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Steroidogenesis in liposomal system containing adrenal microsomal cytochrome *P*-450 electron transfer components

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Key words: Cytochrome *P*-450$_{C21}$; Cytochrome *P*-450$_{17\alpha,lyase}$; NADPH–cytochrome-*P*-450 reductase; Liposome; Steroidogenesis

Purified adrenal microsomal *P*-450$_{C21}$ and / or *P*-450$_{17\alpha,lyase}$ were incorporated with purified NADPH–cytochrome-*P*-450 reductase into liposome membranes composed of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at a molar ratio of 5:3:1. The rate dependences of reduction of liposomal *P*-450$_{C21}$ in the fast phase as well as progesterone hydroxylation activities of *P*-450$_{C21}$ and *P*-450$_{17\alpha,lyase}$ on the reductase concentration in the liposome membranes suggested that electrons were delivered through random collisions between the reductase and cytochrome *P*-450s in the liposome membranes. A rapid exchange of the steroid metabolic intermediate between vesicles was observed in a reaction system consisting of *P*-450$_{C21}$-proteoliposomes and *P*-450$_{17\alpha,lyase}$-proteoliposomes. Using the combined liposomal system, it was definitely proved that androstenedione was formed from progesterone mainly by a successive hydroxylation reaction without the intermediate 17α-hydroxyprogesterone leaving from *P*-450$_{17\alpha,lyase}$. It was also found that 21-hydroxylation of 17α-hydroxyprogesterone into 11-deoxycortisol was inhibited by a physiological concentration of progesterone.

Introduction

Several steroid hormones are synthesized from cholesterol as the starting material in adrenal glands by actions of four species of cytochrome *P*-450 located both in the mitochondria and the endoplasmic reticulum [1]. Pregnenolone is synthesized from cholesterol via the side-chain cleavage reaction catalyzed by *P*-450$_{SCC}$ in the mitochondria and is transferred to the endoplasmic reticulum, where 3β-hydroxy-Δ^5-steroid dehydrogenase-Δ^5-isomerase metabolizes most of the pregnenolone into progesterone. In the endoplasmic reticulum, there are two species of cytochrome *P*-450; *P*-450$_{C21}$ catalyzing steroid 21-hydroxylation and *P*-450$_{17\alpha,lyase}$ catalyzing 17α-hydroxylation and C_{17}–C_{20} bond cleavage of steroids [2–4]. As can be seen in the metabolic pathway of Scheme I, progesterone is hydroxylated either at the 21 or the 17α position resulting in deoxycorticosterone or 17α-hydroxyprogesterone, respectively, and 17α-hydroxyprogesterone might be further hydroxylated at the 21 position into deoxycortisol or converted into androstenedione by C_{17}–C_{20} bond cleavage. The deoxycorticosterone and the deoxycortisol are transferred back into mitochondria and are further metabolized by *P*-450$_{11\beta}$ [5–8]. The species and the amount of steroids secreted from adrenal glands are thus quite dependent on the relative activities of *P*-450$_{C21}$ and *P*-450$_{17\alpha,lyase}$ in the endoplasmic reticulum.

Hydroxylase activity of microsomal cytochrome *P*-450 has been reported to be dependent on the concentration of the reductase in the reaction system [9–13]. The content of the reductase in adrenal microsomes is quite low, which is about 1/4–1/10 of total cytochrome *P*-450 content [14]. It is reasonable to assume that the interaction between the cytochrome *P*-450s and the reductase might be one of the factors in the regulation of the hydroxylation reactions. With respect to the 17α-hydroxyprogesterone, competition for substrate might be occurring between *P*-450$_{C21}$ and *P*-450$_{17\alpha,lyase}$, which would be another factor in adrenal steroidogenesis.

Abbreviations: *P*-450$_{C21}$, cytochrome *P*-450 having steroid 21-hydroxylase activity (P-450XXI); *P*-450$_{17\alpha,lyase}$, cytochrome *P*-450 having steroid 17α-hydroxylase and C17,20-lyase activities (P-450XVII); *P*-450$_{SCC}$, cytochrome *P*-450 having cholesterol desmolase activity (P-450XXII); *P*-450$_{11\beta}$, cytochrome *P*-450 having steroid 11β-hydroxylase activity (P-450XI); *P*-450$_{arom}$, cytochrome *P*-450 having steroid aromatization activity (P-450XIX). The names of gene family are represented in parentheses.

