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Localization of the N-terminal methionine of rat liver cytochrome *P*-450 in the lumen of the endoplasmic reticulum

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Recent cumulative evidence suggests that liver microsomal cytochrome *P*-450 (*P*-450) is exposed to the cytosol with the exception of the N-terminal peptide (amino acid residues 1 to 21), or two peptides (residues 1 to 60). We tested the localization of the N-terminal methionine residue of *P*-450IIB1 of rat liver microsomes in the natural membrane with the site-specific reagent fluorescein isothiocyanate. The N-terminus of isolated *P*-450 was stoichiometrically modified in solution with fluorescein isothiocyanate. In intact microsomes, the N-terminus was not modified but became accessible to the reagent when the membrane was dissolved with Triton X-100. Our results indicate that the N-terminus faces the lumen of the endoplasmic reticulum, and we propose that *P*-450 spans the membrane only once with amino acid residues 1 to 21.

Introduction

The microsomal monooxygenase system comprises several membrane-bound proteins. The membrane topology of cytochrome *P*-450 (*P*-450), the terminal and key enzyme of the microsomal monooxygenase system, is not known with certainty. Models in which a large part of the protein (6 to 10 transmembrane segments) is imbedded in the bilayer were proposed on the basis of hydrophobicity calculations when the amino acid sequence became known [1–4]. More recent data suggested that the main mass of *P*-450 is extra-membranous and directed towards the cytoplasm, the protein spanning the membrane only once or twice. Sakaguchi et al. [5], Monier et al. [6], and Szczesna-Skorupa et al. [7] constructed chimeric proteins to test the insertion of *P*-450 in microsomal membranes. The N-terminal hydrophobic peptide was found to function as a combined insertion-top transfer signal. Evidence was obtained which suggests that the signal peptide spans the membrane. It first enters in a loop configuration and then reorients in a way that the N-terminus is translocated into the lumen. In a different appraoch, De Lemos-Chiarandini et al. [8] used site-specific antibodies directed against rat liver *P*-450IIB1 **. In microsomes, the antibodies recognized all domains of the enzyme except the N-terminal segment (amino acid 1 to 31) and possibly the domain formed by amino acid residues 168 to 185. Structural considerations led Uvarov et al. [9] to conclude that only one N-terminal α-helix incorporates into the membrane. Finally, trypsinolysis studies with rabbit liver microsomes suggested that *P*-450 is bound to the membrane by one or two transmembrane segments, located at the N-terminus [10]. In contrast to these recent results, Nelson and Strobel [11,12] proposed, based mainly on hydrophobicity profiles, that the protein spans the membrane twice, the N-terminal methionine facing the cytosol. Taken that *P*-450 protrudes into the cytosol except with its one or two N-terminal peptide(s) it is possible to determine the true number of membrane-spanning peptides by determining the localization of the N-terminus: In the case of one membrane-spanning peptide, the N-terminus must be

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Abbreviations: FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; kDa, kilodaltons; KP_i, potassium phosphate; *P*-450, cytochrome *P*-450; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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

located at the luminal side of the membrane; if, however, two peptides span the membrane, the N-terminus must face the cytosol. Thermodynamic considerations make the third possibility, namely a localization of the N-terminus in the membrane's lipid bilayer, highly unlikely since cytochrome *P*-450IIB1 is charged at the first two N-terminal amino acid residues. In fact, no one of the above authors propose such a model.

P-450 in solution can be selectively and stoichiometrically modified at the N-terminal amino group with fluorescein isothiocyanate (FITC) [13,14]. Being negatively charged FITC does not penetrate the microsomal [15,16] nor the liposomal membrane [17], and also does not react with phospholipids which could conceivably compete with the protein's amino terminus for FITC [17]. It is thus a useful tool to analyze the localization of the *P*-450 N-terminal methionine in membranes. In fact, we have found that FITC does not react with *P*-450 when the protein is incorporated into liposomes [17] and have therefore concluded that the methionine faces the liposomal vesicles' interior.

Here we describe the reactivity of FITC towards *P*-450 in the natural membrane. We show that the protein is not accessible to FITC in microsomes. FITC can, however, modify the N-terminus when the membrane is dissolved with Triton X-100. From this it appears that the N-terminal methionine of native *P*-450 faces the lumen of the endoplasmic reticulum.

