Cytochrome P-450 Transfer from Adrenocortical Submitochondrial Particles to Liposome Membranes†

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ABSTRACT: The transfer of cytochrome P-450 from bovine adrenocortical submitochondrial particles (smp) to unilamellar liposome membranes was investigated using a table top ultracentrifuge. Submitochondrial particles were incubated with liposome membranes at 25 °C and precipitated by ultracentrifugation at 200000*g* for a few minutes at 25 °C. All liposome vesicles were recovered in the supernatant. Almost no proteins were detected in the supernatant when only smp were incubated and centrifuged. SDS−PAGE revealed one main protein band for the supernatant when smp were incubated with liposome vesicles at 25 °C. This band was reactive to anti-P-450$_{scc}$ IgG. Inaccuracy in time for kinetic studies of the transfer was less than 0.5 min. Transfer of P-450$_{scc}$ from smp to liposome membranes was further demonstrated by the decrease in side-chain cleavage activity of smp for endogenous cholesterol after incubation. Cytochromes P-450 accounted for about 70% of the transferred proteins in the liposome membranes, the amount of which increased exponentially with the incubation time. The inverse value of the relaxation time of the transfer increased linearly with the smp concentration and decreased hyperbolically with the liposome concentration. These results coincide with a mechanism by which cytochrome P-450 dissociates from smp membranes, diffuses, and binds to the liposome membranes. In the transfer of cytochrome P-450, the dissociation from smp membranes was deduced to be the rate-limiting step.

The adrenal cortex synthesizes several steroid hormones from cholesterol with the actions of four molecular species of cytochrome P-450 and 3β-hydroxysteroid dehydrogenase: Δ^4,Δ^5-isomerase (Takemori & Kominami, 1984). The adrenal cortex consists of three histochemically and physiologically distinct zones: zonae glomerulosa, fasciculata, and reticularis. The most potent mineralocorticoid, aldosterone, is synthesized exclusively in zona glomerulosa cells. We reported that the interaction of P-450$_{scc}$[1] with P-450$_{11\beta}$ could contribute to the regulation of aldosterone production of P-450$_{11\beta}$ in the bovine adrenal cortex (Ikushiro et al., 1992). We assumed that the interaction between two mitochondrial cytochrome P-450s differs in these three zones in the adrenal cortex, which might be due to the difference in the concentrations. We attempted to quantify the actual level of P-450$_{11\beta}$ and P-450$_{scc}$ in the mitochondrial inner membranes from the three zones (Ikushiro et al., 1989a). While preparing the inner membranes, we found that the ratio of P-450$_{scc}$ to P-450$_{11\beta}$ decreased. We thought that this might be due to the specific transfer of cytochrome P-450s from the mitrochondrial inner membranes to other membrane systems.

It is generally believed that membrane proteins are not easily released from membranes, but the transfer of several membrane proteins between membrane systems has been reported. Newton et al. (1983, 1988) reported the transfer of band 3, the anion transporter in erythrocyte, from erythrocyte to vesicle membranes. Enoch et al. (1977, 1979) demonstrated the transfer of cytochrome b_5 and NADH−cytochrome b_5 reductase from large unilamellar vesicles to small unilamellar vesicles. Cytochrome b_5 attaches to vesicle membranes only by the C-terminal hydrophobic anchor (Tajima & Sato, 1980; Daily & Strittmatter, 1981; Takagi et al., 1983; Vergeres & Waskell, 1992), whereas NADH−cytochrome b_5 reductase binds to membranes only by the N-terminal anchor (Ozols et al., 1984). Cytochrome b_5 can be stably dissolved in aqueous solution as an octamer (Calabro et al., 1976), and the cytochrome b_5 that dissociates from the octamer can be incorporated into vesicle membranes where the dissociation is the rate-limiting step for the incorporation (Leto & Holloway, 1979). NADPH−cytochrome P-450 reductase forms oligomer aggregates and is fairly stable in aqueous solution (French et al., 1980; Kominami et al., 1984). Kominami et al. (1987) showed the transfer of NADPH−cytochrome P-450 reductase between vesicles using the P-450$_{C21}$ reconstituted system. P-450$_{scc}$ forms an oligomer in aqueous solution without detergent (Shikita & Hall, 1973), and Dhariwal et al. (1991) demonstrated the transfer of adrenal mitochondrial P-450$_{scc}$ between vesicles.

Most of these studies on the transfer of membrane proteins between membrane systems have been performed using small vesicles that could be separated from large particles by gel filtration or density gradient centrifugation. These methods are time consuming and unsuitable for kinetic studies on membrane protein transfer. Table top ultracentrifuge devices

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[1] Abbreviations: smp, submitochondrial particles; P-450$_{scc}$, cytochrome P-450 having cholesterol desmorase activity (P-450 11A1); P-450$_{11\beta}$, cytochrome P-450 having steroid 11β-hydroxylase activity (P-450 11B1); P-450$_{C21}$, cytochrome P-450 having steroid 21-hydroxylase activity (P-450 21A1); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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now provide a centrifugal force of 200000*g* within a few minutes. In this study, we used a table top ultracentrifuge to separate bovine adrenocortical smp from small vesicles and performed detailed kinetic studies on the cytochrome P-450 transfer from smp to vesicle membranes.

