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The spatial arrangement of esterases in the microsomal membrane

Die Anordnung der Esterasen in der mikrosomalen Membran

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Abstract

Rat liver – microsomal esterases – asymmetric distribution

The location of esterases is studied with microsomal vesicles from rat liver using membrane impermeable charged substrates and inhibitors. By comparing esterase activity against charged and uncharged substrates it is shown that microsomal esterases are not latent. Furthermore charged inhibitors were unable to block both esterase – and amidase – activity differently in disrupted and in intact vesicles. From these biochemical studies it was concluded that the major part of the microsomal esterases/amidases is attached to the cytoplasmic side of the microsomes, which was confirmed by electron microscopic studies with enzyme specific staining showing the electron dense reaction products of the microsomal esterases at the cytoplasmic side exclusively.

Introduction

Microsomes isolated by the usual methods from rat liver are closed vesicles, whose inner surface represents the intracisternal side of the endoplasmic reticulum. The outer surface bearing ribosomes in rough microsomes [17, 18], is exposed to the cytoplasm in the intact cell. Very recently it was concluded from iodination studies with lactoperoxidase that most of the microsomal proteins are faced to the cytoplasmic side of the vesicles [15]. Some particular microsomal proteins, however, seem to be located on the inner surface of the microsomal membrane.

There is no doubt about the orientation of microsomal nucleoside diphosphatase [16]: This enzyme shows a high degree of latency (that means the enzymatic activity being greatly stimulated by treatments which affect the lipoprotein structure of the microsomal vesicles). It is also protected from proteolytic attack as well as from inhibition by a specific antibody in intact microsomes but is susceptible to hydrolytic

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enzymes and antiserum after various treatments which affect the integrity of the membrane structure. Especially some phosphohydrolase and phosphotransferase reactions catalysed by microsomal glucose-6-phosphatase are in a latent state [2, 31]. Electronmicroscope [17, 18] and centrifugation studies [19] concerning the trapping of phosphate by Pb^{2+} also favour an intramicrosomal location of this enzyme. Enzyme specific staining of the acyltransferases involved in the acylation of α-glycerophosphate provides evidence for the attachment of this enzyme at the inner surface of the microsomal membrane [13]. UDP-glucuronyl transferase shows also latency and may be another enzyme which is located at the intracisternal side of the microsomal membrane [8].

Digestion of microsomes with proteases neither solubilizes nor inactivates microsomal amidase activity [1] and an antibody to the acetanilide-hydrolyzing esterase fails to react with the membrane bound enzyme. From these observations it was concluded that microsomal esterase may be anchored to the inner surface of the vesicular membrane. If this assumption is correct this enzyme should show latency at least with charged substrates and inhibitors since microsomal vesicles are regarded to be impermeable to anionic compounds [22].

In the present paper we investigated therefore the hydrolysis of especially designed charged substrates in comparison with the uncharged parent compounds in intact microsomes and in preparations previously treated with agents affecting the integrity of the membrane structure. To reveal, whether esterase activity is latent or not, also studies with a charged inhibitor were performed. In addition enzyme specific staining methods were employed to detect, on which side of the microsomal membrane the esterase may be located.

Materials and methods

Microsomes were isolated from a 20 % homogenate of rat liver in 0.25 M sucrose/0.01 M sodium phosphate buffer, *p*H 7.4 by gel filtration [26]. Male Sprague-Dawley rats of body weight 150 to 200 g were used.

Protein was determined according to LOWRY et al. [21], using a calibration curve with crystalline bovine serum albumin (Serva, Heidelberg, Germany). Esterase activity was determined by the following methods: Spectrophotometric determination of liberated 4-nitrophenol or 4-carboxy-2-nitrophenol from 4-nitrophenyl acetate (= nitrophenyl acetate) and 4-carboxy-2-nitrophenyl acetate (= carboxynitrophenyl acetate) at 400 nm. Carboxynitrophenyl acetate was synthesized by acetylation of 4-hydroxy-3-nitrobenzoic acid [7]. The hydrolysis of two esters could be followed very sensitively by fluorometric estimation of 7-hydroxycoumarin [28] and 4-carboxy-7-hydroxycoumarin formed from umbelliferone acetate and 4-carboxyumbelliferone acetate (carboxyumbelliferone acetate), respectively. As the excitation and emission spectra of 7-hydroxycoumarin was only slightly modified by introduction of the carboxy group the same primary and secondary filters could be used for both assays [28]. Carboxyumbelliferone acetate was prepared by acetylation of 4-carboxyumbelliferone, prepared from resorcin and diethyl oxaloacetate [30], acetylation of umbelliferone yielded umbelliferone acetate [27]. The amidase activity of the esterase was determined by measuring the formation of aniline as described [1].