Materials and Methods

Preparation of microsomes. Male Sprague–Dawley rats (150 g) were given 0.1% phenobarbital in their drinking water for 8 days to induce *P*-450. Microsomes were prepared by a standard method [18]. They were suspended in 50 mM potassium phosphate (KP_i), 20% glycerol, 0.1 mM EDTA (pH 7.4), at a concentration of about 40 mg/ml and stored in aliquots at −80 °C. Protein concentration was determined by the Lowry assay using bovine serum albumin as standard.

Enzyme purification. *P*-450IIB1 was purified in an active form by the method of Waxman and Walsh [19] except that Emulgen was removed by detergent exchange while the cytochrome was bound to the hydroxylapatite column. The specific heme content (14 nmol/mg protein) was determined from the CO-reduced difference spectrum as described by Omura and Sato [20].

Modification of P-450 with FITC. *P*-450 in standard buffer (50 mM KP_i, 20% glycerol (pH 7.4)) was modified according to the procedure of Bernhardt et al. [13]. The reaction mixture contained a 35-fold molar excess of FITC (0.7 mM) over *P*-450. The protein concentration was 1 mg/ml. After 2 h at 4 °C the reaction was stopped by removal of free FITC by microcentrifuge filtration on Sephadex G-25 as described by Helmerhorst and Stokes [21]. The degree of labeling was determined using an absorption coefficient ϵ_{496nm} of $7.45 \cdot 10^4\ M^{-1}\,cm^{-1}$ [13].

To test the specificity of the reaction of FITC with the N-terminus, 200 μg of FITC-modified *P*-450 was dialyzed against 50 mM ammonium bicarbonate (pH 8.0) and digested with trypsin (bovine pancreas) (1 μg per 50 μg *P*-450 at time 0 and after 2.5h) at 37 °C over night in 200 μl standard buffer. The tryptic peptides were separated by HPLC on a 250 × 5 mm Nucleosil 300-10 C_{18} column. They 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. A linear gradient from 100% solvent A to 90% solvent B (0.1% TFA in acetonitrile) was applied within 60 min. Peptides were detected at 220 nm. The absorption of FITC in water/acetonitrile/TFA is strongly blue shifted, so that the chromophore was detected at 435 nm. In a different approach, the protein was dansylated as described by Gray [22], hydrolyzed and analyzed for dansyl methionine by HPLC.

For the analysis by gel electrophoresis, 150 μl sodium dodecyl sulfate (SDS) sample buffer (0.5 M sodium phosphate, (pH 7.0), 1.2 ml; 1.5 g SDS; 30 mg dithiothreitol; 10 mg Bromophenol blue; at 20 ml with water) was added to 300 μg (300 μl) modified protein and the sample analyzed on a 12% polyacrylamide gel [23]. The extent of FITC modification was visualized fluorimetrically (excitation at 355 nm) followed by protein staining with Coomassie blue. To test the effect of Triton X-100 on *P*-450 modification by FITC, the same procedure was followed except that Triton (stock solution: 1% in standard buffer) was added to a final concentration of 1 mg/ml.

Modification with FITC in microsomes. Microsomes were suspended in standard buffer at a final concentration of 1 mg protein per ml (total volume: 18 ml) and incubated with 0.7 mM FITC at 4 °C for 2 h.

Partial purification of P-450 after modification with FITC. *P*-450 was enriched from microsomes at 4 °C by an adaptation of the method of West et al. (1979). To remove unreacted FITC, the solution (18 ml) was distributed in six polycarbonate tubes and centrifuged at 70000 rpm for 5 min at 4 °C in a TLA-100.3 fixed angle rotor (Beckman). The yellow supernatant was replaced by standard buffer. The pellets were then suspended with a small hand homogenizer and centrifuged again. This washing procedure was repeated to obtain a colorless supernatant. 150 μl standard buffer and 45 μl sodium cholate solution (20% w/v in buffer) were added to each pellet (3 mg protein), and vortexed to dissolve the membranes. The samples were pooled in one tube (1.25 ml) and 274 μl poly(ethylene glycol) (PEG) 6000 solution (50% w/v in water) was added to reach a 9% PEG concentration. After 15 min the sample was centrifuged as above. To the supernatant (1.5 ml),

308 μl 50% PEG solution was then added to reach a PEG concentration of 16%. After 15 min the sample was centrifuged again. The pellet, which contained the enriched *P*-450, was dissolved in 200 μl standard buffer plus 100 μl SDS sample buffer and analyzed on a 12% polyacrylamide gel as above.