EXPERIMENTAL PROCEDURES

Chemicals. L-α-Phosphatidylcholine (egg yolk, type III), cardiolipin (bovine heart), pregnenolone, and 20α-hydroxycholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). L-α-Phosphatidylethanolamine (egg yolk) was obtained from Lipid Products (Surrey, U.K.). [^{14}C]Dipalmitoylphosphatidylcholine was from DuPont-NEN. (Boston, MA). Cholesterol oxidase from *Nocardia* sp. was from Oriental Yeast Co. (Osaka). All other chemicals were of the best quality commercially available.

Preparation of Submitochondrial Particles (Smp). Submitochondrial particles (smp) were prepared from bovine adrenocortical mitochondria. Fresh bovine adrenal glands (Holstein-Friesian, 4−5 years old) were obtained from a local slaughterhouse. All the preparation procedures were carried out at 4 °C. Tissues from zona fasciculata-reticularis were collected from the bisected adrenals, which were homogenized with a loose Teflon homogenizer in 20 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 0.1 mM EDTA. Mitochondria were obtained by differential centrifugation (2000*g* for 8 min and 5200*g* for 10 min). The mitochondria were suspended to 1.4 mg/mL in 50 mM Tris-HCl buffer (pH 7.3) containing 0.1 mM EDTA and 0.3 M sucrose and sonicated at 160 W for 500 s (W-225, Heat System-Ultrasonic Inc., Farmingdale, NY) in a repeat pulse sequence of 0.5 s of sonication and 0.5 s of cooling. These sonication conditions were selected to maximally destroy the mitochondrial membrane systems, which was detected by the malate dehydrogenase activity (mitochondrial matrix marker enzyme) in the supernatant (Ochoa, 1955). Almost no conversion of cytochrome P-450 to P-420 was detected after the sonication. The suspension was centrifuged at 32000*g* for 30 min, and the precipitate was resuspended in 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 M NaCl for washing out physically attached proteins on the smp surface. This process was repeated three times, and the precipitated smp were resuspended to a protein concentration of about 10 mg/mL in 40 mM Tris-HCl (pH 7.3) containing 0.1 mM EDTA, 160 mM NaCl, and 20% (v/v) glycerol (transfer buffer). The suspension was sedimented once more by centrifugation and suspended in the transfer buffer. The smp were frozen at 77 K until used. This preparation contained 1.0 ± 0.2 nmol of P-450/mg of protein.

Transfer of Membrane Proteins from Smp to Liposomes. Unilamellar vesicles were prepared using phosphatidylcholine, phosphatidylethanolamine, and cardiolipin at a weight ratio of 2:2:1 containing 10 000 cpm of [^{14}C]dipalmitoylphosphatidylcholine/mg of phospholipids as described (Ikushiro et al., 1989b). In a typical transfer experiment, smp containing 1.54 mg of protein were mixed with liposomes containing 1.54 mg of phospholipids in 400 μL (final volume) of transfer buffer and incubated for various periods at 25 °C. This mixture was ultracentrifuged at 25 °C for 3 min (1.5 min of acceleration, 1.5 min at 200000*g*) using a table top ultracentrifuge (HIMAC 100, RP100-AT4, Hitachi, Tokyo). The protein concentration in the supernatant was determined by a BCA protein assay kit using bovine serum albumin as the standard (Pierce, Rockford, IL). The concentration of cytochrome P-450 was estimated from the dithionite-reduced CO difference spectrum using Δε (450 nm − 490 nm) = 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). In some experiments, the concentration of cytochrome P-450 in liposome membranes was estimated from the absorbance at 418 nm using the absorption coefficient of 110 mM^{-1} cm^{-1}. The phospholipid concentration was determined by measuring the level of [^{14}C]dipalmitoylphosphatidylcholine radioactivity.

Assay of Enzymatic Activity. Cholesterol side-chain cleavage activity in the incubated smp with or without liposomes was measured at 37 °C using added 20α-hydroxycholesterol (50 nmol) or endogenous cholesterol in the smp membranes as substrates. The reaction solution in 1 mL of transfer buffer contained 5 nmol of adrenodoxin and 0.2 nmol of NADPH−adrenodoxin reductase and contained either the incubated smp (0.085 mg of protein) or the mixture with liposomes (2.8 mg of phospholipids). The reaction was initiated with 100 nmol of NADPH and terminated by rapid heating of the reaction solution at 90 °C for a few minutes. The produced pregnenolone was converted to progesterone by incubation at 37 °C for 10 min with 0.1 unit of cholesterol oxidase from *Nocardia* sp. (Oriental Yeast Co., Osaka) in 0.1% (w/v) sodium cholate and 0.3% Tween 20 (Ogishima & Okuda, 1986; Sugano et al., 1989). The complete conversion of pregnenolone to progesterone was confirmed under these conditions. Progesterone was extracted with 1 mL of choloroform containing 1 nmol of 17α-hydroxyprogesterone as the internal standard. The steroid was separated by HPLC (CCPM, UV-8000, AS-8000; Tosoh Inc., Tokyo) equipped with a silica gel column (Cosmosil 5SL, 4.6 × 150 mm, Nacalai Tesque Inc., Kyoto) using *n*-hexane/2-propanol/acetic acid [900/65/9.65 (v/v/v)] at a flow rate of 0.6 mL/min (Kominami et al., 1989).

