Biochimica et Biophysica Acta, 1063 (1991) 226–234

ADONIS 000527369100156W

BBAMEM 75183

Chemical modification of rat liver microsomal cytochrome *P*-450: study of enzymic properties and membrane topology

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(Received 8 June 1990)
(Revised manuscript received 16 October 1990)

Key words: Cytochrome *P*-450; Chemical modification; Membrane topology; Substrate binding site

Isolated rat liver cytochrome *P*-450IIB1 was alkylated and acetylated at primary amino groups, and the position of the modified amino acids in the protein was identified. Alkylation of up to nine amino groups did not disturb the interaction of reconstituted *P*-450 and NADPH–cytochrome-*P*-450 reductase in a way that hydroxylation of benzphetamine was altered, whereas deethylation of 7-ethoxycoumarin was gradually reduced in parallel with impaired 7-ethoxycoumarin binding. Acetylation of four lysine residues completely inhibited binding and metabolism of 7-ethoxycoumarin but not of benzphetamine. These results suggest the presence of different substrate binding sites on *P*-450. Exhaustive proteolysis of modified *P*-450 in proteoliposomes liberated all but the N-terminal modified peptide and 85 to 90% of the cytochrome's mass from intact proteoliposomes. These findings further support our previously proposed model of *P*-450 topology (Vergères, G., Winterhalter, K.H. and Richter, C. (1989) Biochemistry 28, 3650–3655), in which *P*-450 is anchored to the membrane with the N-terminal peptide only, the N-terminal methionine facing the lumenal interior.

Introduction

Cytochrome *P*-450IIB1 *** (*P*-450) and NADPH–cytochrome-*P*-450 reductase (reductase) are key enzymes of the hepatic microsomal monooxygenase system, which catalyzes the oxidative and reductive metabolism of endogenous substrates and many xenobiotics [1–3]. Little is known about the role of the various amino acid residues for the structure and function of the monooxygenase system. The importance of electrostatic interactions between *P*-450 and its reductase has been pointed out by Bösterling and Trudell [4]. High ionic strength was shown to interrupt electron flow from the reductase to *P*-450, and the involvement of at least one charge pair for electron transfer was postulated. Crosslinking studies with a water-soluble carbodiimide reagent further supported this hypothesis since covalent complexes between *P*-450 and the reductase could be generated [5,6]. Complex formation involved carboxylic groups of the reductase and amino groups of *P*-450. Accordingly, chemical modifications of carboxylic groups of the reductase led to impaired interactions between the reductase and *P*-450 [6–8]. The modification of *P*-450 amino groups also resulted in a decreased activity of the monooxygenase system. Fluorescein isothiocyanate bound to the amino terminus and to lysine-384 of rabbit *P*-450IIB1 was shown to inhibit the benzphetamine *N*-demethylase activity by 50%. Each modification contributed to 25% to this effect [9–11]. Finally, the modification of seven lysine residues in rabbit *P*-450 by 2-methoxy-5-nitrotropone inhibited the activity towards *p*-nitroanisole by 50% [12]. The model of charge-pairing is generally accepted, but it was also proposed that steric constraints play a role in the binding and electron transfer steps between the reductase and *P*-450 [8].

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*** The nomenclature follows the recommendation of Nebert et al. (1987) DNA 6, 1–11.

Abbreviations: DMF, dimethylformamide; DPH, 1,6-diphenyl-1,3,5-hexatriene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; KP_i, potassium phosphate; *P*-450, cytochrome *P*-450; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; reductase, NADPH–cytochrome-*P*-450 reductase; SDS, sodium dodecyl sulfate.

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Here we describe the covalent modification of microsomal rat liver *P*-450IIB1 at primary amino groups and its influence on the protein's stability and enzymic properties. Tryptic digestion of reconstituted alkylated *P*-450 and identification of modified amino acid residues within tryptic peptides was also used to study the topology of *P*-450 in proteoliposomes.

Materials and Methods

Enzyme purification. *P*-450IIB1 and its reductase were isolated from phenobarbital-induced Sprague–Dawley rats (150 g) supplied by Madoerin AG, Füllinsdorf, Switzerland. Reductase was isolated according to Shephard et al. [13]. *P*-450IIB1 was isolated by the method of Waxman and Walsh [14] except that Emulgen was removed by detergent exchange while the cytochrome was bound to the hydroxylapatite column. Both proteins gave a single band on SDS-polyacrylamide gels. The specific contents were 14 nmol of *P*-450 heme/mg of protein and 51 000 units/mg of protein for the reductase.

Preparation of proteoliposomes. Reconstitution of *P*-450 and reductase in phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (PC/PE/PS) (10 : 5 : 1, w/w) vesicles was performed by a cholate dialysis procedure [15]. The lipid/*P*-450 ratio was 1 : 1 (w/w) unless indicated otherwise. For the determination of hydroxylation activity of the reconstituted monooxygenase system, the molar ratio of *P*-450/reductase was 5 : 1.

Modification of P-450. Acetylation of lysine residues with [^{3}H]acetic anhydride and its quantification have been described [16]. Alkylation was performed by reacting primary amines with formaldehyde in the presence of cyanoborohydride [17]. To the protein (1 mg/ml in 50 mM KP_i or Hepes (pH 7.4), with 20% glycerol) at room temperature, 1 mM [^{14}C]formaldehyde (15 mCi/mmol) from a 100 mM stock solution, and 20 mM sodium cyanoborohydride from a freshly prepared stock solution of 6 mg/ml reaction buffer were added. The reaction time was between 5 min and 5 h. The modification was stopped by removing unreacted formaldehyde and sodium cyanoborohydride by the microfuge desalting method described by Helmerhorst and Stokes [18]. Controls were treated identically without formaldehyde. *P*-450 (0.1–1 nmol) was then mixed with serum albumin (5 mg/ml) and precipitated with 500 µl perchloric acid (12%). After 10 min the suspension was filtered dropwise through a glass filter. The radioactivity remaining on the filter after rinsing with 10 ml perchloric acid (12%) was determined in a liquid scintillation counter.