Modification of microsomes in the presence of Triton X-100. The conditions were the same as above except that the 1 ml solutions contained Triton X-100 (1% stock solution in standard buffer) with concentrations ranging from 0 to 5 mg/ml. The reaction was started by adding microsomes (1 mg protein in 24 μl), and stopped with 150 μl of ice-old TCA (80% w/v in water). The proteins were then pelleted in Eppendorf tubes at 12000 × *g* for 10 min. After removal of the supernatant, the pellet was washed thoroughly with ice-old ethanol to remove traces of TCA and FITC. The pellet was dissolved by sonification (approx. 45 min) in 160 μl standard buffer plus 90 μl SDS sample buffer and analyzed on a 12% polyacrylamide gel.

Effect of Triton X-100 on the structure of microsomes. Solubilization of microsomes was followed by light scattering measurements at 500 nm. Microsomal protein concentration was 1 mg/ml. Triton concentrations ranged from 0 to 5 mg/ml.

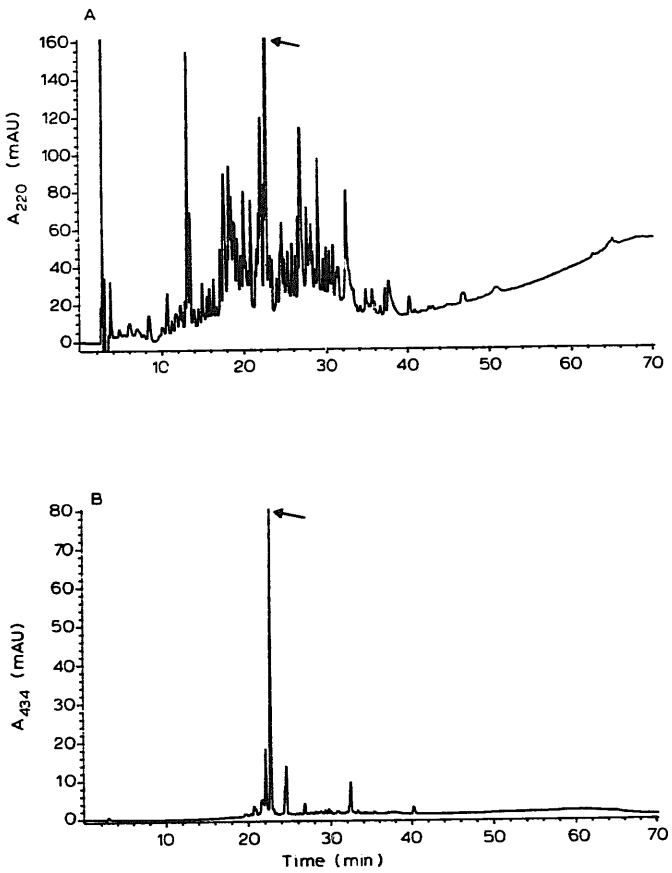

Fig. 1. HPLC of tryptic digest of FITC-modified *P*-450. *P*-450 modified with FITC was exhaustively digested with trypsin. Peptides (200 μg) were separated by reverse phase HPLC. Panel A: Absorption at 220 nm; panel B: Absorption at 435 nm.