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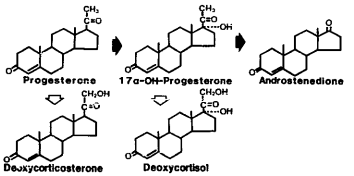

Scheme I. A schematic diagram of progesterone metabolism in adrenocortical microsomes. The black and white arrows indicate reactions catalyzed by $P\text{-}450_{17\alpha,lyase}$ and $P\text{-}450_{C21}$, respectively.

In this study, purified $P\text{-}450_{C21}$ and/or $P\text{-}450_{17\alpha,lyase}$ were incorporated with purified NADPH–cytochrome-*P*-450 reductase into liposome membranes composed of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at a molar ratio of 5:3:1, which were the major phospholipids of bovine adrenal microsomes [15]. The reconstitution of the cytochrome *P*-450 electron-transfer components in the liposome membranes has allowed us detailed investigations concerning various factors affecting adrenal microsomal steroidogenesis.

Materials and Methods

Preparation of enzymes

$P\text{-}450_{C21}$ and NADPH–cytochrome *P*-450 reductase were purified from bovine adrenal microsomes according to the method previously described [2,16,17]. $P\text{-}450_{17\alpha,lyase}$ was purified from guinea pig adrenal microsomes as reported previously [3,4]. All of these enzymes are homogeneous at sodium dodecyl suflate-polyacrylamide gel electrophoresis. Emulgen 913, nonionic detergent, used in the purifications of $P\text{-}450_{17\alpha,lyase}$ and the reductase was removed by extensive washing of the enzymes before the storage at −80 °C [3,17]. The detergent in $P\text{-}450_{C21}$ preparation was removed just before mixing with the phospholipids, because $P\text{-}450_{C21}$ became labile without the detergent during the storage [16]. The complete removal of Emulgen 913 was confirmed by monitoring UV absorption due to the detergent in the sample.

Preparation of proteoliposomes

Proteoliposomes were prepared by the dialysis method [18] from a phospholipid mixture composed of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at a molar ratio of 5:3:1, as described previously [12,19]. More than 80% of each enzyme was found to be located at the external side of the liposomal membranes [12,16,19]. Electron microscopic observations showed that the prepared proteoliposomes were unilamellar vesicles of about 50 nm in the average diameter.

The content of cytochrome *P*-450 in the liposomes was estimated from its CO–dithionite-reduced difference spectrum using $\Delta\varepsilon_{450-490\,nm} = 91\ cm^{-1} \cdot mM^{-1}$ [20] and that of the reductase was determined from its cytochrome-*c* reductase activity after the collapse of the liposomes by 1% sodium cholate in comparison with the activity of the purified reductase in the same condition [12]. The concentrations of $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,lyase}$ residing in the same membrane were determined by solving the following simultaneous equation [19].

$$C(C21) \times 20 + C(17\alpha) \times 53 = \Delta A(DOC)$$

$$C(C21) \times 56 + C(17\alpha) \times 20 = \Delta A(AND)$$

where C(C21) and C(17α) represent the bulk concentrations in mM of $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,lyase}$ in the liposomes, respectively, and ΔA(DOC) and ΔA(AND) are the magnitudes of the type I difference spectra at 25 °C induced by deoxycorticosterone (DOC) and androstenedione (AND), respectively, in 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA and 50 mM NaCl, which will hereafter be referred to as the basal buffer in this article. The absorption coefficients in the above equations were obtained from the difference spectra of the individual proteoliposomes induced by the additions of excess amounts of the steroids at 25 °C. The presence of the reductase in the same membrane did make little observable effects on the coefficients. The liposomes used in this experiment contained 1 mol of each cytochrome *P*-450 per 2500–3000 mol of phospholipids. The cytochrome *P*-450s in the proteoliposomes were considerably stable, of which less than 30% was converted into inactive *P*-420 form during the storage at 0 °C for 48 h. The cytochrome-*c* reductase activity of the reductase in the liposome membranes showed no observable decrease after the storage at 0 °C for 48 h. Stopped-flow measurements were performed at 37 °C in the basal buffer with a double wavelength stopped-flow apparatus (Unisoku Co.) using lights of 450 and 490 nm. The analysis of the stopped-flow data was performed by curve-fitting, using a non-linear least-square method with a personal computer (PC-9801, NEC Inc.). Optical absorption spectra and difference spectra were obtained with a Beckman DU-7 spectrophotometer at 25 °C in the basal buffer.

