

# Determination of the Membrane Topology of the Phenobarbital-inducible Rat Liver Cytochrome P-450 Isoenzyme PB-4 Using Site-specific Antibodies

Carmen De Lemos-Chiarandini, Alan B. Frey, David D. Sabatini, and Gert Kreibich

Department of Cell Biology and the Kaplan Cancer Center, New York University Medical Center, 550 First Avenue, New York 10016

**Abstract.** Fifteen peptides, ranging in length from 6 to 31 amino acids and corresponding in sequence to portions of the major phenobarbital-inducible form of rat liver cytochrome P-450 (P-450 PB-4), were previously synthesized chemically and used to prepare site-specific rabbit antibodies (Frey, A. B., D. J. Waxman, and G. Kreibich, 1985, *J. Biol. Chem.*, 260:15253–15265). The anti-peptide antibodies were affinity purified using Sepharose resins derivatized with the respective peptides and 14 preparations were obtained that in an ELISA assay showed affinities to immobilized P-450 judged to be adequate for binding studies on intact rat liver microsomes. The binding of these antibodies to rough microsomes from the livers of phenobarbital treated rats was assessed using <sup>125</sup>I-labeled IgG and by immunoelectron microscopy employing protein A-gold as a marker. It was found that many of the antibodies bound to the cytoplasmic surface of the membrane but none bound to the luminal face of ruptured or inverted microsomal vesicles or to contaminating membranes of other organelles present

in the preparations. These observations eliminate previously proposed models for the transmembrane disposition of P-450 that postulate the existence of multiple transmembrane domains and the exposure of several polar segments of the polypeptide on the luminal side of the membrane. The fact that an antibody raised to the first 31 residues of P-450 bound well to the purified P-450 but very poorly to rough microsomes, whereas an antibody to a peptide comprising residues 24–38 showed relatively strong binding to intact microsomes, is consistent with the proposal that the amino terminal segment of P-450 extending approximately to residue 20 is embedded in the phospholipid bilayer and the immediately following segment is exposed on the cytoplasmic surface of the membrane. All these results favor a model in which the cytochrome P-450 molecule is largely exposed on the cytoplasmic surface of the endoplasmic reticulum membrane to which it is anchored by its short amino terminal hydrophobic segment.

**I**N the hepatocyte, cytochromes P-450 represent a family of hemoprotein monooxygenases that are located in endoplasmic reticulum (ER)<sup>1</sup> membranes and function in the metabolism of a wide variety of endogenous and exogenous substrates (see Lu and West, 1980; Adesnik and Atchison, 1985; Black and Coon, 1986). The ER membrane provides a framework that facilitates the interaction of cytochrome P-450 molecules with a specific NADPH cytochrome P-450 reductase that transfers electrons to the cytochrome P-450 heme moiety. Both cytochrome P-450 and its reductase are integral membrane proteins partially embedded in the phospholipid bilayer. As is the case with many other ER membrane proteins (Kreibich et al., 1983), the reductase is anchored in the membrane only by a small

hydrophobic segment and the bulk of the protein can be released from the membrane by mild proteolytic digestion (Black and Coon, 1982; Masters and Okita, 1980). The large exposure of the reductase on the cytoplasmic face of the ER membrane indicates that the portion of the cytochrome P-450 molecule that interacts with it must also be exposed on that surface.

Two closely related forms of cytochrome P-450 (PB-4 and PB-5) (Waxman and Walsh, 1982; Ryan et al., 1982a) that accumulate to high levels in the liver of rats treated with phenobarbital (PB) have been widely studied. Direct experimental evidence for the cytoplasmic exposure of functionally important domains of these P-450 polypeptides is provided by the finding that the enzymatic activity associated with these molecules is completely inhibited when anti-P-450 antibodies are added to intact microsomes (Thomas et al., 1977; Ryan et al., 1982b). The extensive exposure of cytochrome P-450 on the cytoplasmic surface of the ER membrane has also been inferred from the sensitivity of the protein to proteolytic digestion of intact microsomes (Nilsson et al., 1978;

Dr. Frey's present address is Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, NJ 08544.

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; PB, phenobarbital; P450 PB-4 and P450 PB-5, cytochrome P-450 form PB-4 and PB-5, respectively.

Cooper et al., 1980) and its accessibility to lactoperoxidase-catalyzed iodination (Welton and Aust, 1974; Rodriguez Boulan et al., 1978). However, unlike the situation with the reductase and other ER membrane proteins (Kreibich et al., 1983), it has not been possible to recover from microsomes treated with proteases large globular fragments of cytochrome P-450 that are spectroscopically recognizable and catalytically active (Nilsson et al., 1978).

The complete amino acid sequence of the phenobarbital-induced cytochrome P-450 of rabbit liver has been determined and, on the basis of a hydrophathy analysis of this sequence, models have been proposed in which the polypeptide spans the lipid bilayer several times and specific portions of the molecule are exposed on the luminal face of the ER (Heinemann and Ozols, 1982; Tarr et al., 1983). Previous immunocytochemical studies (Matsuura et al., 1978, 1979, 1983) have shown extensive binding of antibodies raised against the complete cytochrome P-450 molecule to the cytoplasmic surface of the ER membrane, but no labeling was observed on the luminal face of the membrane. Although these studies suggest that no sizable portion of cytochrome P-450 is exposed on the luminal side of the membrane, it must be recognized that, in general, polyclonal antisera primarily contain antibodies that recognize a small number of dominant epitopes and therefore are not effective probes to localize all parts of a protein molecule. We have therefore used site-specific antibodies raised against 15 synthetic peptides (Frey et al., 1985) that correspond to selected domains of the rat liver cytochrome P-450 PB-4, in binding and immunocytochemical studies, to probe for the exposure of the respective antigenic domains on the cytoplasmic or luminal side of the membranes of rat liver rough microsomes. Our results support a model for P-450 in which the molecule is bound to the membrane only by a small amino terminal anchor, whereas the rest of the molecule is exposed on the cytoplasmic surface of the membrane.

## Materials and Methods

### PB Treatment

Young male Sprague-Dawley rats (50–75 g) were purchased from Charles River Breeding Labs (Wilmington, MA) and fed ad libitum on laboratory chow. PB, dissolved in physiological saline, was administered by intraperitoneal injections (100 mg/kg body wt) once a day for 4 d. Control animals were left untreated.

### Cell Fractionation Procedures

PB-treated animals were fasted for 12–18 h before death by decapitation. Livers were excised and perfused with ice-cold 0.25 M sucrose and liver microsomes were prepared (Adelman et al., 1973) in solutions containing 0.1 mM phenylmethylsulfonyl fluoride (Lewis and Sabatini, 1977). Rough microsomes, separated from smooth microsomes on a discontinuous sucrose gradient, were washed once by sedimentation in 0.25 M sucrose and stored at  $-70^{\circ}\text{C}$  in the same medium. Protein was measured by a modification (Markwell et al., 1978) of the method of Lowry et al. (1951) and by Coomassie Blue binding (Bradford, 1976), using BSA as a standard.

### Preparation of Antibodies against Cytochrome P-450 PB-4

A P-450 preparation (P-450 PB) containing the major PB-inducible isozymes of rat liver, cytochromes P-450 PB-4 (80%) and P-450 PB-5 (20%), was purified from the livers of immature rats as described previously (West et al., 1979; Waxman and Walsh, 1982; Frey et al., 1985). Cytochrome

P-450 PB-4 and PB-5, which have only a 13 amino acid difference in 491 residues (Suwa et al., 1985), are indistinguishable by Ouchterlony immunodiffusion procedures (Ryan et al., 1982a, b). Antibodies against cytochrome P-450 PB were raised in rabbits and IgG fractions of the antisera were purified by ammonium sulfate fractionation (Frey et al., 1985).

### Synthesis of Site-specific Anti-P-450 PB-4 Peptide Antibodies

A hydrophathy profile (Kyte and Doolittle, 1982) for P-450 PB-4 was calculated from the published amino acid sequence (Fujii-Kuriyama et al., 1982; Yuan et al., 1983) using a scan length of nine amino acids. Regions of predominant hydrophobic and hydrophilic character were selected for peptide synthesis.

