

Determination of membrane-bound fragments of cytochrome *P*-450 2B4

Valentin Yu. UVAROV¹, Alexander I. SOTNICHENKO², Elena L. VODOVOZOVA³, Julian G. MOLOTKOVSKY³, Ekaterina F. KOLESANOVA¹, Yuri A. LYULKIN¹, Antony STIER⁴, Volker KRUEGER⁴ and Alexander I. ARCHAKOV¹

¹ The Institute of Biomedical Chemistry, Moscow, Russia

² Research Center of Molecular Diagnostics and Therapy, Moscow, Russia

³ M. M. Shemyakin Institute of Bioorganic Chemistry, Moscow, Russia

⁴ Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

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Membrane-bound sites of cytochrome *P*-450 2B4 (LM2) were determined by means of two different methods, photoactivated binding of membrane phospholipids to the protein and epitope mapping by antibodies. Phospholipids bearing photoreactive labels at different distances from the their polar 'head' were used in the former case. Phosphatidylcholine labelled at the apolar end of the fatty acid chain bound only to the N-terminal region of the hemoprotein. Other phospholipids labelled nearer to the head group bound not only to the N-terminus but also to the segments 273–314 and 427–491. Epitope mapping of the domain next to the N-terminus (residues 21–119) of the isolated hemoprotein was performed with the help of a peptide-scanning method, a programmable peptide synthesis on pins followed by ELISA testing with the polyclonal antiserum against cytochrome *P*-450 2B4. This domain was shown to possess a considerable density of sites with high antigenic activity. No membrane-penetrating part of this domain was found except for the fragment 1–21. A model of structure of *P*-450 2B4 was computed by comparison with the structure of cytochrome *P*-450_{cam} on the basis of an alignment of 47 cytochromes *P*-450 with the former hemoprotein. Major parts of the protein sequences photoreacting with the phospholipid probes, but not the antibody-reactive epitopes of the region 21–119, are located at the membrane-facing side in this model

Though cytochromes *P*-450 are the most thoroughly studied membrane enzymes, there is no agreement on their topology in the lipid bilayer. At present there are several models of the membrane orientation of microsomal cytochrome *P*-450 [1–6] constructed on the basis of various methods of polypeptide chain-structure prediction or some experimental data [7–11]. The aim of our investigation was to examine these models by analysis of the interaction of photoreactive phospholipids bearing the photolabel at different distances from their polar 'heads' with cytochrome *P*-450 2B4 in proteoliposomes and by surface epitope mapping of the 119 N-terminal amino acid residues in the solubilized protein with the help of the peptide-scanning technique (PEPSCAN) [12].

MATERIALS AND METHODS

Materials

Cytochrome *P*-450 2B4 was obtained from phenobarbital-induced rat liver microsomes [13]. Proteoliposomes were prepared as described earlier [14]. Photoreactive lipid was added to the solution of egg phosphatidylcholine, a phospho-

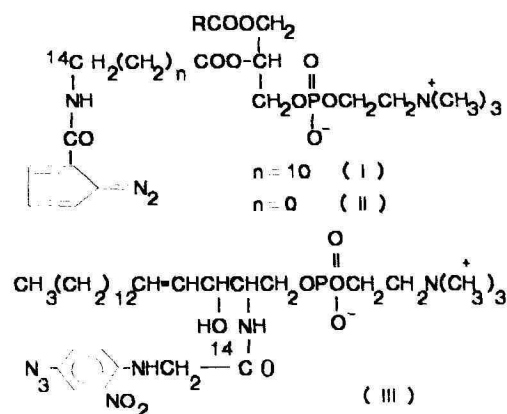


Fig. 1. Photoreactive phospholipids I–III.

lipid probe averaged to 9:1. Fig. 1 shows the structural formulae of the photoreactive phospholipids used.

