Cytochrome P-450$_{scc}$ Induces Vesicle Aggregation through a Secondary Interaction at the Adrenodoxin Binding Sites (in Competition with Protein Exchange)†

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ABSTRACT: Addition of bovine adrenal cytochrome P-450$_{scc}$ to small unilamellar dioleoylphosphatidylcholine vesicles (DOPC-SUV) produces a complex sequence of interactions, indicating exceptional cytochrome mobility. First, cholesterol transfer from cytochrome to vesicles indicated rapid dissociation of P-450$_{scc}$ oligomers and integration of monomers into the membrane (ΔA 390-420 nm; $t_{1/2} \approx 2$ s). After 10-15 s, P-450$_{scc}$-induced aggregation of the vesicles starts, as indicated by increased turbidity (ΔA 448 or 520 nm; complete in 6-8 min). Fluorescence quenching experiments indicate that this aggregation does not lead to measurable vesicle fusion during this period. Aggregation is prevented by mild heat denaturation of P-450$_{scc}$, by addition of anti-P-450$_{scc}$ IgG, and also by 1:1 complex formation with the electron donor adrenodoxin (ADX). P-450$_{scc}$, therefore, links two vesicles through two separate domains involved in, respectively, membrane integration (lipophilic) and ADX binding (charged). Although completely bound by DOPC-SUV, as evidenced by Sephadex elution, P-450$_{scc}$ has access within 1 min to cholesterol in secondary SUV. This is indicated by spectral changes (cholesterol complex formation) and by metabolism of secondary vesicle cholesterol. Since cholesterol equilibrates slowly between vesicles ($t_{1/2} \approx 1$-2 h), these changes arise from P-450$_{scc}$ transfer. This transfer was maximally slowed after a 5-min preincubation with primary vesicles, reflecting more extensive integration into the membrane than is necessary for the rapid initial cholesterol transfer to P-450$_{scc}$. P-450$_{scc}$ transfer probably results from simultaneous interaction of P-450$_{scc}$ with two vesicles that may also initiate aggregation. Weaker integration into primary dimyristoylphosphatidylcholine vesicles facilitates exchange but prevents aggregation. Integration and aggregation are both enhanced by incorporation of 10% phosphatidylinositol into SUV, while exchange is slowed. This mobility of P-450$_{scc}$ is most probably a consequence of the absence of amino-terminal anchoring. P-450$_{scc}$-induced association of inner mitochondrial membrane segments may contribute to the exceptionally vesiculated structure of adrenal and ovarian mitochondria that parallels increased P-450$_{scc}$ content.

Cytochrome P-450$_{scc}$, which catalyzes the conversion of cholesterol to pregnenolone, is located on the inside of the inner mitochondrial membrane in cells of the adrenal cortex and other steroid-producing tissues (Simpson & Boyd, 1966; Omura et al., 1966; Churchill et al., 1978; Mitani, 1979). Cholesterol is almost completely insoluble in water and must be presented to the cytochrome either in nonionic detergents, such as Tween 20, or as a constituent of phospholipid membranes to which P-450$_{scc}$ binds. The kinetics of cholesterol side-chain cleavage (CSCC) can be changed by membrane constituents like cardiolipin (Lambeth, 1981; Pember et al., 1982; Kowluru et al., 1983), which binds cytochrome P-450$_{scc}$ and stimulates cholesterol complex formation (lower K_m), or like spingomyelin (Stevens et al., 1986), which enhances the interaction of cholesterol with the membrane. Phospholipids which contain either unsaturated fatty acids or acidic head groups facilitate cholesterol transfer from vesicles to P-450$_{scc}$ and also stimulate CSCC (Tuckey & Kamin, 1982; Kowluru et al., 1983; Igarishi & Kimura, 1984; Hsu et al., 1985).

Cytochrome P-450$_{scc}$ has a monomeric molecular weight of 48 000 in strong detergents but adopts oligomeric structures (n = 4-8 or 16) in an aqueous environment (Shikita & Hall, 1973). The cytochrome possesses both hydrophobic and hydrophilic domains, the latter containing the heme binding sequence and the surface site for association with adrenodoxin (ADX) (Chashchin et al., 1984, 1985). Chemical cross-linking suggests alternate "head-to-tail" association of these oligomers (Chashchin et al., 1985). It has been shown that dissociation of oligomeric cytochrome b_5 limits incorporation of this protein into vesicles (Leto & Holloway, 1979). Dissociation of oligomeric P-450$_{scc}$ may precede cholesterol transfer from vesicles to the cytochrome. We have suggested elsewhere that acidic lipid head groups may enhance cholesterol transfer and CSCC by enhancing this dissociation (Dhariwal and Jefcoate, unpublished experiments). Liver microsomal P-450 cytochromes also self-associate, and partial perturbation of oligomeric association by detergents has been suggested to play an important role in enzyme activity (Dean & Gray, 1982; Wagner et al., 1984).

Studies with microsomal cytochromes P-450 also indicate effects of phospholipid on the protein secondary structure (Chiang & Coon, 1979). Interaction of two P-450 forms (LM_2 and LM_4) with dilauroylphosphatidylcholine enhanced the circular dichroism spectra in the UV region, indicating an increase in the amount of α-helical content. Tsong and Yang (1978) reported that dimyristoylphosphatidylcholine (DMPC) provides an environment that facilitates a substrate-induced conformational transition of cytochrome P-450$_p$. Although cytochrome P-450$_{scc}$ is typically purified in association with small quantities of phosphatidylcholine and phosphatidylethanolamine (Wang et al., 1974; Hall et al., 1978), little is known of the contribution of such boundary lipids to the structure and function of the cytochrome.

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Although cytochrome P-450$_{scc}$ readily binds to phospholipid vesicles, as judged by the association of cytochrome and vesicles during Sephadex chromatography and following ultracentrifugation (Hall et al., 1979; Seybert et al., 1979; Yamakura et al., 1981; Tuckey & Kamin, 1982), the nature of this interaction is poorly defined. Previously, it was reported that P-450$_{scc}$ reconstituted into sonicated vesicles does not metabolize cholesterol present in other secondary vesicles (Seybert et al., 1979), suggesting that P-450$_{scc}$ was fully integrated into the vesicles. Data presented in this paper show that P-450$_{scc}$ is far more mobile when reconstituted with purified phospholipids. We show that P-450$_{scc}$ binds very rapidly with a high affinity to phospholipid vesicles formed by highly purified dioleoylphosphatidylcholine (DOPC) but there is a subsequent, rapid exchange of the protein between these vesicles. This work also shows that very small amounts of impurities greatly affect the association/dissociation of cytochrome P-450$_{scc}$ with the lipid membranes, which may in part explains the diveristy of previous studies. The paper also describes P-450$_{scc}$-initiated aggregation of phospholipid vesicles that is highly dependent on specific features of the P-450$_{scc}$ and vesicle structure. These interactions provide a basis for understanding the exchange of P-450$_{scc}$ between vesicles.

Experimental Procedures

Materials

Cytochrome P-450$_{scc}$ was purified from beef adrenals by using the procedure of Tsubaki et al. (1987). This procedure, using ADX–Sepharose column chromatography as the final step, produces purified cytochrome P-450$_{scc}$ of specific activity 15 nmol/mg of protein. ADX and ADX reductase were also obtained from beef adrenal by using the procedures of Orme-Johnson and Beinert (1969) and Hiwatashi et al. (1976), respectively, as modified by Hanukoglu et al. (1981a).

[^{3}H]Cholesterol and [^{14}C]cholesterol oleate were obtained from NEN Research Products, DuPont; octyl glucoside, Sephadex 4B, NADPH, terbium chloride, and dipicolinic acid were from Sigma, St. Louis, MO; Sephadex LH-20 was from Pharmacia, Piscataway, NJ. Latex beads were a gift from Seragen Diagnostics, Indianapolis, IN. Lipids were obtained from Serdary Research Labs., London, Ontario, Canada.

