a 470A protein sequencer equipped with a 120A analyzer from Applied Biosystems Instruments (Foster City, CA). Fluorescence measurements were carried out with an Aminco SPF-500 fluorometer equipped with a J4-9501 polarization accessory (American Instrument Co., Silver Springs, MD).

RESULTS

Freeze-fracture microscopy (Figure 1) of P-450 proteoliposomes reveals single-shelled vesicles with diameters between 20 and 60 nm. Comparison between negatively stained electron micrographs (not shown) of P-450 liposomes and P-450 in solution showed a clear background for proteoliposomes, and aggregates in the case of protein in solution, indicating incorporation of the protein into the bilayer.

Figure 2 shows the elution pattern of 20 nmol of free and liposomal P-450 from the Sepharose 4B column. Two well-separated fractions comprising proteoliposomes (fractions 7–10) and free P-450 (fractions 13–15) are distinguishable. With proteoliposomes, no free P-450 is detected. The nonsymmetrical elution pattern of proteoliposomes is an indication of their heterogeneous size: one part of the vesicles elutes with the void volume of the column and the other part somewhat later. Since the exclusion diameter of Sepharose 4B is about 60 nm (Schurtenberger & Hauser, 1984), this finding agrees very well with the vesicle size found in electron micrographs.

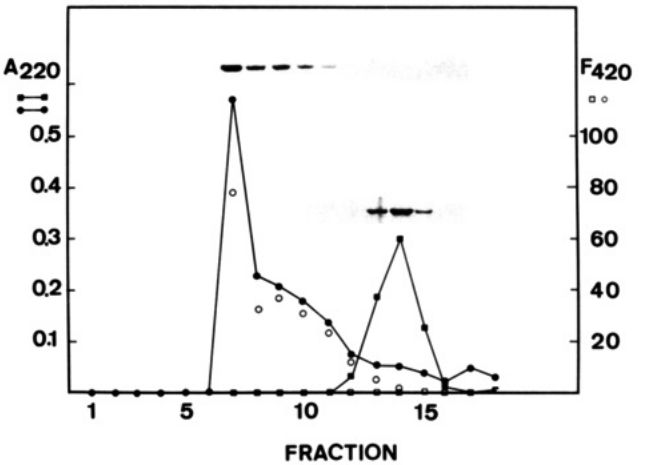

FIGURE 2: Analysis of P-450 incorporation into liposomes. Sepharose 4B gel filtration of free (■) and liposomal (●) P-450 was followed spectrophotometrically at 220 nm and fluorometrically (□, ○) at 420 nm with diphenylhexatriene. The insets above the peaks show SDS-polyacrylamide gels (silver-stained) of the corresponding fractions.

After trypsin treatment of the proteoliposomes, the vesicles were separated from the digested peptides by chromatography on Bio-Gel A-1.5m (not shown). The void volume contained vesicles with associated membrane peptide(s), the main contribution to absorption coming from light scattering by liposomes. The end volume contained peptides liberated from proteoliposomes and trypsin.

The elution pattern from the Sepharose 4B column was essentially the same for undigested and digested proteoliposomes (not shown). The smaller, protein-free liposomes eluted one fraction after proteoliposomes with the peak just after the void volume.

The membrane organization of the various liposomal pools eluting from the Sepharose 4B column was analyzed by DPH fluorescence anisotropy (r^s) measurements at 22 °C. Proteoliposomes, digested proteoliposomes, and liposomes had r^s values of 0.116, 0.116, and 0.103, respectively. They agree very well with those previously reported for proteoliposomes and liposomes (Kunz et al., 1985) and also showed that trypsin digestion leaves the organization of the phospholipid acyl chains unaltered.

The liposomal pool of digested proteoliposomes (void volume of the Bio-Gel A-1.5m column) was dried on a glass filter and subjected to amino acid sequencing. Only one P-450-derived peptide was found. It had the sequence Met-Glu-Pro-Ser-Ile-Leu-Leu-Leu-Leu-Ala-Leu-Leu-Val-Gly-Phe-Leu-Leu-Leu-Leu-Val-Arg, which is that of the first 21 amino acids of P450IIB1. The overall yield of the peptide starting from purified P-450 was greater than 50%. Two peptides not derived from P-450 were also sequenced and could not be sorted out with 100% accuracy. They had a high degree of homology with a "trypsinogen-like proenzyme" peptide and were contributed by the commercially available trypsin preparation.