Phospholipid–protein photoreaction

Photoreactive phosphatidylcholine (I) was synthesized as described earlier [15]. Short-chain phosphatidylcholine (II) was synthesized from [1-¹⁴C]glycine. Lysophosphatidylcholine was acylated with *tert*-butyloxycarbonyl-[1-¹⁴C]glycine via the carbodiimide-coupling procedure in the presence of 4-piperidine-pyridine as a standard catalyst [15]. After re-

Correspondence to V. Y. Uvarov, Institute Biomedical Chemistry, Pogodinskaya 10, 119832 Moscow, Russia

Abbreviations. PEPSCAN, peptide-scanning method; ABTS, 2,2'-azino-di(3-ethylbenzthiazoline sulfonate).

Enzyme. Cytochrome *P*-450 (EC 1.14.14.1).

moving the protective group by trifluoroacetic acid hydrolysis, the 1-acyl-2-[1-¹⁴C]glycyl-*sn*-glycero-3-phosphocholine formed was converted into probe II by coupling with 2-diazo-cyclo-pentadiene carbonic acid (obtained as described in [16]) via the carbodiimide procedure. Photoreactive sphingomyeline (III) was prepared as described earlier [17]. The specific activity of photoreactive phospholipids I–III averaged approximately 50 Ci/mol. All probe syntheses were performed under yellow light.

The proteoliposomes formed were subjected to a 10-min photolysis with a mercury medium-pressure 30-W lamp (with emission maximum at 365 nm) at a distance of 7 cm. The procedure was performed in a pyrex test tube (10 mm in diameter, 2-mm-thick walls) under a stream of argon at 37°C. After the irradiation SDS was added to the water/proteoliposome solution until the ratio SDS/protein became 40:1 (by mass) and the mixture was incubated for 15–20 min at 37°C. Then 2 vol. chloroform/methanol (2:1, by vol.) was added to 1 vol. proteoliposome solution to remove the unbound lipid. After a 6-h incubation at 4°C with periodic shaking, the suspension was centrifuged at 800 *g* for 10 min. The pellet was dissolved in 70% formic acid and BrCN was added to the solution (BrCN/protein methionine molar ratio was 80:1). The reaction mixture was incubated for 18 h in full darkness. The completion of hydrolysis was monitored electrophoretically [18] by disappearance of the band in the 50-kDa region and the appearance of low-molecular-mass fragments. There were no other bands above the low-molecular-mass marker (cytochrome *c*) on the SDS/PAGE plate. The peptide mixture obtained was diluted 20-fold and lyophilized. The lyophilized probe was dissolved in 30% formic acid and isopropanol and subjected to preliminary chromatography on a Superose 12 column (1×30 cm) in the same system.

For further investigations only radioactive fractions were taken. These fractions were chromatographed on an Ultrapore C3 RPSC column (0.46×7.5) using a linear gradient of solvent B [0.2% heptafluorobutyric acid in propanol/acetonitrile (2:1, by vol.)] in solvent A (0.2% heptafluorobutyric acid in water) at an elution rate of 1 ml/min. Radiactive probes were collected and rechromatographed on the same column using the same solvents.

Peptide fragments were subjected to Edman degradation by the automated sequencing method using the Beckman 890C sequencer with program N 102974.

Peptide scanning method (PEPSCAN)

Amino acids N-protected with 9-fluorenylmethyloxycarbonyl (Fmoc) and C-protected as pentafluorophenyl esters, 1-hydroxybenzotriazole, polypropylene pins held on racks in a 96-well format compatible with ELISA plates, polypropylene reaction trays and the software for directing the synthesis and subsequent data analysis were from Cambridge Research Biochemicals. Freund's complete adjuvant, diagnostic antibodies against mouse immunoglobulins labelled with horseradish peroxidase and diammonium 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) (ABTS) were from Calbiochem. Bovine serum albumin and ovalbumin were from Sigma, trifluoroacetic acid, phenol, ethanedithiol, acetic anhydride, dimethylformamide, piperidine and sodium phosphate salts from Merck; Tween 20 and sodium dodecyl sulfate from Serva.