15 peptides (indicated in Fig. 1) were chemically synthesized (Merrifield, 1963) as previously described (Frey et al., 1985). Before injection into rabbits the peptides were conjugated to either Keyhole Limpet Hemocyanin or hen lysozyme as carrier proteins. The site-specific rabbit anti-peptide antibodies were affinity purified on Sepharose 4B derivatized with the respective peptide (Frey et al., 1985).

### ELISA Test for the Binding of Affinity-purified Anti-peptide Antibodies to Purified Cytochrome P-450

The preparation of microplates for the ELISA test has been previously described (Frey et al., 1985). Each well contained cytochrome P-450 PB (0.5  $\mu\text{g}$ ) or 1 mg BSA as immobilized antigen and the anti-peptide antibody diluted with PBS containing 0.1% BSA. The plates were incubated for 1 h at  $37^{\circ}\text{C}$ . After washing, the amount of fixed antibody was measured through the binding of peroxidase-conjugated affinity-purified goat anti-rabbit IgG (1 h incubation at  $37^{\circ}\text{C}$ ) and subsequently reacted with 2,2'-azino-di-C3-ethyl benzthiazolinesulfonic acid (30 min at room temperature). The content of each well was removed, diluted to a final volume of 1 ml, and the  $A_{405}$  was measured. An  $A_{405}$  of 0.11 was obtained from the BSA control and this value was subtracted from those obtained for wells containing the individual antibodies. The binding of each antibody to P-450 PB was graded as: -,  $<0.15 A_{405}$ ; +,  $0.15-0.3 A_{405}$ ; ++,  $0.3-0.6 A_{405}$ , and +++,  $>0.6 A_{405}$ .

### $^{125}\text{I}$ -Labeling of the Anti-peptide Antibodies

Affinity-purified anti-peptide antibodies were iodinated using 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodo-Gen, Pierce Chemical Co., Rockford, IL) and [ $^{125}\text{I}$ ]NaI (carrier-free, 100 mCi/mmol; New England Nuclear, Boston, MA). Aliquots (0.01 ml) of Iodo-Gen dissolved in  $\text{CH}_3\text{Cl}$  (1 mg/ml) were dried in the bottom of glass test tubes with a gentle stream of  $\text{N}_2$ . The tubes were then rinsed five times with PBS (pH 7.4) and the antibody solutions were added. [ $^{125}\text{I}$ ]NaI neutralized immediately before use with an equal volume of 0.1 N HCl was then added to each antibody sample. The reaction was allowed to proceed for 45 min on ice with occasional mixing. Unincorporated [ $^{125}\text{I}$ ]NaI was removed by chromatography on a Sephadex G-25 column using PBS as elution buffer. Incorporation of  $^{125}\text{I}$  in the excluded fraction was determined by gamma counting after TCA precipitation. The specific activities of the iodinated IgGs ranged from 1.3 to  $10.6 \times 10^3$  cpm/pmol. Solutions of the  $^{125}\text{I}$ -labeled antibodies in 30% glycerol were stored at  $-20^{\circ}\text{C}$ .

### Binding of Radiolabeled Antibodies to Rough Microsomes

Aliquots (0.5 ml) of a microsome suspension (2 mg/ml) in a buffer containing 0.1 M sodium phosphate buffer (pH 7.5), 0.16 M NaCl, and 0.25 M sucrose were incubated for 1 h at  $0^{\circ}\text{C}$  with increasing amounts of  $^{125}\text{I}$ -labeled anti-peptide IgGs (1–15), with pre-absorbed anti-7 antibody, or pre-immune IgG. The microsomes were recovered by sedimentation (30 min at 40,000 rpm in a rotor; model SW56, Beckman Instruments, Inc., Fullerton, CA) through a 20% sucrose cushion containing 0.5 M NaCl. Radioactivity in the membrane pellets was determined by gamma counting.

### Conjugation of Colloidal Gold Particles to Protein A

Colloidal gold particles, 10 nm in diameter, were purchased from Janssen Life Sciences Products (Beerse, Belgium). The pH of the colloidal gold was adjusted to 7.4 with 0.01 M  $\text{K}_2\text{CO}_3$  and the conjugation of protein A

Met Glu Pro Ser Ile Leu Leu Leu Leu Ala Leu Leu Val Gly Phe Leu Leu Leu Val 20  
 Arg Gly His Pro Lys Ser Arg Gly Asn Phe Pro Pro Gly Pro Arg Pro Leu Pro Leu Leu 40  
 Gly Asn Leu Leu Gln Leu Asp Arg Gly Gly Leu Leu Asn Ser Phe Met Gln Leu Arg Glu 60  
 Lys Tyr Gly Asp Val Phe Thr Val His Leu Gly Pro Arg Pro Val Val Met Leu Cys Gly 80  
 Thr Asp Thr Ile Lys Glu Ala Leu Val Gly Gln Ala Glu Asp Phe Ser Gly Arg Gly Thr 100  
 Ile Ala Val Ile Glu Pro Ile Phe Lys Glu Tyr Gly Val Ile Phe Ala Asn Gly Glu Arg 120  
 Trp Lys Ala Leu Arg Arg Phe Ser Leu Ala Thr Met Arg Asp Phe Gly Met Gly Lys Arg 140  
 Ser Val Glu Glu Arg Ile Gln Glu Glu Ala Gln Cys Leu Val Glu Glu Leu Ser Arg 160  
 Gln Gly Ala Pro Leu Asp Pro Thr Phe Leu Phe Gln Cys Ile Thr Ala Asn Ile Ile Cys 180  
 Ser Ile Val Phe Gly Glu Arg Phe Asp Tyr Thr Asp Arg Gln Phe Leu Arg Leu Leu Glu 200  
 Leu Phe Tyr Arg Thr Phe Ser Leu Leu Ser Ser Phe Ser Ser Gln Val Phe Glu Phe Phe 220  
 Ser Gly Phe Leu Lys Tyr Phe Pro Gly Ala His Arg Gln Ile Ser Lys Asn Leu Gln Glu 240  
 Ile Leu Asp Tyr Ile Gly His Ile Val Glu Lys His Arg Ala Thr Leu Asp Pro Ser Ala 260  
 Pro Arg Asp Phe Ile Asp Thr Tyr Leu Leu Arg Met Glu Lys Glu Lys Ser Asn His His 280  
 Thr Glu Phe His His Glu Asn Leu Met Ile Ser Leu Leu Ser Leu Phe Phe Ala Gly Thr 300  
 Glu Thr Ser Ser Thr Thr Leu Arg Tyr Gly Phe Leu Leu Met Leu Lys Tyr Pro His Val 320  
 Ala Glu Lys Val Gln Lys Glu Ile Asp Gln Val Ile Gly Ser His Arg Leu Pro Thr Leu 340  
 Asp Asp Arg Ser Lys Met Pro Tyr Thr Asp Ala Val Ile His Glu Ile Gln Arg Phe Ser 360  
 Asp Leu Val Pro Ile Gly Val Pro His Arg Val Thr Lys Asp Thr Met Phe Arg Gly Tyr 380  
 Leu Leu Pro Lys Asn Thr Glu Val Tyr Pro Ile Leu Ser Ser Ala Leu His Asp Pro Gln 400  
 Tyr Phe Asp His Pro Asp Ser Phe Asn Pro Glu His Phe Leu Asp Ala Asn Gly Ala Leu 420  
 Lys Lys Ser Glu Ala Phe Met Pro Phe Ser Thr Gly Lys Arg Ile Cys Leu Gly Glu Gly 440  
 Ile Ala Arg Asn Glu Leu Phe Leu Phe Phe Thr Thr Ile Leu Gln Asn Phe Ser Val Ser 460  
 Ser His Leu Ala Pro Lys Asp Ile Asp Leu Thr Pro Lys Glu Ser Gly Ile Gly Lys Ile 480  
 Pro Pro Thr Tyr Gln Ile Cys Phe Ser Ala Arg 491

**Figure 1.** Location of the 15 antigenic peptides within the sequence of rat liver cytochrome P-450 PB-4. The sequences corresponding to the 15 peptides that were synthesized and used to obtain site-specific antibodies are underlined and numbered sequentially beginning at the amino terminus. The amino acid sequence of cytochrome P-450 PB-4 is that deduced from the nucleotide sequence of a cDNA clone (Fujii-Kuriyama et al., 1982; Yuan et al., 1983).