Assay of Cholesterol Content in Smp and Liposome Membranes. Cholesterol contents in smp and liposome membranes were determined according to the method of Gamble et al. (1978). Smp (1.54 mg) in the transfer buffer were incubated with or without 1.54 mg of liposomes at 25 °C for various periods and ultracentrifuged at 200000*g*. Cholesterol in the liposome membranes (supernatant) or smp membranes (precipitates) was extracted three times with 1 mL of choloroform/methanol solution [2/1 (v/v)], and the collected extract was dried under N_2 gas. The organic solvents were completely removed by heating the samples in an oven at 105 °C for 20 min. The extracted cholesterol was dissolved with 150 μL of ethanol and was diluted with 150 μL of 100 mM potassium phosphate buffer (pH 7.4) containing 0.25% (w/v) Triton X-100 and 10 mM sodium cholate. To this solution was added 1.425 mL of 100 mM potassium phosphate buffer (pH 7.4) containing 0.2 unit of cholesterol oxidase, 2 units of horseradish peroxidase (Sigma, Type X), and 0.4 mg of *p*-hydroxyphenylacetic acid (Sigma). The mixture was incubated at 37 °C for 1 h, and the fluorescence was measured at 415 nm with excitation at 325 nm using a fluorescence spectrometer (Hitachi MPF-4). The intensity of the fluorescence was compared with that of the standard cholesterol solution treated in the same way.

Other Methods. SDS−PAGE was performed using tricine buffer according to the method of Schaegger and Von Jagow (1987). The concentration of polyacrylamide in the separa-

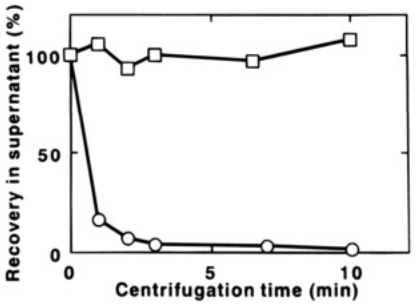

FIGURE 1: Ultracentrifugation of smp and liposome membranes at 200000*g*. Bovine adrenocortical smp (1.54 mg of protein) and liposomes (1.54 mg of phospholipids) in 0.4 mL of transfer buffer were centrifuged separately at 25 °C for the periods indicated on the horizontal axis which includes the acceleration but not deceleration time. Residual smp in the supernatant were determined by measuring the concentration of cytochrome P-450 and are represented by open circles. The recovery of liposome membranes in the supernatant was estimated from the level of [^{14}C]dipalmitoylphosphatidylcholine radioactivity in the membranes and is represented by open squares.

tion and stacking gels was 10% and 4%, respectively. Electrophoresis was performed at 20 mA for 30 min and then at 80 mA for 1 h. Proteins were silver stained or Western blotted as described (Shinzawa et al., 1988) using a semidry blotting device (Biocraft Co., Tokyo) with anti-P-450$_{scc}$ IgG and anti-P-450$_{11\beta}$ IgG. These antibodies were prepared from rabbit sera as described (Kominami et al., 1983). The IgG binding was detected by staining with horseradish peroxidase-labeled anti-rabbit sera IgG, 4-chloro-1-naphthol and H_2O_2. Optical spectra were measured in transfer buffer using a Beckman DU-7. The kinetic data were calculated by a personal computer, PC-9801 VM (NEC, Tokyo).

RESULTS

Detection for Membrane Protein Transfer. To obtain kinetic data on the transfer of membrane proteins from smp to liposome membranes, it is necessary to quickly separate liposomes from smp. We used a table top centrifuge to achieve this, since only 1.5 min was required for acceleration to a force of 200000*g* and only 1.5 min for deceleration. Figure 1 shows the time dependence of smp precipitation at a centrifugal force of 200000*g*. Almost all the smp were precipitated after 3 min of centrifugation, including acceleration (not including deceleration), in which cytochrome P-450 in smp was chosen as a marker of smp concentration. A very small amount of cytochrome P-450 (about 1% of the cytochrome P-450 content of the original smp) was detected in the supernatant after an extended period of centrifugation of the smp incubated without liposomes. The liposomes did not precipitate under these centrifugation conditions.

In a typical transfer experiment, we preincubated the smp and liposome solutions separately at 25 °C for 3 min in a temperature-regulated water bath. These solutions were mixed by gentle pipetting, incubated for various periods at 25 °C with gentle shaking, and subsequently centrifuged at 25 °C for 3 min at 200000*g*. The incubation time for the transfer was defined as the duration from the mixing to the initiation of rotation plus 30 s, hereafter. The time for evacuation of the centrifuge chamber was included in the incubation time, which is about 1 min. About half of the smp precipitated within 30 s after the start of the centrifugation. We confirmed that no membrane proteins were transferred from the precipitated smp to the liposome membranes. One minute after the start of centrifugation, most smp precipitate even if the force does not reach 200000*g*, indicating that the inaccuracy of the incubation time is below 0.5 min. Radioactive phospholipids were undetectable in the smp precipitate after a 30-min incubation with liposomes, indicating that there was no fusion between vesicle and smp membranes.

