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INVESTIGATION OF THE TRANSVERSE TOPOLOGY OF THE MICROSOMAL MEMBRANE USING COMBINATIONS OF PROTEASES AND THE NON-PENETRATING REAGENT DIAZOBENZENE SULFONATE

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Summary

Intact microsomal vesicles from rat liver were subjected to combined treatment with trypsin and an unspecific protease and were also examined after reaction with the chemical probe *p*-diazobenzene sulfonate. In addition, the latency of various enzymes in intact microsomal vesicles has been investigated. All microsomal electron transport enzymes studied, i.e. NADH-ferricyanide and cytochrome *c* reductases, cytochrome b_5, NADPH-cytochrome *c* reductase and cytochrome *P*-450, were either solubilized or inactivated by one or both treatments. The experimental data indicate that UDPglucuronyl-transferase is also localized at the outer surface of microsomes. In contrast, a number of hydrolytic enzymes are apparently located inside the permeability barrier of the membrane and presumably at the inner surface. Under conditions where the levels of electron transport enzyme activities or amounts are changed, such as in newborn rats and rats treated with phenobarbital or methylcholanthrene, the intramembranous position of these enzymes is the same as in control adult rats. This indicates that the enzyme molecules are not relocated after their insertion into the membrane.

Introduction

It has become increasingly clear during the past decade that virtually all biological membranes are asymmetric in the transverse plane with respect to their protein components, i.e. the protein composition of the outer half of the membrane is not the same as that of the inner half [1]. One of the classical methods of examining such asymmetry in the case of closed vesicles is by treatment with proteases [2].

We have previously carried out an extensive study of the transverse protein

topology of rat liver microsomes using proteolysis [3]. This was possible because of the thorough investigation by Ito and Sato [4] which demonstrated that only the outer surface of intact microsomal vesicles is susceptible to proteolytic attack. However, as with all methods, this one has its limitations. For example, trypsin attacks only peptide bonds on the carboxyl side of arginine and lysine residues. In addition, the three-dimensional structures of a protein and/or its structural relationship to other membrane components may sterically hinder hydrolytic attack.

Here we have used two approaches to get around some of these limitations. On the one hand, we have investigated the transverse protein topology of rat liver microsomes using a combination of trypsin and an unspecific protease. An unspecific protease would be expected to hydrolyze most of the peptide bonds exposed at the vesicle surface. On the other hand, we have employed a totally different method, i.e. treatment of intact microsomes with the non-penetrating chemical reagent diazobenzene sulfonate. This reagent does not readily cross membranes, presumably because of the negative charge on its sulfonate group, and the azo moiety of this compound reacts with a number of groups on proteins, including sulfhydryl, amino, and hydroxyl groups [5,6]. Diazobenzene sulfonate has a molecular weight of 186, so that steric hindrance of its reaction with functional groups on proteins would be expected to be much less than the possible steric hindrance of protease attack on peptide bonds.

It was also necessary to do latency studies in combination with these investigations. The substrates and cofactors used in assaying many microsomal enzymes, including NADH, NADPH, cytochrome *c*, ferricyanide, and nucleoside phosphates, are not thought to penetrate into intact microsomes [7]. Thus, if microsomal enzymes whose substrates or cofactors do not penetrate the membrane are assayed in the absence of membrane-disrupting agents (e.g. detergents), activity which may be present at the inner surface of the vesicles will not be detected.

Materials and Methods

Animals and microsomes. Male Sprague-Dawley rats weighing 160—180 g were used routinely except for methylcholanthrene treatment, for which rats with an average weight of 90 g were used. All rats were starved for 20 h before decapitation and liver microsomes were prepared as described previously [8]. Phenobarbital (8 mg/100 g body weight) was injected intraperitoneally once daily for 3 days prior to killing. Methylcholanthrene (2 mg/100 g body weight) injections into the peritoneal cavity were made on days 1 and 3 and microsomes prepared on day 4. When newborn rats were used they were taken away from their mothers and kept at 30°C in a moist atmosphere for 12 h before killing. Microsomes were then prepared in the usual fashion. In all cases the total microsomal pellet from 2 g wet weight of liver was suspended in 10 ml of 0.15 M Tris · HCl, pH 8.0, and centrifuged at 105 000 × *g* for 60 min in order to remove adsorbed proteins [8]. The washed pellet was resuspended in 0.25 M sucrose at a concentration equivalent to 1 g of liver wet weight per ml.