Methods

Preparation of Vesicles. Small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) of specified composition were prepared by, respectively, sonication and octyl glucoside dialysis, as described elsewhere (Dhariwal & Jefcoate, 1989).

Purification of Dioleoylphosphatidylcholine. Purification of DOPC was necessary since small amounts of impurities in the commercially available lipid greatly affect the rate of P-450$_{scc}$ transfer between vesicles (Dhariwal and Jefcoate, unpublished experiments). For example, lysophosphatidylcholine (0.1–1%) substantially inhibits P-450$_{scc}$ transfer when present in DOPC preparations. The purification was based on the established methods for silicic acid chromatography of phosphatides (Lea et al., 1955; Hanahan et al., 1957; Zilversmit et al., 1961; Newman et al., 1961; Chang & Sweely, 1963). Briefly, silicic acid (4 g) was packed into a column (5 cm × 1 cm) and washed thoroughly with chloroform, and DOPC (20 mg) was loaded onto the column and washed with 50 mL of chloroform. DOPC was eluted with 50 mL of methanol–chloroform (1:1). Contaminants, including lysophosphatidylcholine, were eluted with 100% methanol. The resulting purified lipird was then dried under N_2, weighed, and redissolved in 100% chloroform at 5 mg/mL concentration. The yield was 65%.

Association of P-450$_{scc}$ with Vesicles by Gel Exclusion Chromatography. Cytochrome P-450$_{scc}$ (0.4 μM) was incubated with phospholipid vesicles (600 μM) and 0.5 μM ADX at 37 °C for 5 min. After the incubation, the reaction mixture was immediately chilled and chromatographed on a Sepharose 4B column (1.5 cm × 20 cm) preequilibrated with 20 mM HEPES buffer (pH 7.2) containing 0.1 mM DTT. The column was precoated with 1.0 mg of PC at 4 °C; 1-mL fractions were collected. The distribution and recovery of vesicles were determined from the level of [^{3}H]cholesterol added in the vesicles prior to vesicle formation. The concentration of cytochrome P-450$_{scc}$ in each fraction was determined by the reduced CO difference spectrum using an ΔA(450–490 nm) = 91 cm^{-1} mM^{-1} (Omura & Sato, 1964). The sizes of SUV and LUV were established by chromatography on the Sepharose 4B column at 4 °C; elution profiles were determined by monitoring OD_{280}. The column void volume was determined by blue dextran exclusion, and two different size marker latex beads (38 and 85 nm) were used to further define the elution characteristics of the column (Boni & Rando, 1985).

Cholesterol Exchange. Intervesicle cholesterol exchange was measured by the method of Bar et al. (1986). DOPC–SUV were generated as previously described. The donor vesicles (600 μM) contained cold cholesterol (60 μM), [^{3}H]cholesterol (1 × 10^6 cpm), and 15 mol % phosphatidic acid. The acceptor vesicles (600 μM) contained [^{14}C]cholesterol oleate as a nonexchangeable marker. The two vesicle samples were incubated for various times at 37 °C, and 100-μL aliquots were applied to a DEAE-Sepharose CL-6B column (1 cm × 0.6 cm). The column was eluted with 1.0 mL of 100 mM HEPES buffer, pH 7.3, containing 50 mM KCl. The eluant was collected and counted in 3.5 mL of scintillation fluid. The experiment was also carried out with P-450$_{scc}$ (0.6 μM), first incubated with donor vesicles for 5 min in the presence of 0.8 μM ADX.

Cholesterol Side-Chain Cleavage (Cytochrome P-450 Exchange). CSCC assays were carried out under varying preincubation and incubation conditions. In the first set of experiments, the time of preincubation of cytochrome P-450$_{scc}$ with primary vesicles (P) was varied. The reaction was subsequently initiated simultaneously with the addition of secondary vesicles (S). In the second set of experiments, the preincubation was 5 min, and the incubation period was varied. In the third set of experiments, the preincubation period was 5 min, followed by the addition of an equal amount of secondary vesicles and preincubation for a further 5 min. The incubation initiated by NADPH was for 2 min. ADX was present in all the preincubation samples. Following are the reactant concentrations used: ADX reductase, 0.7 μM; ADX, 7 μM; P-450$_{scc}$, 0.5 μM; NADPH, 2.5 mM; phospholipid, 600 μM; cholesterol, 60 μM; [^{3}H]cholesterol, 20 000 cpm/reaction volume (in vesicle P or S); the reaction volume was 200 μL. The reaction was terminated with 0.2 mL of ice-cold ethanol. Pregnenolone was extracted with 2 mL of methylene chloride and separated from unreacted cholesterol on LH 20 mini columns (Hanukoglu & Jefcoate, 1980).

Spectral Assay for Cytochrome P-450$_{scc}$ Exchange. All spectra were performed on an Aminco-Chance DW-2c spectrophotometer in either dual wavelength or split-beam modes. A typical assay contained 20 mM HEPES, 50 mM KCl, and 600 μM phospholipid. The assay was started by the addition of 100 nM P-450$_{scc}$, and changes in absorption at 420–390 nm were followed in the dual-wavelength mode at 37 °C. The absolute spectra were conducted under similar conditions in the split-beam mode. P-450$_{scc}$ (high spin) was initially added

to cholesterol-free primary vesicles (600 μM) of appropriate composition, and the spectra were followed in the dual-wavelength mode (ΔA 390–420 nm) for 5 min (dissociation) or until no further change was observed. Absolute spectra was determined at the end of each reaction. Secondary vesicles (600 μM) containing 600 μM cholesterol were than added to the cuvette, and the reversal of the spectra (i.e., low- → high-spin change or association) was followed. The final absolute spectra were determined as before. Cholesterol-free cytochrome was generated as described elsewhere and used in the association experiments (Hanukoglu et al., 1981b). The first-order rate constants for the reaction were determined as previously described (Kowluru et al., 1983). The spin-state change was calculated from the change in ΔA (390–420 nm) relative to the fully low-spin state of the cytochrome in 0.3% Tween 20. The percentage high spin is given by $[\Delta A/(1.1 \times [P450])] \times 10^{-3}$, which is based on $\Delta E = 110\ cm^{-1}\ mM^{-1}$ for a complete spin-state change.

Aggregation of Lipid Vesicles. Aggregation of small unilamellar lipid vesicles was monitored as the change in absorbance at 448 nm, an isosbestic point for cytochrome P-450_{scc}–cholesterol complex formation, and at 520 nm. Lipid vesicles (600 μM) were added to both the reference and sample cells, and a baseline was obtained. Cytochrome P-450_{scc} (0.3 μM) was then added to the sample compartment, and the ensuing absorbance change was recorded. Where appropriate, 0.5 μM ADX and 25 μg of anti-cytochrome P-450_{scc} IgG were added to the vesicles prior to the addition of the cytochrome. Denatured cytochrome (P-420) was obtained by heating at 50 °C for 5 min. Rates of aggregation refer to the linear portion of the aggregation plot (Figure 7).

Vesicle Fusion Measurements. The fusion assay of liposomes was carried out by the method of Hoeckstra et al. (1985). Briefly, vesicles (SUV) were made as before in the presence of (a) 2.5 mM terbium chloride ($TbCl_3$) and (b) 50 mM dipicolinic acid (DPA, sodium salt). The nonencapsulated materials were separated by Sephadex G-75 column chromatography (20 cm × 1.5 cm). The buffer used for this experiment was 20 nM HEPES (at pH 7.2) with 50 mM KCl. Fusion was investigated with a 1:1 mixture of vesicle a and vesicle b at a lipid concentration of 600 μM in a volume of 1 mL. The formation of a highly fluorescent $Tb(DPA)_3^{3-}$ complex resulting from the mixing of the aqueous contents inside the fusing vesicles produces a 10^4-fold enhancement of the Tb fluorescence (Oreland et al., 1973; McCauley, 1976) and was followed by exciting the sample at 276 nm and monitoring at 545 nm using a cutoff filter at >530 nm in an Aminco-Bowman spectrofluorometer. A positive control was conducted by adding 5 μL of 20% sodium cholate to the vesicle mixture. Vesicle association was initiated by adding 0.3 μM cytochrome P-450_{scc} and followed for 60 min. The assay is discussed at length by Wilschut and Papahadjopoulos (1979) and Wilschut et al. (1980).