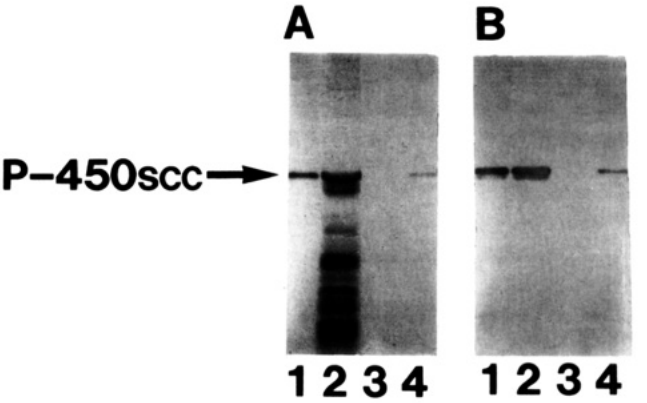

FIGURE 2: SDS−PAGE (A) and Western blotting using anti-P-450$_{scc}$ IgG (B) of the transferred proteins from smp to liposome membranes. Smp containing 1.54 mg of protein were incubated with and without liposome membranes (1.54 mg of phospholipids) in 0.4 mL of transfer buffer at 25 °C for 5 min and subsequently centrifuged at 200000*g* for 3 min. In lane 2, $^1/_{400}$th of the original smp solution was applied, and $^1/_{400}$th of the supernatant after the incubation without or with liposomes, respectively, was applied in lanes 3 and 4. Lane 1 is for purified bovine adrenocortical P-450$_{scc}$ (0.1 μg).

SDS−PAGE and Western Blotting of the Transferred Membrane Proteins. Figure 2A shows the results of SDS−PAGE of the transferred proteins from smp to liposome membranes. Lanes 3 and 4 show proteins in the supernatant fractions after smp were incubated at 25 °C for 5 min without and with liposomes, respectively. Almost no protein bands are detected in lane 3, but there is one main protein band with several other faint bands in lane 4. This shows that some membrane proteins can move from smp to liposome membranes. There are many membrane proteins in the smp membranes as evidenced by lane 2, but only limited species move to liposome membranes in which the main protein band appears at a position similar to that of P-450$_{scc}$ in lane 1. The protein (BCA method) and cytochrome P-450 concentrations (dithionite-reduced CO difference spectrum) in the supernatant after the incubation showed that cytochrome P-450 accounted for about 70% of the protein. The major cytochrome P-450s in adrenocortical mitochondrial membranes are P-450$_{scc}$ and P-450$_{11\beta}$, which have a similar molecular mass (Suhara et al., 1978). Western blotting (Figure 2B) shows that anti-P-450$_{scc}$ IgG binds to the main protein band in lane 4 whereas anti-P-450$_{11\beta}$ IgG does not (data not shown). This demonstrates that the major protein which moves from smp to the liposome membranes is P-450$_{scc}$. About 10−20% of the proteins that immigrated after a long incubation, such as 1 h at 25 °C, were P-450$_{11\beta}$ because the proteins in the supernatant showed a little reactivity with anti-P-450$_{11\beta}$ IgG. The amount of P-450$_{scc}$ in lane 4 in Figure 2B could be roughly estimated to be $^1/_{10}$th to $^1/_{20}$th of that in lane 2, which agrees with the data in Figure 4 under the assumption that the ratio of P-450$_{scc}$ to P-450$_{11\beta}$ is 1.0 in the original smp (Hanukoglu & Hanukoglu, 1986).

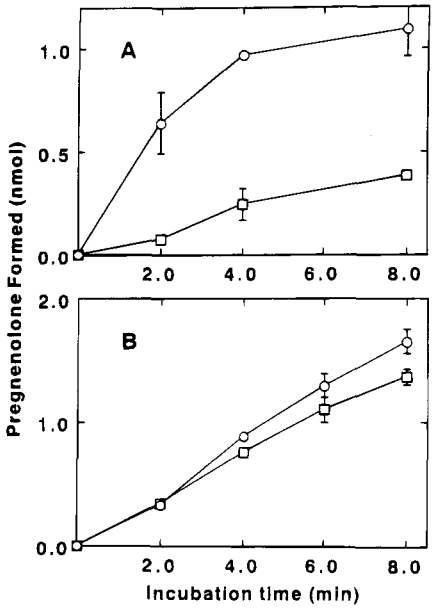

FIGURE 3: Cholesterol side-chain cleavage activity of the transferred P-450$_{scc}$ for the endogenous cholesterol in smp membranes (A) and for exogenous 20α-hydroxycholesterol (B). The open circles show the activity of smp (0.085 mg of protein) after the incubation of smp alone at 25 °C for 30 min. The open squares show the activity of the mixture of smp (0.085 mg of protein) and liposome membranes (2.8 mg of phospholipids) after the incubation. The enzyme reaction was carried out at 37 °C for the periods indicated on the horizontal axis. The vertical axis shows the amount of produced pregnenolone. The assay conditions are described in Experimental Procedures.

