Biochimica et Biophysica Acta 905 (1987) 143–150
Elsevier

BBA 73781

Two modes of binding of adrenal NADPH–cytochrome *P*-450 reductase to liposomal membranes

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(Received 6 July 1987)

Key words: NADPH–cytochrome *P*-450 reductase; Liposome; Electron transfer; Cytochrome $P\text{-}450_{C21}$; (Bovine adrenocortical microsome)

NADPH-cytochrome *P*-450 reductase, purified from bovine adrenocortical microsomes, was shown to bind in two different modes to liposomal membranes composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1. As demonstrated by Ficoll density gradient centrifugation and HPLC gel filtration, the cholate dialysis method made the reductase bind tightly to the liposomal membranes, while the incubation with the preformed vesicles made the reductase bind loosely to the membranes. From the experiments of electron transfer to $P\text{-}450_{C21}$ residing at the other vesicles, the loosely bound reductase was found to be transferable between the vesicles, whereas the tightly bound reductase was not readily transferred. The rates of the binding and the release of the loosely bound reductase to and from the membranes were measured with the stopped-flow method by observing the reduction of $P\text{-}450_{C21}$ embedded in the vesicles. These kinetic studies showed that the rate-limiting step of the reductase transfer between the vesicles was the release of the reductase from the membranes. The reductase in both binding modes well supported the steroid 21-hydroxylase activity.

Introduction

NADPH-cytochrome *P*-450 reductase is a membrane protein localized predominantly at the endoplasmic reticulum of a variety of cells [1,2] and is responsible for electron transfer from NADPH to cytochrome *P*-450 [3,4] as well as to cytochrome b_5 [5] and to heme oxygenase [6]. This enzyme consists of a hydrophilic functional domain and a hydrophobic tail anchoring to the membranes [7–9]. The complete amino acid sequence of the hepatic reductase has been determined by microsequence analysis [10] and also by cDNA sequencing [11]. The hydrophobic tail plays important roles in the interaction with the membranes and with cytochrome *P*-450 [12,13]. Without the tail the reductase can neither transfer electrons to cytochrome *P*-450 nor bind to membranes. Yang et al. [14] reported that incubation of the purified hepatic reductase with the microsomes enhanced the catalytic actions of the cytochrome *P*-450's. The reductase was suggested to be a rate-limiting component in the hepatic microsomal system by Miwa et al. [15,16], who obtained experimental results that the reductase might form

Abbreviations: $P\text{-}450_{C21}$, cytochrome *P*-450 having steroid 21-hydroxylase activity; $P\text{-}450_{17\alpha,lyase}$, cytochrome *P*-450 having steroid 17α-hydroxylase and C17,20-lyase activities; $P\text{-}450_{scc}$, cytochrome *P*-450 having cholesterol desmolase activity; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine.

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an equimolar complex with cytochrome *P*-450 during the electron transfer reaction.

NADPH-cytochrome *P*-450 reductase also plays an important role in adrenocortical microsomes in which two different cytochrome *P*-450 species, *P*-450_{C21} [17,18] and *P*-$450_{17\alpha,\text{lyase}}$ [19,20], accept electrons from NADPH via the reductase. The relative activities of the cytochrome *P*-450's in the adrenocortical microsomes have a crucial role in the regulation of the biosynthesis of the steroid hormones [21]. There is a possibility that the individual activities of these cytochrome *P*-450's might be controlled by their relative strength of the interaction with the reductase [22]. In this context, it is quite important to study the interaction of the reductase with the cytochrome *P*-450 of adrenocortical microsomes. We have been investigating their interaction using detergent-solubilized [23] and liposomal-reconstituted systems. The incorporation of *P*-450_{C21} into the liposomes could be achieved by the cholate dialysis method [24] but not by incubating *P*-450_{C21} with the preformed vesicles because of the quite low solubility of *P*-450_{C21} in the absence of detergent.