Results

The association of cytochrome P-450_{scc} with phospholipid vesicles during ultracentrifugation and Sepharose chromatography indicates a high affinity by the cytochrome for the phospholipid membrane (Hall et al., 1979; Seybert et al., 1979; Yamakura et al., 1981; Kowluru et al., 1983). The extent of association of cytochrome P-450_{scc} with small and large unilamellar vesicles (SUV and LUV) was compared by elution from Sepharose 4B. SUV and LUV have been measured from electron microscopy to be 30 and 150 nm in diameter, respectively (Dhariwal & Jefcoate, 1989). When chromatographed separately, cytochrome P-450_{scc} was retained at approximately twice the elution volume of SUV (Figure 1). When preincubated with SUV or LUV formed with DOPC, P-450_{scc} coeluted exactly with the vesicles. In both elutions, relatively little additional P-450_{scc} eluted at the position where unbound cytochrome was expected, and 60–70% of added P-450_{scc} was recovered from the column; lipid recovery was >90%.

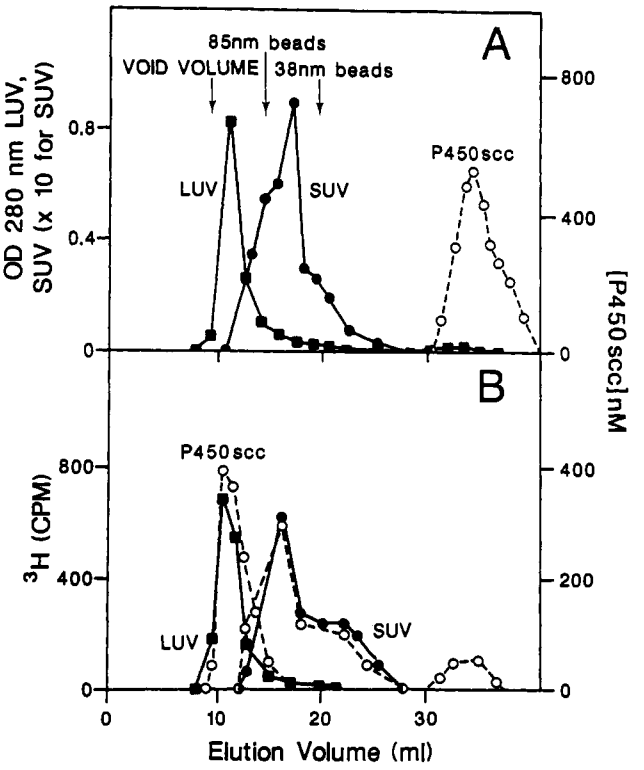

FIGURE 1: (A) Elution profiles of DOPC–LUV (■), DOPC–SUV (●), each 300 nmol, and cytochrome P-450_{scc} (0.4 nmol) (○) were obtained separately on a Sepharose 4B column (1.5 cm × 20 cm) characterized with blue dextran and latex beads of 85- and 38-nm diameter. Vesicles were prepared, as described under Experimental Procedures, with the addition of [^{3}H]cholesterol as a marker in 20 mM HEPES–100 mM NaCl. (B) Reconstituted vesicles (SUV or LUV), 300 nmol, were incubated with 4 nmol of cytochrome P-450_{scc} and 3.5 mol of adrenodoxin in 0.5 mL of HEPES–NaCl buffer for 5 min at 37 °C. Reaction mixtures for SUV and LUV were chilled and applied separately on the Sephadex column. Fractions (1 mL) were collected, and cytochrome P-450_{scc} content was determined spectrophotometrically, as described under Experimental Procedures. The profile of the vesicle elution was measured by monitoring [^{3}H]cholesterol.

Table I: Cholesterol Transfer in Phosphatidylcholine Vesicles[a]

vesicle composition	$t_{1/2}$ (h)	$t_{1/2}$ (h) + P-450_{scc}
DOPC–SUV	1.1 ± 0.05	1.3 ± 0.1
DOPC–LUV	2.4 ± 0.12	2.2 ● 0.15
DMPC–SUV	6.8 ± 0.16	7.1 ± 0.40
DMPC–LUV	10.1 ● 0.16	9.9 ± 0.42

[a] Donor vesicles (600 μM) contained cold cholesterol (60 μM), [^{3}H]cholesterol, and 15 mol % PA. Identical acceptor vesicles contained [^{14}C]cholesterol oleate as a nonexchangeable marker. The two vesicles were incubated at 37 °C, aliquots were drawn at intervals and applied to a DEAE-Sepharose column, and separation of donors and acceptors was achieved as described under Experimental Procedures. P-450_{scc} (0.6 μM) was incubated with the donor vesicles in the presence of 0.8 μM ADX for 5 min prior to the initiation of the exchange reaction. Each data point is a mean of three experimental determinations.

Intervesicle Exchange of Cholesterol. Cholesterol exchanges very slowly between phospholipid vesicles, as indicated by the half-time for exchange between phospholipid vesicles (McLean & Phillips, 1981; Bittman et al., 1985; Bar et al., 1986; Thomas & Pozansky, 1988a,b). Results in Table I

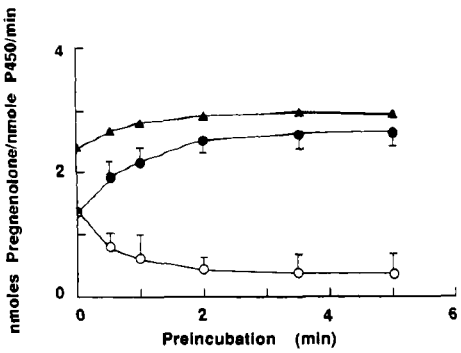

FIGURE 2: Effects of preincubation time on the CSCC in primary and secondary DOPC–SUV. Cytochrome P-450$_{scc}$ (0.5 μM) was preincubated with primary vesicles, 600 μM, in the presence of 7 μM ADX for varying times at 37 °C prior to the addition of secondary vesicles, differing only in [^{3}H]cholesterol content. The CSCC reaction was initiated concomitantly with the addition of secondary vesicles, 600 μM. Reactant concentrations were as follows: NADPH, 2.5 mM; [^{3}H]cholesterol, 20 000 cpm/200-μL reaction volume (in vesicle P or S); ADX reductase, 0.7 μM. The reaction was terminated after 1 min with 0.2 mL of ice-cold ethanol. Pregnenolone was determined as described under Experimental Procedures. Each data point is the mean of three experimental determinations. Total metabolism (▲), metabolism in primary vesicles (●), and metabolism in secondary vesicles (○) are shown.

confirm these rates and indicate that the rate of cholesterol transfer in DOPC–SUV and DMPC is independent of P-450$_{scc}$ at concentrations used in protein exchange and activity assays. It is important to indicate that the donor vesicles contain 15% phosphatidic acid, which, as will be shown later, slows P-450 association and dissociation. The half-time for cholesterol exchange in DOPC–SUV was 60–80 min while that for LUV was 135–150 min. However, changing the lipid to DMPC increased the half-times for each exchange by 5–6-fold. This decrease in cholesterol exchange rate with large vesicles has been reported recently and is thought to be related to the curvature of the membrane (Fugler et al., 1985; Thomas & Pozansky, 1988b).

Metabolism of Cholesterol in Secondary Vesicles. We have used cholesterol metabolism as a means to probe the exchange of P-450$_{scc}$ between vesicles. Since cholesterol exchanges very slowly, cytochrome P-450$_{scc}$ reconstituted with one set of vesicles (primary vesicles, P) can only metabolize [^{3}H]-cholesterol in a second set of vesicles (secondary vesicle, S) during a 10-min period following exchange of the cytochrome between the vesicles. Rates of [^{3}H]cholesterol metabolism that exceed the slow exchange rate then provide a measure of P-450 exchanging from the primary vesicle to the secondary vesicle.