(Sigma Chemical Co., St. Louis, MO) to the colloidal gold particles was carried out as described (Horisberger and Rosset, 1977; Roth et al., 1978) with the modification that three to four times more protein A gold than was necessary for the stabilization of the colloidal gold suspension was used. After 1 min, polyethylene glycol 20,000 M (0.1% final concentration; Fisher Scientific Co., Pittsburgh, PA) was added. The suspension was gently shaken for 15 min, sedimented at 60,000 g for 1 h at 4°C, resuspended in PBS containing 0.1% polyethylene glycol 20,000 M, and centrifuged at 60,000 g for 1 h or at 6,700 g overnight. The sediment, resuspended in PBS containing 0.03% polyethylene glycol 20,000 M and 0.02% Na azide, was stored at 4°C. Before use the suspension was diluted 1:3 with PBS containing 50 µg/ml BSA and 0.03% polyethylene glycol 20,000 M.

### Immunogold Labeling of Rat Liver Rough Microsomes

The rough microsome suspension was diluted to 10 mg protein/ml and 0.1 ml aliquots were used. The microsomes were recovered by sedimentation for 2 min in an Eppendorf microcentrifuge (model 5412; Brinkmann Instruments Co., Westbury, NY) and resuspended in PBS. The resuspended microsomes were fixed for 2 h at 4°C with 3 vol of PLP (3% paraformaldehyde, 0.075 M lysine-HCl, 0.01 M Na-m-periodate, 0.037 M Na phosphate buffer, pH 7.4 [McLean and Nakane, 1974]). The fixed microsomes were sedimented and resuspended in PBS containing 10 mM glycine, incubated for 10 min in this medium to block free aldehyde groups, and sedimented. This procedure was repeated three times and the fixed samples were finally resuspended in 0.1 ml PBS. The anti-P-450 IgG or the affinity-purified anti-peptide antibody (25–250 µg) was then added in 0.1 ml of PBS. The samples were incubated with shaking overnight at 4°C. The microsomes were sedimented, resuspended in PBS, and washed three times in the medium to remove unbound antibody. Controls were incubated with pre-immune rabbit IgG instead of the specific antibody. For an additional control, the anti-P-450 and anti-peptide antibodies (150 µg/ml) were incubated overnight at 4°C with rough microsomes (3 mg) to remove the reactive antibodies.

The microsomes were then sedimented in an Eppendorf centrifuge and the supernatants were used in the immunolabeling procedure as described above. After antibody binding, the microsomes were recovered by sedimentation in the Eppendorf centrifuge and resuspended in the protein A-gold conjugate suspension by gentle tapping. After incubation at room temperature for 2.5 h, followed by washing with PBS (eight times for 2 min), the microsomes were refixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h or overnight, washed with the same buffer (3 × 10 min), and fixed with 1% OsO<sub>4</sub> in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h. Sections of epon-embedded material were stained with uranyl acetate and lead citrate and viewed with a Philips 301 electron microscope at 80 kV.

### Quantitation of Immunogold Labeling of Rat Liver Rough Microsomes

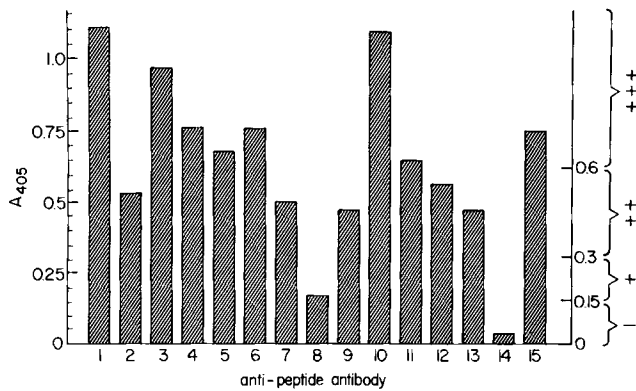
The perimeters of rough microsomes and the numbers of gold particles on cross sections of ribosome-bearing membranes were measured on electron micrographs of randomly chosen fields at a final magnification of 62,500 and recorded using a Hipad digitizer pad (Bausch and Lomb, Inc., Rochester, NY) and a stereological analysis program (Black and Cornacchia, 1986). For each antibody tested a total rough microsomal membrane length of 30 µm, obtained from ~50 ribosome-bearing vesicles was measured. The labeling density (Table II) is expressed as the number of gold particles per micrometer of membrane.

### Results

Fifteen peptides that represent different segments of the P-450 polypeptide were synthesized by standard procedures (Merrifield, 1963) and injected into rabbits to obtain antibodies specific for different sites in the P-450 molecule (Frey et al., 1985). The peptides and the antibodies to them are designated with consecutive numbers (1–15) according to the position of the peptide along the P-450 polypeptide, beginning at the amino terminal end (Fig. 1). Three of the peptides (1, 8, and 12, corresponding to residues 1–31, 173–184, and 290–302, respectively), contain portions of the main hydrophobic domains in the P-450 polypeptide whereas five others (2, 7, 9, 11, and 13) represent largely hydrophilic segments adjacent to those hydrophobic domains (Fig. 1). Peptide 2 (residues 24–38) partially overlaps with peptide 1 and contains the hydrophilic sequence immediately adjacent to the amino terminal hydrophobic domain within peptide 1. Of the remaining seven peptides, five represent regions interspersed between other segments along the first half of the P-450 molecule (3 [40–48], 4 [61–72], 5 [93–98], 6 [108–116], and 10 [211–233]), and two correspond to hydrophilic regions in the COOH-terminal half of the polypeptide (14 and 15 [residues 318–392 and 398–408, respectively]).

In a previous study (Frey et al., 1985), these site-specific anti-peptide antibodies were employed to localize functionally important regions of the P-450 molecule that participate in substrate binding or in the interaction with the NADPH cytochrome P-450 reductase, and to demonstrate the conservation of specific domains of the polypeptide among different P-450 isozymes. These antibodies were now used to study the topology of the P-450 polypeptide with respect to the phospholipid bilayer in the ER membrane.

The capacity of the anti-peptide antibodies to recognize antigenic sites exposed on the surface of the P-450 PB molecule was measured in an ELISA employing chromatographically pure cytochrome P-450 and antibodies purified by affinity chromatography to the corresponding peptide. Except for antibodies to peptide 14, which showed no binding, and antibodies to peptide 8, which bound very weakly, it was found



**Figure 2.** Reactivity of the anti-peptide antibodies to purified cytochrome P-450 PB. The binding of each antibody to immobilized cytochrome P-450 was measured in an ELISA test (see Materials and Methods). The numbers in the abscissa identify the rabbit anti-peptide antibodies and the adsorbance values in the ordinate correspond to the amount of each antibody bound to the cytochrome P-450, determined from the color reaction yielded by the peroxidase-conjugated goat anti-rabbit second antibody. The brackets on the right ordinate define the system used to grade the binding. The ability of each anti-peptide antibody to bind to P-450 was graded as follows: -, <0.15  $A_{405}$  (not significant); +, 0.15–0.3  $A_{405}$ ; ++, 0.3–0.6  $A_{405}$ ; +++, >0.6  $A_{405}$ .

that all other antibodies recognized the intact P-450 molecule (Fig. 2, Table I). To determine the extent to which the antigenic sites for these antibodies correspond to portions of the P-450 molecule exposed on the surface of the ER membrane, the binding of each antibody, labeled with  $^{125}\text{I}$ , to intact rat liver rough microsomes was measured. In this assay, after incubation with the labeled antibodies, the microsomes were recovered by sedimentation through a heavy sucrose cushion containing a high salt concentration. The highest levels of saturable binding were obtained with antibodies to peptides 4, 6, 7, and 15 (Fig. 3, Table I). All other antibodies showed lower, yet significant (at least twofold over background) levels of binding except anti-peptide 14, which behaved like the pre-immune serum and was, therefore, used as a control in all other experiments. The specificity of the binding was demonstrated by the fact that antibody preparations treated with the respective peptide antigens to preadsorb the specific IgGs showed only background levels of binding to rough microsomes (see Fig. 3 for anti-peptide 7 preadsorbed). These observations strongly suggest that the antigenic sites recognized in the P-450 molecule by anti-peptide antibodies 4, 6, 7, and 15 are directly accessible on the cytoplasmic face of the ER membrane. The fact that some antibodies gave lower levels of binding to intact microsomes than expected from their capacity to bind to the purified P-450 molecule in the ELISA test (Table I, e.g. 1, 3, 5, and 10) raised the possibility that the corresponding antigenic sites are lumenally located or at least partially embedded in the lipid bilayer. Alternatively, such sites may be exposed on the cytoplasmic surface of the microsomes but could be sterically hindered from the antibodies by other ER membrane proteins.