Enzyme Activity of Transferred P-450$_{scc}$. The cholesterol side-chain cleavage activity of P-450$_{scc}$ of smp after a 30-min incubation at 25 °C was assayed using the endogenous cholesterol in the smp membranes as substrate. The smp were mixed with adrenodoxin and NADPH–adrenodoxin reductase, and the reaction was started by addition of NADPH. The production of pregnenolone was almost linear for 4 min (Figure 3A). The side-chain cleavage activity of P-450$_{scc}$ in this smp was about 2.9 ± 0.3 nmol of pregnenolone formation/mg of protein of smp/min. When smp of 0.31 mg of protein were incubated with 10 mg of liposomes (phospholipid weight) in 400 μL of transfer buffer at 25 °C for 30 min (not separated from liposomes), the side-chain cleavage activity decreased to a quarter of that in the original smp. The side-chain cleavage activity of the incubated mixture was measured using 20α-hydroxycholesterol, a water-soluble substrate for P-450$_{scc}$. As shown in Figure 3B, the pregnenolone formation from 20α-hydroxycholesterol by the smp is about same as that when incubated with liposomes. This shows that the decrease in pregnenolone production in Figure 3A is not due to the inactivation of P-450$_{scc}$ in smp during the incubation with liposomes but is the result of P-450$_{scc}$ transfer to liposome membranes. The decrease in pregnenolone production of smp in Figure 3A cannot be attributed to the dilution of the endogenous cholesterol in the liposome membranes. Cholesterol transfer from smp to liposome membranes was estimated after incubation of 1.54 mg of smp with 1.54 mg of liposome membranes at 25 °C for 30 min. The decrease of cholesterol content in the smp was less than 5% of the total cholesterol of the smp. The visible-range spectrum of the transferred proteins was essentially that of the low-spin cytochrome P-450.

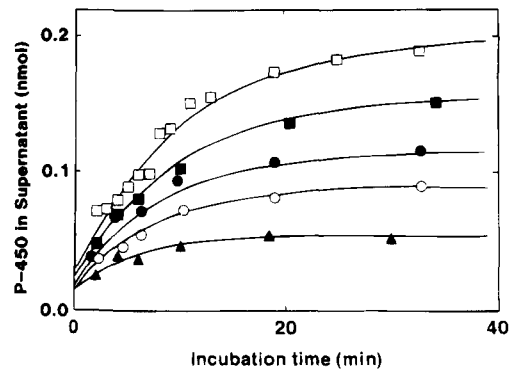

FIGURE 4: Transfer of cytochrome P-450 from smp to liposome membranes. Submitochondrial particles (1.54 mg of protein) were incubated in 0.4 mL of transfer buffer at 25 °C for the periods indicated on the horizontal axis in the presence of 0.308 mg (▲), 0.616 mg (○), 0.924 mg (●), 1.23 mg (■), and 1.54 mg (□) of liposome membranes and subsequently centrifuged at 200000*g*. The amount of cytochrome P-450 in the supernatant was estimated from the absorption at 418 nm. The solid lines from the bottom to the top show theoretical curves with relaxation times of 5.5, 7.5, and 8.5, 10.5, and 10.5 min, respectively, which were determined by least squares fittings of the data. The experimental conditions are described in Experimental Procedures. The ratio of the transferred P-450$_{scc}$ to the P-450$_{scc}$ in the original smp could be roughly estimated by dividing the values on the *y* axis with 0.77 nmol under an assumption that the ratio of P-450$_{scc}$ to P-450$_{11\beta}$ is 1.0 in smp.

Kinetics of Cytochrome P-450 Transfer. The major proteins transferred from smp to liposome membranes were cytochrome P-450s. The amount of transferred cytochrome P-450 was estimated from the Soret absorption at 418 nm, which was proportional to the amplitude of the dithionite-reduced CO difference spectra at each point in Figure 4. The side-chain cleavage activity for 20α-hydroxycholesterol in the supernatant was parallel to the cytochrome P-450 concentration. The amount of cytochrome P-450 in the supernatant increased exponentially with the incubation period. The amount of transferred cytochrome P-450 to liposome membranes after 30 min of incubation increased with the amount of liposomes in the system. The solid lines in Figure 4 are theoretical curves drawn with relaxation times which fit the observed points by the nonlinear least squares method. The relaxation time of cytochrome P-450 transfer was dependent on the amount of both smp and liposomes. Figure 5 shows the inverse value of the relaxation time, τ^{-1}, plotted against the liposome and smp concentrations, in which τ^{-1} increased with the latter (Figure 5B) but decreased with an increase in the former (Figure 5A). The maximum amount of transferred cytochrome P-450 could be estimated from the exponential curve in each experiment in Figure 4 and was also dependent on the amount of both smp and liposomes in the system. The dependences were illustrated in Figure 6 with the theoretically derived curves based on eq 10 in the Appendix.

DISCUSSION

The transfer of several membrane proteins between membrane systems has been reported. Among them, cytochrome b_5 has been studied in detail (Enoch et al., 1979; Greenhut et al., 1986). The transferable cytochrome b_5 in vesicle membranes is thought to have a hairpin-like C-terminal structure which might be anchored in the membranes (Tajima & Sato, 1980; Takagi et al., 1983). Cytochrome b_5 forms a stable octamer in aqueous solution (Calabro et al.,