In order to test the exchangability of P-450$_{scc}$, the cytochrome was preincubated with primary vesicles (P) for varying periods prior to addition of an equal concentration of secondary vesicles (S) and concomitant initiation of metabolism (Figure 2). In various experiments, [^{3}H]cholesterol was added to either the primary or the secondary vesicles. An increase in preincubation time increased metabolism in the primary vesicle and decreased metabolism in the secondary vesicle, while total metabolism remained constant. This time-dependent difference probably reflects integration of P-450$_{scc}$ into the primary vesicle.

When this procedure was applied to primary and secondary DOPC–SUV, the difference between primary and secondary metabolism became maximum after a 5-min preincubation. When this optimum preincubation was used, pregnenolone formation from the secondary DOPC–SUV exhibited a lag time of nearly 1.0 min (Figure 3A). Primary metabolism progressively decreased, while secondary metabolism increased

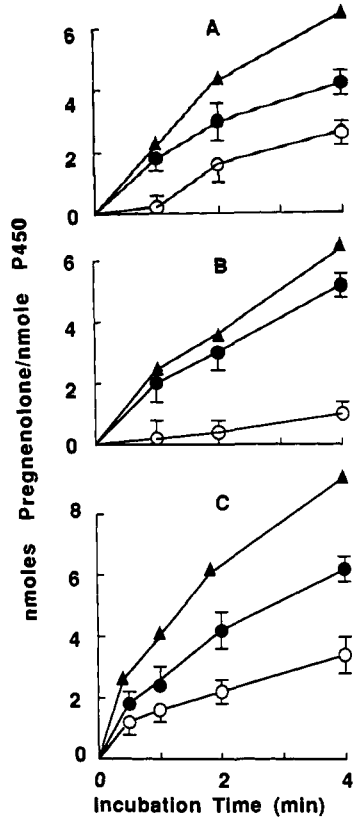

FIGURE 3: Effects of 10% (w/w) phosphatidic acid in primary vesicles and of vesicle size on the CSCC in primary and secondary lipid vesicles. (A) Homologous conditions (SUV): cytochrome P-450$_{scc}$ was preincubated with primary DOPC–SUV for 5 min, and CSCC was initiated concomitantly with the addition of secondary DOPC–SUV. (B) Heterologous conditions (SUV): cytochrome P-450$_{scc}$ was preincubated with DOPC/PA–SUV, and CSCC was initiated with the addition of secondary DOPC–SUV. The reaction was terminated after varying incubation times. (C) Homologous conditions (LUV): cytochrome P-450$_{scc}$ was preincubated with primary DOPC–LUV, and CSCC was initiated with the addition of secondary DOPC–LUV. Pregnenolone was determined as described under Experimental Procedures. Reaction conditions were the same as those described in Figure 1. Total metabolism (▲), metabolism in primary vesicles (●), and metabolism in secondary vesicles (○) are shown.

until, after 4–5 min, rates in the two vesicles became nearly equal. Total metabolism from the two vesicles remained nearly linear.

By varying the composition of the primary vesicle, while leaving the secondary vesicle unchanged, we tested the effect of vesicle composition specifically on P-450$_{scc}$ dissociation rates. The kinetics of cholesterol metabolism in primary (P) and secondary vesicles (S) were measured in parallel incubations in which [^{3}H]cholesterol was present in, respectively, the primary or secondary vesicles. We show that phosphatidic acid slows dissociation of P-450$_{scc}$ from the primary vesicle. Thus, inclusion of 15% phosphatidic acid in the primary vesicles, while maintaining DOPC–SUV as the secondary vesicle (Figure 3B), considerably increased the lag time (3 min) in the secondary vesicle metabolism. Total metabolism, however, remained unchanged. The size of the vesicles also affected P-450$_{scc}$ exchange when DOPC–LUV were used for both primary and secondary vesicles. Biphasic kinetics were observed for both vesicles but with secondary metabolism diminished during the first 2 min (Figure 3C). Total metabolism was also higher, probably due to residual octyl glucoside which increases CSCC activity (Dhariwal & Jefcoate, 1989). The absence of a delay in secondary metabolism suggests more rapid P-450$_{scc}$ exchange than for DOPC–SUV. The biphasic

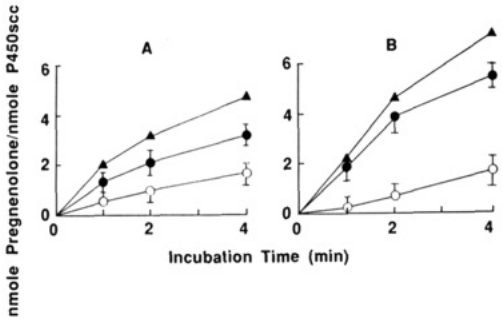

FIGURE 4: Effect of 10% (w/w) phosphatidylinositol in primary vesicles on the CSCC in primary and secondary DMPC–SUV. (A) Homologous conditions: cytochrome P-450$_{scc}$ was preincubated with DMPC–SUV for 5 min, and CSCC was initiated concomitantly with the addition of secondary DMPC–SUV. (B) Heterologous conditions: cytochrome P-450$_{scc}$ was preincubated with primary DMPC/phosphatidylinositol–SUV, and CSCC was initiated with the concomitant addition of secondary DMPC–SUV (S). Reaction conditions were as in Figure 1. Each data point is the mean of three experimental determinations. Total metabolism (▲), metabolism in primary vesicles (●), and metabolism in secondary vesicles (○) are shown.

kinetics are consistent with a small proportion of a more reactive cholesterol pool in each vesicle that is turned over rapidly relative to the exchange time. Interestingly, this pool represents one cholesterol per P-450$_{scc}$ in the primary vesicle and 0.5 in the secondary vesicle, which only receives half of the total cytochrome at equilibrium. This suggests a rapid initial cholesterol turnover in each vesicle that cannot be sustained in subsequent cycles. When the phospholipid in primary and secondary SUV was changed to DMPC (Figure 4A), the lag was again indiscernible, and the kinetics closely resembled those for DOPC–LUV. The lag period was restored by inclusion of 10% w/v of phosphatidylinositol in the primary vesicles, which also enhanced total activity by 50% (Figure 4B).

The relative affinities of cytochrome P-450$_{scc}$ for different vesicles have also been determined. The primary CSCC activity is proportional to the amount of P-450$_{scc}$ bound to the primary vesicle; thus, redistribution of P-450$_{scc}$ to a secondary vesicle decreases this activity in proportion to the affinity of P-450$_{scc}$ for the secondary vesicle. Competitive effects shown in Figure 5 indicate that the cholesterol content of DOPC–SUV does not affect the affinity of cytochrome P-450$_{scc}$ for the vesicle. This redistribution of P-450$_{scc}$ between cholesterol-containing and cholesterol-free vesicles is also supported by the spectral data presented in Figure 5B. From the spectra of P-450$_{scc}$ bound to each vesicle separately, we calculated the distribution of the cytochrome between mixtures of cholesterol-containing and cholesterol-fre DOPC–SUV. After 5 min, the enzyme distributed equally between these vesicles, irrespective of which type of vesicle was added first to P-450$_{scc}$.