Enzyme assays. 7-Ethoxycoumarin *O*-deethylase activity was measured according to Ullrich and Weber [19] in the presence of a NADPH-regenerating system (5 mM glucose 6-phosphate, 2.5 units glucose-6-phosphate dehydrogenase) except that *P*-450 was used in vesicles instead of microsomes. Benzphetamine hydroxylation was determined according to Gut et al. [15]. Substrate-induced difference spectra in the Soret region were recorded in tandem cuvettes with vesicles in 100 mM KP_i (pH 7.4), containing 20% glycerol.

Characterization of modified amino acids. Modified amino acids were characterized as follows: *P*-450 was cleaved by trypsin (see below) into small peptides. Under the conditions employed, each resulting peptide contained only one modified residue. It should be noted that trypsin does not recognize modified lysines. Peptides were separated by high-performance liquid chromatography (HPLC) (see below) and analyzed for amino acid composition and amino terminal residues. The amino acid composition and sequence of the peptides were compared with the sequence of whole *P*-450 to localize modified amino acids. For digestion (37 °C) with trypsin, *P*-450 (1 mg/ml) was first dialyzed against 50 mM ammonium bicarbonate, pH 8.0, and then trypsin (10 µg/ml) was added. Another 10 µg/ml were added after 4 h. After digestion over night, the peptides were concentrated to 30 mg/ml under a stream of nitrogen. It is important to note that tryptic cleavage and all following steps were performed in polypropylene tubes since cytochrome *P*-450-derived peptides stick to glass.

Tryptic peptides were separated on a 250 × 5 mm LiChrosorb RP 8 10 µm column (Kontron Analytic) in a Hewlett Packard 1090 Liquid Chromatography. The HP 1090 was used with a HP-85 B personal computer and a diode array detector. The peptides (100–500 µg) were injected directly into the column equilibrated with 0.1% TFA (solvent A), and eluted at 60 °C with a flow rate of 1 ml/min. First a linear gradient from 100% solvent A to 35% acetonitrile/water/TFA (90 : 10 : 0.1) (solvent B) was applied within 40 min, and then a linear gradient to 100% solvent B in 30 min. Peptides were detected at 220 nm and collected in 0.5 min fractions which were analyzed for radioactivity. Labelled peptides were re-chromatographed on the same system. To this end, they were concentrated in polypropylene tubes under nitrogen and injected into the column equilibrated with the starting mixture of solvent A and B. The solvent and gradient systems used for re-chromatography were principally the same as above. The gradients started 5% below the expected elution and were less steep.

Preparation of samples for amino acid analysis. Peptides were dried on the bottom of a glass tube and hydrolyzed in vacuo in the gas phase with 6 M HCl for 18 h at 110 °C, HCl/TFA = 2 : 1 for 1 h at 157 °C, or with HCl/propionic acid = 1 : 1 at 110 °C for 18 h, or with 6 M HCl + 5% TFA 110 °C for 18 h. For each series of peptides to be hydrolyzed a sample of whole *P*-450 was included. The hydrolysates were subse-

quently dried in vacuo. A maximal amount of 40 nmol amino acids was dissolved in a glass tube of 5 mm diameter in 20 μl 50 mM sodium bicarbonate buffer (pH 8.2), which was adjusted with bicarbonate when necessary. Dabsyl chloride (160 nmol) in acetonitrile was then added. The tubes were sealed by a stopper, and heated to 70 °C. After 15 min the samples were cooled to room temperature and diluted with 140 μl 50 mM KP_i (pH 7), with 50% ethanol, and, when necessary, with up to 600 μl starting buffer.

Separation of dabsyl amino acids. Dabsyl amino acids were separated on a Nucleosyl 100-5 C18 column (5 × 250 mm) at 55 °C with a flow rate of 1 ml/min. Solvent C consisted of 4% DMF in 20–50 mM sodium acetate (pH 5.8–6.5), depending on the condition of the column. Solvent D was 40 ml DMF per 1 L acetonitrile. For elution, first a linear gradient of 15–30% solvent D was applied within 32 min and then a linear gradient of 30–80% solvent D over the next 20 min. Detection was at 420 nm.

Analysis of the amino terminus. The analysis of rechromatographed peptides was performed according to the method of Gray [20]. Dansylated amino acids were identified on thin-layer chromatography micropolyamide foils. The amino acids were extracted with 30 μl ethanol (95%) and spotted near the corner of a 5 × 5 cm foil. On the reverse side of the foil a standard mixture of dansylated amino acids was applied. Separation in the first dimension was with 1.5% formic acid, in the second dimension with benzene/acetic acid (9 : 1, v/v).