All enzyme assays were performed at *p*H 7.4 in 0.01 M sodium phosphate buffer containing 0.25 M sucrose at a temperature of 20° C. To avoid uncertainties from incomplete ionisation of phenols or quenching of fluorescence a known amount of product was added for calibration to the cuvettes after each assay. Initial reaction velocities were determined using an external recorder.

For electron microscopic investigations microsomes were prepared from fasted rats by gel filtration in 0.01 M cacodylate buffer, *p*H 7.0, containing 0.14 M NaCl. One ml of microsomal

suspension (3 mg of protein/ml) was spun down several times in an Eppendorf centrifuge model 3200 to form a pellet. The supernatant was removed and the pellet was fixed in 2,5 % glutaraldehyde in 0,03 M cacodylate buffer *p*H 7.0 for 60 min. at room temperature. After postfixation in 1 % osmium tetroxide in the same buffer the pellets were dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate in the conventional manner and examined with a Siemens Elmiskop 101. Thiophenol acetate, the substrate used for electron microscopic observation of esterase activity, was prepared from thiophenol and acetyl chloride [10]. Enzyme specific staining [29] was achieved by preincubation of the unfixed microsomal pellets in 5 mM gold sodium thiosulphate (Sanocrysin®, a gift from Ferrosan, Copenhagen, Denmark) dissolved in 0.01 M cacodylate buffer *p*H 7/0.14 M NaCl for five minutes at the temperature of melting ice. After addition of 10 µl thiophenyl acetate (destilled just before use to remove traces of thiophenol from this unstable ester and diluted 1/1000 by sonification in incubation buffer), the pellets were shaked gently for ten minutes at 30° C, stopped with 2,5 % glutaraldehyde and processed as described above. For controls microsomes were incubated 15 min. before centrifugation with 1 mM bis(4-nitrophenyl)phosphate.

All reagents not specified in detail were the best grade available and were used without further purification.

Results

In order to find a method to disrupt microsomal membranes avoiding an inactivation of microsomal esterases we undertook preliminary experiments. Many treatments commonly used for this purpose including taurocholate, deoxycholate, acetone and alkali treatment, or sonication were accompanied by considerable inactivation of the enzyme activity. Among the compounds tested only Triton X 100 caused no inactivation of the microsomal esterase. Figure 1 shows the insensitivity of the enzyme to various concentrations of Triton X 100.

Since the detergent did not inactivate the microsomal esterase, it was expected, that the activity to the uncharged substrate (nitrophenyl acetate) was unaffected. Surprising, however, was the finding that even the hydrolytic activity against the charged substrate carboxynitrophenyl acetate was unchanged upon addition of different concentrations

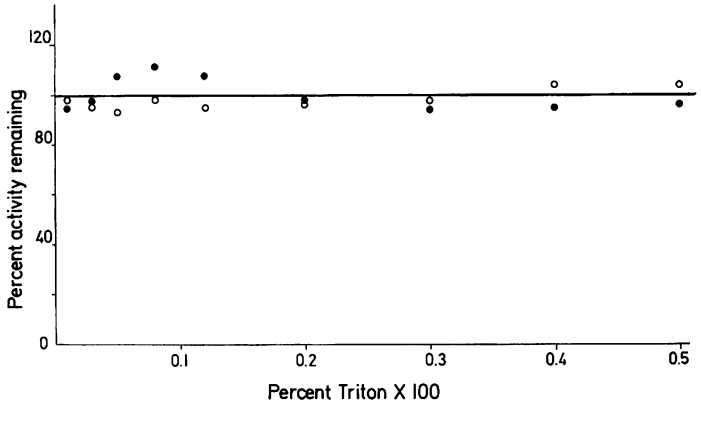


Fig. 1. Effect of Triton X 100 on the esterase activity of microsomes. – *Open circles:* Hydrolytic activity against carboxynitrophenylacetate (10^{-4} M). – *Closed circles:* Hydrolytic activity against nitrophenylacetate (10^{-4} M).