In this paper, the binding modes of the adrenal NADPH-cytochrome *P*-450 reductase to liposomal membranes were studied for the samples prepared by the incubation with preformed vesicles and by the cholate dialysis method. The reductase prepared by the former method bound loosely to the liposomal membranes and was transferable between vesicles, whereas the reductase incorporated by the latter method was not released readily from the membranes. The reductases in both preparations were active in electron transport from NADPH to *P*-450_{C21} and well supported the steroid 21-hydroxylase activity of *P*-450_{C21}.

Experimental procedures

NADPH-cytochrome *P*-450 reductase and *P*-450_{C21} were purified from bovine adrenocortical microsomes according to the methods previously described [18,25], both being homogeneous in SDS-polyacrylamide gel electrophoresis. The following two methods were applied for the binding of the reductase to liposomal membranes: incubation of the reductase with the preformed vesicles and the cholate dialysis method. In the former method, the detergent-free reductase was mixed with the preformed vesicles in 50 mM Tris-HCl buffer (pH 7.2) containing 50 mM NaCl and 0.1 mM EDTA, which was used as the basal buffer in this experiment. The vesicles were prepared by dialyzing phospholipid solution consisting of 6.5 nmol PC from egg yolk (Sigma), 3.9 nmol PE from egg yolk (Sigma), and 1.3 nmol PS from bovine brain (Sigma) in 1 ml of 1% (w/v) sodium cholate and 0.1 mM EDTA. The incubation of the reductase with the preformed vesicles was performed for 1 h at 25 °C. In the latter method, the detergent-free reductase was mixed with the phospholipid in 1% sodium cholate solution and subsequently dialyzed for 12 h at 4 °C against 50 mM Tris-HCl buffer (pH 7.2) containing 200 mM NaCl and 0.1 mM EDTA. In order to remove the residual cholate completely, the dialyzed solution was applied to Toyo Soda HPLC system (CCPM and UV8000) with a column of Toyo Soda TSK PWH (0.75×7.5 cm), previously equilibrated with the dialysis buffer. The liposome fractions were detected by ultraviolet scattering at 300 nm and also by the radioactivities of [^{14}C]DPPC (New England Nuclear) which had been mixed with the phospholipids before the dialysis. The concentration of the reductase in the prepared sample was determined from its cytochrome *c* reductase activity, after solubilization of the vesicles with 1% sodium cholate, in comparison with that of the reductase of known concentration. *P*-450_{C21} was incorporated into the liposomal membranes by the cholate dialysis method, in which Emulgen 913 in the purified preparation had been removed before mixing with the phospholipid solution by extensive washing of *P*-450_{C21} on hydroxylapatite [18]. The concentration of *P*-450 was determined from its CO-reduced difference spectrum [26]. The electron microscopic observations showed all the prepared liposomes and proteoliposomes to be unilamellar vesicles of about 50 nm (average diameter). The topology of the reductase, incorporated to the liposomal membranes by the cholate dialysis method, was inferred from the electrophoresis pattern at SDS-polyacrylamide gel electrophoresis after proteolysis of the reductase [7] and about 80% of the reductase was found to be located at the external side of the vesicles. More than 80% of *P*-450_{C21} incorporated into the vesicles was located

at the external side of the vesicles, as judged from the reducibility upon the external addition of the reductase and excess NADPH. The reduction of $P\text{-}450_{C21}$ by the reductase was measured in the basal buffer by observing the increase of the difference in the absorbance between 450 and 490 nm in the presence of CO and the oxygen scavenging system, consisting of 50 mM β-glucose, 1 unit glucose oxidase from *Penicillum amagasakiense* (Nagase Biochemicals) and 30 units bovine liver catalase (Boehringer). The assay procedure of progesterone 21-hydroxylase activity has been described previously [17]. Optical spectra measurements were carried out at 25 °C with Beckman DU-7 spectrophotometer and the kinetics of $P\text{-}450_{C21}$ reduction was studied at 25 °C with a double wavelength stopped-flow apparatus (450 and 490 nm) made by Unisoku Co. using the basal buffer. Density gradient centrifugations with Ficoll (Pharmacia), 3–10% (w/v) in the basal buffer, were performed at 190 000 × *g* at 4 °C for 12 h with a Hitachi 70P72 centrifuge using an RPS 50-2 rotor. Analytical gel filtrations were performed at 25 °C with the Toyo Soda HPLC system equipped with a Toyo Soda TSK-GEL G-5000PW column (0.75 × 60 cm) using the basal buffer.