Hexapeptides linked to pins were synthesized by the active-ester-coupling procedure using the protected amino

acid in the presence of an equimolar amount of 1-hydroxybenzotriazole as described in the manufacturer's manual (Cambridge Research Biochemicals Co.) and in [19]. Anchoring of peptides to the pins is achieved through the spacer arm, β -alanine, which is attached to the pins in a Fmoc-protected form by the manufacturer. Briefly, following the deprotection of the amino group with 20% piperidine in dimethylformamide, the coupling step was allowed to proceed for 18 h, using 30 mM solutions of protected amino acid esters and 1-hydroxybenzotriazole. An additional step of washing the pins with 30 mM 1-hydroxybenzotriazole was introduced prior to the coupling step to increase coupling efficiency as described in [19]. A GNET sequence program run on the IBM-PC was used to create the synthesis schedule and direct the addition of the correct amino acid to each pin on each day. At the completion of the sixth coupling step, the pin-attached peptides were finally deprotected on the N-terminal α -amino group and acetylated by acetic anhydride. The side-chain deprotection was carried out by a 4-h treatment of pins with a mixture of trifluoroacetic acid/phenol/ethanedithiol (95:2.5:2.5, vol./mass/vol.).

Antiserum to purified cytochrome *P*-450 2B4 was raised in mice. Mice were injected intraperitoneally with 20 μ g cytochrome *P*-450 2B4 once a month for 6 months. The first three injections were made in a Freund's complete adjuvant, the last three in phosphate-buffered saline. Mice were bled through the eye sinus (under ether anaesthesia) 10 days after the last immunization. Blood was left for coagulation and then centrifuged at 3000 *g* for 15 min to obtain the serum. Cytochrome *P*-450 2B4 antiserum thus obtained was tested for cytochrome *P*-450 2B4 whole molecule specificity by the Ouchterlony test [20] and ELISA [21] and was found to have a titer of 1:800.

ELISA testing of pin-linked peptides was performed as described in [19, 21] with ABTS as a dye reagent in a final concentration 0.025%. Non-specific binding of Rossmann-domain-derived peptides with immunoglobulins, defined by incubating pin-linked peptides with control serum (from intact mice), was subtracted from the total binding defined by incubation with the antiserum, to obtain the specific binding of peptides with cytochrome *P*-450 2B4-specific antibodies. Cytochrome *P*-450 2B4 antiserum and control serum dilutions were 1:200, second antibody preparation dilution was 1:15000, incubation time with ABTS and H₂O₂ for colour development was 10 min. Before and between each ELISA testing, the pins were incubated for 30 min in hot (60°C) disruption buffer (1% SDS, 0.1% 2-mercaptoethanol in 0.1 M sodium phosphate pH 7.2) under sonication, washed repeatedly with hot water and then with boiling methanol and dried.

Molecular graphic modelling

A modelling of the tertiary structure of cytochrome *P*-450 2B4 was performed by a computer-assisted comparison with the known tertiary structure of cytochrome *P*-450_{cam} [23] using an alignment of the primary structure of the latter protein with the primary structures of 47 membrane-bound cytochromes *P*-450 as described [24]. On this alignment a basic skeleton of cytochrome *P*-450 2B4 was built using the program SYBYL (Tripos Associates Inc., St Louis, Missouri) on a Graphics work station of Evans and Sutherland 350. Side chains of cytochrome *P*-450_{cam} were exchanged with the corresponding side chains of cytochrome *P*-450 2B4. The program controls and corrects the molecular overlap by