It should be noted that even the highest level of antibody binding measured in this test (20 pmol of IgG/ $\mu\text{g}$  of microsomal protein at an input of 75 pmol) represents only a small fraction of the number of binding sites theoretically available

**Table I.** Summary of Data on the Binding of the Anti-peptide Antibodies to Purified Cytochrome P-450 and to Rough Microsomes

Anti-peptide antibody No.*	Binding to purified P-450 PB†	Binding to RM	
		[ $^{125}\text{I}$ ]IgG‡	EM immunocytochemistry
1 (1–31)	+++	+	(+)
2 (24–38)	++	+	++
3 (40–48)	+++	+	+
4 (61–72)	+++	+++	+++
5 (93–98)	+++	+	(+)
6 (108–116)	+++	+++	+++
7 (122–131)	++	+++	+++
8 (173–184)	+	+	(+)
9 (186–193)	++	+	+++
10 (211–223)	+++	+	+
11 (225–232)	+++	++	+++
12 (290–302)	++	+	+
13 (315–323)	++	+	+++
14 (381–392)	–	–	–
15 (398–408)	+++	++	+++

The density of labeling (gold particles per micrometer of microsome perimeter) was measured on micrographs of rough microsomes processed by the immunogold technique. The grading system (from – to +++) is described in the legend to Table II.

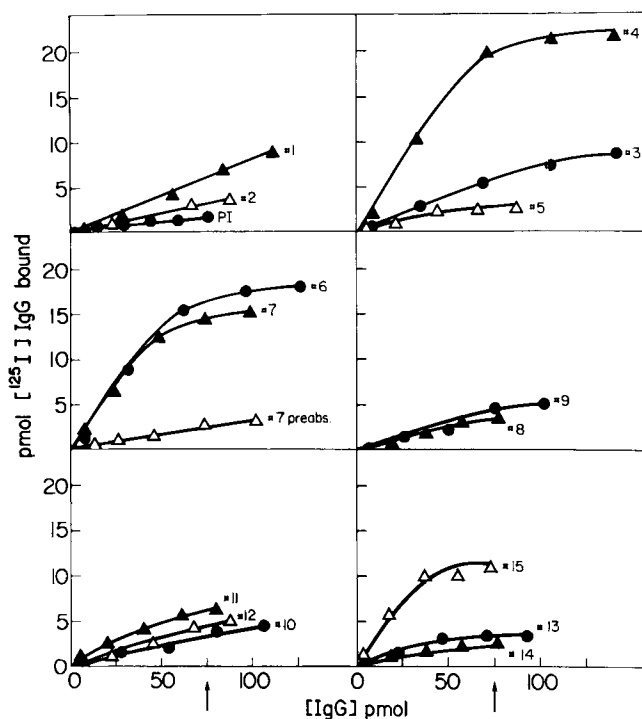
\* The numbers, 1–15, assigned to the different anti-peptide antibodies are the same used to designate the peptides that served as antigens. The numbers in parentheses correspond to the amino acid residues that define the position of the peptides within the sequence of cytochrome P-450 PB-4 (see also Fig. 1).  
 † The binding determined by the ELISA test was graded from – to +++, as indicated in the legend to Fig. 2.

‡ The binding of  $^{125}\text{I}$ -labeled anti-peptide IgGs to rat liver rough microsomes was quantitated from the data in Fig. 3, measured at a 75 pmol of IgG. It is indicated as follows: –, <3 pmol  $^{125}\text{I}$  IgG/mg RM; +, 3–6 pmol/mg; ++, 6–12 pmol/mg; +++, >12 pmol/mg.

in the microsomes (1.1 nmol P-450/mg microsomal protein). This is likely to reflect, at least in part, the loss of weakly bound antibody molecules during the centrifugation of the microsomes through the sucrose cushion containing high salt that had to be employed to separate them from the unbound antibodies. It is also possible that within the native P-450 molecule, polypeptide segments bearing antigenic sites can attain several conformations, only some of which may be recognized by the antibodies (Shinnick et al., 1983).

An immunoelectron microscopic approach was used to ascertain more directly whether each epitope in the cytochrome P-450 molecule recognized by the antibodies was exposed on the cytoplasmic or the luminal side of the ER membrane. Rat liver rough microsomes obtained from PB-treated animals were first incubated with the anti-peptide antibody and subsequently with the protein A-gold conjugate that served as an electron dense marker. In electron micrographs, the gold particles are easily distinguished from the membrane-bound ribosomes that serve as a marker for the cytoplasmic side of the membrane. In intact microsomal vesicles, the antibodies and the gold particles have access only to the cytoplasmic surface of the membranes. Rough microsome preparations, however, always contain small numbers of ruptured or inverted vesicles and in these cases the luminal side of the membrane is accessible to the antibodies and the gold particles.

Intense labeling of the cytoplasmic membrane surfaces was obtained with anti-peptide antibodies 2, 4, 6, 7, 9, 11, 13, and 15 (Fig. 4, Tables I and II). Interestingly, some of these



**Figure 3.** Binding of  $^{125}\text{I}$ -labeled anti-P-450 peptide antibodies to rough microsomes from the livers of PB-treated rats. Aliquots of suspensions of rough microsomes from the livers of control or PB-treated rats were incubated with the individual  $^{125}\text{I}$ -labeled IgGs (as indicated in Materials and Methods). The numbers (1 to 15) next to each curve are those assigned to the respective anti-peptide antibody (see Figs. 1 and 7). The arrow on each abscissa (75 pmol) indicates the IgG concentration used to compare the binding of different antibodies. The symbols are used to differentiate anti-peptide antibodies in each panel.

antibodies (e.g., 9 and 13) had given only weak binding in the biochemical assay, but the strong labeling they gave in the immunoelectron microscopy assay left no doubt as to their specificity and the cytoplasmic exposure of their respective antigenic domains. Very low labeling was obtained with antibody 8, and as expected, pre-immune serum and anti-peptide 14 gave no significant labeling. Antibodies 1, 3, 5, 10, and 12, which in the ELISA test bound efficiently to the purified P-450 but only poorly to the microsomes in the biochemical assay, gave significant levels of binding (at least 2 times above background, Table II) in the immunoelectron microscopic assay, and there was no doubt that their antigenic sites were only present on the cytoplasmic face of the membrane. None of the antibodies bound to luminal membrane surfaces, which were clearly exposed in ruptured (Fig. 4, *arrows*) or inverted microsomes (Fig. 4, *asterisks*). The specificity of the binding was clearly demonstrated by the fact that in all cases preincubation of the anti-peptide antibody with intact microsomes to remove the specific antibodies abolished the binding to the subsequent test microsomes (e.g., Fig. 5). Moreover, no binding was observed to mitochondria or plasma membrane fragments that contaminated the rough microsome samples (Figs. 4–6).