The rate of association of P-450$_{scc}$ with vesicles can be directly measured from the spectral changes caused by the ensuing fast cholesterol transfer. When P-450$_{scc}$–cholesterol was mixed with DOPC–SUV in a stopped-flow spectophotometer, we observed a very rapid transfer of cholesterol, as indicated by the high- to low-spin spectral change. Providing that DOPC was prepurified, this first-order spectral change and the reverse transfer using cholesterol-free P-450$_{scc}$ and cholesterol-containing SUV (1:1 cholesterol–DOPC) were each complete in 10 s and showed similar first-order kinetics (Dhariwal and Jefcoate, unpublished experiments). As previously reported (Kowluru et al., 1983), this cholesterol transfer process was independent of P-450$_{scc}$ and vesicle concentrations in the range of these experiments and is probably limited by a minimal integration of monomer P-450$_{scc}$ into the vesicle

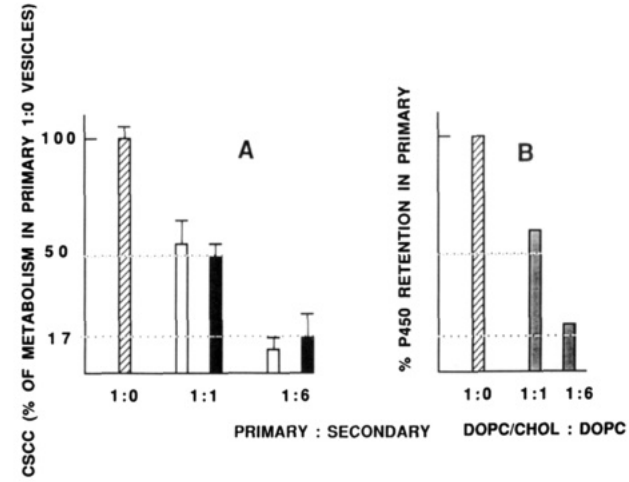

FIGURE 5: Cytochrome P-450$_{scc}$ distribution between two DOPC vesicles. (A) Cholesterol metabolism in primary vesicles inhibited by increasing amounts of secondary vesicles. Cytochrome P-450 (0.3 μM) was incubated with 600 μM DOPC–cholesterol (1:1) in the presence of 0.5 μM ADX for 5 min (primary) and the CSCC reaction initiated (hatched bar). Secondary vesicles [with (black bars) and without (open bars) cholesterol] were added independently as an equal ratio and 6-fold excess to DOPC–CHOL primary vesicles. After 5 min, CSCC was initiated and terminated after 2 min. Reaction conditions were the same as described in Figure 1. Each data point is the mean of three experimental determinations. (B) Distribution of cytochrome P-450$_{scc}$ between cholesterol-containing DOPC–SUV (hatched bar) and cholesterol-free DOPC–SUV (stippled bars). Cytochrome P-450$_{scc}$ (0.3 μM) was incubated with each type of vesicle (600 μM) separately in the presence of 0.5 μM ADX for 5 min, and the absolute spectra were obtained. Equimolar DOPC–SUV was then added to DOPC–CHOL–SUV and incubated for a further 5 min, and the absolute spectra were determined. This was repeated with a 6-fold excess of DOPC vesicles. Percent distribution is based on the amount of cytochrome in the high-spin form determined as described under Experimental Procedures.

(Figure 6). We next carried out this same experiment using reconstituted vesicular P-450$_{scc}$ instead of the soluble preparation. It was reasoned that a slow component to cholesterol transfer in an experiment using cholesterol-free and cholesterol-containing vesicles would correspond to the rate of intervesicle P-450 transfer. We reconstituted P-450$_{scc}$ with cholesterol-containing SUV (>90% high-spin P-450$_{scc}$) and then followed the spectral change following addition of an equal concentration of cholesterol-free vesicles. A small proportion of the spectral change occurred within the mixing time (~10 s) and presumably reflected peripherally associated P-450$_{scc}$. However, most of the change (>80%) occurred at a substantially slower rate (3–4 min^{-1}) than for direct mixing of P-450$_{scc}$–cholesterol with DOPC–SUV (10 min^{-1}) (Figure 6). When the vesicles were added in the reverse order, the final spectral change was also reversed, but a similarly decreased cholesterol transfer rate was observed. Inclusion of 10% phosphatidic acid in the primary reconstitution greatly decreased the rate of this intervesicle P-450$_{scc}$ transfer, just as we observed from the CSCC kinetics.

These rates of intervesicle cholesterol complex formation are fully consistent with the intervesicle P-450$_{scc}$ transfer measured from CSCC kinetics. This equilibration of complex formation is complete in 40–60 s, which is comparable to the time seen for the onset of CSCC in secondary vesicles (Figure 2A). The enhanced delay in P-450$_{scc}$ transfer when phosphatidic acid is present in the primary vesicle is also similar, quantitated either by these spectral changes or by activity measurements in the secondary vesciles.

Mechanism of Intervesicle Transfer. A rapid direct dissociation of P-450$_{scc}$ from DOPC–SUV to a free solution form

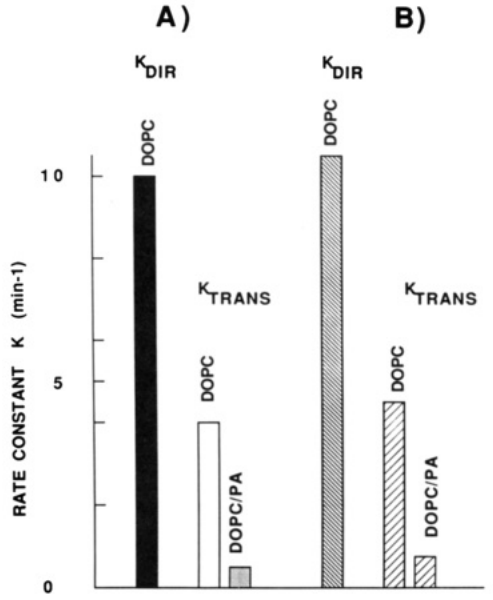

FIGURE 6: Rates of dissociation/association of P-450$_{chol}$ and P-450$_{free}$ in DOPC vesicles. Cytochrome P-450$_{scc}$ (0.1 μM) was either added directly (K_{DIR}) to primary lipid vesicles (600 μM) (rates determined in a stopped-flow spectrophotometer) or incubated first with primary vesicles for 5 min followed by the addition of equimolar secondary vesicles (rates determined at 390–420 nm in the dual-wavelength spectrophotometer). (A) Cytochrome P-450$_{scc}$ was added directly to DOPC–SUV (K_{DIR}) or first incubated with DOPC–CHOL–SUV, followed by the addition of DOPC–SUV (open bar) or DOPC–CHOL–10% (w/w)–PA–SUV (stippled bar), K_{TRANS}. (B) Cholesterol-depleted cytochrome P-450$_{scc}$ was added directly to DOPC–CHOL–SUV or first incubated with DOPC–SUV or DOPC–PA–SUV, followed by the addition of DOPC–CHOL–SUV. The first-order rate constants were determined by extrapolation of the first-order plots to zero time.

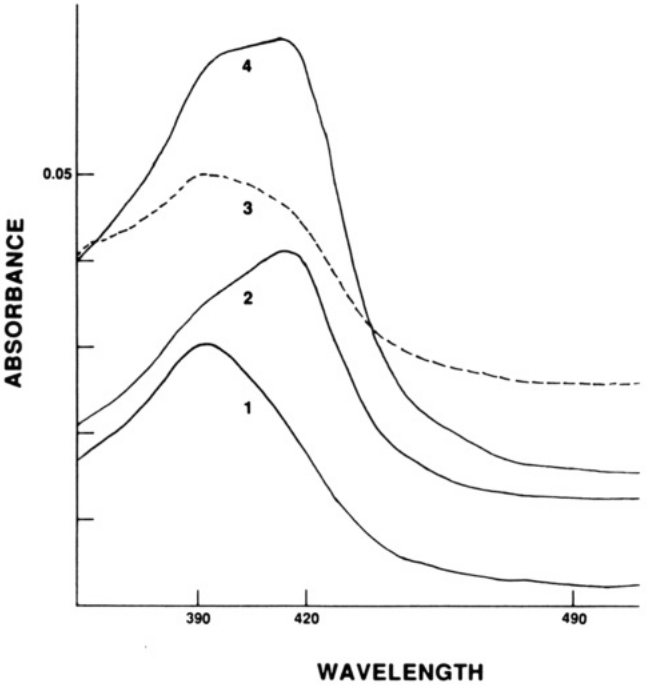

FIGURE 7: Exchange of membrane-associated and soluble cytochrome P-450$_{scc}$. Cytochrome P-450$_{scc}$ (0.3 μM) was incubated with 60 μM DOPC–SUV in the presence of 0.5 μM ADX for 5 min (spectrum 2), followed by a further addition of 0.3 μM cytochrome P-450$_{scc}$ and incubation for another 5 min (spectrum 4). Spectrum 3 is the difference between spectra 4 and 2. For comparison, a spectrum of cytochrome P-450$_{scc}$ (0.3 μM) in 20 mM HEPES–50 mM KCl, pH 7.2, buffer is shown (spectrum 1).