Treatment of microsomes with reagents. Treatment with proteases was

carried out with 50 mM KCl, 50 mM Tris · HCl, pH 7.5, 0.25 M sucrose and 18 mg microsomal protein, in a final volume of 3 ml. Trypsin (Boehringer, Mannheim) and/or unspecific protease (Type VII from *Bacillus amyloliquefaciens*, Sigma, St. Louis) were added and incubation was carried out for 10 min at 37° C unless otherwise indicated. Controls were incubated in the absence of proteases. After protease treatment the microsomes were collected by dilution with 5 ml cold 0.25 M sucrose and centrifugation for 60 min [9]. Pellets were routinely resuspended in 3 ml 0.25 M sucrose.

p-Diazobenzene sulfonate was prepared as described previously [10] and dissolved in 50 mM phosphate buffer, pH 7.5, to give a concentration of 20 mM. The preparation of diazobenzene sulfonate was kept on ice and used within 30 min after being dissolved in the buffer. For treatment the incubation contained 18 mg microsomal protein, 50 mM KCl, 50 mM Tris · HCl, pH 7.5, and 0.25 M sucrose in a final volume of 3 ml. The incubation was started by addition of diazobenzene sulfonate and carried out in an ice-water bath for 2 min with gentle shaking. The treatment was terminated by adding 5 ml cold 0.25 M sucrose containing 13 mM $CaCl_2$ and 8 mM $MgCl_2$ and centrifuging at 25 000 × *g* for 15 min [9].

Using the conditions given above interaction of diazobenzene sulfonate is dependent on the protein amount present, the time of incubation and the temperature. The amount of reagent was not limiting. The interaction of diazobenzene sulfonate with the active groups of protein could be easily followed at 400 nm.

Chemical and enzyme analysis. Enzyme assays were always carried out on microsomes prepared the same day. Protein was determined by the biuret reaction [11]. Phospholipid content was analyzed as described previously [12].

Intramicrosomal water space inaccessible to dextran of 70 000 molecular weight was estimated by the ultracentrifugation procedure [7]. The amount of heme was measured spectrophotometrically after converting the heme into pyridine-hemochromogen in the presence of 0.1 M NaOH and 20% pyridine as described by Omura and Sato [13]. The difference in extinction between 557 and 575 nm in the $Na_2S_2O_4$-reduced minus oxidized difference spectrum of the hemochromogen was used.

The various enzyme activities were determined by previously described methods [3,9,14—17]. The amount of cytochrome b_5 was determined by difference spectroscopy using $Na_2S_2O_4$ as reducing agent [15]. An extinction coefficient of 185 mmol/l per cm was used in the calculations. For determination of the amount of cytochrome *P*-450 the samples were reduced by $Na_2S_2O_4$ and after bubbling with carbon monoxide the spectrum was taken [16]. An extinction coefficient of 91 mmol/l per cm was used for calculation of cytochrome *P*-450 amount.

Results

Latency studies with microsomes

We assayed the activities or amounts of various microsomal enzymes in the presence of different concentrations of detergent. Latency was observed only for β-glucuronidase, *p*-nitrophenyl-proprionate esterase, glucose-6-phosphatase,

mannose-6-phosphatase and IDPase and these enzymes were latent both before and after trypsin treatment. In addition, the level of cytochrome *P*-450 which can be measured in microsomes treated with low concentrations of deoxycholate [3] or subjected to the Tris-water-Tris washing procedure [8] (both of which preparations should be permeable to dithionite [7]) is the same as the level found in control microsomes. These results agree well with the results obtained with proteolysis and diazobenzene sulfonate treatment (see below) and also with the literature [18—20].