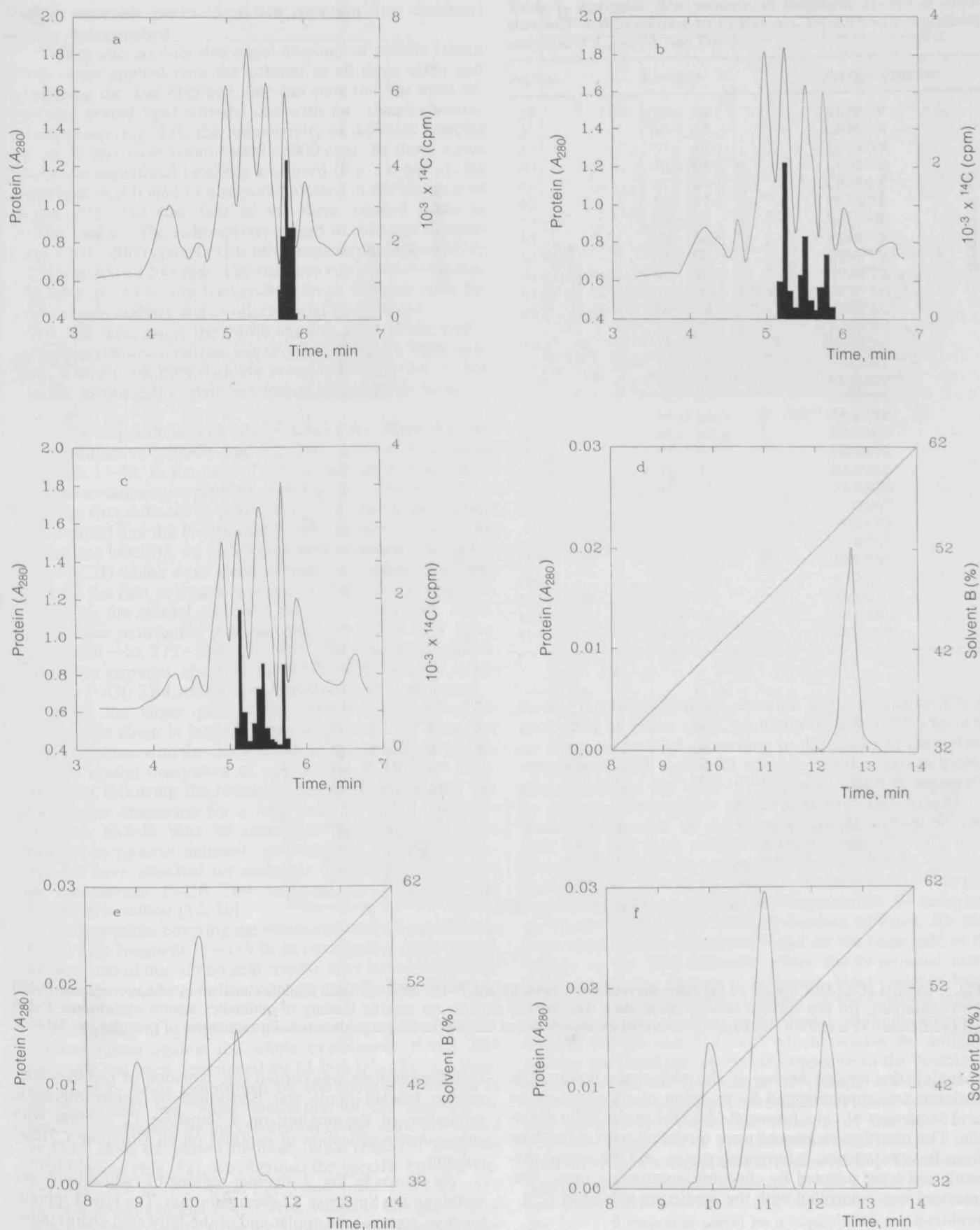


Fig. 2. Chromatographic separation of BrCN peptides of modified cytochrome P-450 2B4. (a–c) Separation of peptides on Superose 12: complexes with photoreactive phospholipids of type I (a), II (b) or III (c). (d–f) Chromatography of the peptides bound to photoreactive lipids I (d), II (e) or III (f) on an Ultrapore C3 RPSC column. The experiment is fully described in Materials and Methods.