The liposomal pool of digested proteoliposomes was also dissolved in formic acid/ethanol, applied to a Sephadex LH-60 column, and analyzed on a 16.5% Tricine-SDS-polyacrylamide gel (Figure 3). Coomassie blue staining reveals a protein band, representing less than 5% of the starting material, in the high molecular weight region (void volume of the Sephadex LH-60 column). When analyzed on a 12% gel (not shown), this protein banded in the region of P-450. It may be inverted liposomal P-450 or another trypsin-insensitive protein. In the low molecular weight region, one peptide with an apparent molecular mass of about 4.7 kDa and two faint bands of 6.8

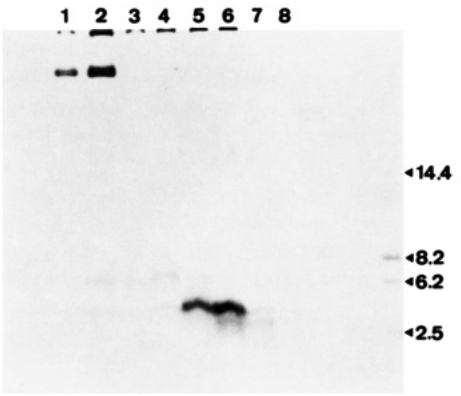

FIGURE 3: SDS–polyacrylamide gel electrophoresis of the membrane anchor of P-450. The fractions of the Bio-Gel A-1.5m column containing digested proteoliposomes were pooled, dialyzed, precipitated with trichloroacetic acid, dissolved in 600 μL of formic acid/ethanol (1:2.8 v/v), and separated on a Sephadex LH-60 column. The 30-min fractions were dried with nitrogen and analyzed on a 16.5% Tricine gel. Lanes 1–8 cover molecular masses ranging from 10 kDa (void volume) to ca. 2 kDa. Late fractions (not shown) contained phospholipids and were not stainable with Coomassie blue. Numbers to the right refer to molecular mass (in kDa) standards.

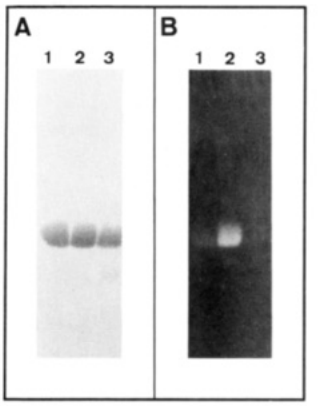

FIGURE 4: Modification of P-450 with fluorescein isothiocyanate. Modification of the N-terminal methionine by FITC was analyzed on a 12% SDS–polyacrylamide gel for P-450 in solution (lane 2) and for P-450 in proteoliposomes (lane 3). In a control experiment (lane 1), mercaptoethanol and acetic acid were added to the protein in solution before FITC. Panel A shows Coomassie blue staining of the different protein samples. FITC was excited at 355 nm and its fluorescence photographed with a yellow filter (panel B).

and 3.5 kDa are seen. The various bands most probably reflect different aggregation states of a single peptide (see below). The mass of the major peptide was also estimated from its elution time on the LH-60 column to be 2.4 kDa. We interpret this apparent discrepancy to be due to the nonstatistical distribution of amino acids in low molecular weight peptides and the resulting unusual binding of SDS. The pooled low molecular weight peptides of the Sephadex column were sequenced and found to be made up exclusively (>95%) of amino acid residues 1–21 of P450IIB1. Late fractions of the gel (not shown in Figure 3) contained phospholipids and were not Coomassie blue stainable.

We excluded the possibility that peptides, originally present in the extramembranous domain of proteoliposomes, dissolve in the membrane after trypsin treatment as follows: we digested P-450 in solution and added the peptide mixture to protein-free liposomes. The membrane peptides were than analyzed as described under Materials and Methods. No peptide(s) could be found on the 16.5% Tricine gel.

The localization of the N-terminus of P-450 in proteoliposomes was analyzed by labeling with FITC (Bernhardt et al., 1983, 1984). Figure 4 shows Coomassie blue staining and fluorescence of different P-450 bands on a 12% SDS–polyacrylamide gel. P-450 in solution is labeled very efficiently with FITC (panel B, lane 2), whereas P-450 in vesicles (panel B, lane 3) is labeled as weakly as the control (panel B, lane 1). Thus, the N-terminus is protected from reaction with FITC in proteoliposomes.