Results

The binding of the reductase to the liposomal membranes

When the reductase alone was centrifuged on the Ficoll density gradient (3–10%, w/v), the sedimentation pattern detected by cytochrome *c* reductase activity showed two peaks around fraction 9 (approx. density 1.040) and fraction 14 (approx. density 1.014), as shown in Fig. 1(a). The peaks varied in their relative peak heights upon alternation of the concentration of the applied reductase, suggesting that the two peaks might be attributed to the oligomer and the monomer forms of the reductase [27,23]. Fig. 1(b) shows the pattern of the reductase incubated with the preformed vesicles composed of PC, PE, and PS at the molar ratio of 5 : 3 : 1, in which the reductase was detected around fraction 15 (approx. density 1.011) and [^{14}C]DPPC radioactivities, representing the phospholipid concentration, showed a peak around

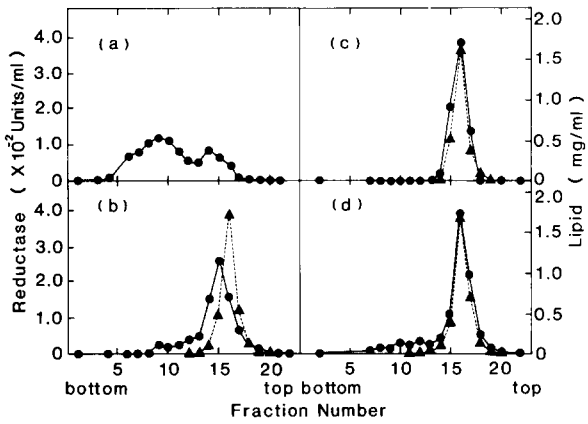

Fig. 1. Sedimentation pattern of NADPH-cytochrome *P*-450 reductase and the vesicles on the Ficoll density gradient (3–10%, w/v). (a), 0.1 nmol unbound reductase; (b), 0.1 nmol reductase previously incubated with 0.5 mg of the preformed vesicles composed of PC, PE, and PS at the molar ratio of 5 : 3 : 1; (c), 0.1 nmol reductase incorporated into 0.5 mg of the vesicles by the cholate dialysis method; (d), 0.1 nmol reductase previously incubated with 0.5 mg of the preformed vesicles containing 0.04 nmol $P\text{-}450_{C21}$. ●———●, cytochrome *c* reductase activity; ▲------▲, concentration of the phospholipids. One unit of the cytochrome *c* reductase activity corresponds to 1 μmol cytochrome *c* reduced per min. See the text for details.

fraction 16 (approx. density 1.008). The difference of the peak position of the reductase between (a) and (b) in Fig. 1 indicates that the reductase interacts with the preformed vesicles. When the incubation of the reductase with the preformed vesicles and the subsequent centrifugation were performed in the presence of a higher salt concentration (200 mM NaCl), the sedimentation pattern of the reductase was similar to that in Fig. 1(b). This suggests that the interaction of the reductase with the preformed vesicles is not mainly governed by the electrostatic force. The density gradient centrifugation of the reductase, incubated with vesicles prepared from PC alone, showed that the interaction of the reductase with PC vesicles was weaker than that with the vesicles composed of PC, PE, and PS. When the reductase was incubated with the preformed vesicles containing $P\text{-}450_{C21}$ in which $P\text{-}450_{C21}$ and the reductase were present at the molar ratio of 1 : 2.5, almost all the reductase was located at the liposome fraction (Fig. 1(d)). A similar result was obtained with the system containing $P\text{-}450_{C21}$ and the reductase at a 1 : 10 molar ratio, indicating that $P\text{-}450_{C21}$ in