In addition, no latency could be demonstrated for any of the electron transport enzymes after phenobarbital or methylcholanthrene treatment. This indicates that all the newly synthesized enzymes are distributed in the same membrane compartment as in the control membranes. Also, the decrease in NADH-cytochrome *c* reductase activity caused by phenobarbital cannot be explained by a relocation of this enzyme.

These results provide evidence concerning the topology of the different microsomal enzymes (see Discussion). In addition, they indicate that all of the electron transport enzymes can be fully measured in intact microsomes. All of the latent enzymes were measured by titration with detergent until maximal activity was obtained. This was necessary because the sensitivity of these enzymes to detergents varied considerably when the microsomes were treated in different ways.

The effect of protease or diazobenzene sulfonate treatment on microsomal membrane integrity

When studying the transverse topology of closed vesicles with proteases or a non-penetrating reagent it is important to be certain that the permeability barrier of the membrane is not damaged by the treatment. Otherwise, the proteases or the reagent may gain access to the inner surface of the membrane as well.

Here we have used three different criteria for the intactness of the microsomal membrane. First, when assayed at low substrate concentrations, the latency of mannose-6-phosphatase seems to be a good test for the intactness of the microsomal permeability barrier [17]. Secondly, IDPase is readily solubilized from rat liver microsomes when the permeability barrier is destroyed sufficiently for it to be able to cross the membrane [3,21], which seems to indicate that IDPase is loosely bound to the inner surface of the membrane or is soluble in the lumen. Finally, the intramicrosomal water volume that is inaccessible to Dextran of 70 000 molecular weight is an indication of the impermeability of the membrane to this molecule [7].

The effect of protease treatment on these parameters is shown in Table I. It can be seen that trypsin or unspecific protease alone at a concentration of 50 μg per mg microsomal protein liberates about one third of the microsomal protein and that incubation with both of these enzymes together does not solubilize much additional protein. Nor do these relatively low amounts of proteases release membrane phospholipid. Table I also demonstrates that the microsomes as isolated are almost all intact (94% latency of mannose-6-phosphatase). Treatment with 25 μg each of trypsin and unspecific protease reduces this latency about 5%, lowers the activity of IDPase 10%, and probably

TABLE I

EFFECT OF PROTEOLYTIC AND DIAZOBENZENE SULFONATE TREATMENT ON MICROSOMAL MEMBRANE INTEGRITY

Microsomes were incubated in the presence of 50 mM KCl, 50 mM Tris · HCl, pH 7.5, 0.25 M sucrose and an amount of trypsin and/or unspecific protease per mg protein given in the table. Incubations were performed for 10 min at 37°C. The microsomes were sedimented after incubation by ultracentrifugation and the resuspended fraction was used for the various measurements. Diazobenzene sulfonate treatment of microsomes was performed at 0°C for 2 min as described in Materials and Methods. The fractions were recovered after incubation by centrifugation and washed by recentrifugation in 0.15 M Tris · HCl buffer, pH 8. Mannose-6-phosphatase activity was determined on the resuspended fractions both without and with applying a Triton treatment according to Materials and Methods. IDPase activity was measured in the presence of 0.1% Triton.

Treatment	Protein (mg/ g liver)	Phospholipid (mg/ g liver)	Mannose-6-phosphatase (μmol P_i/min per mg phospholipid)		Latency (%)	IDPase (μmol P_i/min per mg phospholipid)	Intramicrosomal water (μl/mg dry wt.)
			+ Triton	— Triton			
None	17.4	6.4	0.80	0.05	94	1.64	1.24
Trypsin, 50 μg	11.5	6.5	0.75	0.06	92	1.54	1.41
Unspecific protease, 50 μg	11.4	6.3	0.77	0.05	94	1.56	1.38
Trypsin, 25 μg + unspecific protease, 25 μg	11.0	6.3	0.75	0.08	89	1.52	1.28
Trypsin, 150 μg + unspecific protease, 150 μg	10.7	5.9	0.62	0.13	79	1.30	1.02
Diazobenzene sulfonate, 0.75 mM	16.7	6.3	0.75	0.09	88	1.54	1.16
Diazobenzene sulfonate, 3.0 mM	16.2	6.1	0.67	0.15	78	1.23	0.84