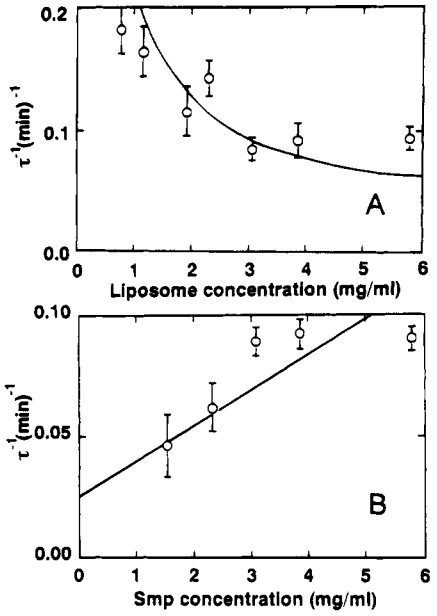

FIGURE 5: Effects of smp and the liposome concentration on the inverse value of the relaxation time for cytochrome P-450 transfer from smp to liposome membranes. (A) Smp (1.54 mg of protein) were incubated in 0.4 mL of transfer buffer with various amounts of liposome membranes. The horizontal axis shows the concentration of liposomes during the incubation. (B) Liposome membranes (1.54 mg) were incubated in 0.4 mL of transfer buffer with various amounts of smp. The horizontal axis shows the protein concentration of smp. The experimental conditions are the same as those in Figure 4. The lines show the theoretical curves using eq 9 with the kinetic parameters shown in the text.

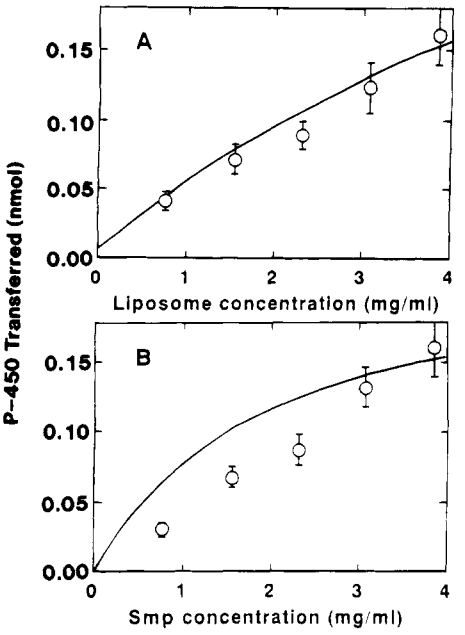

FIGURE 6: Effects of liposome and smp concentrations on the maximum amount of cytochrome P-450 transferred to the liposome membranes after the incubation of smp with liposome membranes at 25 °C. (A) Smp (1.54 mg) were incubated with various amounts of liposomes as indicated on the horizontal axis in 0.4 mL of transfer buffer at 25 °C, and the maximum amount of cytochrome P-450 transferred was estimated from the exponential curves. (B) Liposome membranes (1.54 mg) were incubated with various amounts of smp. The solid lines show the theoretical curves drawn using eq 10 in the Appendix with the kinetic parameters shown in the text. It is assumed that one-third of cytochrome P-450 in smp is transferable from smp to liposome membranes.

1976), and the dissociation of cytochrome b_5 from the octamer has been reported to the rate-limiting step for the binding to vesicle membranes (Leto & Holloway, 1979). Details of the transfer kinetics were not investigated, since no means of rapidly separating the membrane systems were available at that period. However, table top ultracentrifuges can now provide a centrifugal force of 200000g within a few minutes. As shown in Figure 1, liposome vesicles can be separated from smp in 3 min. If the vesicles are incubated with smp at the same temperature at which ultracentrifugation proceeds, the inaccuracy in the time for the kinetic studies is less than 0.5 min. Half of the smp precipitates in 0.5 min, and no protein is transferred from the precipitated smp to the vesicle membranes.

There is no fusion of vesicles and smp membranes, based upon the fact that there was no radioactivity in the smp precipitates after an incubation with radioactive liposome membranes. This is also confirmed from the result of almost no decrease of cholesterol in smp membranes during the incubation. Thus, the membrane protein transfer cannot be attributed to simple membrane fusion. There are other experimental results which confirm that the transfer is not due to simple fusion. If the transfer was indeed through simple fusion, the proteins in the vesicle membranes would be primarily the same as those involved in smp. The SDS–PAGE profile of smp was completely different from that of the vesicles containing transferred proteins (Figure 2). SDS–PAGE revealed that the major transferred protein has a molecular mass similar to that of P-450$_{scc}$, and Western blotting with anti-P-450$_{scc}$ IgG showed that it contained P-450$_{scc}$. The protein concentrations showed that 70% of transferred proteins were cytochrome P-450. P-450$_{scc}$ in smp can catalyze the side-chain cleavage of the endogenous cholesterol in smp, but transferred P-450$_{scc}$ in liposome membranes cannot catalyze the cholesterol metabolism. This result shows that P-450$_{scc}$ metabolizes cholesterol in the same membranes but cannot metabolize the cholesterol either in the aqueous phase or in the other membrane systems. Almost no cholesterol was transferred from smp to liposome membranes under our experimental conditions. The half-time of cholesterol transfer between vesicle membranes has been reported to be 1–2 h at 37 °C (Dhariwal et al., 1991).