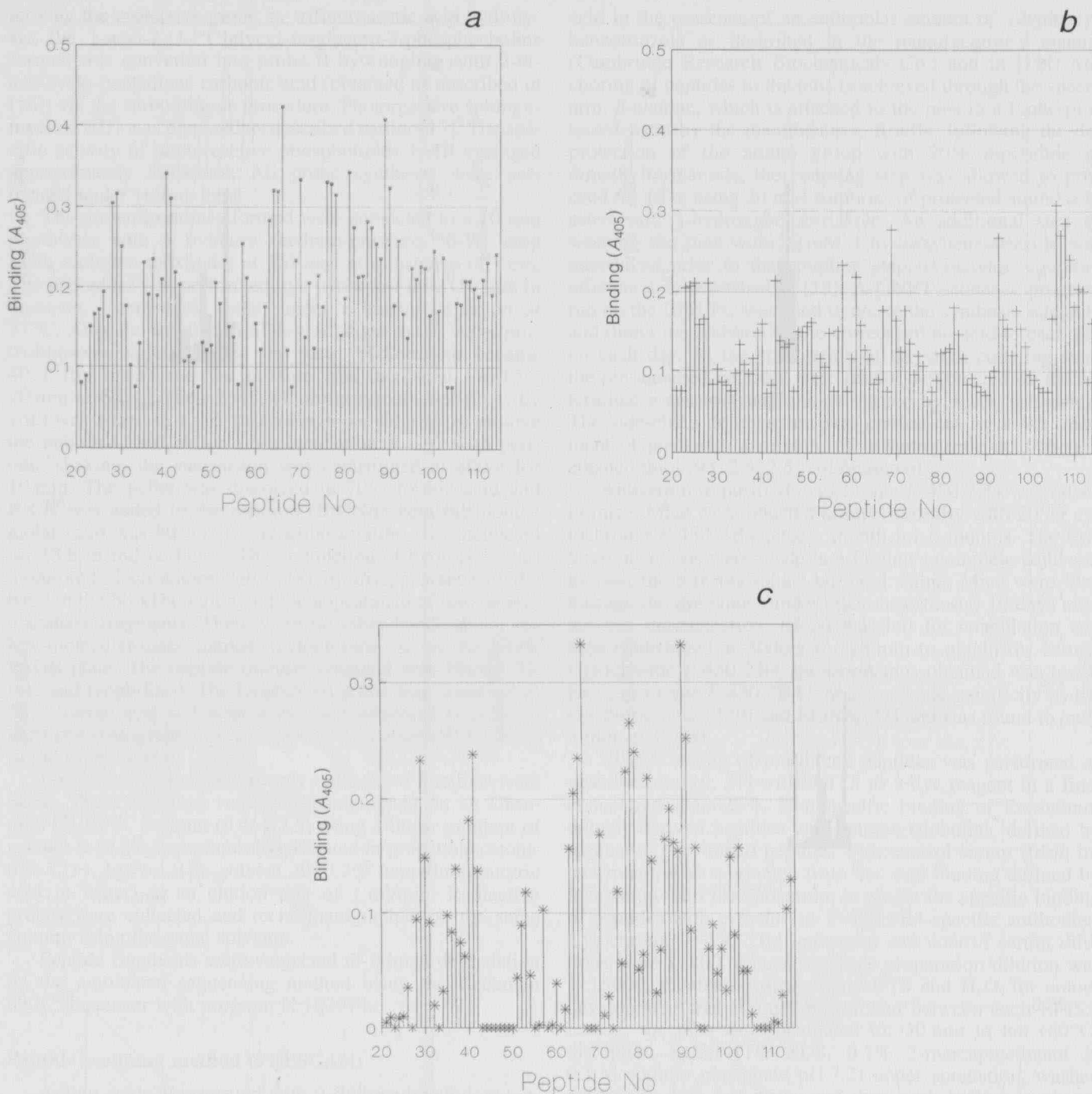


Fig. 3. Results of ELISA testing of peptides derived from cytochrome *P*-450 2B4. (a) Total binding (incubation with cytochrome *P*-450 2B4 antiserum); (b) non-specific binding (incubation with control serum); (c) specific binding of antibodies against cytochrome *P*-450 2B4 (difference of a and b). Binding was measured as absorbance at 405 nm in the peroxidase test. For structures of peptides see Table 1.

changing the torsion angles of the side-chain bonds. The skeleton was supplemented by insertion of six short amino acid sequences of cytochrome *P*-450 2B4 in the basic skeleton. The insertion sequences were modelled with algorithms from Bayes statistics, information theory and the neural network and were adapted by choosing appropriate turns. The insertion was controlled with the prediction published [23].