Since the proteoliposomes have a 60-fold molar excess of amino groups over P-450, and since FITC may conceivably partition into the lipid bilayer, we had to exclude a possible competition for FITC between phospholipids and P-450. We therefore incubated FITC with protein-free liposomes and analyzed them on Bio-Gel A-1.5m (not shown). All FITC eluted in the end volume and not with liposomes, excluding the above possibilities.

Discussion

The use of liposomes for reconstitution of biological functions and topological studies is well accepted (Eytan, 1982). If one of the tasks of the membrane is to facilitate the interaction of proteins, it is plausible that liposomes bind membrane proteins in a native state, i.e., in the correct topology. The preserved enzymatic activity of reconstituted P-450 has previously been documented (Gut et al., 1982). We therefore conclude that proteoliposomes are a valid model to investigate the membrane topology of P-450.

Quality of Proteoliposomes. Sepharose 4B chromatography has been used by others to test the incorporation of P-450 into liposomes (Ingelman-Sundberg & Glaumann, 1980; Yamakura et al., 1981). In our proteoliposome preparation, all protein is incorporated at a lipid to protein ratio of 5 to 1 (w/w). At a ratio of 1 to 1, the protein was incorporated incompletely (not shown). The proteoliposomes elute partly with the void volume but also later, in agreement with the size distribution found in electron micrographs. The smaller proteoliposomes still elute prior to free P-450 which in solution forms hexameric aggregates (Dean & Gray, 1982).

Trypsin can cause membrane disorder (Hu et al., 1986). The size distribution of vesicles after digestion was unchanged as seen by Sepharose 4B chromatography, excluding phenomena such as aggregation or multilayer formation. Interestingly, liposomes are smaller than digested proteoliposomes. This indicates that the increased diameter of our proteoliposomes is not due to the protein extruding from the vesicles. Rather, the protein seems to cause the formation of larger liposomes during cholate dialysis.

The order of the membrane lipid phase is affected by both lipid peroxidation and the presence of P-450 (Eichenberger et al., 1982; Kunz et al., 1985). Since trypsin can induce lipid peroxidation (Cooper et al., 1981) which in turn can make liposomes leaky (Richter, 1987), we analyzed the integrity of the lipid bilayer with DPH steady-state fluorescence polarization (r^s) measurements. The r^s values were unchanged after trypsin treatment, excluding trypsin-induced lipid peroxidation and gross alterations of the interactions between phospholipid acyl chains and the protein's membrane domains.

Analysis of Membrane Peptides. The analysis of membrane-bound peptides after trypsinolysis requires techniques capable of dissolving and separating phospholipids from hydrophobic peptides. Such techniques are not widespread, and we therefore discuss several aspects of methods used in the present investigation.

Chromatography in conventional protein chemistry involves aqueous buffer or the use of detergents, both being unable to monomerize phospholipids. Sephadex LH-60 tolerates organic

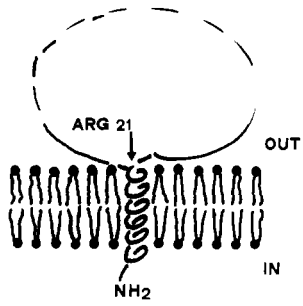

FIGURE 5: Proposed membrane topology of P-450. The protein spans the bilayer only once, the N-terminus facing the liposomal interior, which corresponds to the lumen of the endoplasmic reticulum. The arrow shows the location of arginine-21, which is one site for trypsin digestion. The remainder of the protein is located on the extravesicular side, which corresponds to the cytosol.

solvents and has been used to isolate hydrophobic membrane peptides (Brunner et al., 1985; Roberts & Rosenberry, 1986). With this material, we were able to separate the membrane-bound peptide from phospholipids, a procedure which made the analysis on a SDS–polyacrylamide gel and sequencing possible. Furthermore, buffer systems containing glycine without urea are not able to resolve small peptides well. With tricine, we obtained a better resolution and sharp bands even with the smallest membrane peptides (3–2 kDa).

Exposure of the N-terminal peptide to the solvents during Sephadex LH-60 chromatography in formic acid/ethanol led to formylation of the serine residue in position 4. Since after the third cycle the serine amino group is free, the formyl group shifts from the hydroxyl to the amino group and blocks the peptide (Smyth et al., 1963). We reversed the blocking by treatment of the peptide with methanol/hydrochloric acid prior to sequencing.