Proteolytic digestion of P-450 proteoliposomes. *P*-450 alkylated in solution for 5 h was reconstituted into PC/PE/PS vesicles in 50 mM potassium phosphate (KP_i), 0.1 mM EDTA, 20% glycerol (pH 7.4), (standard buffer). Incorporation was analyzed on a Sepharose 4B column (43 × 2 cm, equilibrated with standard buffer plus 100 mM KCl) at a flow of 12 ml/h. The 45-min fractions were analyzed for protein content at 220 nm and by liquid scintillation counting, and for liposomes by fluorescence intensity measurements in the presence of 2 μM diphenylhexatriene (DPH) as described [21]. Contributions due to light scattering and absorption by liposomes were corrected for. Cleavage of peptides facing the outside of the vesicles was done with trypsin (bovine pancreas) (1 μg per 50 μg *P*-450 at time 0 and after 2.5 h) at 37 °C over night in standard buffer. The vesicles were then dialyzed against 80 mM KP_i (pH 7.4) with 10% glycerol for 3 h, and then against 10 mM KP_i (pH 7.4) for 2 h. They were then pelletted at 110000 × *g* for 2 h, and the radioactivity in the supernatant and pellet was determined. Alternatively, the membrane-bound peptides were analyzed as follows [22]: The liberated peptides were separated from the vesicles on a Bio-Gel A-1.5m column (29 × 2 cm, equilibrated with standard buffer plus 100 mM KCl). Flow rate was 12 ml/h. The 30 min-fractions were analyzed for peptides at 220 nm, by liquid scintillation counting, and with the bicinchoninic acid assay. Contributions by light scattering and absorption were again corrected for. Peptides remaining associated with liposomes after proteinase digestion were analyzed after dialysis against water and lyophilization. They were taken up in formic acid/ethanol (1 : 2.8, v/v) and applied onto a Sephadex LH-60 column (68 × 2 cm). Flow rate was 7 ml/h. Fractions were analyzed for radioactivity. An aliquot of the radioactive pool containing low molecular weight peptides was analyzed by HPLC as described above.

Materials

Emulgen was a kind gift from Kao Atlas Chemicals, Tokyo, Japan. Egg PC, egg PE and bovine spinal cord PS, all Grade I, were purchased from Lipid Products, Nutfield, U.K. DPH, dansyl chloride, dabsyl chloride (particles insoluble in acetonitrile removed) and 7-hydroxycoumarin were from Fluka, Buchs, Switzerland. 7-Ethoxycoumarin was prepared from 7-hydroxycoumarin according to the method of Ullrich and Weber [19]. [^{14}C]Formaldehyde (15 mCi/mmol), and [^{3}H]acetic anhydride (500 mCi/mmol) were obtained from Amersham, U.K. Micropolyamide foils were supplied by Schleicher and Schuell GmbH, Dassel, F.R.G. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase and NADPH were from Boehringer, Mannheim, F.R.G. Trypsin was bought from Worthinton Biochemical Corporation, Freehold, NJ, U.S.A. Bio-Gel A-1.5m was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Sephadex LH-60 and Sepharose 4B were from Pharmacia, Uppsala, Sweden. Bicinchoninic acid came from Pierce, Rockford, IL, U.S.A. Benzphetamine was from Applied Science Laboratories, Inc., State College, PA, U.S.A.

Results

Reductive alkylation

Fig. 1 shows the time course of reductive alkylation of *P*-450 by formaldehyde in the presence of cyanoborohydride. The extent of modification was calculated from ^{14}C incorporation into the protein as determined by acid precipitation of the protein and filtration. Up to 6 nmol primary amino groups per nmol *P*-450 can be modified without heme loss or denaturation as analyzed by heme-CO spectroscopy. Alkylation for 18 h introduces 10.7 nmol formaldehyde per nmol *P*-450 and is accompanied by partial conversion to *P*-420. 7-Ethoxycoumarin (0.5 mM) or reductase (1 nmol/nmol *P*-450) do not influence the modification.

Alkylated *P*-450 (0–6 nmol formaldehyde per nmol protein) and native reductase were reconstituted into PC/PE/PS liposomes. The activity of this monooxygenase system towards benzphetamine and 7-ethoxycoumarin was measured against a control treated in

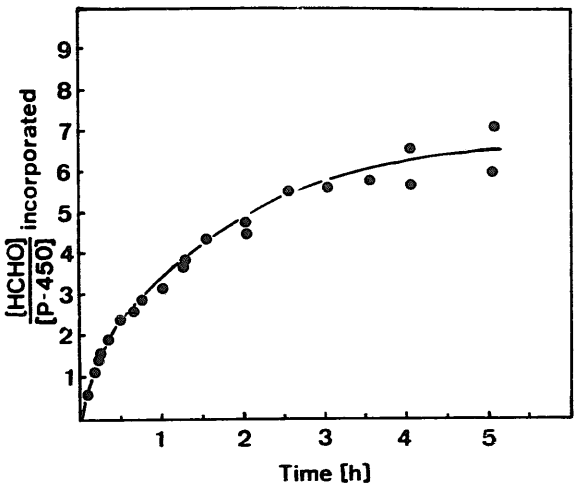

Fig. 1. Reductive alkylation of *P*-450. To the protein (1 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4), with 20% glycerol, 1 mM [^{14}C]formaldehyde was added at 25°C, followed by freshly prepared sodium cyanoborohydride (20 mM). Portions were removed at the indicated times, and radiolabel incorporation was measured after precipitation and filtration of the desalted samples.

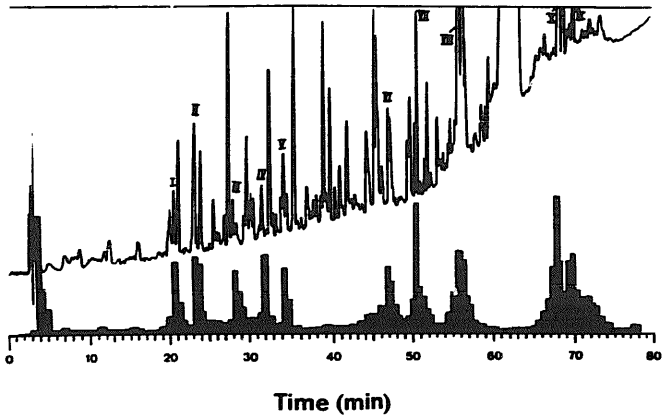

Fig. 3. HPLC of tryptic digest of 500 μg alkylated *P*-450 (6 nmol alkyl residues/nmol *P*-450). Peptide separation was performed on a RP8, 10 μm (5×250 mm) column at a flow rate of 1 ml/min at 60°C. Solvent A was 0.1% TFA, solvent B acetonitrile water/TFA (90:10:0.1). At 5 min after injection a gradient of 0–100% solvent B in 75 min was started. Peptides were recorded at 220 nm (upper part). Of each fraction collected at 30-s intervals 5% were analyzed for radioactivity (lower part).

the same manner without adding formaldehyde. Fig. 2 shows that the reactivity towards benzphetamine is unaltered whereas the dealkylation of 7-ethoxycoumarin is significantly inhibited. The inhibition does not correlate linearly with the extent of modification since one nmol of formaldehyde can be introduced without loss of reactivity.