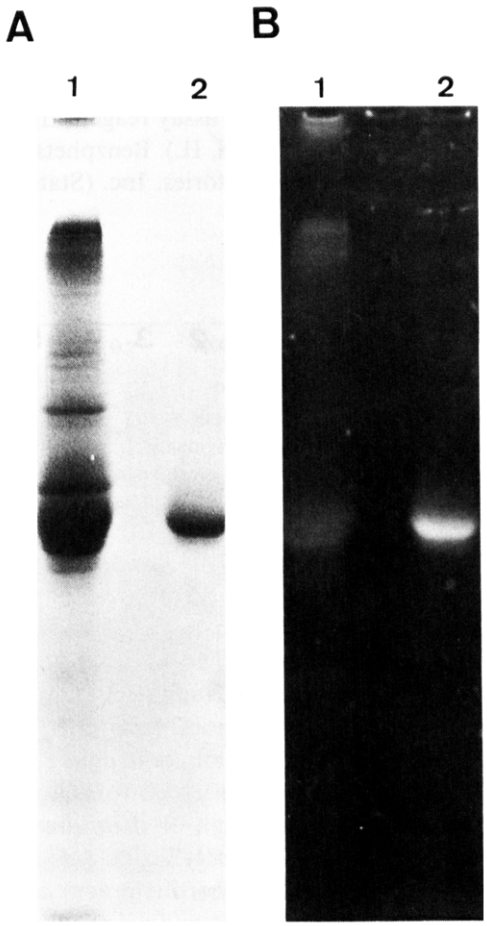

Fig. 2. SDS-polyacrylamide gel electrophoresis of *P*-450 modified in solution and in microsomes. Microsomes (1 mg protein/ml) were incubated with FITC (0.7 mM) at 4°C for 2 h and *P*-450 was enriched by a 9 to 16% PEG 6000 cut. 10 μg *P*-450 as determined from the CO-reduced difference spectrum was analyzed on a 12% gel (lane 1). 1 mg/ml *P*-450 was modified under the same conditions and 10 μg was analyzed on the gel (lane 2). Panel A: Coomassie blue staining. Panel 13: FITC fluorescence, excited at 355 nm and recorded through a yellow filter.

Effect of Triton X-100 on P-450 hydroxylation activity. 7-Ethoxycoumarin *O*-dealkylation was determined fluorimetrically according to Ullrich and Weber [25]. The reaction mixture contained 2.4 ml microsomal solution (1 mg protein per ml standard buffer) with Triton concentrations ranging from 0 to 5 mg/ml. 5 μl glucose 6-phosphate (500 mM stock solution in standard buffer), 5 μl glucose 6-phosphate dehydrogenase (1 mg/ml), and 200 μl 7-ethoxycoumarin (2 mg/2.5 ml standard buffer) (prepared as described in Ref. 25) were added. The reaction was started with 5 μl NADPH (200 mM stock solution in standard buffer). Umbelliferone (10 μl from a 1 mM stock solution in ethanol) was added to the solution as standard after 15 min. Fluorescence was recorded at 455 nm with excitation at 370 nm. Benzphetamine hydroxylation was determined according to Gut et al. [37].

Materials

Fluorescein isothiocyanate (isomer I) was from Fluka (Buchs, CH), Triton X-100 from Serva (Heidelberg, F.R.G.), and the BCA protein assay reagent from Pierce Chemical Company (Rockford, IL). Benzphetamine was from Applied Science Laboratories, Inc. (State College, PA). Emulgen 911 was a kind gift from Kao Atlas Chemicals (Tokyo, Japan). High-performance liquid chromatography (HPLC) was done in a Hewlett-Packard 1090 Liquid Chromatograph. Fluorescence measurements were carried out with an Aminco SPF-500 fluorimeter (American Instrument Company, Silver Springs,