Besides Sephadex LH-60 chromatography, phospholipids were removed by drying digested proteoliposomes on glass filters. The success of this procedure was checked by incorporating radiolabeled PC in proteoliposomes. More than 70% of the applied proteoliposomes bound to the filter. By washing with hexane, all phospholipids were removed whereas the peptide still bound to the glass. The yield of >50% for the peptide as determined by sequencing underlines the usefulness of this technique.

Localization of the N-Terminus. Since FITC, under suitable conditions (pH 7.45), modifies the N-terminus of P-450 selectively and stoichiometrically (Bernhardt et al., 1983), and since membranes are impermeable to this reagent (Nilsson et al., 1973), we used it to analyze the orientation of the N-terminus in proteoliposomes. FITC did modify the N-terminal methionine of the protein in solution but not when incorporated into vesicles. Since FITC also does not dissolve in liposomes and does not react with phospholipid amino groups, we conclude that the N-terminus of P-450 faces the inside of the vesicles.

Membrane Topology of P-450. In the past, different models for the topology of liver microsomal P-450 have been proposed. On the basis of hydrophobicity profiles, models were suggested in which P-450 spans the membrane 8–9 times (Heinemann & Ozols, 1982; Tarr et al., 1983). Recently, another model, also based mainly on hydrophobicity profiles, was published in which the protein spans the bilayer twice, the N-terminus being on the cytoplasmic side (Nelson & Stobel, 1988). Here we show that after exhaustive trypsinolysis of proteoliposomes only the first 21 amino acid residues are membrane-associated. Since the main mass of P-450, including the heme, is known to be on the cytoplasmic side (Kunz et al., 1985; De Lemos-Chiarandini et al., 1987) and since the N-terminus is inaccessible to FITC in proteoliposomes, we propose that P-450 spans the membrane only with the N-terminal peptide, the first amino acid residue facing the liposomal lumen (Figure 5). This topology is consistent with two other recent studies. In one, site-specific antibodies were directed against P450IIB1 in rat liver microsomes (De Lemos-Chiarandini et al., 1987). The antibodies recognized all domains of the enzyme except the N-terminal segment (amino acid residues 1–31) and possibly the domain formed by amino acid residues 168–185. In the other study, chimeric proteins were constructed to test the insertion of rabbit liver microsomal P-450 into the endoplasmic reticulum (Sakaguchi et al., 1987). The N-terminal peptide containing residues 1–29 was found to function as an insertion signal and stop–transfer sequence. Neither of these studies determined the location of the N-terminal amino acid. Our proposal is also consistent with the data of Monier et al. (1988), which demonstrate that the amino-terminal segment of P-450 has both insertion and halt–transfer functions and, in addition, localize the N-terminal methionine on the luminal side of the microsomal membrane. Our findings do not support the recently proposed hairpin model of Nelson and Strobel (1988) which is based mainly on hydrophobicity calculations and positioning of the N-terminus on the cytosolic side of the membrane. Indeed, the second putative membrane-spanning domain is not particularly hydrophobic, and no experimental evidence is available for its localization in the membrane. As to the localization of the N-terminus, the data of Bernhardt et al. (1983, 1984), which where used as an argument by Nelson and Stobel (1988), are certainly not in conflict with a positioning on the luminal side of the membrane.

Obviously, only a small fraction of the hepatic microsomal protein's mass is in contact with the hydrophobic core of the membrane. Nevertheless, P-450 rotates relatively slowly in liposomal and microsomal membranes, with an apparent rotamer diameter of about 4 nm (Kawato et al., 1982; Gut et al., 1982, 1983). From this, it appears that the rotamer comprises not only the membrane-spanning peptide, which, in the case of an α-helix, has a diameter of about 0.6 nm. The slow rotation may be due to electrostatic interaction of the main protein body with the bilayer surface, particularly the negative charges contributed by PS (Ingelman-Sundberg et al., 1980, 1981). The membrane topology of P-450 in adrenal cortex mitochondria may be significantly different as deduced from trypsinolysis studies in the natural membrane and proteoliposomes (Lombardo et al., 1986) and from mobility measurements of P-450 in the natural membrane (Kawato et al., 1988). These studies suggest that this cytochrome may be deeply imbedded in the bilayer.

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Registry No. P-450, 9035-51-2.

REFERENCES

Arinc, E., Rzepecki, L. M., & Strittmatter, P. (1987) *J. Biol. Chem. 262*, 15563–15567.