P-450 modified by up to 5 nmol formaldehyde per nmol protein yields a full type I spectral change with benzphetamine. 7-Ethoxycoumarin-induced spectral changes are normal up to 2.4 nmol formaldehyde per nmol *P*-450. Further modification reduces them (not shown).

We have previously shown [16] that lysine-specific acetylation modifies about 2.8 residues. When incorporated into vesicles, the modified *P*-450 showed full activity and substrate-induced spectral changes with benzphetamine. With 7-ethoxycoumarin, neither enzymatic activity nor substrate-induced spectral changes were detected.

Separation of peptides by HPLC and assignment of modified peptides

Fig. 3 shows the elution of peptides derived from 500 μg *P*-450 with 6 nmol alkyl groups per nmol protein after trypsin digestion. Fig. 4 shows the analogous profile of *P*-450 modified with 3 nmol acetyl groups per nmol of protein. To identify the position of modified residues within the primary structure, fractions with labelled peptides were concentrated, re-chromatographed, and hydrolyzed. Since cleavage with 6 M HCl at 110°C for 18 h does not completely hydrolyze hydrophobic regions in a protein (Brunner, J., personal

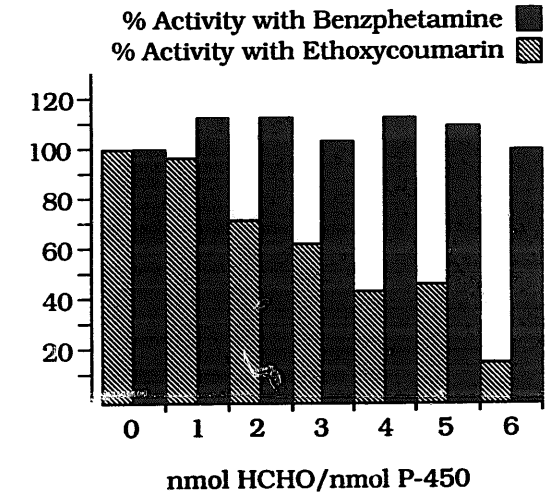

Fig. 2. Activity of *P*-450, alkylated at primary amino groups, towards benzphetamine and 7-ethoxycoumarin. For details, see Methods.

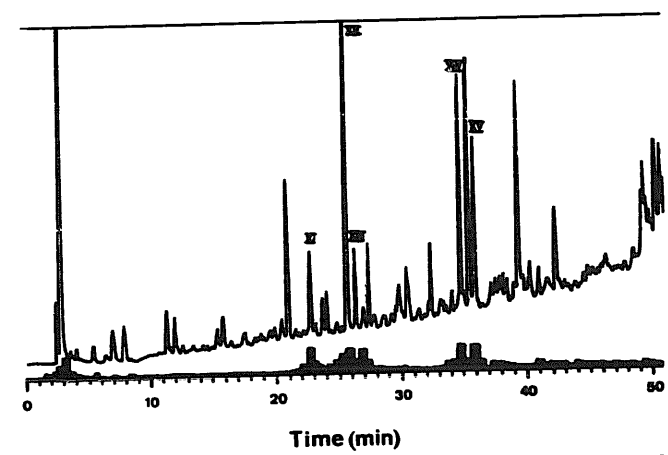

Fig. 4. HPLC of tryptic digest of 400 μg *P*-450 acetylated to 3 nmol label/nmol *P*-450. Conditions were the same as described in the legend to Fig. 3.

TABLE I

Amino acid composition of tryptic peptides alkylated at primary amino groups [a]