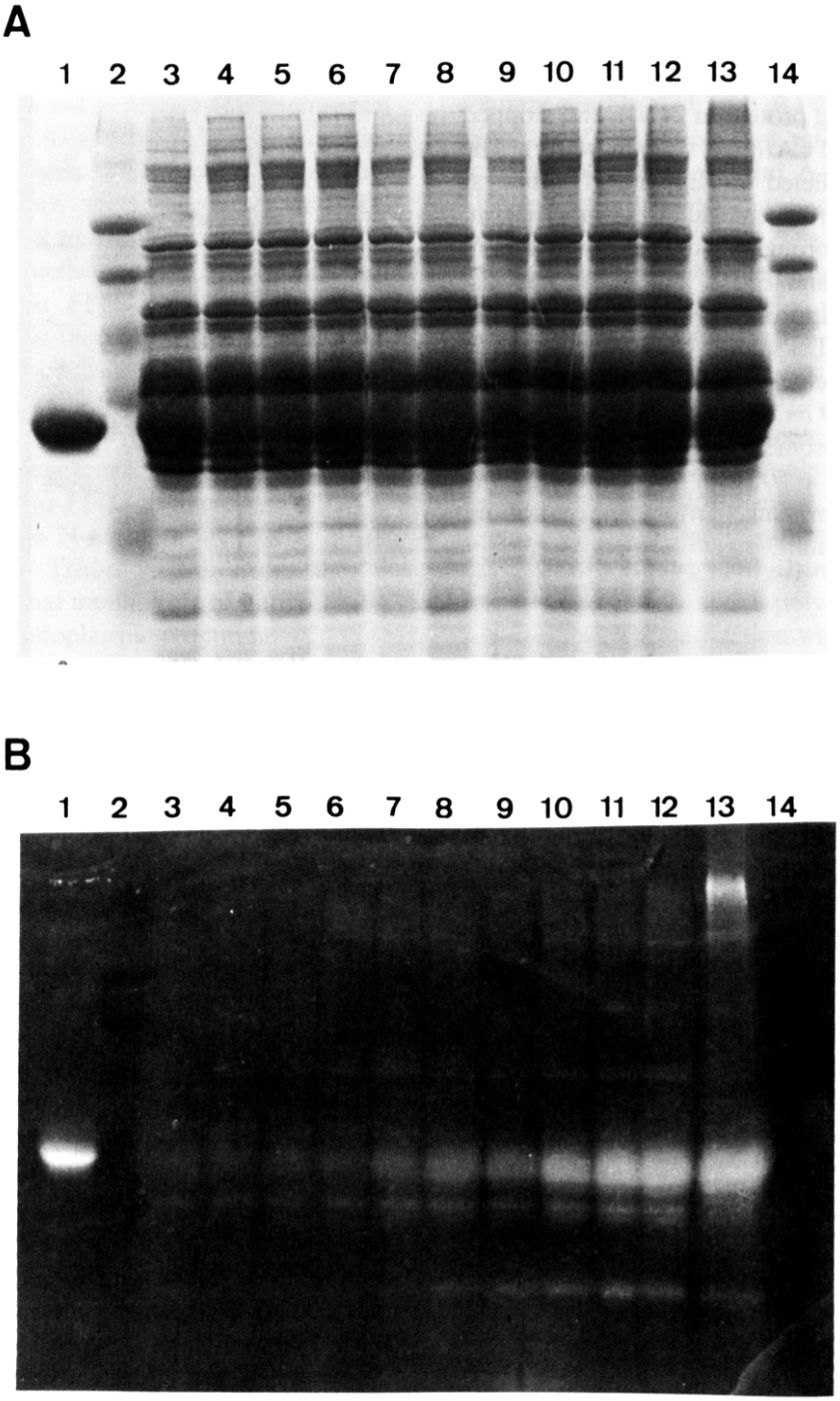

Fig. 3. Effect of Triton X-100 on modification of microsomal *P*-450 by FITC. Microsomes (1 mg protein/ml) were incubated with 0.7 mM FITC at 4°C for 2 h at various Triton concentrations. The reaction was stopped by addition of ice-cold TCA, followed by centrifugation of the protein to remove unreacted FITC. The detergent concentration ranged from 0 to 5 mg/ml as follows: 0; 0.05; 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 5.0 mg/ml for lanes 3 to 13, respectively. Lane 1 contains as a reference 10 μg *P*-450 modified in solution. Lanes 2 and 14 contain prestained molecular weight markers of 36.5, 48.5, 58, 84, 116 kDa. Panel A: Coomassie blue staining. Panel B: FITC fluorescence, excited at 355 nm and recorded through a yellow filter.

MD). Centrifugation was done with a TL-100 Ultracentrifuge from Beckman Instruments (Palo Alto, CA).