Bernhardt, R., Ngoc Dao, N. T., Stiel, H., Schwarze, W., Friedrich, J., Jänig, G., & Ruckpaul, K. (1983) *Biochim. Biophys. Acta 745*, 140–148.

Bernhardt, R., Makower, A., Jänig, G., & Ruckpaul, K. (1984) *Biochim. Biophys. Acta 785*, 186–190.

Brunner, J., Franzusoff, A. J., Lüscher, B., Zugliani, C., & Semenza, G. (1985) *Biochemistry 24*, 5422–5430.
Cooper, M. B., Estall, M. R., & Rabin, B. R. (1981) *Biochem. J. 196*, 585–589.
Dean, W. L., & Gray, R. D. (1982) *J. Biol. Chem. 257*, 14679–14685.
De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D., & Kreibich, G. (1987) *J. Cell Biol. 104*, 209–219.
Eichenberger, K., Böhni, P., Winterhalter, K. H., Kawato, S., & Richter, C. (1982) *FEBS Lett. 142*, 59–62.
Eytan, G. D. (1982) *Biochim. Biophys. Acta 694*, 185–202.
Gum, J. R., & Strobel, H. W. (1981) *J. Biol. Chem. 256*, 7478–7486.
Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1982) *J. Biol. Chem. 257*, 7030–7036.
Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1983) *J. Biol. Chem. 258*, 8588–8594.
Heinemann, F. S., & Ozols, J. (1982) *J. Biol. Chem. 257*, 14988–14999.
Helmerhorst, E., & Stokes, G. B. (1980) *Anal. Biochem. 104*, 130–135.
Hu, L., Ho, R. J. Y., & Huang, L. (1986) *Biochem. Biophys. Res. Commun. 141*, 973–978.
Ingelman-Sundberg, M., & Glaumann, H. (1980) *Biochim. Biophys. Acta 599*, 417–435.
Ingelman-Sundberg, M., Johansson, I., Brunström, A., Ekström, G., Haaparanta, T., & Rydström, J. (1980) in *Biochemistry, Biophysics and Regulation of Cytochrome P450* (Gustafsson, J. A., Carlstedt-Duke, J., Mode, A., & Rafter, J., Eds.) pp 299–206, Elsevier/North-Holland, Amsterdam.
Ingelman-Sundberg, M., Haaparanta, T., & Rydström, J. (1981) *Biochemistry 20*, 4100–4106.
Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1982) *J. Biol. Chem. 257*, 7023–7029.
Kawato, S., Mitani, F., Iizuka, T., & Ishimura, Y. (1988) *J. Biochem. (Tokyo) 104*, 188–191.
Kunz, B. C., Rehorek, M., Hauser, H., Winterhalter, K. H., & Richter, C. (1985) *Biochemistry 24*, 2889–2895.
Lombardo, A., Laine, M., Defaye, G., Monnier, N., Guidicelli, C., & Chambaz, E. M. (1986) *Biochim. Biophys. Acta 863*, 71–81.
Monier, S., Van Luc, P., Kreibich, G., Sabatini, D. D., & Adesnik, M. (1988) *J. Cell Biol. 107*, 457–470.
Müller, M., Meister, N., & Moor, H. (1980) *Mikroskopie 36*, 129–140.
Nebert et al. (1987) *DNA 6*, 1–11.
Nelson, D. R., & Strobel, H. W. (1988) *J. Biol. Chem. 263*, 6038–6050.
Nilsson, R., Peterson, E., & Dallner, G. (1973) *J. Cell Biol. 56*, 762–776.
Richter, C. (1987) *Chem. Phys. Lipids 44*, 175–189.
Roberts, W. L., & Rosenberry, T. L. (1986) *Biochemistry 25*, 3091–3098.
Sakaguchi, M., Mihara, K., & Sato, R. (1987) *EMBO J. 6*, 2425–2431.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Schurtenberger, P., & Hauser, H. (1984) *Biochim. Biophys. Acta 778*, 470–480.
Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227–234.
Tarr, G. E., Black, S. D., Fujita, V. S., & Coon, M. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6552–6556.
Tennyson, J., & Holloway, P. W. (1986) *J. Biol. Chem. 261*, 14196–14200.
Waxman, D. J., & Walsh, C. (1982) *J. Biol. Chem. 257*, 10446–10457.
Yamakura, F., Kido, T., & Kimura, T. (1981) *Biochim. Biophys. Acta 649*, 343–354.