Amino acid (AA)	Peptide No.									
	I [b]	II	III	IV	V	VI	VII	VIII	IX	X
Asp + Asn	1.7 (2)	0.3	1.0 (1)	0.3	1.1 (1)	5.4 (6)	2.0 (2)	1.9 (2)	0.2	0.2
Glu + Gln	3.6 (3)	0.3	1.0 (1)	1.0 (1)	1.7 (1)	1.9 (2)	2.9 (3)	3.2 (4)	0.9 (1)	0.2
Ser						2.8 (3)	0.2	4.1 (5)	n.d.	1.1 (1)
Thr	0.5	0.5	0.7 (1)	0.8 (1)	1.1 (1)	0.3	0.3	2.7 (5)	0.9 (1)	0.3
Gly	1.0 (1)	0.3	1.0 (1)	0.9 (1)	1.7 (2)	1.2 (1)	1.0 (1)	1.3 (1)	1.1 (1)	1.3 (1)
Ala	0.3	1.1 (1)	0.4	0.8 (1)	0.6	2.8 (3)	0.4	1.3 (1)	1.3 (1)	0.1
Arg	1.1 (1)	2.0 (2)	0.5 (1)	1.0 (1)	1.0 (1)	0.3	1.3 (1)	0.9 (1)	1.2 (1)	0.9 (1)
Pro	n.d.	0.3	1.1 (2)	1.0 (1)	1.0 (1)	2.8 (3)	0.5	0.3	1.0 (1)	0.9 (1)
Val	0.6 (1)	0.7	0.5 (1)	0.2	1.3 (2)	0.2	0.8 (1)	0.7	2.4 (2)	0.2
Met	n.d.	n.d.	n.d.	0.8 (1)	0.1	n.d.	n.d.	0.9 (1)	1.1 (1)	n.d.
Ile	2.9 (3)	0.1	1.0 (1)	0.3	0.5	0.4	2.7 (3)	1.2 (1)	1.1 (1)	0.3
Leu	1.9 (2)	2.0 (1)	1.9 (2)	0.4	1.4 (1)	3.2 (3)	2.4 (2)	4.7 (5)	10.8 (10)	0.4
Phe	n.d.	0.6	0.4	1.6 (2)	0.8 (1)	2.9 (3)	0.2	2.5 (3)	0.9 (1)	0.2
Cys	n.d.	n.d.	0.2	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	1.7 (1)	0.2 (1)	0.6 (1)	0.2 (1)	0.4 (1)	1.2 (2)	n.d. (1)	1.0 (1)	n.d.	0.7 (1)
His	1.4 (2)	0.1	0.4	0.3	1.0 (1)	2.8 (3)	1.9 (2)	2.8 (4)	n.d.	1.1 (1)
Tyr	0.7 (1)	0.1	1.3 (2)	0.2	0.9 (1)	0.9 (1)	0.8 (1)	0.6	n.d.	0.2
No. of AA	17	6 [c,d]	14	12	14	30	17	34	21	6
Amino term.	Asx	n.d.	Gly	Ser	Glx	Ser	Asx	Glx	n.d.	Gly
Position [e]	237–253	121–125	379–392	423–434	60–73	393–422	237–253	275–308	1–21	22–27
AA modified	Lys-251	Lys-121	Lys-384	Lys-433	Lys-1	Lys-421	Lys-251	Lys-276	Met-1	Lys-25

[a] The numbers of each amino acid residues derived from the known sequence [26] is given in parenthesis. The alkylated lysine residues are resistant to hydrolysis [37] and are not identified.
[b] Peptides are numbered according to Fig. 3.
[c] Tryptophan is destroyed during hydrolysis.
[d] Arg-Arg bonds are slowly cleaved by trypsin.
[e] According to Fujii-Kuriyama et al. [26].
n.d., not determined.

communication), four different hydrolysis methods were compared in the present study (c.f. Methods). Hydrolysis with 6 M HCl containing 5% TFA in the gas phase gave the best results. All amino acid analyses of peptides were compared with an analysis of *P*-450 as a standard. In Table I the amino acid composition of tryptic peptides containing alkylated residues is shown, in Table II that of acetylated peptides. Each set of experiments, i.e., labelling of *P*-450, separation, re-chromatography of tryptic peptides and amino acid analysis was performed three times for alkylation and two times for acetylation. Alkylated or acetylated peptides eluted from the column at the same positions, indicating that the modifications altered the chromatographic behavior of the peptides in the same way.

The composition of the peptides allowed assignment within the sequence of *P*-450. Analysis of dansylated amino termini was taken as an additional proof of the peptides' identity (Tables I and II).

Time course of peptide modification

Incorporation of one nmol alkyl groups per nmol *P*-450 alters neither hydroxylation activity (c.f. Fig. 2) nor substrate binding. This indicated that one residue not involved in substrate binding is modified much faster than all the others. In order to test this possibility, *P*-450 was alkylated to different extents and cleaved with trypsin. Peptides were separated by HPLC, and the extent of modification of each peptide was calculated (Fig. 5). A maximum of one nmol label was found per nmol peptide. Alkylation for 15 min introduced 0.6 nmol alkyl residues into one nmol of the N-terminal peptide IX (c.f. Table I) and less than 0.2 nmol alkyl groups into the other peptides. Thus, the N-terminal methionine becomes alkylated much faster than the intra-chain lysines. The α-amino group is, therefore, not essential for substrate binding or interaction with the reductase (c.f. Fig. 2).

Proteolytic digestion of P-450 in proteoliposomes and peptide analysis

Modified *P*-450 was used to study the topology of the cytochrome in membranes. To this end, *P*-450 was alkylated at peptides I to X (c.f. Table I) and reconstituted into PC/PE/PS (10 : 5 : 1) liposomes. Successful reconstitution was verified by gel chromatography on Sepharose 4B column (Fig. 6). The buffer contained 100 mM KCl to prevent a possible superficial binding

TABLE II

Amino acid composition of tryptic peptides acetylated at lysines [a]

Amino acid (AA)	Peptide No.				
	XI [b]	XII	XIII	XIV	XV
Asp + Asn	1.1 (1)	1.0 (1)	0.9 (1)	1.0 (1)	1.0 (1)
Glu + Gln	0.1	0.8 (1)	0.1	1.8 (2)	0.9 (1)
Ser	0.1	0.3	0.1	0.8 (1)	0.3
Thr	0.1	0.8 (1)	2.2 (2)	0.8 (1)	0.7 (1)
Gly	1.8 (2)	1.9 (2)	0.1	0.2	1.8 (2)
Ala	0.1	0.2	0.1	0.8 (1)	0.3
Arg	1.2 (1)	1.0 (1)	1.2 (1)	0.9 (1)	1.1 (1)
Pro	0.2	1.2 (1)	0.1	0.8 (1)	1.4 (1)
Val	0.1	1.8 (2)	1.4 (1)	0.7 (1)	2.1 (2)
Met	1.1 (1)	n.d.	1.4 (1)	0.8 (1)	n.d.
Ile	0.1	0.5	0.1	1.5 (2)	0.6
Leu	0.1	1.2 (1)	0.2	0.6	1.3 (1)
Phe	1.3 (1)	0.8 (1)	1.1 (1)	0.1	0.9 (1)
Cys	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	1.1 (1)	0.7 (1)	1.0 (1)	0.8 (1)	0.8 (1)
His	n.d.	0.8 (1)	n.d.	0.7 (1)	0.9 (1)
Tyr	n.d.	0.6 (1)	n.d.	0.8 (1)	0.7 (1)
No. of AA	7	14	8	15	16
Amino term.	Asx	Glx	Val	Ser	Glx
Position [c]	134–140	60–73	371–378	344–368	60–73
AA modified	Lys-139	Lys-61	Lys-373	Lys-345	Lys-61

[a] The numbers of each amino acid residues derived from the known sequence [26] are given in parenthesis. The acetylated lysine residues are hydrolyzed and are identified as lysine residues.