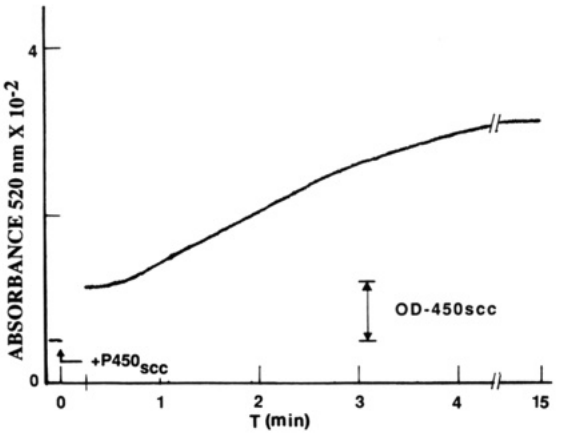

FIGURE 8: Cytochrome P-450$_{scc}$-induced aggregation of DOPC–SUV. Cytochrome P-450$_{scc}$ (0.3 μM) was added to DOPC–CHOL–SUV (1:1) (600 μM) and the change in turbidity monitored at 448 nm as a function of time. The optical increase observed upon the addition of cytochrome P-450$_{scc}$ (0.3 μM) alone is indicated by the arrow.

apparently does not readily occur. We examined equilibration between P-450$_{scc}$ bound to cholesterol-free DOPC–SUV and excess unassociated P-450$_{scc}$–cholesterol oligomers. Cholesterol-complexed P-450$_{scc}$ (0.3 μM) was added to cholesterol-free DOPC–SUV at a concentration (60 μM) corresponding to near-saturation of the binding of the cytochrome to the vesicle. Cholesterol was almost completely transferred from the cytochrome to the vesicle, as seen by the change to low-spin P-450$_{scc}$ (Figure 7). Although the cholesterol content of the vesicle is still extremely low (1/200), a further addition of this same amount of cholesterol-complexed P-450$_{scc}$ retained cholesterol. The final spectrum shifted to a more high-spin state, and the subtraction spectrum suggested that the added P-450$_{scc}$ had remained nearly fully high spin. Thus, complexed cholesterol in the second addition of P-450$_{scc}$ did not transfer to the vesicle, presumably because there are no available sites for P-450$_{scc}$. Thus, SUV-bound P-450$_{scc}$ from the first addition does not readily dissociate and exchange with the additional oligomeric P-450$_{scc}$. This unexpected result indicats that monomeric P-450$_{scc}$ bound to DOPC–SUV only dissociates by direct transference to acceptor vesicles.

A possible mechanism of this P-450$_{scc}$ exchange was indicated by the observation that P-450$_{scc}$ also induces aggregation of DOPC–SUV vesicles. This presumably occurs through simultaneous interaction of the protein with two vesicles (Figure 8). This was observed by means of increased light scattering from the vesicles at wavelengths (448 and 520 nm) unaffected by spin-state changes in P-450$_{scc}$. This aggregation was completed in about 6–8 min but interestingly exhibited a delay of 20–30 s prior to onset. This delay was only a little longer than the 10 s required for cholesterol transfer from P-450$_{scc}$ to the vesicles that is measured from spin-state change, and that probably reflects minimal insertion of the protein into the vesicle. Aggregation was specific, as evidenced by a loss of activity upon mild denaturation of P-450$_{scc}$ to P-420 (50 °C/5 min) and by inhibition with anti-P-450$_{scc}$ IgG. P-450$_{scc}$ could not be replaced by a different P-450 cytochrome (rat P4501A1) that also associates with DOPC–SUV. More significantly, aggregation was blocked by a concentration of ADX (0.5 μM) that is sufficient to form a 1:1 complex with P-450$_{scc}$ (Figure 9). Although cholesterol has been reported to be essential for calcium phosphate induced aggregation of phospholipid vesicles (Ohki & Leonards, 1984), it has no effect on the rate or extent of aggregation in this study.

Vesicle Fusion. Aggregation is a prerequisite step in vesicle fusion but does not always lead to fusion. Many agents have

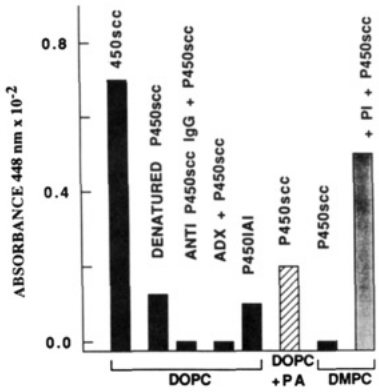

FIGURE 9: Cytochrome P-450$_{scc}$ induced initial rates of aggregation of lipid vesicles under various conditions. Lipid and cytochrome P-450$_{scc}$ concentrations were as in Figure 8. Cytochrome P-450$_{scc}$ was denatured by heating at 50 °C for 5 min. For complete inhibition, the IgG concentration was 80 μg mL^{-1}; the ADX concentration was 0.5 μM. The P4501A1 concentration was 1.0 μM. The amount of phosphatidic acid and phosphatidylinositol in lipid vesicles was 10% (w/w). The rates of aggregation were determined from the linear portion of the trace, such as in Figure 8.

been reported in the literature which promote vesicle aggregation, for example, myelin basic protein (Lampe et al., 1983; Surewicz et al., 1986), tubulin (Kumar et al., 1982), lectins and calcium ions (Hoeckstra et al., 1985), lectin (Orr et al., 1979), and calcium ions (Ohki & Leonards, 1984; Bakas & Disalvo, 1988). The possibility of fusion was investigated by using a fluorescent assay which monitors the mixing of aqueous contents of liposomes. One set of vesicles contained terbium chloride ($TbCl_3$) while the other contained dipicolinic acid (DPA). A highly fluorescent chelation complex, $Tb(DPA)_3^{3-}$, is formed upon mixing of the contents of the vesicles (600 μM) if vesicle fusion allows transfer of the intravesicle contents. No increase in fluorescence, and therefore no fluorescence, was detected over a period of 1 h in the presence of 0.3 μM cytochrome P-450$_{scc}$. Addition of 0.1% sodium cholate led to 70% fusion, as compared to the fluorescence exhibited by sonicated, detergent-containing vesicles (extent of fusion = 100%). Spontaneous fusion or partial fusion does not occur in these vesicles for up to at least 7 days at 4 °C (Parente & Lentz, 1984) and, therefore, was not present in these freshly prepared vesicle samples.

Discussion

The interaction of P-450$_{scc}$ with phospholipid vesicles is particularly interesting, both because the substrate cholesterol is only available through the membrane and also because this cytochrome lacks a hydrophobic amino terminus that anchors microsomal cytochromes P-450 to the membrane (Edwards et al., 1989). Cytochrome P-450$_{scc}$ is also predominantly bound within the matrix of adrenal mitochondria to 60–100-nm unilamellar vesicles (Farkash et al., 1986). Such small unilamellar phospholipid vesicles (SUV) prepared either by cholate dialysis in the presence of cytochrome P-450$_{scc}$ or by sonication (Hall et al., 1979; Hsu et al., 1985) bind cytochrome P-450$_{scc}$ in ratios of up to 1 cytochrome molecule per 100–200 molecules of phospholipid. The present study establishes that P-450$_{scc}$ binds in these proportions with high affinity to SUV formed from DOPC but also exchanges rapidly between vesicles. Larger vesicles formed by octyl glucoside dialysis (DOPC–LUV; 150-nm diameter versus 30 nm) and SUV formed with phosphatidylcholines containing saturated side chains exhibit even more rapid exchange of P-450$_{scc}$, while phosphatidic acid greatly slows exchange. In this paper, we have used several approaches to show that the basis of this mobility is a very specific capacity of P-450$_{scc}$ to span between two vesicles using two distinct binding domains on the protein. It is important to indicate that small amounts of impurities in commercial lipids decrease the cytochrome mobility considerably. Thus, 0.01% lysophosphatidylcholine substantially reduces both the association and the dissociation rates (Dhariwal and Jefcoate, unpublished data).