Results

Rat liver microsomal *P*-450IIB1 was modified stoichiometrically (1.1 ± 0.1 nmol FITC per nmol *P*-450) in solution. Fig. 1 shows HPLC patterns of a tryptic digest of the modified protein with detection of peptides at 220 nm (panel A). Analysis at 435 nm (panel B) reveals the presence of one major modified peptide (arrows) and several minor peaks, in keeping with the specificity of the modification reported by Bernhardt et al. [13,14]. The peak eluting at 40.2 min was caused by heme (absorption maximum around 400 nm). The other peaks may be due to incompletely hydrolyzed peptides, minor modifications of reactive groups such as lysine or cysteine residues, or reagent impurities (for example FITC isomer II) present in the commercially available compound as shown by reverse phase HPLC. The modified protein was also dansylated, hydrolyzed, and the amino acids were analyzed by reverse phase HPLC (not shown). Dansyl methionine was detected with unmodified but not with FITC-modified *P*-450, showing that the N-terminal methionine was blocked by reaction with FITC.

Fig. 2 shows the extent of modification by FITC as measured by fluorescence. Lane 1 contains *P*-450 (10 μg as judged from the heme content of 4.2 nmol per mg protein) enriched from microsomes by PEG precipitation after exposure to FITC. Lane 2 contains 10 μg of the purified protein which had been exposed to FITC in solution. Clearly, *P*-450 is heavily modified by FITC in solution but not when membrane-bound. The background fluorescence of the membrane-bound protein may be due to leakiness of some microsomal vesicles and/or minor modifications of reactive groups such as lysine or cysteine residues.

To further test whether the intact membrane renders the N-terminus of *P*-450 inaccessible to FITC, we analyzed the effect of the detergent Triton X-100 on the extent of *P*-450 modification in microsomes (Fig. 3). Lane 1 shows a *P*-450 standard modified in solution with FITC. Lanes 3 to 13 contain equal amounts of proteins of microsomes modified with FITC in the presence of increasing concentrations of Triton (0 to 5 mg/ml). In the Coomassie blue-stained gel (panel A), microsomal *P*-450 clearly shows up as the main band which migrates as the *P*-450 standard. The fluorescence pattern (panel B) reveals very little modification of *P*-450 at low Triton concentrations (lanes 3 to 6; 0 to 0.2 mg/ml) as compared with the protein modified in solution. At higher Triton concentrations (lane 7; 0.3 mg/ml) a fluorescent band, comigrating with *P*-450, appears the intensity of which increases dramatically between 0.3 and 0.6 mg Triton/ml (lanes 7 to 10). Higher Triton concentrations do not result in a significant augmentation of the modification. Lane 13, being the last on the gel, is broader and the apparent higher fluorescence is therefore an artifact. To be able to document a good fluorescence signal photographically, relatively high amounts of protein (200 μg) were applied to the gel. However, fluorescence could be detected by eye already with 40 μg. Under these conditions, it was evident that only *P*-450IIB1, well separated from other proteins, was modified by FITC at higher Triton concentrations (not shown). To exclude a possible effect of Triton on *P*-450 reactivity due to a change in structure, the protein in solution was incubated with FITC in the absence and in the presence of Triton (1 mg/ml).

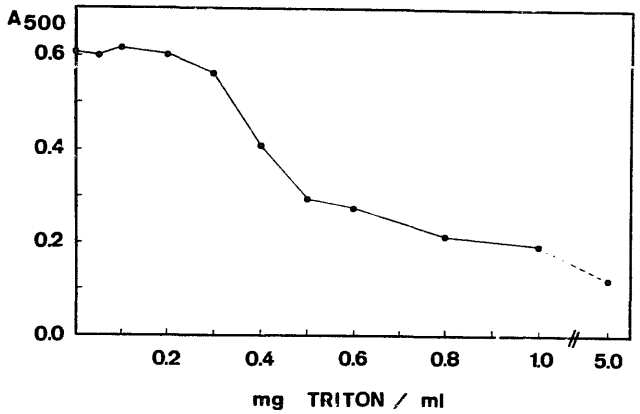

Fig. 4. Effect of Triton X-100 on microsomal membrane integrity. Microsomes (1 mg protein/ml) were incubated with various Triton concentrations ranging from 0 to 5 mg/ml at 4°C for 20 min. Light scattering was measured at 500 nm as a function of the detergent concentration.