[b] Peptides are numbered according to Fig. 4.

[c] According to Fujii-Kuriyama et al. [26].

n.d., not determined.

of some *P*-450 to the liposomes. The protein co-migrated with the liposomal fractions as revealed by DPH fluorescence intensity measurements and liquid scintillation counting, whereas free *P*-450 eluted later as shown by absorption measurements at 220 nm. Proteoliposomes were then subjected to exhaustive proteolysis [22]. To ascertain their intactness during the protease treatment use was made of the impermeability of intact liposomes to ascorbate at 0 °C [23]. For this, the spin label 4-*N*, *N*-dimethyl-*N*-hexadecyl-ammonium-2,2,6,6-tetramethyl-piperidine-1-oxyl iodide was co-reconstituted into *P*-

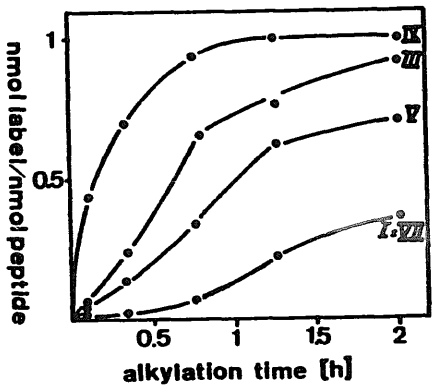

Fig. 5. Time course of peptide modification. Roman numbers correspond to those of Fig. 3 and Table I. The five peptidees not shown behaved like peptide V.

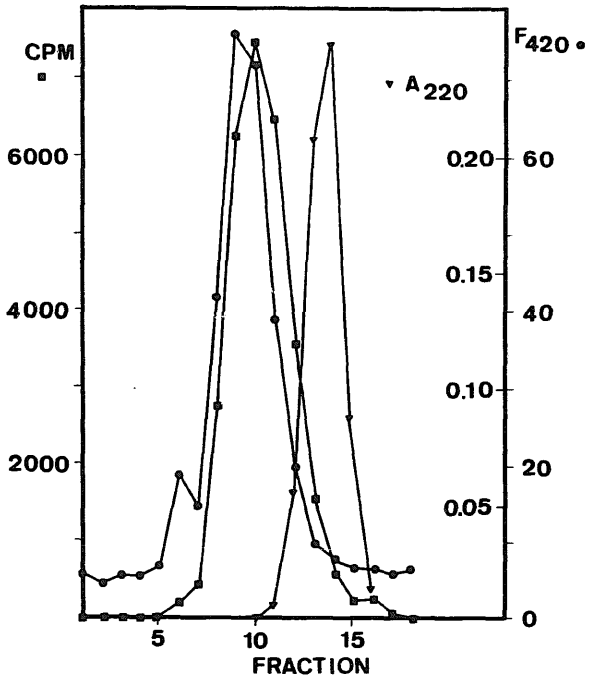

Fig. 6. Incorporation of alkylated *P*-450 into liposomes. Alkylated *P*-450 was reconstituted into liposomes as described in Methods and analyzed on a Sepharose 4B column by DPH fluorescence intensity (●) measurements (λ_{ex} = 357 nm; λ_{em} = 420 nm) and liquid scintillation counting (■). All protein was recovered in the liposomal fractions. The elution peak for *P*-450 in solution measured spectroscopically at 220 nm (▼) is also shown.

450 proteoliposomes (lipid/spin label = 100 : 1). Ascorbic acid (25 mM) reduced only the nitroxide label facing the outside of both the control and proteinase-treated proteoliposomes indicating their integrity. Further proof of the intactness of the liposomal membrane bilayer came from the finding that the fluorescence

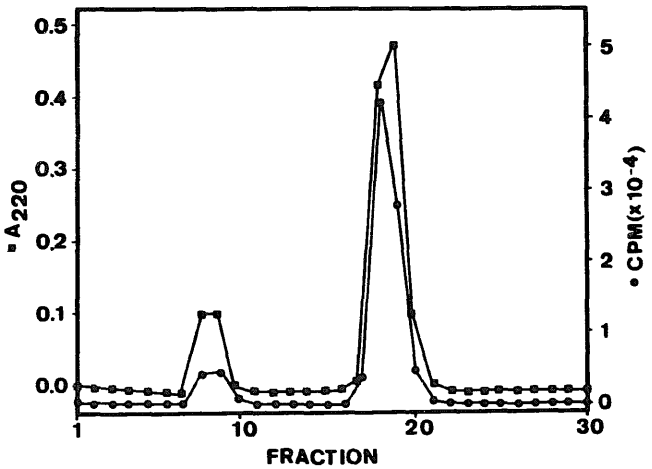

Fig. 7. Separation of trypsin-liberated peptides from liposomes. Trypsin-digested proteoliposomes were applied to a Bio-Gel A-1.5m column equilibrated with standard buffer plus 100 mM KCl. The fractions were analyzed for peptides at 220 nm and for radioactivity. Fractions 8 to 10 contain liposomes with peptides and protein which are not released by trypsin. The second pool (fractions 17–21) contains trypsin-liberated peptides (M_r < 10000).