RESULTS AND DISCUSSION

After formation of the lipid-protein covalent complexes (see Materials and Methods), BrCN hydrolysis of the modi-

fied hemoprotein was carried out. Isolation of peptides containing labeled lipids was performed by chromatographic separation of the mixture on a Superose 12 column with subsequent purification of peptides on an Ultrapore C3RSC column.

As shown in Fig. 2, distinct regions of radioactivity are visible in the Superose 12 chromatogram. The type I (photo-reactive group at the apolar end of the fatty acid chain) phosphatidylcholine binding site is represented by a narrow peak with a retention time of 6 min. In the case of lipids with surface photoreactive groups, three closely located but

clearly separable peaks (5–6 min retention time distance) can be distinguished.

Taking into account that equal amounts of protein (about 7 mg) were applied onto the column in all three cases and comparing the data obtained, one can state that the most effectively bound lipid was the one with the 'deep' photoreactive group (Fig. 2a); the radioactivity of different samples varied in this case within 4000–6000 cpm. In those cases where the superficial labelling was used (Fig. 2b and c), the most lipid was bound to a segment located in the amino acid region 273–313 (the first of the three labeled peaks in Fig. 2b and c). The radioactivity varied in different samples over 1500–2000 cpm. In two other regions the radioactivity level was 700–1200 cpm. The radioactivity of other chromatographic peaks was at background level. Samples with the highest radioactivity were collected and lyophilized.

At the next stage, the purification of each of the radioactive fractions was carried out on an Ultrapore C3RSC column. The elution time data are presented in Fig. 2d–f. For peptide identification five N-terminal amino acids were sequenced.

In the experiment with 'deep' label I we found that the only radioactive peptide (see Fig. 2d) was the N-terminal fragment 1–46. In the case of the surface label II, we purified three radioactive peptides (see Fig. 2e): residues 273–314 (the first radioactive peak), 427–491 (the second radioactive peak) and the N-terminal 1–46 (the third one). Using the surface label III, we also found three radioactive peptides (see Fig. 2f) which were identical with the peptides bound to label II: the first radioactive peptide in Fig. 2f is the peptide 273–314, the second 427–491 and the third 1–46. Thus, using three radioactive phospholipids we found only three peptides (1–46, 273–314, 427–491) that reacted with them. The other peptides obtained by BrCN hydrolysis of cytochrome *P*-450 2B4 were not radioactive in all three cases.

Thus, the larger part of the cytochrome *P*-450 2B4 polypeptide chain is located in the water solution. This fact fully correlates with the calculations we published earlier [4].

The spatial orientation of cytochrome *P*-450 2B4 fragment just following the N-terminal hydrophobic anchor has been under discussion for a long time [4, 9, 10]. Surface-oriented, mobile sites of membrane proteins have been thought to possess antigenic activity [19, 28, 29]. Therefore we have searched for antigenic (B-epitope) activity in the cytochrome *P*-450 2B4 fragment 21–119 using the PEPSCAN method [12, 19].

Hexapeptides covering the whole structure of cytochrome *P*-450 2B4 fragment 21–119 in an overlapping manner with a frame step of one amino acid residue have been synthesized for epitope scanning on the pins as described in Materials and Methods.