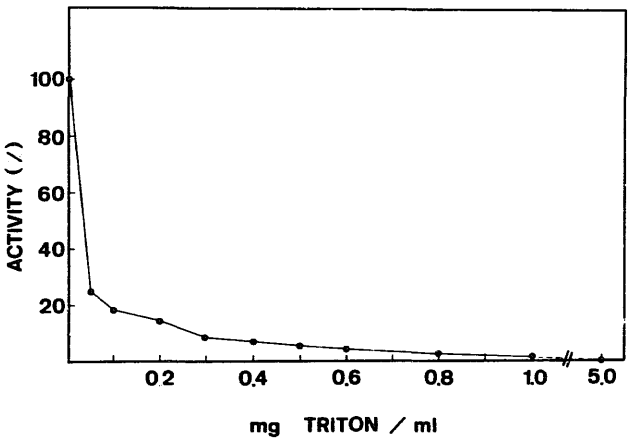

Fig. 5. Effect of Triton X-100 on *P*-450 activity. To 2.4 ml microsomes (1 mg protein/ml), containing various Triton concentrations ranging from 0 5 mg/ml, 5 μl glucose 6-phosphate (500 mM), 5 μl glucose 6-phosphate dehydrogenase (1 mg/ml), 200 μl 7-ethoxycoumarin (2 mg/2.5 ml) were added. The reaction was started by the addition of 5 μl NADPH (200 mM). Fluorescence was recorded at 455 nm with excitation at 370 nm. The *O*-dealkylation of 7-ethoxycoumarin (in percent relative to the sample without Triton, 0.5 nmol umbelliferone/min per mg protein) is expressed as a function of the detergent concentration.

Analysis on a 12% gel (not shown) revealed the same extent of modification.

Light scattering of microsomes as a function of Triton concentration is shown in Fig. 4. Scattering remains constant up to 0.2 mg Triton/ml, decreases between 0.3 and 0.6 mg/ml and reaches its minimum at 5 mg Triton. Light scattering is decreased by 50% at a Triton concentration of about 0.4 mg/ml.

Fig. 5 shows dealkylation of 7-ethoxycoumarin by microsomes as a function of Triton. The enzymatic activity decreases very rapidly to reach a value < 5% of the control. At 0.05 mg/ml, it has decreased already by 75%.

Discussion

The pK values of the ϵ-amino group of lysine residues are between 9.5 and 10.5. FITC reacts with deprotonated amino groups. These groups, therefore, can be modified at pH values above 9 [26]. Under appropriate conditions (FITC concentration, pH 7.4) the α-amino group of the N-terminal methionine of the *P*-450IIB1 isozyme from rabbit liver is modified stoichiometrically and specifically [13,14]. Spectroscopic quantification of covalently bound FITC at 496 nm, analysis of a tryptic digest and of the N-terminal methionine, and fluorescence detection on a gel show that also purified rat liver microsomal *P*-450IIB1 is similarly modified under these conditions.

Electrophoresis of phenobarbital-induced microsomes on a SDS gel reveals well separated bands in the 50 kDa molecular mass region [27,28]. They are mainly *P*-450IIB1, which represents more than 55% of the total microsomal *P*-450, and epoxide hydrolase which migrates faster than *P*-450IIB1. In addition, comparison of uninduced and phenobarbital-induced microsomes at the position of *P*-450IIB1 shows that other proteins are present only at background levels in induced microsomes. For these reasons, the reactivity of FITC towards *P*-450IIB1 could be studied by analysis of the entire or partially purified microsomal protein population on SDS gels.