The observations made with the site-specific antibodies were extended using antisera that were obtained from rabbits immunized with the complete P-450 polypeptide and had been characterized by determining their reactivity toward the

**Table II. Labeling Density of Rat Liver Rough Microsomes Incubated with Different Anti-peptide Antibodies and Protein A-Gold\***

Antibody <sup>‡</sup>	Density <sup>§</sup>	Score <sup>  </sup>
	<i>Gold particles/μm</i>	
Pre-immune	0.6	–
Preabsorbed anti-7	0.3	–
Anti-P-450 PB	16.6	+++
1	0.8	(+)
2	2.5	++
3	1.8	+
4	5.0	+++
5	0.9	(+)
6	11.2	+++
7	10.9	+++
8	0.9	(+)
9	9.1	+++
10	1.7	+
11	11.5	+++
12	1.7	+
13	11.2	+++
14	0.2	–
15	9.4	+++

\* Rough microsomes from the livers of PB-treated rats were incubated with the specific antibody and processed for the immunogold labeling technique as described in Materials and Methods.

<sup>‡</sup> The numbers, 1–15, assigned to the different anti-peptide antibodies are those used to designate the peptides that served as antigens (see Fig. 1).

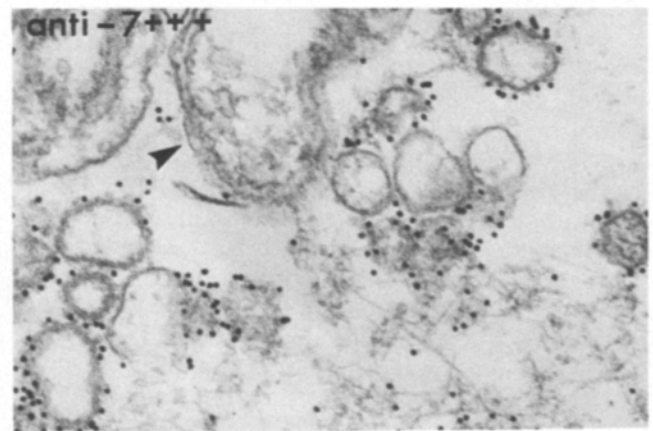
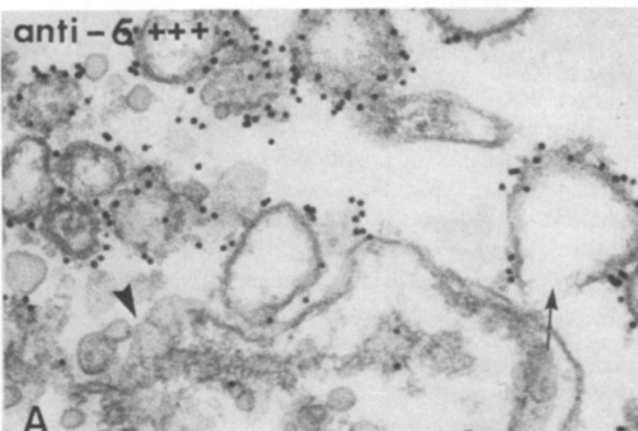
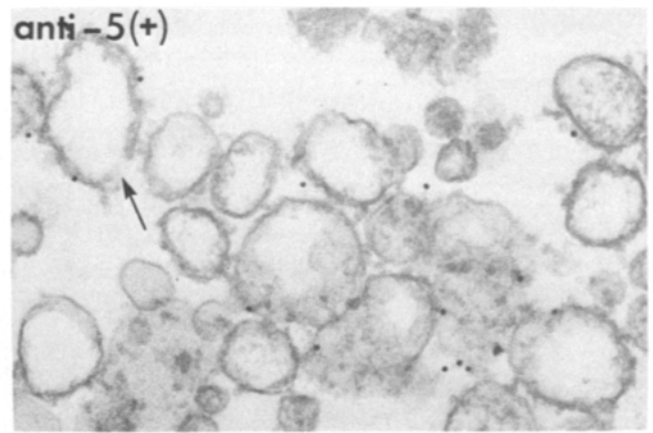
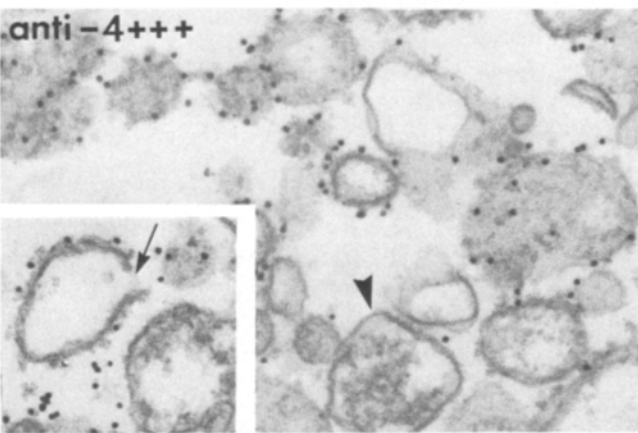
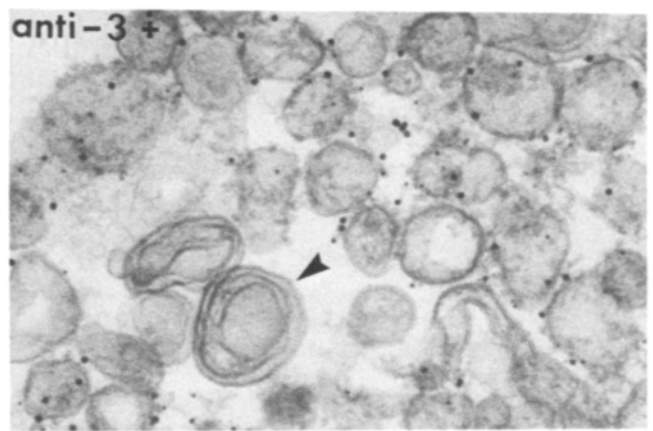
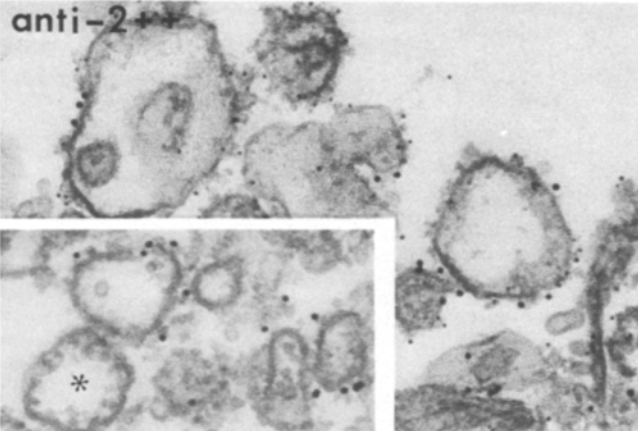
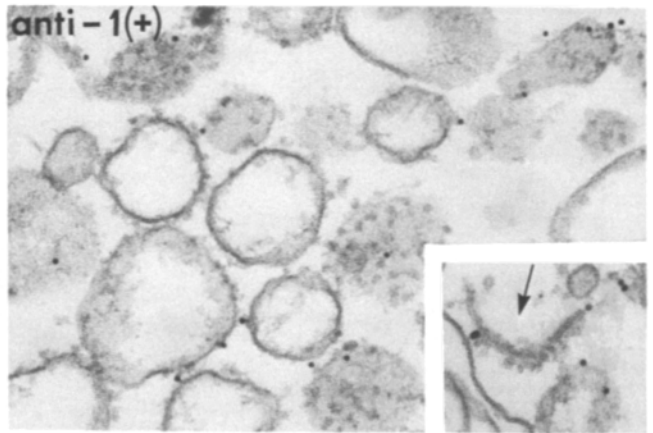
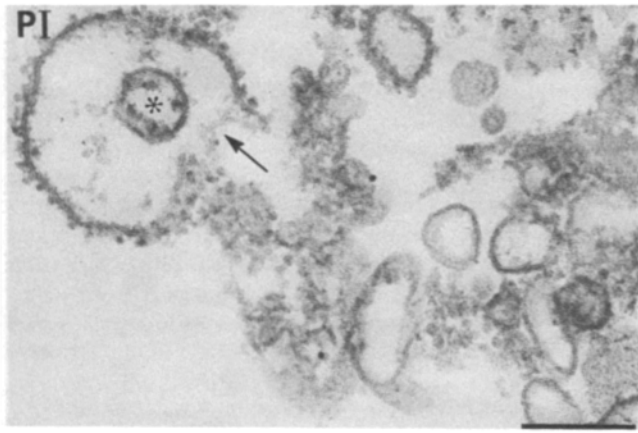
<sup>§</sup> The background labeling of 0.6 gold particles per micrometer, obtained for pre-immune IgG, was subtracted from all particle density values, except from the values obtained with “pre-immune IgG” and “preabsorbed anti-7.” Density values listed are the average of two independent experiments using the same preparations of antibody and rough microsomes. Particle densities between experiments differed at most by 35%.