Two theoretical models are applicable to the protein transfer in this experiment. In the vesicle collision model, the transfer occurs via collision between smp and liposome vesicles (Figure 7A). Pm and Pl represent cytochrome P-450 in smp and in liposome vesicles, respectively. The concentration of the transferred cytochrome P-450, [Pl], increases exponentially with the relaxation time according to the equation (see Appendix)

$$1/\tau = k_{+1}[\mathrm{L}] + k_{-1}[\mathrm{smp}]$$

where L represents liposome membranes. The inverse of the relaxation time would be linearly dependent on both [L] and [smp] in this model, which does not coincide with the observed decrease with the increase in [L] (Figure 5A).

Another model of protein transfer is that of protein diffusion (Figure 7B), where cytochrome P-450 in smp (Pm) dissociates from the smp membranes and becomes free cytochrome P-450 (Pf) in aqueous solution. The free cytochrome P-450 moves and binds to the liposome membranes. To obtain a mathematical solution of the rate equations for this mechanism, we assume a rapid equilibrium

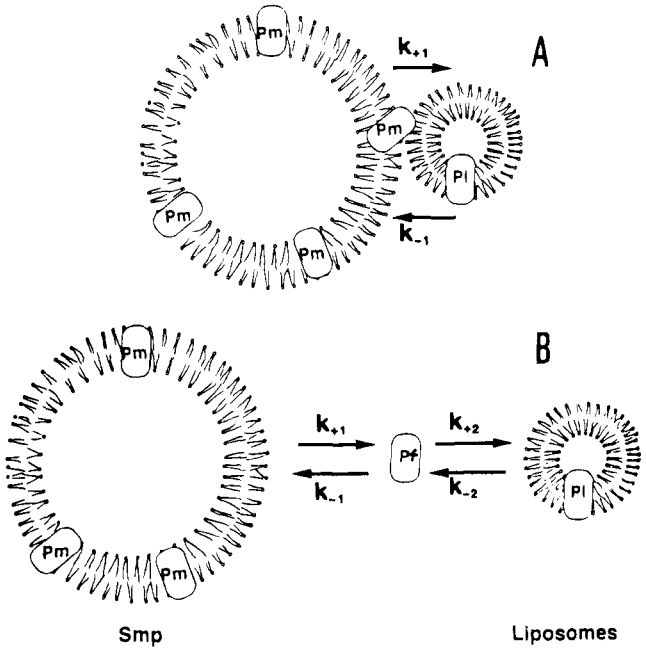

FIGURE 7: Reaction scheme for cytochrome P-450 transfer from smp to liposome membranes: (A) vesicle collision model; (B) protein diffusion model. Pm, Pl, and Pf represent cytochrome P-450 in smp, in liposome membranes, and in the aqueous phase, respectively.

for the binding of Pf to liposome membranes. Tuckey and Kamin (1982) measured the rate of P-450_{scc} binding from aqueous solution to liposome membranes. The binding was in the second-order kinetics, and the rate was dependent on the concentration of liposome membranes. The half-time of the binding to the liposome membranes was reported to be 1−2 min at 25 °C for various phospholipid liposome membranes. In our previous study (Ikushiro et al., 1992), most of P-450_{scc} in aqueous solution binds within 2 min to liposome membranes having the same phospholipid composition as used in this study. P-450_{scc} is in oligomeric forms in aqueous solution (Shikita & Hall, 1973), and the P-450_{scc} that dissociates from the oligomer binds to the liposome membranes. The above assumption of a rapid equilibrium in P-450_{scc} binding to liposome membranes is not unreasonable in comparison with the relaxation time of the transfer of cytochrome P-450 from smp to liposome membranes which is in the range of 10−20 min at 25 °C in this experiment. Under this assumption of a rapid equilibrium of Pf binding to liposome membranes, the inverse of the relaxation time is expressed by the equation (see Appendix)

$$1/\tau = k_{+1} + k_{-1}[\text{smp}]/(1 + \text{Kb}[\text{L}])$$

where Kb stands for the binding constant of cytochrome P-450 to liposome membranes, which is equal to k_{+2}/k_{-2}. The inverse of the relaxation time must increase linearly with the increase in [smp] in this model, which agrees well with the result of Figure 5B. The inverse relaxation time must decrease with the increase in [L], which coincides well to the experimental results in Figure 5A.

Dhariwal et al. (1991) reported P-450_{scc} transfer between phospholipid vesicles. They detected P-450_{scc}-induced vesicle aggregation for some phospholipids but excluded a possibility of the transfer through the vesicle fusion. These authors found poor exchange between P-450_{scc} in vesicles and additional soluble P-450_{scc} and therefore proposed that P-450_{scc} might be transferred through the transient aggregation of vesicles, rather than direct dissociation. Kinetic equations of the diffusion model in this study do not necessarily contradict their mechanism since the transfer could occur only after P-450_{scc} transition to the loosely bound state. This state could not be indistinguishable from complete release of P-450_{scc} in the kinetic equation for the diffusion model. Dissociation is certainly favored by the fact that P-450_{scc} is relatively stable in an aqueous solution without detergents.

Using equations for the diffusion model, we estimated k_{+1}, k_{-1}, and Kb to be 0.028 ± 0.008 min^{-1}, 0.7 ± 0.2 min^{-1} (mg of smp protein/mL)$^{-1}$, and 12.5 ± 5 (mg of phospholipid/mL)$^{-1}$, respectively. The binding constant of Pf to smp membranes, k_{-1}/k_{+1}, becomes 25 ± 7 (mg of smp protein/mL)$^{-1}$. The binding constant of Pf to smp seems similar to that to liposome membranes. The lipid content in smp might be 20% of the protein concentration, which means lipids in smp have 10 times more affinity for Pf. This hypothetical high affinity of Pf to smp might be due to some interaction of cytochrome P-450 with other mitochondrial proteins.