We have compared the modification of *P*-450 in solution and in microsomes. To this end, we enriched *P*-450 from microsomes after incubation with FITC. The amount of *P*-450 in the 9 to 16% PEG fraction was calculated from the heme-CO difference spectrum which detects only the holoenzyme. The amount of *P*-450 to be modified in solution was calculated from a protein assay (holo-plus apoenzyme) so that the microsomes-derived sample contained *at least* as much *P*-450 as the protein in solution. Isolated but not membrane-bound cytochrome fluoresces intensely. This indicates that the N-terminal methionine is very well protected from FITC modification when the protein is anchored to the microsomal membrane. Triton X-100 added to microsomes abolishes protection at a concentration of 0.3 mg detergent per mg protein (Fig. 3). Since FITC reacts with *P*-450 and since there is some unspecific adsorption of FITC to microsomes, a direct determination of FITC penetration through the microsomal membrane induced by Triton is difficult and was not performed. However, light scattering measurements show that at 0.3 mg Triton per mg of protein the membrane also begins to desintegrate (Fig. 4), in keeping with the above conclusion that modification of the N-terminal methionine is prevented by an intact membrane.

A similar approach, i.e., labelling with FITC in the natural membrane, has been made recently with rabbit liver microsomal *P*-450 LM2 [29]. FITC labelled almost exclusively a protein with a molecular mass corresponding to that of *P*-450 as analyzed by SDS-polyacrylamide gel electrophoresis and fluorimetry. Manual microsequencing of the labelled protein revealed a modified methionine at the N-terminus. The authors concluded that the N-terminus of *P*-450 LM2 faces the cytosolic side of the endoplasmic reticulum. However, since no quantitative information on the labelling efficiency and specificity was given nor leakiness of the membrane controlled, this conclusion does not seem to be experimentally substantiated at the present time.

As is evident from our data and those of Bernhardt et al. [29], FITC labels essentially no other protein besides *P*-450, even when the membrane has been made leaky with Triton. This may be due to a particular reactivity of the N-terminus of *P*-450. At pH 7.4 FITC does not modify lysine residues. At higher pH some lysine residues of *P*-450 are reactive to FITC [13,14] and one would expect to see additional modification of many microsomal proteins. The selective modification of *P*-450 at pH 7.4 may also be due to blocked N-termini of most of the other proteins. Up to 80 to 90% of soluble proteins are blocked at their N-terminus [30,31]. Also membrane proteins, among them microsomal cytochrome b_5 [32,33], cytochrome-b_5 reductase [34], and NADPH–cytochrome-*P*-450 reductase [35], are acylated at their N-terminus [36], but the extent of N-terminal modification of most microsomal membrane proteins is presently unknown. Finally, *P*-450 is clearly the protein of highest concentration in phenobarbital-induced microsomes (see Fig. 3). It is conceivable that some other proteins are modified by FITC at pH 7.4 but they escape detection on the gel due to their low concentration.

P-450 forms self-aggregates and complexes with other proteins in the microsomal membrane [37,38]. Conceivably, protection from modification of the N-terminus could be due to steric hindrance by *P*-450 aggregation or interaction with with other proteins, for example reductase. Detergents have been used to perturb the catalytic activities of the monoxygenase system [39–42]. We used Triton as a probe to test whether

protein interactions prevent FITC from reacting with the N-terminus. With 0.05 mg Triton/ml, the microsomal membrane integrity is completely preserved (Fig. 4), whereas *P*-450 has lost most (75%) of its activity (Fig. 5). The inhibitory effect of Triton is not due to changes in the secondary or tertiary structure of the proteins but to a disruption of the functional complex [42]. At this detergent concentration the N-terminal methionine is not modified (Fig. 3, panel B) ruling out steric hindrance by protein aggregation. In line with this, Triton (1 mg/ml) did not influence the modification of the protein in solution. To test whether the 7-ethoxycoumarin dealkylase activity is particularly sensitive to Triton we also measured the effect of this detergent on the benzphetamine demethylase activity (not shown). We obtained essentially the same results as with 7-ethoxycoumarin dealkylation, although the amount of Triton needed to inhibit the activity is slightly higher. 63% of the activity is lost at 0.2 mg Triton/ml which is still well below the concentration of Triton needed to modify *P*-450 (Fig. 3).

Our results indicate that the N-terminal methionine of microsomal rat liver *P*-450 is located on the luminal side of the endoplasmic reticulum. Our data are fully consistent with the conclusion that *P*-450 spans the membrane only once [5–9,17]. Hydrophobicity calculations [1,2,11,12] apparently do not differentiate between hydrophobic peptides in the membrane or in the core of *P*-450.

Acknowledgements

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