the liposomes might induce the binding of the reductase to the membranes. The reductase incorporated into the liposomal membranes by the cholate dialysis method was located at the same position as the liposomes on the density gradient (Fig. 1(c)). From the results shown in Fig. 1(a), (b), and (c), it can be suggested that the reductase can bind to the preformed vesicles during the incubation but the interaction is not so strong as that in the sample prepared by the cholate dialysis method. The modes of the reductase binding to the membranes were further examined by HPLC gel filtration. The chromatogram of the reductase incorporated to the vesicles by the cholate dialysis method is depicted in Fig. 2(a), where the reductase was detected at the same position as the liposomes. When the reductase alone was submitted to gel filtration, only 5% of the applied reductase was recovered around the position shown by an open arrow in Fig. 2(a), which might be due to the adsorption of the reductase to the column.

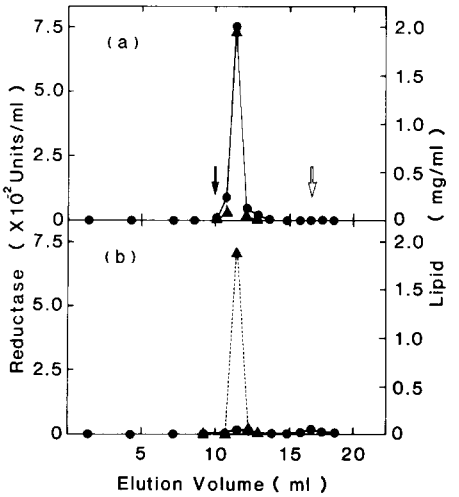

Fig. 2. Gel filtrations of NADPH-cytochrome *P*-450 reductase and the vesicles. (a), 0.1 nmol reductase incorporated into 0.3 mg of vesicles prepared by the cholate dialysis method; (b), 0.1 nmol reductase previously incubated with 0.3 mg of the preformed vesicles. The closed arrow shows the position of the void volume of the column and the open arrow shows the position where the unbound reductase is recovered. ●———●, cytochrome *c* reductase activity; ▲------▲, concentration of the phospholipids. The chromatography was performed at 25 °C at the flow rate of 0.6 ml/min with a Toyo Soda TSK-GEL G-5000PW column. The composition of the phospholipids was the same as that in Fig. 1.

As shown in Fig. 2(b), almost no reductase was recovered at the position of liposomes when the reductase, incubated with preformed vesicles, was applied to the column, suggesting that the reductase interacts weakly with the membranes. These results are consistent with those in Fig. 1.

Transfer of the reductase between the vesicles

The transfer of the reductase between the vesicles could be detected by its reduction activities toward $P\text{-}450_{C21}$ in the other separated vesicles. The experiments in Fig. 3 were performed in the presence of CO but in the absence of the oxygen-scavenging system, where the oxygen in the sample solution was removed with repetitions, more than ten times, of the evacuation and the subsequent flushing of CO, because the scavenging system gradually reduced $P\text{-}450_{C21}$ in the presence of NADPH without the reductase. When 2.8 pmol of the reductase were preincubated with preformed vesicles containing 280 pmol of $P\text{-}450_{C21}$ and subsequently mixed with NADPH, about 90 pmol of $P\text{-}450_{C21}$ were reduced within 10 min as shown by line (a) in Fig. 3. Since one vesicle can be estimated to contain about 7 molecules of $P\text{-}450_{C21}$ under the experimental condi-