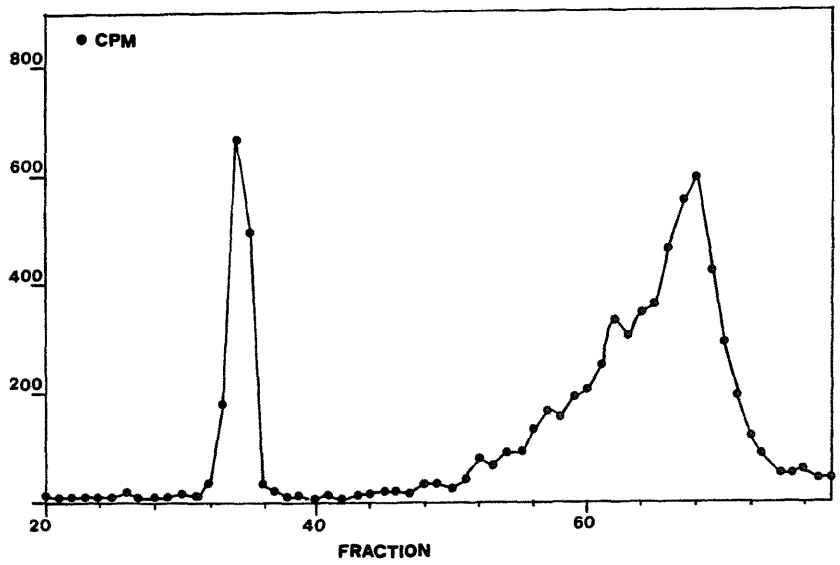

Fig. 8. Analysis of liposomes-associated peptide after proteolysis. The material associated with liposomes after trypsin-treatment (fractions 8 to 10 in Fig. 7) was analyzed on a Sephadex LH-60 column. The first peak contains undigested *P*-450 as revealed by SDS-polyacrylamide gel electrophoresis. The molecular weight of the second peak was estimated to be 3000.

anisotropy of DPH in proteinase-treated proteoliposomes was 0.115 at room temperature, a value very similar to the one reported previously for proteoliposomes containing *P*-450 [21]. Comparison of this r_s value with the one of protein-free liposomes (0.103) [21] gives additional proof that *P*-450 does not simple stick to the bilayer surface but penetrates the membrane, thereby disturbing the order of the fatty acyl chains.

Three different approaches were used to determine the extra-membranous parts of the protein: (1) Proteoliposomes containing labelled cytochrome were centrifuged after proteolysis. About 6% of the radioactivity remained associated with the vesicles, whereas about 84% was recovered from the supernatant. (2) Proteinase-treated proteoliposomes were analyzed by SDS-polyacrylamide (12%) gel electrophoresis. All peptides migrate with the front, i.e., had a M_r smaller than 10000, whereas about 7% of the protein was undigested as shown by scanning densitometry of the gel. (3) The peptides liberated by trypsin were separated from the vesicle-associated peptides and protein by gel chromatography on a Bio-Gel A-1.5m column (Fig. 7). 10–15% of the total protein mass was found in the void volume while the remaining eluted in a single peak of material of small molecular weight. 10% of the radioactivity remained associated with the membrane, indicating that most of the labelled peptides are accessible to trypsin attack.

To identify the membrane-associated labelled peptide(s) in proteinase-treated proteoliposomes, the material remaining associated with liposomes after proteinase digestion was analyzed on a LH-60 column (Fig. 8). It gave a single sharp peak in the void volume corresponding to the intact protein as shown by SDS-polyacrylamide gel electrophoresis, and a well resolved broad peak of material with a M_r of about 3000. It was subjected to HPLC analysis (Fig. 9). Most of the radioactivity is associated with peptide IX, the hydrophobic segment next to the N-terminus in *P*-450 (c.f. Fig. 3 and Table I).

Discussion

The *P*-450IIB1 isozyme used in this study was isolated essentially according to Waxman and Walsh [14]. The alkylated peptides No. I and VII despite their different retention times during HPLC apparently have the same amino acid composition and N-terminus (Ta-

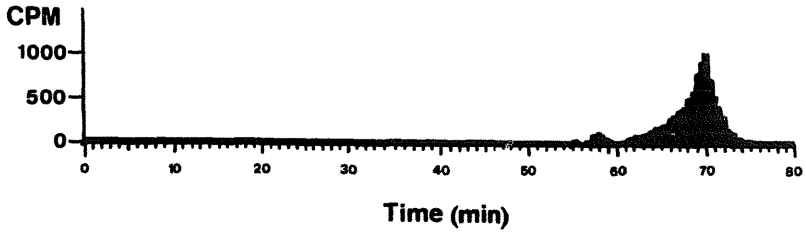

Fig. 9. Identification of membrane-associated labelled peptide after trypsin-treatment of proteoliposomes. Low molecular weight peptide(s) from the LH-60 column (c.f. Fig. 8) were analyzed by HPLC as described in Fig. 3.

ble I). The same holds true for the acetylated peptides No. XII and XV (Table II). This difference may be attributed to a modification of at least one of the peptides of each pair, e.g., double labelling, or microheterogeneity of the *P*-450 preparation. The former is unlikely, since in each case one nmol label per nmol peptide is found. As to the latter, the *P*-450IIB1 gene family has been reported to contain different isozymes which could be separated by isoelectric focusing [24,25]. This microheterogeneity could be due to slightly different sequences or posttranslational modifications. Indeed, the peptides contain a tyrosine residue, a possible site for phosphorylation. The putative posttranslational modification would be labile in 6 M HCl since no modified amino acid is found after hydrolysis of the peptides. However, the method used in this study, i.e., amino acid analysis in combination with determination of the N-terminus allows the identification of a peptide within a known sequence but is not appropriate for more detailed investigations.

According to the published sequence [26], residue 392 should be a leucine. In our protein it is replaced by an arginine (Table I), the presence of which gives rise to peptides III and VI. This pecularity may originate from the strain used in this study.