The ability of synthesized pin-linked peptides to bind antibodies raised against the whole cytochrome *P*-450 2B4 molecule has been determined by ELISA (Fig. 3a–c), binding capacity being determined by absorbance at 405 nm in the peroxidase test. After subtraction of the non-specific binding determined by the incubation of the same pin-linked peptides with the serum of intact mice (Fig. 3b) from the total binding (Fig. 3a), we obtained the specific binding values (Fig. 3c). As seen from Fig. 3 and Table 1, the 21–119 domain of cytochrome *P*-450 2B4, which is thought to possess a supersecondary structure similar to the Rossmann domain of dehydrogenases [4, 31], has a rather high density of sites with antigenic activity. The B-epitopes found on the N-terminal domain of cytochrome *P*-450 2B4 are almost uni-

Table 1. Antigenic determinants of fragment 21–119 of cytochrome *P*-450 2B4 found by PEPSCAN. Peptides were considered antigenic if $A_{405} \geq 0.1$ (see Fig. 3c).

Peptide	Residues	Peptide structure
29	29–34	RLPPGP
30	30–35	LPPGPS
37	37–42	LPVLGN
40	40–45	IGNLLQ
41	41–46	GNLLQM
42	42–47	NLLQMD
53	53–58	RSFLRL
57	57–62	RLREKY
63	63–68	GDVFTV
64	64–69	DVFTVY
65	65–70	VFTVYL
66	66–71	FTVYLG
70	70–75	LQSRPV
74	74–79	PVVVLC
76	76–81	VVLCGT
77	77–82	VLCGTD
78	78–83	LCGTDA
81	81–86	TDAIRE
82	82–87	DAIREA
85	85–90	REALVD
86	86–91	EALVDQ
87	87–92	ALVDQA
88	88–93	LVDQAE
89	89–94	VDQAEA
90	90–95	DQAEAF
92	92–97	AEAFSG
100	100–105	KIAVVD
102	102–107	AVVDPI
113	113–118	VIFANG
114	114–119	IFANGE

formly incorporated in its structure and are separated from each other by rather short, primarily hydrophobic, sites with no antigenic activity. According to the model of the tertiary structure of 2B4 almost all epitopes are the surface-located sites except for the 100–107 fragment which is apparently an antigenic determinant of the denatured 2B4 (results not shown). Moreover, all the most accessible surface-oriented and, thus, the most evident antigenic sites [28–30] (protruded loops) of the N-terminal domain of 2B4, i.e. the fragments 29–36, 53–58, 81–86, 89–94 and 113–119, do possess the B-epitope properties, the fragment 89–94 being one of the two most readily antibody-binding epitopes. No antigenic determinants have been found on the same side of the surface of the 2B4 molecule where the N-terminal membrane-penetrating domain is localized. Most epitopes found in the 21–119 domain are situated on the sidelong surface of the molecule according to the model, while the fragments 63–71, 81–86 and 113–119 which possess the antigenic activity are localized on the side opposite to the N-terminal membrane-penetrating domain. Our results of epitope scanning are in good agreement with earlier estimation of space accessibility and presumably cytoplasmic location of the 61–72 and 108–116 fragments of cytochromes *P*-450 relative to 2B4 with the help of antipeptide antibodies [8, 32]. Therefore, the Rossmann domain of cytochrome *P*-450 2B4 seems to contain many surface-localized and accessible sites. No site of the 21–119 fragment could be a candidate for a membrane-penetrating helix.

In addition, our results on antigenic activity estimation are in good agreement with computer modelling of cyto-