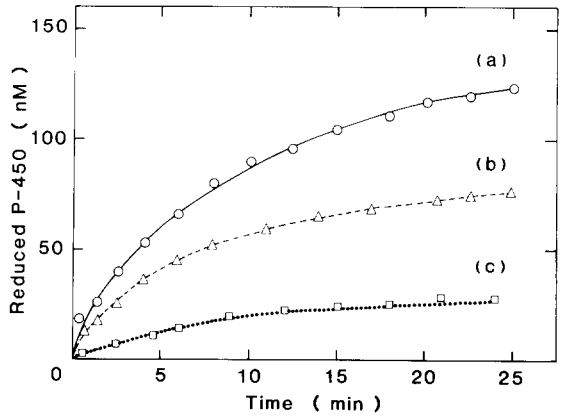

Fig. 3. Transfer of NADPH-cytochrome *P*-450 reductase between the vesicles. (a), 2.8 pmol reductase incubated previously with 0.8 mg of the vesicles containing 280 pmol $P\text{-}450_{C21}$ were mixed with 150 nmol NADPH and the reduction of $P\text{-}450_{C21}$ was measured at 25 °C. (b), the $P\text{-}450_{C21}$-free vesicles (0.8 mg) were further added to the system in (a). (c), 2.8 pmol reductase incorporated into 0.04 mg vesicles by the cholate dialysis method were reacted with 280 pmol $P\text{-}450_{C21}$ in the separated vesicles (0.8 mg). The composition of the phospholipids was same as that in Fig. 1.

tions of line (a) from the calculation using the average diameter of the vesicles and the molar ratio of $P\text{-}450_{C21}$ to the phospholipids [28], the results shown by line (a) in Fig. 3 mean that one reductase molecule interacts with at least more than four vesicles containing $P\text{-}450_{C21}$ within 10 min. When $P\text{-}450_{C21}$-free vesicles were added to the system to double the number of vesicles, the observed rate of the reduction was decreased significantly (line (b) in Fig. 3). These results suggest that the reductase binds not only to the vesicles containing $P\text{-}450_{C21}$ but also to the $P\text{-}450_{C21}$-free vesicles. The reductase incorporated into the vesicles by the cholate dialysis method showed a much slower reduction rate of $P\text{-}450_{C21}$ in the separated vesicles (line (c) in Fig. 3), indicating that the reductase in this preparation is less easily transferable between vesicles.

It is interesting to determine whether the rate-limiting step in the transfer of the loosely bound reductase between vesicles is the binding to or the release from the vesicles. A solution containing a small amount of the unbound reductase and a large amount of NADPH was mixed rapidly with vesicles containing $P\text{-}450_{C21}$ and the initial rates in the reduction of $P\text{-}450_{C21}$ were measured. Because of the fast electron transfer from the reductase to $P\text{-}450_{C21}$ [23,29], the reduction must reflect the binding of the reductase to $P\text{-}450_{C21}$ proteoliposomes. The initial rates of the reduction of $P\text{-}450_{C21}$ were increased with the amount of $P\text{-}450_{C21}$ proteoliposomes (line (a) in Fig. 4), showing the second-order kinetic process. Similar kinetics was observed when various amounts of the reductase were reacted with a constant amount of $P\text{-}450_{C21}$ proteoliposomes. In order to measure the rate of the release of the reductase from the vesicles, a solution containing $P\text{-}450_{C21}$ proteoliposomes, with which a small amount of the reductase had been previously incubated for the binding to the proteoliposomes, was mixed rapidly with $P\text{-}450_{C21}$ proteoliposomes. The observed reduction of $P\text{-}450_{C21}$ indicates that the reductase was released from the original vesicles and subsequently bound to the other $P\text{-}450_{C21}$ proteoliposomes. As illustrated by the line (b) in Fig. 4, the rates were independent of the amount of the $P\text{-}450_{C21}$ proteoliposomes mixed afterwards and were slower than those of line (a). This indicates