Chemical modification of cytochrome P-450

In the course of this study, 11 out of 24 lysines were modified by acetylation or reductive alkylation. Modification of *P*-450 affects the binding and metabolism of 7-ethoxycoumarin but not of benzphetamine. This argues against a decreased rate of electron transfer due to the modified amino groups. The different sensitivities of the substrates to modification of *P*-450 indicates different modes of substrate binding. It would, therefore, be of interest to examine the possible inhibitory action of one substrate to the other in the native and modified enzyme. According to Bernhardt et al. [9] the binding of the rather bulky, negatively charged fluorescein isothiocyanate to the amino terminus and to Lys-384 of rabbit cytochrome *P*-450IIB1 leads to steric hindrance of groups involved in the interaction of *P*-450 and the reductase or blocks charge pair interactions. In our study, the introduction of a small electroneutral group into the N-terminus and Lys-384, corresponding to Lys-384 in rabbit *P*-450IIB1, did not alter the benzphetamine-*N*-demethylase activity. Therefore, these two residues do not seem to be directly involved in the interaction of *P*-450 with the reductase. Some of the alkylated residues, the N-terminal methionine not included, seem to be necessary for proper binding of ethoxycoumarin to the cytochrome. On the other hand, at least one of the acetylated lysines is necessary for ethoxycoumarin binding. Its identity is presently uncertain. Lys-139 is situated close to the highly conserved region near the N-terminus of *P*-450. This region lies in a hydrophilic domain composed of two strains of α-helices [27], the modified lysine being situated between them. It is not very likely that the area of 7-ethoxycoumarin binding is hydrophilic and highly conserved among various *P*-450 species some of them not being involved in the metabolism of this compound. It seems, therefore, unlikely that Lys-139 is important for 7-ethoxycoumarin binding. The most likely candidates are Lys-345 and/or Lys-373. They both lie near the heme-binding cysteine. This domain contains a hydrophobic region probably shielded from the surrounding water by charged amino acids. A hydrophobic region within a hydrophilic domain would be a good candidate for substrate binding. In the vicinity of these two acetylated lysines, lysines in position 384, 421, and 433 become alkylated (see below). This might explain why 7-ethoxycoumarin binding and hydroxylation is highly sensitive to alkylation.

P-450 topology in membranes

Based on sequencing studies, Heinemann and Ozols [28] and Tarr et al. [29] predicted secondary structures and proposed that eight or nine hydrophobic regions of *P*-450 span the membrane. In the last few years, site-specific antibodies [30] and chimeric proteins [31,32,33] were used to probe the topology of *P*-450 in the microsomal membrane. It was proposed that *P*-450 is largely exposed on the cytoplasmic surface of the microsomal membrane to which it is anchored by the N-terminal segment. Using proteolysis, we have recently analyzed the membrane topology of *P*-450IIB1 in liposomes and concluded that *P*-450 is anchored to the membrane only via the N-terminal peptide comprising amino acid residues 1 to 21, the N-terminal methionine facing the lumenal interior [22]. Brown and Black [34] came to similar conclusions using proteolysis in microsomes. They could, however, not decide if one or two peptides span the membrane. Here we use the alkylated enzyme for topological studies. The modified *P*-450 fully retains its benzphetamine-*N*-demethylase activity, suggesting that its tertiary structure is not significantly changed by the alkylation. In addition, modified amino acids are charged and therefore unlikely to be situated in the membrane bilayer. We therefore incorporated alkylated *P*-450 in liposomes and used this system as a model to analyze the membrane topology of the protein. We show that after exhaustive proteolytic digestion of modified *P*-450 reconstituted into proteoliposomes 10 to 15% of the protein's mass and about 10% of the radioactivity remain associated with the membranes. About 7% of the protein's mass is accounted for by undigested *P*-450 which most likely represents the small proportion of molecules which are reconstituted in opposite direction and face the liposomal interior. The fact that this population of *P*-450 is not cleaved by the proteinase gives further evidence that most of the protein's mass

protudes on one side of the membrane. The other 3 to 8% correspond largely to the hydrophobic membrane-spanning segment immediately following the N-terminus (amino acid residues 1 to 21). The peptides containing Lys-25, -61, -122, -251, -276, -384, -421, and -433 must, therefore, lie on the outer side of the liposomes. Lys-384, -421, and -433 are located near Cys-436 which binds the heme iron [35]. These findings, therefore, support the proposed localization of the active site of *P*-450 in the cytosolic space [21]. The fact that Lys-25 and Lys-61 are accessable to proteinase is highly important for correctly assigning the localization of the N-terminal domain since it shows that the second α-helix of *P*-450 (amino acid residues 30 to 46) cannot span the bilayer. This domain must be located on the cytosolic side of the membrane, ruling out models in which the protein penetrates the bilayer twice (amino acid residues 1 to 61) and in which the N-terminal methionine faces the cytosol [36]. Finally, when the proteolytic digest of proteoliposomes was analyzed by HPLC without any previous separation on the Bio-Gel column, the radioactivity elution profile obtained by HPLC was the same as for the modified protein digested in solution (c.f. Fig. 3). This shows that trypsin cleaves *P*-450 in the same way when the protein is in solution or in liposomes, suggesting that, in proteoliposomes, most of the protein's mass is situated in the extravesicular space.

These results further support our recently proposed topology model of microsomal *P*-450 [22] in which the N-terminal hydrophobic segment comprising amino acid residues number 3–21 spans the membrane with the N-terminus facing the liposomal interior.

Acknowledgements

This work was supported by a very generous financial help of Solco Basel AG, and by the Schweizerischer Nationalfonds, grants 3.199-085 and 3.471-086. We thank Dr. H. Hauser for help with the spin label experiment.

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