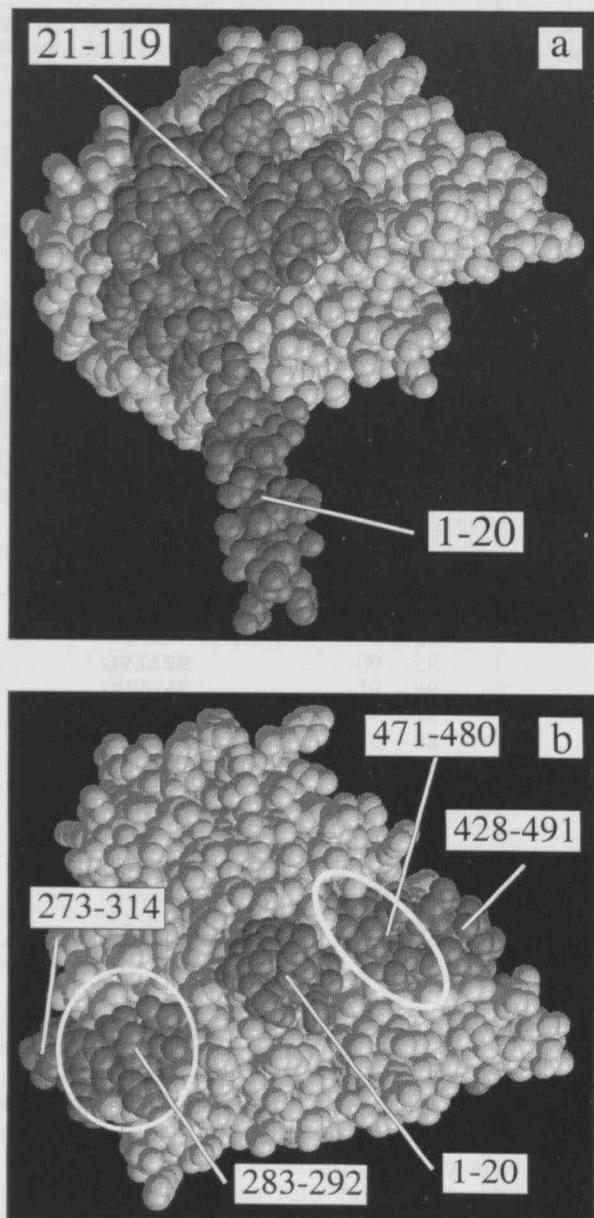


Fig. 4. Computer model of cytochrome *P*-450 2B4. (a) Regions 1–20 and 21–119; (b) regions 1–20, 273–314 and 428–491.

chrome *P*-450 2B4 tertiary structure (Fig. 4). Apparently, all sites most active in the PEPSCAN testing are located on the surface of the model.

Thus, our data make it evident that only the N-terminal sequence 1–20 of the cytochrome *P*-450 2B4 molecule is deeply embedded in the membrane or even penetrates the membrane. This conclusion is in agreement with results of the comprehensive proteolysis proteoliposomes containing cytochrome *P*-450 2B4 [33]. We found that only the N-terminal 21-amino-acid fragment remained bound to the liposomes. The two segments partially embedded in the membrane were released from the membrane to the soluble fraction during such treatment. The presence of two hydrophobic segments partially embedded in the membrane might explain the fact that a third of the cytochrome *P*-450 2B4 lacking the N-terminal fragment (obtained by Dr Pernecky and colleagues) remained bound to membrane [34]. Furthermore, computer-assisted modelling of the cytochrome *P*-450 2B4

structure on the basis of a comparison with the structure of cytochrome *P*-450_{cam} shows (Fig. 4) that major parts of the sequences 21–119, 273–314, and 428–491 are located at the same site of cytochrome *P*-450 2B4 as the hydrophobic N-terminal helix. As other parts of the fragments (273–314, 428–491) extend to the substrate- and heme-binding regions, it may be speculated that structural changes of the membrane surface influence the catalytical activity of cytochrome *P*-450 2B4 [25–27].

Using the model presented in Fig. 4, we have found that the sites located in the regions of residues 283–292 and 471–480 are the most probable candidates for the interaction with the membrane. We suggest that the membrane regulation is most likely to be brought about through the first site because, as seen from the model presented, this site is a butt-end region of α -helix which immediately recedes into the hemoprotein molecule, to the heme location site. The site of the polypeptide chain preceding the α -helix region is a β -turn. The potential site of membrane binding located in the vicinity of the C-terminus is represented by a random conformation; therefore the changes in the lipid bilayer cannot be transmitted as effectively over the long distance upon initiation in this region.

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