of the detergent. To compare the influence of detergent upon the hydrolysis of uncharged and charged substrates by microsomal esterases the initial reaction velocities were determined at different substrate concentrations. Reciprocal plots of the data obtained are presented in Figures 2 and 3.

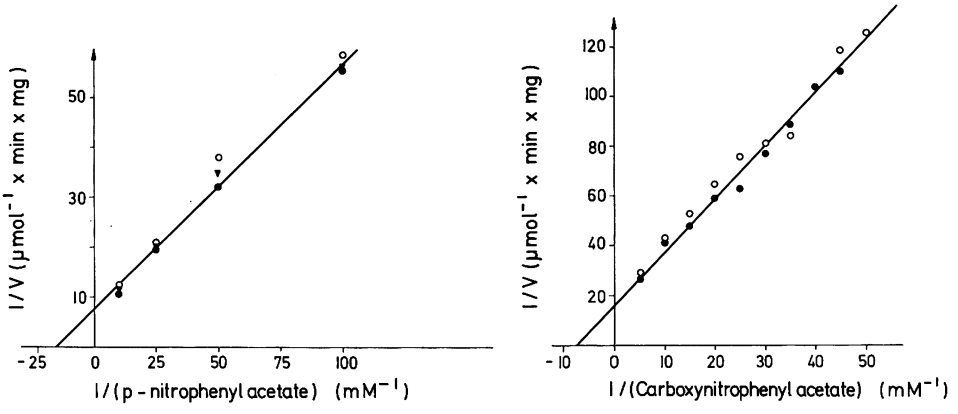


Fig. 2. Plot of reciprocal initial reaction velocity against reciprocal millimolar concentration of nitrophenylacetate in the absence (o) and in the presence of 0.1 % (●) or 0.5 % (▼) Triton X 100.

Fig. 3. Plot of reciprocal initial reaction velocity against reciprocal millimolar concentration of carboxynitrophenylacetate in the absence (o) and in the presence (●) of 0.1 % Triton X 100.

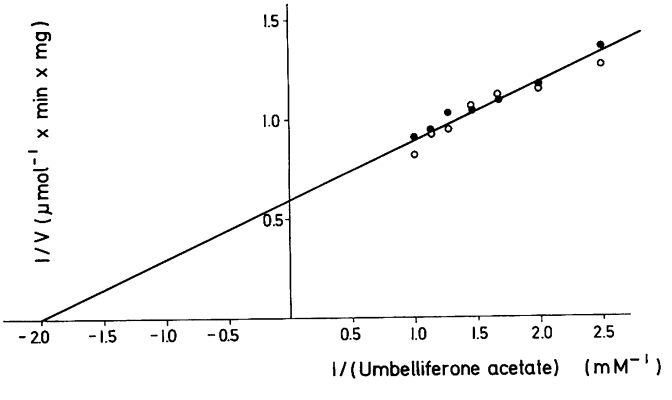


Fig. 4. Kinetics of umbelliferoneacetate hydrolysis in the presence (●) and in the absence (o) of 0.1 % Triton X 100.

The kinetic constants are unchanged by Triton X 100 for charged and uncharged substrates. The affinity of the enzyme for a substrate does not seem to be changed greatly by the introduction of a negative charge. In contrast the apparent maximal velocity is more than doubled with nitrophenyl acetate compared to carboxynitrophenyl acetate.

to measure esterase activity against charged and uncharged substrates fluorometrically. Preliminary experiments again showed no change in esterase activity using concentrations of Triton X 100 up to 1 %. Lineweaver-Burk plots of the hydrolysis of umbelliferone acetate and carboxyumbelliferone acetate by microsomal esterases in the presence and in the absence of detergent are shown in Figures 4 and 5.