TABLE II

EFFECT OF PROTEASE AND DIAZOBENZENE SULFONATE TREATMENT ON ELECTRON TRANSPORT ENZYMES

The enzyme activities and amounts were determined as earlier on the resuspended portions previously subjected to proteolytic treatment. When diazobenzene sulfonate pretreatment was performed the fractions rewashed by alkaline Tris buffer were used for measurement of enzyme activities and amount.

Treatment	NADPH-cytochrome *c* reductase *	Cytochrome *P*-450 **	NADH-ferricyanide reductase *	Cytochrome b_5 **	NADH-cytochrome *c* reductase *
None	0.11	1.67	3.58	1.95	1.73
Trypsin, 50 μg	0.01	0.82	2.40	0.50	0.41
Unspecific protease, 50 μg	0.01	0.80	2.50	0.48	0.39
Trypsin, 25 μg + unspecific protease, 25 μg	0.01	0.35	0.66	0.25	0.15
Trypsin, 150 μg + unspecific protease, 150 μg	0.01	0.13	0.46	0.15	0.05
Diazobenzene sulfonate, 0.75 mM	0.01	0.27	0.28	2.01	0.12
Diazobenzene sulfonate, 3.0 mM	0.0	0.09	0.15	1.78	0.07

* μmol NADPH or NADH oxidized/min per mg phospholipid.

** nmol cytochrome *P*-450 or b_5 per mg phospholipid.

decreases the intramicrosomal water somewhat. (Protein is solubilized and thereby the intramicrosomal water space/mg dry wt. is increased. For this reason treatment with 50 μg trypsin should be used as reference.) Treatment with greater amounts of the proteases affects these parameters somewhat more adversely; but even with 150 μg each of trypsin and unspecific protease the microsomal vesicles remain about 80% intact, although a significant release of phospholipid is observed. In addition Table I illustrates the effect of diazobenzene sulfonate treatment on our three criteria for the intactness of the membrane permeability barrier. This treatment does not remove any phospholipid from the vesicles, though we have found that the reagent seems to react with about 10% of the phospholipid molecules. 0.75 mM diazobenzene sulfonate decreases the latency of mannose-6-phosphatase about 6%, decreases IDPase activity 6%, and reduces intramicrosomal water volume only 6%. 3.0 mM diazobenzene sulfonate affects these parameters somewhat more seriously. If mannose-6-phosphate (molecular weight 260, negatively charged) cannot cross the microsomal membrane, it seems unlikely that diazobenzene sulfonate (molecular weight 186, negatively charged) can.

Localization of the electron transport enzymes

Five microsomal electron transport enzymes are either solubilized or inactivated by treatment of intact microsomes with trypsin and/or unspecific protease (Table II). NADPH-cytochrome *c* reductase is completely solubilized in an active form by all of the treatments. Cytochrome b_5 is somewhat more resistant, but can be completely solubilized in an active form (as measured spectrally) by both enzymes together. As expected, NADH-cytochrome *c* reductase activity is decreased to about the same extent that cytochrome b_5 is solubilized. Both cytochrome *P*-450 and NADH-ferricyanide reductase are only partially affected by treatment with either trypsin or unspecific protease alone, but both components almost completely disappear as a result of the combined action of these proteases. Neither NADH-ferricyanide reductase activity nor cytochrome *P*-450 (as measured spectrally) can be recovered in the supernatant. The effect of diazobenzene sulfonate treatment confirms the findings with proteases, except in the case of cytochrome b_5 (Table II). This reagent inactivates NADPH-cytochrome *c* reductase, NADH-ferricyanide reductase, and cytochrome *P*-450 apparently without liberating them. The fact that cytochrome b_5 is not inactivated may be because no functional groups on this protein that can be attacked by diazobenzene sulfonate are exposed to the aqueous phase. It is also possible that the reagent inactivates cytochrome b_5 as an electron carrier without this effect being reflected in the difference spectrophotometric assay.