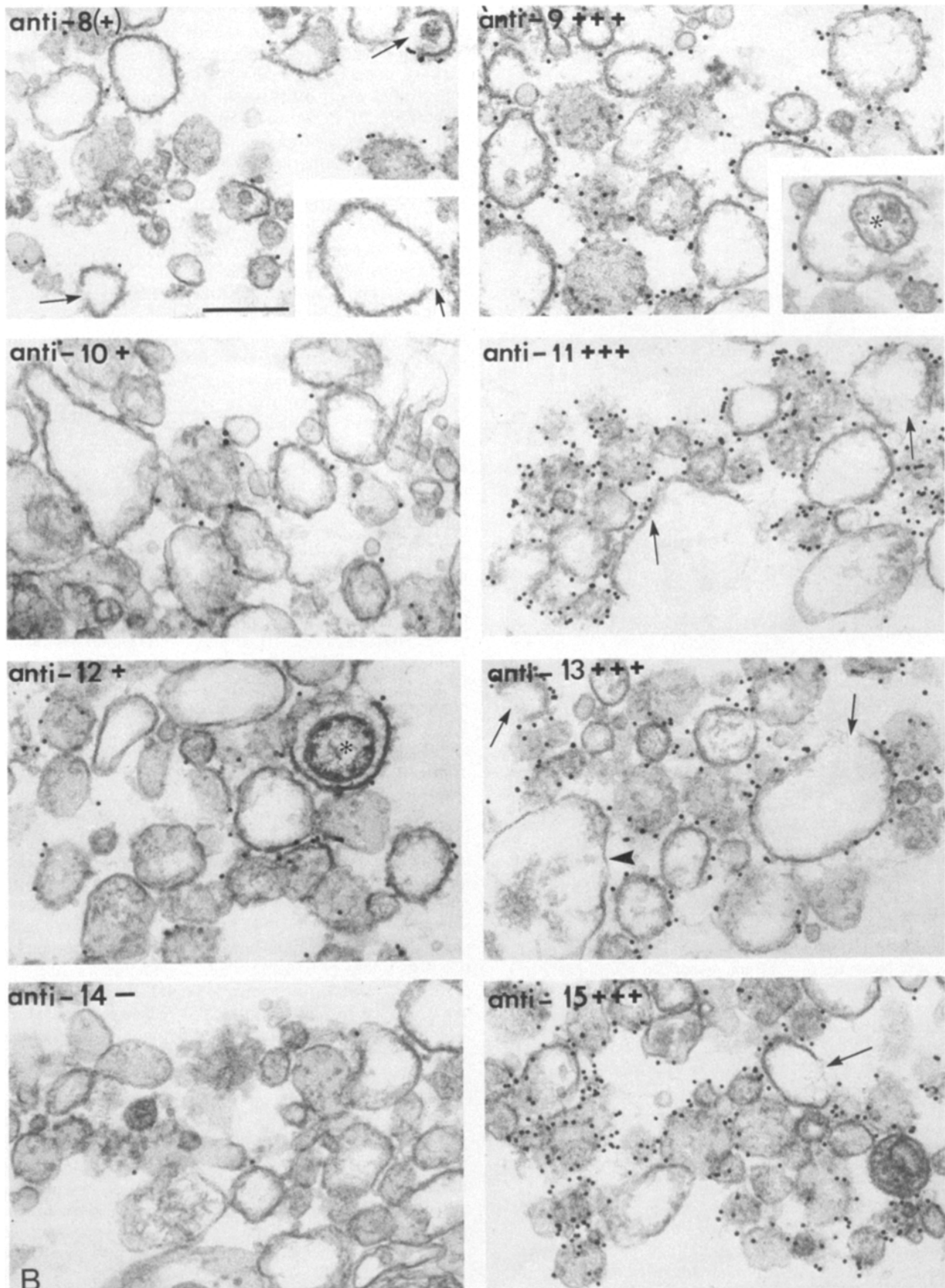
<sup>||</sup> The following scoring system was used for particle densities: –, <0.5; (+), 0.5–1; +, 1–2; ++, 2–4; +++, >4.

various synthetic peptides (Frey et al., 1985). Six samples of these antisera were available and all of these recognized peptide 2. One of them also recognized peptide 9 and another, peptide 15 (Frey et al., 1985). When the specific antibodies were removed from the anti P-450 antisera by adsorption to the respective immobilized peptides, however, the antisera still recognized the intact P-450 molecule. Therefore, the antisera also contained antibodies to P-450 epitopes not present in the repertoire of peptides used in this study. With all these antisera, exclusive labeling of the cytoplasmic surface of microsomal membranes was observed (Fig. 6), although it was clear that the luminal face in many ruptured microsomal vesicles was accessible to the antibodies. The density of labeling of the cytoplasmic face obtained with the antisera raised against the complete P-450 was higher than that obtained with any of the anti-peptide antibodies (Table II). These observations confirm those of other investigators (Matsuura et al., 1978, 1979, 1983) who, using antisera to the whole P-450 molecule, found no labeling of the luminal face of microsomal membranes.

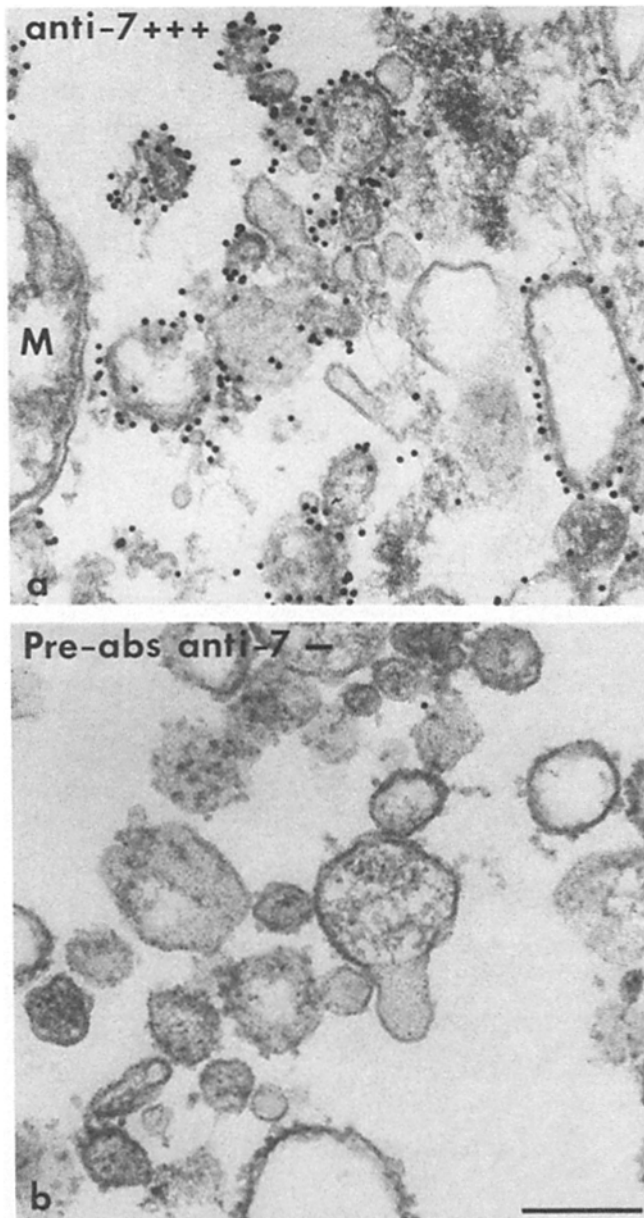
## Discussion

Although a rigorous determination of the transmembrane disposition of a membrane protein generally requires the use of macromolecular probes, such as antibodies and proteases, and nonpenetrating or selective labeling reagents, in recent