The affinity of the microsomal esterases for these two substrates also was nearly the same. The apparent maximal velocity was even 40 times higher with the uncharged substrate compared with the charged one. But again with this couple of substrates neither K_m nor V_{max} was changed by detergents indicating that the esterase activity of microsomes is not latent.

Negatively charged inhibitors also should have a limited access to the active site of an enzyme located inside a membrane barrier. We expected, that after disruption of the membrane, bis(4-nitrophenyl)phosphate, a well known esterase inhibitor, would be more powerful than in intact microsomes. However, as shown in Figure 6 the time course of inhibition of microsomal esterases was unchanged in Triton X 100 compared to the assays in absence of detergent.

It is known, that microsomal esterases from rat liver are heterogeneous. Some enzymes are able to split esters exclusively and others can also cleave amides [3, 4, 5]. Unfortunately we could not repeat the described experiments with charged and uncharged amides since we did not measure any hydrolytic activety against negatively charged amides. The experiment concerning the inhibition of amidase activity by a charged inhibitor presented in Figure 7 led to the conclusion that the major part of the acetanilide hydrolyzing esterases is also accessible and therefore located on the outer surface of the microsomes.

The experiments described with charged substrates and a charged inhibitor suggest that microsomal esterases are attached to the cytoplasmic side of the microsomal vesicles and are not excluded from the surrounding medium by a membrane which is impermeable for charged substances. This finding could be confirmed by enzyme specific staining of microsomal esterases using thiophenyl acetate as a substrate. In preliminary studies we were able to follow the splitting of this ester by microsomes spectrophoto-

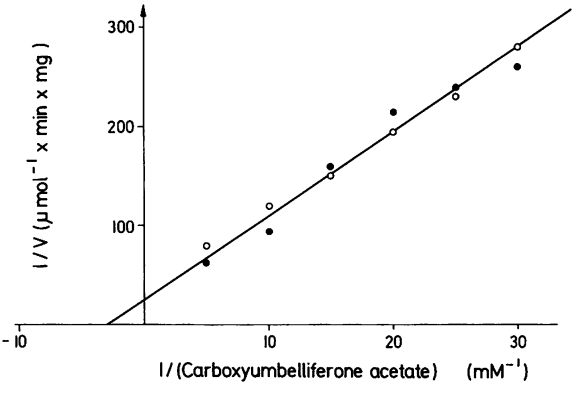


Fig. 5. Plot of reciprocal initial reaction velocity against reciprocal millimolar concentration of substrate (carboxyumbelliferoneacetate). *Closed circles* represent assays in 0.1 % Triton X 100, *open circles* control assays without detergent.

metrically based on the production of a yellow color with 5,5'-dithiobis-2-nitrobenzoate [10]. This enzymatic hydrolysis could be completely inhibited by 1 mM bis(4-nitrophenyl)phosphate. As shown in Figures 8 a and b the reaction product (Au^+-trapped thiophenol) appears outside the microsomal vesicles exclusively. In preparations of microsomes previously treated with the inhibitor nearly no deposits can be seen in the sections (Fig. 8 c).