Release of heme by treatment of microsomes with proteases

It is well known that treatment of microsomes with proteases can be used to solubilize a portion of cytochrome b_5 containing the heme group and portions of NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase molecules containing the flavin moieties [22—25]. It was of interest to ask whether treatment of microsomes with trypsin and unspecific protease together removes a portion of the cytochrome *P*-450 molecule containing the heme group, even

TABLE III

RELEASE OF HEME BY PROTEOLYTIC TREATMENT OF INTACT MICROSOMES

Microsomes were incubated in the absence (none) or in the presence of proteolytic enzymes (trypsin + unspecific protease) for 10 or 20 min at 37°C. After incubation the membranes were sedimented by centrifugation and the amount of heme was estimated by difference spectroscopy both in the supernatant and in the pellet.

Treatment	Incubation time (min)	Heme *		Recovery (%)
		Pellet	Supernatant	
None	20	17.1	0.4	97
Trypsin, 25 μg + unspecific protease, 25 μg	10	7.3	10.8	101
Trypsin, 25 μg + unspecific protease, 25 μg	20	6.4	11.6	100

* nmol heme in microsomes isolated from 1 g liver.

though this hypothetical portion cannot be detected using the standard spectrophotometric assay for cytochrome *P*-450. Microsomes were subjected to proteolysis and the total heme content was measured both in the pellet and the supernatant after ultracentrifugation. Virtually all microsomal heme is in cytochromes *P*-450 and b_5 [13,26]. About 60% of this heme is recovered in the supernatant after protease treatment (Table III), and this amount corresponds to the heme content of cytochrome b_5. Thus, treatment with trypsin and unspecific protease does not seem to release a portion of the cytochrome *P*-450 molecule containing the heme group.

The topology of various microsomal hydrolase and transferase activities

Table IV shows the effects of treating intact microsomes with trypsin and unspecific protease or with diazobenzene sulfonate on various hydrolase and transferase activities. ATPase, AMPase, β-glucuronidase, esterase, and glucose-6-phosphatase activities are little affected by either of these treatments. This indicates that the proteins catalyzing these activities may be at the inner surface of the vesicles or deeply buried in the membrane. However, even though UDPglucuronic acid transferase activity is not much affected by proteolysis, this treatment does eliminate the requirement for detergent to obtain full activity. This may mean that the transferase is exposed at the outer surface of the vesicles but "covered" in such a way that substrate penetration to the active site is limited. Protease treatment could remove the barrier to substrate penetration without attacking the enzyme itself. Diazobenzene sulfonate inhibits UDPglucuronic acid transferase activity about 50%, which is consistent with this interpretation.

Enzyme topology after induction and during development after birth

It seems possible that changes in membrane structure are involved in the induction of the hepatic endoplasmic reticulum brought about by certain xenobiotics. One possibility is that the intramembraneous localization of the induced enzymes differs from that of these same enzymes in control microsomes. In order to investigate this question, microsomes were prepared

TABLE IV

HYDROLASE AND TRANSFERASE ACTIVITIES IN MICROSOMES TREATED WITH PROTEOLYTIC ENZYMES AND DIAZOBENZENE SULFONATE

After preincubation of microsomes with proteolytic enzymes or diazobenzene sulfonate the enzyme activities were measured as described earlier [3,17].