The capacity of P-450$_{scc}$ to move between vesicles has been demonstrated by studies of cholesterol SCC kinetics. We have shown that P-450$_{scc}$ optimally reconstituted with one set of vesicles can both bind and metabolize cholesterol from a second set of vesicles added after completion of the reconstitution. This exchange can take from 30 s to 5 min, depending on the composition and size of the vesicles. These studies have made use of the extremely low cholesterol exchangeability between vesicles. We have confirmed previous rates for cholesterol exchange between SUV (Bittman et al., 1985; Bar et al., 1986, 1987) and LUV (Fugler et al., 1985; Thomas & Pozansky, 1988b). These rates ($t_{1/2}$ = 1.5–10 h) are slower for LUV than for SUV (2 times for DOPC; 1.5 times for DMPC) and are also decreased about 5-fold when DOPC is replaced by DMPC. P-450$_{scc}$ both complexes cholesterol and readily transfers cholesterol to vesicles but, nevertheless, does not affect intervesicle cholesterol exchange. These exchange kinetics, however, utilized phosphatidic acid in either donor or acceptor vesicles to allow separation. This modification greatly slows the exchange of P-450$_{scc}$ between vesicles (see Figure 6) and thus decreases potential contributions by P-450$_{scc}$ to the cholesterol exchange.

Spectrophotometric measurements of cholesterol–P-450$_{scc}$ complex formation directly confirm this transfer of P-450$_{scc}$. While soluble P-450$_{scc}$ exchanges cholesterol rapidly with DOPC–SUV (k = 10 min^{-1}, complete in 10 s), membrane-bound P-450$_{scc}$ gains access to cholesterol in DOPC–SUV much more slowly. This intervesicle cholesterol–P-450$_{scc}$ complex formation exhibited rates that implicate this step as a potential cause of the lag period for metabolism of cholesterol in these same secondary vesicles. Thus, for DOPC–SUV, intervesicle complex formation occurs with $t_{1/2}$ = 15–20 s, comparable to the 40–60-s lag period for metabolism of secondary cholesterol. The 5-min reconstitution time is required for a maximum lag period and probably corresponds to more extensive insertion of cytochrome into the vesicle than is necessary for the far faster cholesterol exchange between soluble P-450$_{scc}$ and the same vesicle (Kowluru et al., 1983).

The more rapid P-450$_{scc}$ exchange seen with DMPC–SUV and DOPC–LUV ($t_{1/2}$ < 15 s) suggests that the exchange becomes faster when reconstitution involves either saturated phosphatidylcholines or the much larger LUV. For DMPC–SUV, this enhanced exchange may result from the weaker binding of P-450$_{scc}$ to the reconstituted vesicle that is indicated by dissociation during Sephadex chromatography. Although there was no evidence for weak binding by P-450$_{scc}$ to DOPC–LUV during Sephadex elution, transfer of cholesterol from soluble P-450$_{scc}$ to DOPC–LUV only approaches SUV rates at very high cytochrome concentrations, suggesting distinct surface interactions (Dhariwal and Jefcoate, unpublished experiments).

This vesicular P-450$_{scc}$ can gain access to the secondary vesicle either by an initial dissociation from the primary vesicle or by interacting simultaneously with both primary and secondary vesicles. The mechanism of P-450$_{scc}$ exchange has been further established by optical changes that follow the addition of P-450$_{scc}$ to DOPC–SUV. Although soluble P-450$_{scc}$ loses cholesterol to the vesicle within 10 s with a simultaneous

reverse type I spectral change, impurities such as lysophosphatidylcholine and phosphatidic acid that are present in variable amounts in many commercial preparations produce a much slower change which can be accelerated by thin-layer purification of the lipid (Dhariwal and Jefcoate, unpublished experiments). These impurities may thus account for major differences between these and earlier reports of these interactions (Seybert et al., 1979; Kido et al., 1981; Hsu et al., 1985; Kowluru et al., 1983). This process is independent of P-450$_{scc}$ and vesicle concentrations in the range of these experiments, suggesting a rate-limiting step *after* association of P-450$_{scc}$ with the vesicle. Since P-450$_{scc}$ is oligomeric in solution (Shikita & Hall, 1973), this slow step could involve oligomer dissociation at the vesicle surface and a sufficient monomer insertion into the vesicle to allow cholesterol transfer. This cholesterol transfer is followed by increases in turbidity which electron microscopy shows to be vesicle aggregation (Dhariwal and Jefcoate, unpublished experiments). This aggregation exhibits a delay before onset (20–30 s) that is only slightly longer than the time required for the minimal P-450$_{scc}$–vesicle interaction detected by the spectrally determined cholesterol transfer. Possibly both cholesterol transfer and aggregation may need protein insertion into the vesicle as a first step. This aggregation proceeds for 4–5 min but does not lead to sufficient vesicle fusion to produce the mixing of intravesicular contents that has been reported for several protein lipid aggregation processes (Blume, 1982; Kumar et al., 1982; Blumenthal et al., 1983; Elamrani & Surewicz et al., 1986; Meers et al., 1988). This lack of membrane fusion is also consistent with the lack of accelerated cholesterol exchange which would occur rapidly between fused vesicles.

This P-450$_{scc}$-promoted aggregation appears to require interactions of hydrophobic and polar domains of the protein with vesicles and to be highly specific to the cytochrome structure. Thus, aggregation is prevented by mild denaturation, is not duplicated by rat microsomal P4501A1, and is blocked or reversed by anti-P-450$_{scc}$ IgG. The need for a stable insertion interaction is suggested by the failure of DMPC–SUV to aggregate and by the ability of phosphatidylinositol, when included in these vesicles, to facilitate both insertion and aggregation. These changes correlated closely with differences in the retention of P-450$_{scc}$ in the vesicles during Sephadex elution and with the various experiments that have examined intervesicle exchange of P-450$_{scc}$. The requirement for a specific polar domain is indicated by the inhibition of aggregation at ADX concentrations that form a 1:1 complex close to the heme group. Complex formation most probably involves an interaction of carboxyl groups from ADX with two conserved, positively charged regions on P-450$_{scc}$ (Lys-377; Lys-381, Arg-385) (Lambeth & Kriengsiri, 1985; Tsubaki et al., 1989). This domain must also in some way bind phosphatidylcholine head groups of the secondary vesicle to promote aggregation.