**Figure 4.** Immunoelectron microscopic localization of anti-P450-peptide antibodies bound to rough microsomes. After incubation with the affinity-purified anti-peptide antibodies (anti-1 to anti-7 in *A* and anti-8 to anti-15 in *B*) or with preimmune rabbit IgG (*PI*, first panel in *A*), the rough microsomes were labeled with protein A-gold (10 nm) complexes and prepared for routine electron microscopy. The anti-peptide antibody used is indicated by the number in the upper left-hand corner of each panel. Labeling densities are scored from – (not significant) to +++ (dense labeling), as explained in Tables I and II. The arrows point to ruptured microsomal vesicles, and the asterisks label inverted rough microsomes. Note that the luminal sides of microsomal membranes are not labeled. Membranes of organelles contaminating the microsomal preparations are also unlabeled (*arrowheads*). Bars, 0.2  $\mu$ m.



**Figure 5.** Specificity of the binding of anti-peptide antibodies to the cytoplasmic surface of rat liver microsomes. The rough microsomes were incubated with the affinity-purified anti-peptide 7 IgG (a), or with the supernatant (b) obtained after the anti-peptide 7 IgG was preincubated with rough microsomes to preadsorb the specific IgGs. Note that whereas the mitochondrial membrane (M) is unlabeled, the cytoplasmic surface of the rough microsomes (a) is densely labeled, and that preincubation with rough microsomes abolishes the binding (b). Bar, 0.2  $\mu$ m.

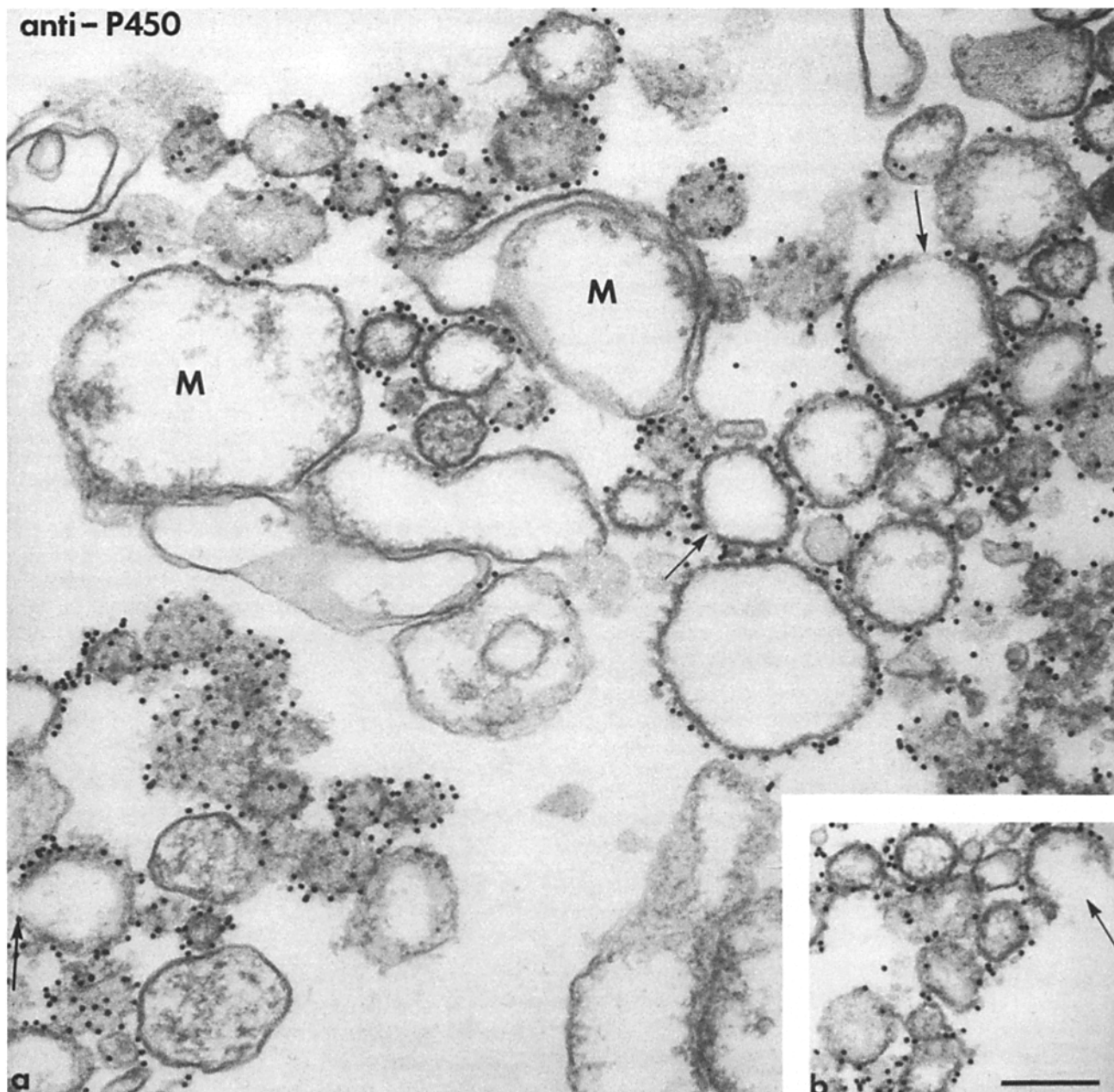
years the availability of complete primary sequences for numerous membrane proteins has allowed tentative predictions of their transmembrane disposition to be made on the basis of hydrophathy plots (Hopp and Woods, 1981; Kyte and Doolittle, 1982). Thus, several authors have suggested, assuming that intra- and interhelical ion pairs neutralize charges in putative transmembrane domains, that the rabbit PB-inducible cytochrome P-450 polypeptide traverses the membrane at least eight times (Heinemann and Ozols, 1982; Tarr et al., 1983; Ozols et al., 1985). The hydrophathy profile

of the rat liver P-450 PB-4 studied here closely resembles that of the major PB-induced form (isozyme 2) present in rabbit liver, and corresponding putative membrane-spanning domains in the rat and rabbit P-450s are easily identified, although they vary somewhat in hydrophobicity and precise length (Fig. 7). In the rat P-450 PB-4, the four polar regions that would join the transmembrane domains on the luminal side of the ER membrane (Tarr et al., 1983) are represented in peptides 2, 6, 9, 12, 13, and 15. We have shown, however, both through the binding of radiolabeled antibodies and, more convincingly, by immunoelectron microscopy, that these peptides are accessible to antibodies in intact microsomes and are therefore exposed on the cytoplasmic surface of the membrane, which is contrary to the models proposed for the transmembrane disposition of the rabbit P-450. An analysis of the hydrophobicity and length of the postulated eight transmembrane domains of the rabbit P-450 isozyme 2 (Tarr et al., 1983) shows, in fact, that only the first and fifth of these domains comprise more than 17 amino acids and have mean hydrophobicities of at least 1.6, which are regarded as minimum conditions necessary to provide for the stable association of a polypeptide with the lipid bilayer (Kyte and Doolittle, 1982). Further evidence against the existence of multiple transmembrane domains is provided by the fact that the hydrophobic photoactivatable reagent 3-(trifluoromethyl)-3-(*m*[<sup>125</sup>I]-iodophenyl) diazirine, when applied to rat liver microsomes, labeled the putative substrate binding site of P-450 PB-4 but none of the putative transmembrane domains (Frey et al., 1986).