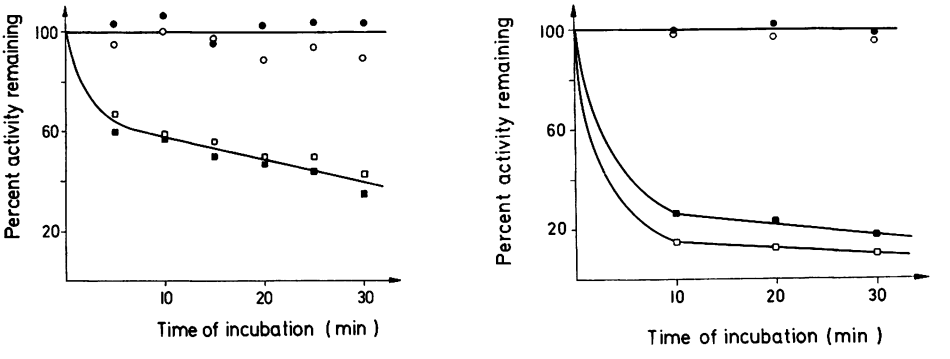


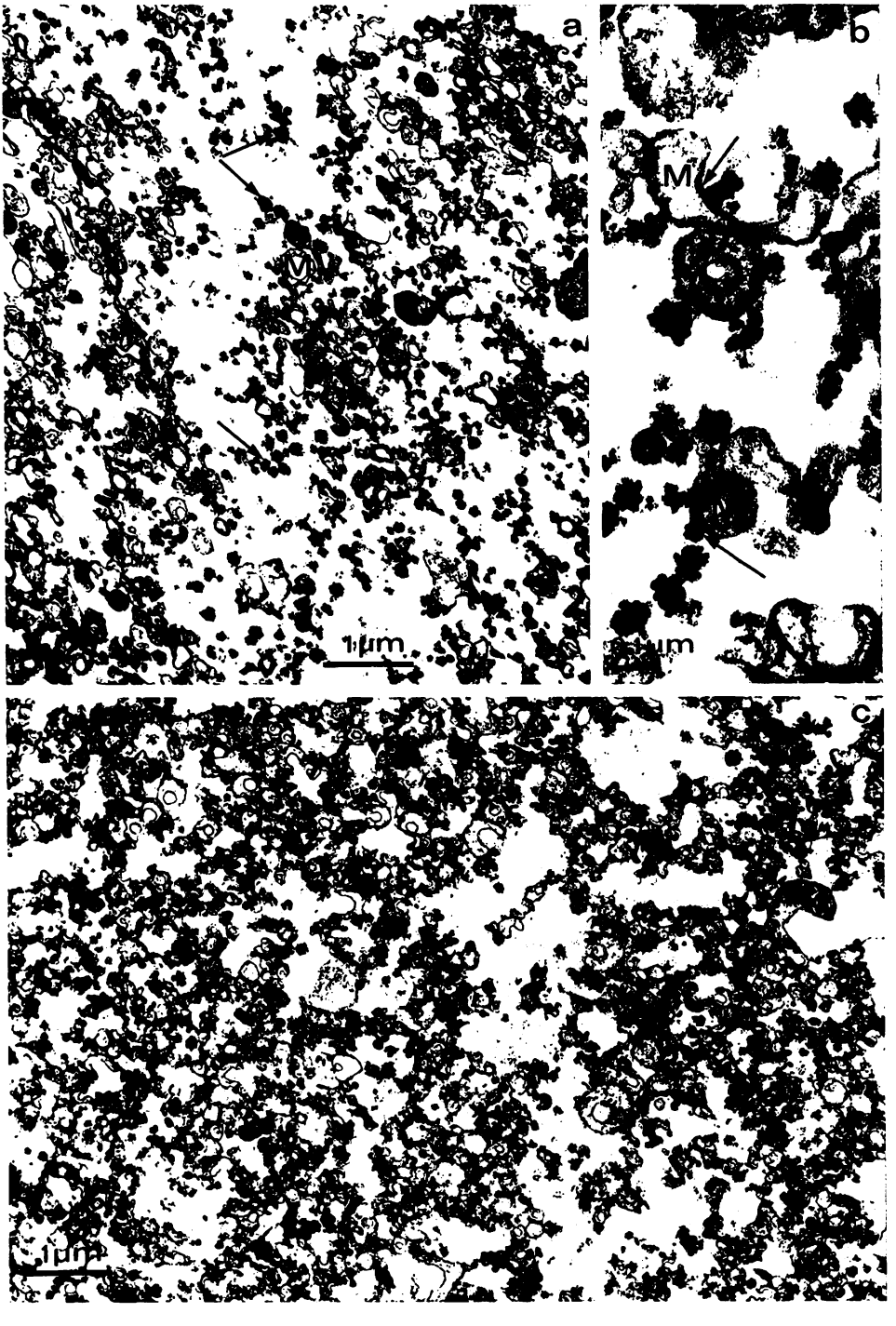
Fig. 6. Inhibition of microsomal esterase (substrate 10^{-4} M p-nitrophenylacetate by bis(p-nitrophenyl)phosphate. Circles represent control values without detergent (o) and in the presence of 0.1 % Triton X 100 (●), *squares* represent inhibition of microsomal esterases (0.43 mg/ml) after supplementation of suspension with 1,31 µ mole inhibitor per ml in the absence (□) and in the presence (■) of 0.1 % Triton X 100.