The maximum amounts of cytochrome P-450 transferred to liposome membranes can be estimated from the exponential curves in Figure 4, which are dependent on both smp and liposome concentration (Figure 6). The dependence can be theoretically described in eq 10 in the Appendix. To fit the observed values, we have to estimate the amount of transferable cytochrome P-450 in smp, $[P_0]$. The solid lines in Figure 6 were drawn under the assumption that one-third of cytochrome P-450 in the smp would be transferable from smp to liposome membranes. It has been reported that the ratio of P-450_{scc} to P-$450_{11\beta}$ in bovine adrenal cortex is almost 1:1 (Hanukoglu & Hanukoglu, 1986), and almost the same ratio has been observed in this laboratory (data not shown). The transferable P-450_{scc} can be calculated to be two-thirds of P-450_{scc} in the original smp, which agrees with the decrease of cholesterol metabolizing activity of the smp incubated with a large amount of liposomes where the weight ratio of vesicles to smp was 33 (Figure 3A). P-450_{scc} transfer was much lower when analyzed by immunoblotting (5−10%, Figure 2B) or optical spectra of heme protein (10−20%, Figure 4) where the weight ratio of vesicles to smp was 0.6−1.5. About one-third of P-450_{scc} in smp was not transferable in this experiment. There might be some immobilizing mechanism for the P-450_{scc} in smp, such as the interaction with other proteins in smp membranes (Ikushiro et al., 1992).

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APPENDIX

Vesicle Collision Model. In the vesicle collision model, cytochrome P-450s in smp, Pm, are transferred to liposome membranes via collision of vesicles with smp, as shown in Figure 7A. The increase in the concentration of transferred cytochrome P-450 in liposome membranes, d[Pl]/dt, is

represented by the equation

$$d[Pl]/dt = k_{+1}[Pm][L] - k_{-1}[Pl][smp] \quad (1)$$

where [Pm], [L], [Pl], and [smp] refer to the concentration of cytochrome P-450 in smp, the liposome concentration (mg of phospholipids/mL), cytochrome P-450 in liposome membranes, and smp (mg of protein/mL), respectively. All concentrations are expressed as the apparent concentration where absolute amounts (mol or mg) are divided by bulk volume (Kominami et al., 1986). The liposome and smp concentrations are assumed to be sufficient for the transfer, so no change in the concentration of smp and liposomes can occur during this process. The total concentration of transferable cytochrome P-450 in the system, $[P_0]$, is equal to [Pm] + [Pl], and eq 1 is converted to eq 2.

$$-d[Pm]/dt = [Pm](k_{+1}[L] + k_{-1}[smp]) - k_{-1}[P_0][smp] \quad (2)$$

The following equation can be derived:

$$[Pl] = [P_0] + a/b - ([P_0] + a/b)\exp(-bt) \quad (3)$$

where $a = -k_{-1}[P_0][smp]$ and $b = k_{+1}[L] + k_{-1}[smp]$. The inverse of relaxation time, τ^{-1}, is expressed in the equation:

$$\tau^{-1} = K_{+1}[L] + k_{-1}[smp] \quad (4)$$

Protein Diffusion Model. In this model, cytochrome P-450s dissociate from smp membranes, and the free cytochrome P-450s (Pf) diffuse and bind to liposome membranes, as shown in Figure 7B. The binding of cytochrome P-450s to liposome membranes is assumed to be in rapid equilibrium with a binding constant of Kb:

$$Kb = k_{+2}/k_{-2} = [Pl]/([Pf][L]) = ([P_0] - [Pm] - [Pf])/([Pf][L]) \quad (5)$$

$$[Pf] = ([P_0] - [Pm])/(Kb[L] + 1) \quad (6)$$

The decrease of Pm can be described by the equation:

$$-d[Pm]/dt = (k_{+1} + k_{-1}[smp]/(1 + Kb[L]))[Pm] - k_{-1}[smp][P_0]/(1 + Kb[L]) \quad (7)$$

The observed concentration in supernatant after centrifugation is [Pf] + [Pl] in this model, which can be expressed by the equation:

$$[Pl] + [Pf] = [P_0] - [Pm] = [P_0] + c/f - ([P_0] + c/f)\exp(-ft) \quad (8)$$

where $f = k_{+1} + k_{-1}[smp]/(1 + Kb[L])$ and $c = -k_{-1}[smp]\text{-}[P_0]/(1 + Kb[L])$. The inverse of relaxation time, τ^{-1}, is

$$\tau^{-1} = k_{+1} + k_{-1}[smp]/(1 + Kb[L]) \quad (9)$$

The maximum concentration of cytochrome P-450 in the supernatant after an extended incubation is described by the equation:

$$([Pl] + [Pf]) = \alpha[smp]k_{+1}(1 + Kb[L])/(k_{+1}(1 + Kb[L]) + k_{-1}[smp]) \quad (10)$$

The specific content of transferable cytochrome P-450 in the original smp is represented by α and $[P_0]$ becomes α[smp].

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