Treatment	ATPase *	AMPase *	UDP glucuronic acid transferase **		β-Glucuronidase ***	*P*-Nitrophenyl-propionate exterase ***	Glucose-6-phosphatase *
			+ Triton	— Triton			
None	0.36	0.21	0.018	0.006	0.19	23.2	1.24
Trypsin, 25 μg + unspecific protease, 25 μg	0.29	0.18	0.014	0.011	0.15	20.5	0.93
Trypsin, 50 μg + unspecific protease, 50 μg	0.26	0.19	0.013	0.011	0.15	21.0	0.84
Diazobenzene sulfonate, 0.75 mM	0.26	0.17	0.010	0.007	0.19	21.2	1.01

* μmol P_i/min per mg phospholipid.
** μmol *P*-nitrophenyl conjugated/min per mg phospholipid.
*** μmol *P*-nitrophenyl liberated/min per mg phospholipid.

TABLE V

ACCESSIBILITY OF MICROSOMAL ELECTRON TRANSPORT ENZYMES TO PROTEASES AND DIAZOBENZENE SULFONATE AFTER INDUCTION WITH PHENOBARBITAL OR METHYLCHOLANTHRENE AND IN NEWBORN ANIMALS

The microsomal fraction from phenobarbital- and methylcholanthrene-treated and from newborn rats were treated with proteolytic enzymes (trypsin, 25 μg + unspecific protease, 25 μg per mg protein) or diazobenzene sulfonate (0.75 mM) as described in Materials and Methods. The values are given as percent where compared to that found in control microsomes prepared from non-treated adult rats.

Rats	Treatment	Cytochrome *P*-450	NADH-ferricyanide reductase	NADPH-cytochrome *c* reductase	Cytochrome b_5	NADH-cytochrome *c* reductase	Phospholipid
							g liver
Phenobarbital treated	None	281	69	212	125	56	204
	Proteolytic	60	21	17	13	7	200
	Diazobenzene sulfonate	78	3	13	119	3	198
Methylcholanthrene treated	None	240	104	106	99	96	114
	Proteolytic	49	32	7	11	5	109
	Diazobenzene sulfonate	68	3	5	111	9	116
Newborn, 1 day old	None	20	22	94	30	20	50
	Proteolytic	2	3	5	2	1	52
	Diazobenzene sulfonate	3	2	3	31	1	47
Newborn, 6 days old	None	65	64	98	55	35	86
	Proteolytic	16	17	5	6	2	89
	Diazobenzene sulfonate	11	4	1	52	2	87

from the livers of rats which had been induced with phenobarbital or methylcholanthrene and the isolated vesicles were treated with proteases or diazobenzene sulfonate.

The induction of the endoplasmic reticulum by phenobarbital involves an increase in the amounts of cytochrome *P*-450 and of NADPH-cytochrome *c* reductase, a slight increase in the amount of cytochrome b_5, and a decrease in NADH-ferricyanide and -cytochrome *c* reductases. Methylcholanthrene, on the other hand, seems only to increase the microsomal levels of cytochrome *P*-450. All of these enzymes were accessible to combined proteolytic treatment and to diazobenzene sulfonate in intact microsomes (Table V). In several cases the removal and/or inactivation was somewhat less than in the control; but the basic pattern was the same. Thus, these findings indicate that induction does not affect the localization of these enzymes in the membrane of the endoplasmic reticulum. In newborn rats the microsomal electron transport enzymes, even those in the same chain, increase to adult levels with different time courses [27]. In order to examine the intramembraneous localization of these enzymes during early development, microsomes from 1- and 6-day-old rats were treated with proteases or diazobenzene sulfonate (Table V). It can be seen that the localization of electron transport components in these microsomes is the same as in microsomes from adult animals. In fact, proteolytic and diazobenzene sulfonate treatment are even more effective in decreasing the levels of electron transport components in microsomes from newborn rats.

Discussion

The lack of latency observed with NADH-ferricyanide reductase activity, NADH- or NADPH-cytochrome *c* reductase activities, and cytochrome *P*-450 suggest that the enzymes involved have substrate- or dithionite-binding sites exposed at the external surface of intact microsomes. In the case of the latent microsomal enzymes interpretation is more difficult, since detergents may stimulate the activity of membrane-bound enzymes in ways other than disrupting the permeability barrier to substrate. However, these latency findings are fully consistent with the observations with proteases and diazobenzene sulfonate (see below).