Fig. 7. Inhibition of microsomal acetanilide-hydrolyzing esterases (substrate 25 mM acetanilide) by bis(p-nitrophenyl)phosphate. *Circles* represent control values without detergent (o) and in the presence of 0.6 % Triton X 100 (●), *squares* show inhibitor of microsomal esterases (0,36 mg/ml) after supplementation of the suspension with 0,3 µ mole inhibitor per ml in the absence (□) and in the presence (■) of 0,6 % Triton X 100.

Discussion

The experiments described were designed to determine the localization of esterases in the microsomal vesicles. The results obtained demonstrate that esterase activity is not latent in microsomes. However, latency of membrane bound enzymes in microsomes is considered to indicate the presence of a permeability barrier and a location of the latent enzyme inside the vesicle [2, 8, 13, 16, 31]. In agreement with our cytochemical demonstration of esterolytic enzymes it may be concluded therefore that microsomal esterases are attached to the cytoplasmic side of the microsomal vesicles.

Fig. 8 a. Thin section of a microsomal fraction previously treated with thiophenyl acetate, showing besides microsomes (M) the reaction product of the microsomal esterases, the Au^+-trapped thiophenol, as electron dense particles *(arrows)*. – 12 000 ×. – **b.** In higher magnification the reaction product is clearly visible outside the microsomes (M), partly contacting the microsomal membranes *(arrows)*. – 50 000 ×. – **c.** Microsomal fraction after inhibition of the enzymatic hydrolysis of thiophenyl acetate by bis-p-nitrophenyl-phosphate. Nearly no esterase reaction products are visible. – 12 000 ×.


a
M
1 µm
b
M
µm
c
1 µm

This interpretation is at variance with immunological experiments reported by AKAO and OMURA [1] showing that a precipitating antibody prepared to purified acetanilide-hydrolyzing esterase did not interact with the enzyme associated with microsomal vesicles [1]. The resistance of microsomal acetanilide-hydrolyzing esterase to proteolysis also suggested that this enzyme is not exposed to the outside medium [1].

However, protease treatment may be no ideal tool for studying the sideness of esterases since soluble and membrane bound acetanilide-hydrolyzing esterase is insensitive to proteolytic attack [1]. Furthermore enzymatic activity against aromatic esters, which are split by all species of microsomal esterases [5], is neither inactivated nor solubilized during digestion of microsomes with trypsin in concentrations up to 0,1 %[2]), although it is known from experiments described in this paper, that main esterase activity is localized on the outer surface of the microsomal vesicle.

The conflicting results obtained by AKAO and OMURA [1] and by us could be due to the heterogeneity of microsomal esterases. From rat liver five esterases could be differentiated during one purification procedure [3, 4, 5]. Other preparations of microsomal esterases from rat liver are described in the literature [1, 11, 12, 20] but the relations to the five mentioned above are not clear [5].

Two of these enzymes variants hydrolyze carboxyl esters only (designated E_1 and E_2), whereas the remaining three exhibit both esterase and amidase activities. The controverse conclusions drawn in this work compared to interpretations arising from immunological and digestion experiments [1] may be explained by different distribution of the esterase variants on both sides of the microsomal membrane. In this model, according to the experiments reported in this publication, the two esterases exhibiting hydrolyzing activity against carboxyl esters (E_1, E_2) are located on the outer surface of the microsomal vesicles. One of the esterase variants exhibiting amidase activity seems to be linked to the inner surface of the vesicular membrane as supported mainly by the finding that an antibody to the esterase fails to react with the microsome-bound enzyme [1], while others, as concluded above from studied with a membrane impermeable inhibitor of amidase activity, together with NADH-cytochrome b_5 reductase, NADPH-cytochrome c reductase, cytochrome b_5 [14, 23, 24, 25] and the esterases E_1 and E_2 are facing the cytoplasmic side of the microsomal membrane.

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