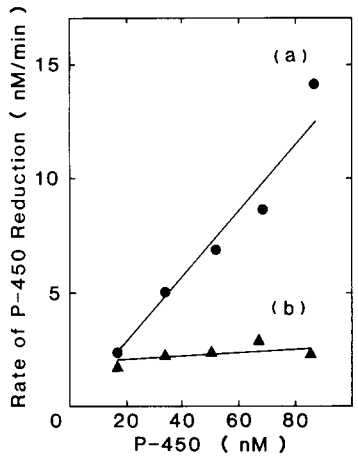

Fig. 4. The kinetics of the binding and the release of NADPH-cytochrome *P*-450 reductase to and from the vesicles. The details are described in the text. (a), various amounts of $P\text{-}450_{C21}$ proteoliposomes were mixed with 1.48 nM reductase and 150 μM NADPH, and the initial rate in the reduction of $P\text{-}450_{C21}$ was measured at 25 °C. (b), 17 nM $P\text{-}450_{C21}$ in 0.03 mg/ml of the vesicles, previously incubated with 1.48 nM reductase and 150 μM NADPH, was mixed with various amounts of $P\text{-}450_{C21}$ proteoliposomes. The composition of the phospholipids was same as that in Fig. 1.

that the release of the reductase from the original vesicles is the rate-limiting step in the transfer of the reductase between the vesicles (see Discussion).

Loosely bound reductase can support the hydroxylase activity of $P\text{-}450_{C21}$ proteoliposomes

Progesterone 21-hydroxylase activity of the $P\text{-}450_{C21}$ proteoliposomes was measured after the incubation with the reductase. The activity was dependent on the molar ratio of the reductase to $P\text{-}450_{C21}$, as shown in Table I. The observed activity of the system containing the reductase and $P\text{-}450_{C21}$ at the molar ratio of 1 : 1 was quite similar to the value of 10.5 mol/min per mol *P*-450, observed for the sample containing both $P\text{-}450_{C21}$ and the reductase at an equimolar ratio in the same vesicles prepared by the dialysis method. On the other hand, the hydroxylase activity was not detected for the sample in which $P\text{-}450_{C21}$ proteoliposomes and the reductase proteoliposomes were mixed together, each prepared by the cholate dialysis method. This result shows that the reductase incorporated to the vesicles by the cholate dialysis method can not readily transfer between the vesicles.

TABLE I

DEPENDENCE OF PROGESTERONE 21-HYDROXYLASE ACTIVITY OF $P\text{-}450_{C21}$ PROTEOLIPOSOMES ON THE MOLAR RATIO OF NADPH-*P*-450 REDUCTASE TO $P\text{-}450_{C21}$

NADPH-*P*-450 reductase was incubated with $P\text{-}450_{C21}$ proteoliposomes at the indicated molar ratio before the reaction. The reaction was carried out at 25 °C in the basal buffer. The details are described in the text.

	Reductase/$P\text{-}450_{C21}$ (mol/mol)			
	0.5	1.0	2.0	4.0
Activity (mol/min per mol *P*-450)	4.4 ± 0.3	8.5 ± 0.5	13.0 ± 1.0	23.0 ± 5.0

Discussion

It is quite clear that there are two modes of binding for adrenal NADPH-cytochrome *P*-450 reductase to the liposomal membranes. Black and Coon speculated two possible binding modes for the hepatic reductase to the membrane of the endoplasmic reticulum, based on its amino acid sequence of the hydrophobic tail [9]. One form was assumed to have the hydrophobic tall spanning the lipid bilayer of the membranes with the N-terminal exposed from the internal side of the membrane and the hydrophilic functional domain exposed from the other side of the membrane. The other form might have the tail with a hairpin loop and both the N-terminal and the functional domain might be located at the external side of the membrane. Two similar modes of binding to the membranes were proposed for cytochrome b_5, but the experimental results do not completely confirm the two binding modes [30–32]. Two such binding modes for a single protein were shown experimentally for the H-$2K^k$ glycoprotein using fluorescently labeled compound [33]. The N- and C-terminals of the glycoprotein were located at different sides of the native plasma membrane but the glycoprotein, incorporated by the cholate dialysis method to liposomal membrane, had both terminals at the same side with a hairpin loop. The two binding modes of the reductase in the present study might be similar to those proposed by Black and Coon [9], but the configuration of the tail of the reductase cannot be discussed from the present results.