We found that none of the 15 anti-peptide antibodies against various portions of P-450 showed any binding to the luminal face of microsomal membranes and that many showed strong binding to the cytoplasmic surface. These results are, therefore, consistent with a model in which nearly the entire protein is exposed on the cytoplasmic surface of the ER membrane. Indeed, on the basis of the fact that P-450 is synthesized in membrane-bound ribosomes and contains at its amino terminus an extremely hydrophobic segment that is retained in the mature protein (Bar-Nun et al., 1980) we have previously suggested that this segment functions as a signal that initiates cotranslational insertion of the protein into the membrane, but also halts translocation of downstream sequences and serves as a permanent anchor for the protein. In this regard, the behavior of the antibodies to peptide 1 (residues 1-31) and peptide 2 (residues 24-38) is of particular interest. Antibodies to peptide 1 bound strongly to the purified P-450, but very weakly to the cytoplasmic surface of the rough microsomes, and not at all to the luminal side of the microsomal membranes. On the other hand, antibodies to peptide 2, which partially overlaps with peptide 1, clearly labeled the surface of intact microsomes. Thus, the very weak binding of anti-peptide 1 antibodies to the rough microsomes may represent the recognition of epitopes that are also present in peptide 2 (residues 24-31). These data are therefore consistent with a disposition of P-450 in which the amino terminal segment of the molecule (including approximately the first 20 residues) is embedded in the phospholipid bilayer and the immediately following segment is exposed on the cytoplasmic side.

We cannot establish, however, whether the charged extreme amino terminus of P-450 is located on the luminal side and therefore the first 20 residues completely span the ER



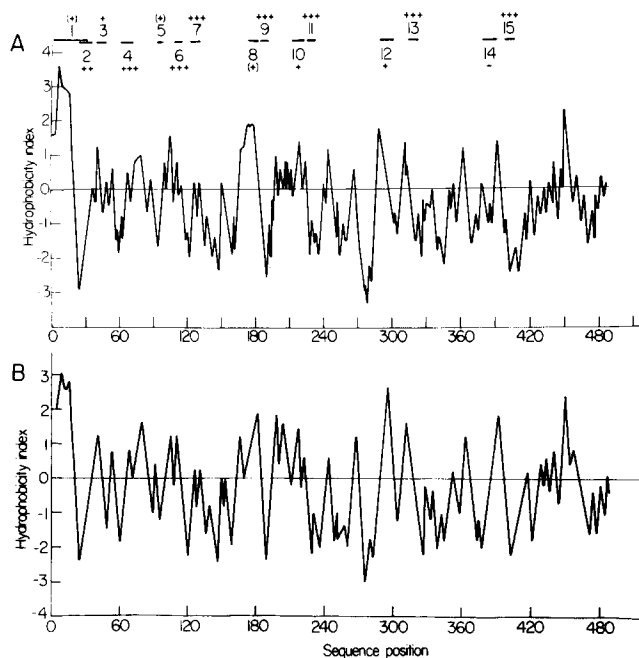


**Figure 6.** Binding of antibodies raised to the purified P-450 PB. The rough microsomes were incubated with a rabbit anti-P-450 PB antibody raised against the complete P-450 polypeptide. Labeling occurs exclusively on the cytoplasmic surfaces to which ribosomes are bound. The arrows point to the luminal surfaces of ruptured rough microsomal vesicles, which are free of label, as is the surface of contaminating mitochondrial membranes (*M*). Similar results were obtained with all six different antisera. Bar, 0.2  $\mu$ m.

membrane, or whether this portion of the polypeptide forms a loop within the membrane and therefore does not completely traverse the bilayer. A loop configuration for the amino terminal region of P-450 is consistent with the proposal (Bar-Nun et al., 1980) that during synthesis of the polypeptide this portion of the molecule functions as a non-cleavable insertion signal, since it is thought that in many cases insertion signals assume a loop disposition (Inouye and Haleboua, 1980; Steiner et al., 1980; Sabatini et al., 1982). On the other hand, it is clear that the insertion signal in rhodopsin, which is near but not at the extreme amino terminus, also displays combined insertion and halt transfer properties, and does not adopt a loop disposition. This signal

traverses the membrane only once, since the region of rhodopsin preceding the signal is exposed on the exoplasmic face of the photoreceptor membrane, which corresponds to the luminal side of the ER (Friedlander and Blobel, 1985). In a configuration analogous to that of rhodopsin, P-450 would, therefore, have its extreme amino terminus exposed on the luminal side of the ER membrane.

A second segment of the P-450 polypeptide, which extends from residues 168 to 185 and is partially contained in peptide 8, could, from its average hydrophobicity and length, also be considered as a potential membrane-embedded domain. Unfortunately, antipeptide 8 antibodies bound weakly to the purified P-450 polypeptide and gave only slightly higher than



**Figure 7.** Conservation of hydrophobic and hydrophilic regions of the rat and rabbit liver PB-inducible P-450s. The hydropathy plots shown correspond to the sequences of the rat liver cytochrome P-450 PB-4 (A) (Fujii-Kuriyama et al., 1982) and the PB-inducible rabbit liver isozyme 2 (B) (Heinemann et al., 1982). Polypeptide segments of high average hydrophobicity are represented by peaks above the mid-line and those of prevalent hydrophilicity by troughs. The segments within the sequence of P-450 PB-4 that correspond to the peptides used to prepare the site-specific antibodies are indicated by bars at the top of the graph. The numbers near the bars identify the peptides (see also Fig. 1). The relative reactivity of the antipeptide antibodies against P-450 epitopes exposed in rough microsomes, determined by immunoelectron microscopy, is indicated. These range from - (not significant) to +++ (dense labeling) (see Tables I and II).

background binding to the intact microsomes. We cannot, therefore, directly establish whether this portion of the molecule is embedded in the membrane. If this were the case, however, the polypeptide could not traverse the membrane completely, since antipeptide antibodies corresponding to both the preceding and following portions of the P-450 molecule intensely labeled the cytoplasmic microsomal surface. We must therefore conclude that if the segment between residues 168 and 185 is membrane embedded, it too must assume a loop disposition. In addition, it is worth noting that an analysis of the primary sequence of P-450 in terms of possible amphipathic secondary structure (Schiffer and Edmundson, 1967; Kaiser and Kézdy, 1984) suggests that, although less hydrophobic in its overall character, the portion of the P-450 PB-4 molecule that extends from amino acids 206 and 224 (represented in peptide 10) could form an amphipathic helix. This segment could therefore interact directly with the ER membrane and be partially embedded in the lipid bilayer. Such disposition would be consistent with the fact that antibodies to peptide 10 reacted weakly with intact microsomes but bound intensely to purified P-450.

Previous studies have shown that several of the antipeptide antibodies utilized here (4, 6, and 7) inhibit the function of P-450 as a mixed function oxidase when added to a system

in which the P-450 molecule and its reductase are incorporated together in lipid micelles (Frey et al., 1985). This inhibition most likely reflects antibody-induced changes in the substrate-binding site of P-450 or an interference with the capacity of P-450 to interact with its reductase. Since the reductase is almost totally exposed on the cytoplasmic side of the membrane, these observations were consistent with the suggestion, now confirmed directly, that the antigenic sites in peptides 4, 6, and 7 are exposed on the cytoplasmic side of the ER membrane. Similar considerations suggest that cysteine residue 436, which is thought to provide the heme-binding site in cytochrome P-450 (Gotoh et al., 1983; Adesnik and Atchison, 1985), is also exposed on the cytoplasmic side of the membrane since during electron transfer the heme interacts directly with the flavine mononucleotide in the reductase, which must be exposed on that side of the membrane.

The results presented here, therefore, provide strong support for a model for the disposition of P-450 with respect to the lipid bilayer in which at least the portion of the molecule that follows the amino terminal putative insertion-stop transfer signal and extends to residue 168 is completely exposed on the cytoplasmic surface of the ER. Even though it is possible that other relatively uncharged segments of the polypeptide (in particular the 168-185 segment and possibly the amphipathic helical region in residues 206-224) also serve as additional anchoring domains, these could not fully traverse the ER membrane.

This paper is dedicated to the memory of Dr. Dante Chiarandini.

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