Intervesicle exchange of P-450$_{scc}$ probably occurs without dissociation of P-450 monomers. We have provided evidence that P-450$_{scc}$ reconstituted with DOPC–SUV does not exchange readily with soluble oligomeric P-450$_{scc}$. This suggests that the dissociation of free P-450$_{scc}$ monomers is highly unfavorable and is unlikely to provide a mechanism for rapid intervesicle–P-450$_{scc}$ exchange. More probably, a bridging complex of P-450$_{scc}$ with two vesicles forms the key intermediate, both in P-450$_{scc}$ exchange and in aggregation. However, two observations indicate that these processes represent competing pathways for such complexes. P-450$_{scc}$ exchange occurs rapidly (e.g., DMPC–SUV) when aggregation is absent and

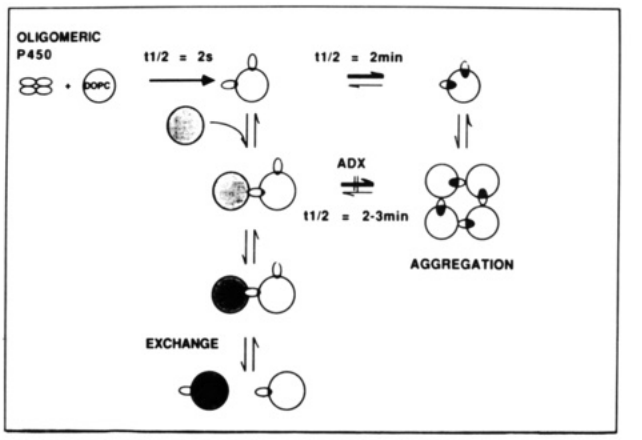

FIGURE 10: Diagrammatic representation of cytochrome P-450$_{scc}$ interactions with DOPC–SUV. Oligomeric P-450$_{scc}$ initially interacts with DOPC–SUV with a $t_{1/2}$ = 2 s. Deeper insertion of the cytochrome into the membrane occurs with a $t_{1/2}$ = 2 min. When a secondary vesicle (shaded) is presented to P-450-containing vesicles, exchange of the enzyme (which is independent of ADX) occurs readily. Cytochrome P-450$_{scc}$, once associated with the vesicles, can also induce the vesicles to aggregate, which is inhibited and reversed by the presence of ADX and anti-P-450$_{scc}$ antibody. The shaded portion of the enzyme represents the extent of insertion in the membrane.

more slowly when aggregation is effective (DMPC–phosphatidylinositol–SUV or DOPC–SUV). Conversely, P-450$_{scc}$ exchange occurs readily in the presence of ADX, as determined by intervesicle complex formation or secondary cholesterol SCC while ADX blocks aggregation. This facile exchange of P-450$_{scc}$ between vesicles may be a consequence of the exceptional lack of amino-terminal hydrophobic helices (Edwards et al., 1989). However, the exchange of other P-450 cytochromes has not been examined, and recent work indicates both rapid and slow interactions with SUV that may parallel those reported here (Causey et al., 1990).

A possible reconciliation of these opposite trends is to consider P-450$_{scc}$ exchange the outcome of unstable tertiary vesicle-P-450$_{scc}$ complexes, while aggregation is favored by more substantial integration of P-450$_{scc}$ into the vesicle. In this mechanism (Figure 10), the formation of tertiary complexes occurs readily for DOPC–SUV and DMPC–SUV, even in the presence of ADX. Exchange occurs through rearrangement of the ternary complex such that the locations of integral and polar interactions are reversed prior to dissociation of the complex. Such a rearrangement should be slowed by a more stable integration of P-450$_{scc}$ into the primary vesicle (DOPC) and enhanced by weak integration (DMPC). The selective inhibition of P-450$_{scc}$-induced aggregation by ADX but not by P-450$_{scc}$ exchanges can be reconciled if ADX accelerates P-450$_{scc}$ dissociation without affecting association. Less stable ternary complexes may be sufficient for P-450$_{scc}$ exchange, while only a more stable complex through the ADX binding site provides the stability for forming an aggregate. Since there is neither mixing of intravesicle contents nor enhanced cholesterol exchange, direct contact between the individual vesicles is apparently low.

Adrenal fasciculata and ovarian luteal cells contain mitochondria with exceptionally vesiculated inner membranes (Giacomelli et al., 1965; Friend & Brassil, 1970; Blanchette, 1966; Milner, 1972; Kahri & Leonards, 1968; Nussdorfer, 1986; Sabatini & DeRobertis, 1961). This multivesicular structure occurs in proportion to the incorporation of cytochrome P-450$_{scc}$ into the mitochondria under hormonal activation (Farkash et al., 1986) but is readily reversed by modification of the ionic environment of the mitochondria. This unusual structure may be stabilized by associations between

the vesicle surfaces mediated by the large amounts of P-450$_{scc}$ present in the bovine adrenal mitochondria (2% of total protein) (Hanukoglu & Hanukoglu, 1986). Such a dynamic set of contacts may also greatly facilitate the distribution of cholesterol from the inner membrane to these vesicles. Preliminary studies in this laboratory show that P-450$_{scc}$ rapidly aggregates vesicles comprised of adrenal mitochondrial phospholipids and that adrenal mitochondrial vesicles also aggregate through a P-450-dependent process.

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Inhibition of Mitochondrial Calcium Ion Transport by an Oxo-Bridged Dinuclear Ruthenium Ammine Complex†

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ABSTRACT: Ruthenium red is a well-known and effective inhibitor of the mitochondrial Ca^{2+} uniporter; however, Reed and Bygrave [(1974) *FEBS Lett. 46*, 109–114] tentatively attributed this inhibition to a colorless impurity present in commercial samples of ruthenium red (RR). This component has now been isolated and a derivative, $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2Cl_3$, structurally characterized. The active species in solution appears to be the symmetrical oxo-bridged ion, $[X(NH_3)_4Ru\text{–}O\text{–}Ru(NH_3)_4X]^{3+}$, where X = Cl^- or OH^-. Its absorption spectrum shows a maximum at 360 nm. The dinuclear ruthenium ammine complex inhibits Ca^{2+}-stimulated respiration of rat liver mitochondria with an I_{50} of 3.5 pmol/mg of protein compared to the value of 60 pmol of RR/mg of protein. The inhibition by the dinuclear compound is noncompetitive with Ca^{2+}. Respiration-linked swelling of mitochondria induced by Cd^{2+} also responds similarly to both the dinuclear complex and RR. A close correlation was observed between binding to mitochondria as monitored with ^{103}Ru-labeled dinuclear complex and inhibition of Ca^{2+} transport. A Scatchard plot yielded estimates of maximum specific binding and dissociation constant of 7.5 pmol/mg of protein and 1.3 nM, respectively. The inhibitor has the characteristics of a satisfactory affinity ligand for purification of the uniporter.

Ruthenium red is a cytological stain widely used for acidic mucopolysaccharides and selectively staining mitochondria for both visible and electron microscopy (Clarke, 1980). It has the structure $[(NH_3)_5Ru^{III}\text{–}O\text{–}(NH_3)_4Ru^{IV}\text{–}O\text{–}Ru^{III}(NH_3)_5]^{6+}$ and specifically inhibits respiration-driven uptake of Ca^{2+} by mitochondria (Lehninger et al., 1967; Moore, 1971; Vasington et al., 1972; Reed & Bygrave, 1974a) as well as the Ca^{2+} release mechanism of the sarcoplasmic reticulum (Antoniu et al., 1985; Chiesi et al., 1988). Reed and Bygrave (1974b) noted that commercial preparations of ruthenium red which are often less than 20% pure, produced stronger inhibition than purified preparations and used thin-layer chromatography to partially separate an active component that absorbed strongly around 355 nm. Experiments on similarly purified radiolabeled ruthenium red preparations showed that these nearly colorless fractions had a higher inhibitory activity per atom of ruthenium than ruthenium red itself (Reed & Bygrave, 1974b). In this paper, we report the synthesis and isolation of the inhibitor $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2Cl_3$, obtained by the reaction of the active ion (presumbly $[X(NH_3)_4Ru\text{–}O\text{–}Ru(NH_3)_4X]^{3+}$, where X = Cl^- or OH^-) with formate.

Experimental Procedures

Ruthenium red was obtained from Eastman Kodak Co., ruthenium chloride from AESAR, CM-cellulose from Whatman and Bio-Gel P-6 from Bio-Rad. $^{103}RuCl_3$ was supplied by NEN Research Products (Du Pont). Arsenazo[1] III supplied by Aldrich Chemical Co. was purified by passage through a column of Chelex 100 (Bio-Rad). The absorption spectra were measured in the Perkin-Elmer 557 double-beam, dual-wavelength spectrophotometer.

Rat liver mitochondria were prepared according to Johnson and Lardy (1967). Oxygen consumption was measured po-

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[1] Abbreviations: Arsenazo, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthylenebis(azo)]bis(benzenearsonic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ru360, the dinuclear ammine complex; Tris, tris(hydroxymethyl)aminomethane; RR, ruthenium red.

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