The interaction of the reductase with the preformed PC vesicles was significantly weaker than that with the vesicles composed of PC, PE, and PS, as shown by density gradient centrifugation. The dependence of the interaction of cytochrome b_5 with the membrane on its composition was also reported, in which the transferable cytochrome b_5 bound more tightly to the microsomes than to the PC vesicles [34]. Ingelman-Sundberg and Glaumann [35] showed that almost all the hepatic reductase, incubated with the preformed PC vesicles, was removed by gel filtration. In contrast, Duppel et al. [36] reported that the hepatic reductase was not removed from the vesicles during gel filtration when the reductase was incubated with liposomes prepared from the extracted microsomal lipids.

Leto and Holloway [37] showed in their kinetic study of cytochrome b_5 binding to liposomal membranes that the binding proceeded in first order kinetics, from which it was inferred that only the monomer form of cytochrome b_5, dissociated from the oligomer form, could bind to the membranes and its dissociation might be the rate-limiting step of cytochrome b_5 binding to the membranes. The kinetics of the reductase binding to the liposomal membranes in this study is apparently in second-order kinetics and the reductase may be in the equilibrium of the monomer-oligomer forms [23,27]. Second-order kinetics indicates that the rate-limiting step of the reductase binding is not the dissociation process of the reductase from the oligomer form, but is related to the collisions between the reductase and the vesicles. Second-order kinetics was also reported for the binding of $P\text{-}450_{scc}$ to liposomal membranes [38]. On the other hand, the rate of the release of the reductase from liposome membranes is not dependent on the amount of additional $P\text{-}450_{C21}$ proteoliposomes, which indicates that the rate-limiting step of the transfer of the reductase between the vesicles might be the release from the original vesicles. If fast release of the reductase from the original vesicles and slow binding of the reductase to the other vesicles were assumed in the experiment of line (b) in Fig. 4, the released reductase would bind to the other *P*-

450_{C21} proteoliposomes in second-order kinetics and the observed rate of reduction of $P\text{-}450_{C21}$ would be dependent on the amount of additional $P\text{-}450_{C21}$ proteoliposomes.

Gum and Strobel [8] showed that the hydrophobic segment of the hepatic reductase did not prevent the binding of the reductase to the hepatic cytochrome *P*-450, suggesting that the tail, the hydrophobic segment, does not bind to cytochrome *P*-450. They deduced the role of the tail in the interaction with cytochrome *P*-450, i.e., that the tail might support the topological orientation of the reductase in the membrane for facilitating the interaction. However, Black et al. [39] reported that the tail segment of the hepatic reductase could bind to cytochrome *P*-450. In cytochrome b_5 the hydrophobic tail is not necessarily required for the interaction with NADH-cytochrome b_5 reductase [40]. The tails of the reductases in the two binding modes in this study must have different configurations from each other in the membrane, but both forms are active for electron transfer toward $P\text{-}450_{C21}$ and can support the hydroxylase activity of $P\text{-}450_{C21}$. These results suggest that the shape of the hydrophobic tail in the membrane does not affect the interaction with $P\text{-}450_{C21}$ very much.

It is important to distinguish whether the reductase at the native membranes is readily released from the membrane or not. When the adrenocortical microsomes were mixed with $P\text{-}450_{C21}$ proteoliposomes in the presence of NADPH and CO in a preliminary experiment, significant amounts of $P\text{-}450_{C21}$ in the vesicles were found to be reduced and the rate of the reduction was similar to that of the case where one third of the reductase in the microsomes was in loosely bound form. We cannot conclude from this result that one third of the reductase at the endoplasmic reticulum is bound loosely to the membranes, but we must now examine the possibility that the loosely bound reductase might occur during the preparation of microsomes.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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