When using proteases or non-penetrating reagents to examine the outer surface of membrane vesicles, it is important to be certain that the treatment used does not disrupt the permeability barrier of the membrane so that the proteases or reagent gain access to the inner surface as well. We have used three criteria for the intactness of microsomal vesicles, the latency of mannose-6-phosphatase activity, the solubilization of nucleoside diphosphatase from the microsomes, and the volume of intramicrosomal water inaccessible to dextran of 70 000 molecular weight. Under the conditions used here treatment with trypsin and unspecific protease or with diazobenzene sulfonate had only minor effects on these parameters.

In all cases, with the exception of cytochrome b_5, the results obtained with the proteases and with diazobenzene sulfonate agreed remarkably well. Since these two different approaches have different problems and limitations, this agreement strengthens the conclusions arrived at. In agreement with earlier

findings [3] cytochrome b_5 and NADPH-cytochrome *c* reductase were found to be exposed to a large extent at the outer surface of the microsomal vesicles. A new finding is the observation that treatment both with trypsin and unspecific protease and with diazobenzene sulfonate drastically reduces the level of microsomal cytochrome *P*-450 which could be detected with difference spectroscopy. Previously, we reported that treatment of intact microsomes with trypsin, chymotrypsin, or unspecific protease alone affected only about 1/3 of the cytochrome *P*-450 [3]. Apparently, a combination of proteases or a non-specific chemical reagent is necessary to completely denature this protein. This is also valid for NADH-ferricyanide reductase. Thus, some portion of almost all the cytochrome *P*-450 molecules seems to be exposed at the cytoplasmic surface of the endoplasmic reticulum. This portion does not seem to contain the heme group, since treatment of microsomes with trypsin and unspecific protease does not solubilize the heme from cytochrome *P*-450.

Another indication that a portion of the cytochrome *P*-450 molecule is exposed at the outer surface of microsomes is the common use of dithionite to reduce this cytochrome in intact microsomes. Studies on the permeability of rat liver microsomal vesicles suggest that dithionite should not be able to penetrate into them [7]. In addition, increasing concentrations of dithionite cause an osmotic response with microsomes, i.e. the intramicrosomal water space decreases (Nilsson, O., unpublished observations). This also indicates that dithionite is not crossing the membrane.

It seems not unreasonable that a portion of the cytochrome *P*-450 molecule is at the cytoplasmic surface of the endoplasmic reticulum. NADPH-cytochrome *P*-450 reductase, which donates an electron to the cytochrome, is located at this surface and indeed, the catalytically active portion of the reductase molecule seems not to interact with the phospholipid bilayer of the membrane [2]. If the active site of cytochrome *P*-450 is included in that part of the molecule which is at the cytoplasmic surface, the apparent requirement that substrates of this enzyme be hydrophobic may be due to the hydrophobic nature of the binding site rather than to its localization "inside" the phospholipid bilayer or at the luminal surface of the membrane.

Treatment of intact microsomes with trypsin and unspecific protease and with diazobenzene sulfonate revealed that various hydrolase and transferase enzymes, including ATPase, AMPase, β-glucuronidase, esterase, and glucose-6-phosphatase, are not exposed at the outer surface of microsomes, at least not in such a way that they are readily accessible to protease or chemical attack. The situation is more complex for UDPglucuronic acid transferase. In this case treatment with trypsin and unspecific protease seems to remove some "barrier" preventing access of substrate to the active site of the enzyme. This suggests that UDPglucuronic acid transferase is on the surface but covered by some other protein component of the membrane. Diazobenzene sulfonate can get through this cover partially to denature about 50% of the transferase.

The same approaches demonstrated that the topology of the electron transport components in the endoplasmic reticulum is the same in newborn as in adult rats and that this topology is not altered by induction of the hepatic endoplasmic reticulum with phenobarbital or methylcholanthrene. This suggests that the electron transport components are synthesized completely at

the cytoplasmic surface of the endoplasmic reticulum and incorporated directly, rather than entering the lumen and then being translocated to the surface again.

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