Enzyme assay

Progesterone hydroxylase activity of the proteoliposomes was measured aerobically at 37 °C, as described previously [11]. The steroids extracted from the reaction solution were separated by HPLC system (HLC 803 and UV-8, TOSOH Inc.) with a silica gel column (0.46 × 15 cm, Cosmosil 5SL, Nakarai Chemcial Co.)

using a solvent system of *n*-hexane/isopropanol/acetic acid (93:7:1, v/v) and quantified from their peak areas relative to that of the internal standard (spironolactone), using calibration curves of the relative peak areas versus the known amount of the steroids. The data accumulation and distal calculations were performed with an OBA-3 system consisting of an auto sampler (AS-80, TOSOH Inc.), a personal computer (PC-9801, NEC Inc.) and the HPLC system.

Materials

Sodium cholate, L-α-phosphatidylcholine from egg yolk (Type III), L-α-phosphatidylethanolamine from egg yolk, L-α-phosphatidylserine from bovine brain, spironolactone, deoxycortisol and androstenedione were obtained from Sigma Chemcial Co., St. Louis, MO. Progesterone, deoxycorticosterone, butylated hydroxytoluene, and acetic acid (HPLC grade) were from Nakarai Chemical Co., Kyoto and 17α-hydroxyprogesterone was from Fluka AG. Buch. Dithiothreitol and NADPH were obtained from Boehringer, Mannheim, F.R.G. Normal hexane (HPLC grade) and isopropanol (HPLC grade) were from Cica-Merk, Tokyo. All other chemcials used in this experiment were of the best grade commercially available.

Results

Stopped-flow experiments of the reduction of P-450$_{C21}$ in the liposomes

The dependence of the rate of *P*-450$_{C21}$ reduction on the reductase content in the liposome membranes was measured with a stopped-flow method. The molar ratio of *P*-450$_{C21}$ to the phospholipids was kept in the range of 1/2500–1/3000, but that of the reductase to the phospholipids was altered. When liposomes containing *P*-450$_{C21}$ and the reductase were mixed rapidly with an excess amount of NADPH in the presence of CO, the absorbance at 450 nm increased biphasically as shown in Fig. 1 (inset). The absorbance change could always fit a sum of two first-order reaction kinetics. The relative portion of the fast phase to the overall absorption change was not much dependent on the reductase content in the proteoliposomes, which was about 60–70%. The rate of the fast-phase reduction increased almost linearly with the molar ratio of the reductase to *P*-450$_{C21}$ in the liposomes at least up to 2.0 (Fig. 1) but the rate of the slow-phase reduction did not alter much with the reductase content which was around 0.5–0.2 s^{-1}. The linear increase of the rate of the fast-phase reduction of *P*-450$_{C21}$ with the reductase content suggests that *P*-450$_{C21}$ might be accepting electrons from the reductase through random collisions between them in the membranes. Similar experiments could not be performed for liposomes containing *P*-450$_{17\alpha,lyase}$ because of the lability of the *P*-450-CO complex.

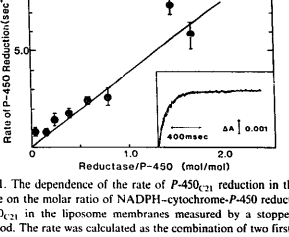

Fig. 1. The dependence of the rate of *P*-450$_{C21}$ reduction in the fast phase on the molar ratio of NADPH–cytochrome-*P*-450 reductase to *P*-450$_{C21}$ in the liposome membranes measured by a stopped-flow method. The rate was calculated as the combination of two first-order reactions by curve fittings using a nonlinear least-square method. The reaction was initiated by a rapid mixing of the liposomes containing the reductase and *P*-450$_{C21}$ at various molar ratio with an excess amount of NADPH in the basal buffer containing 30 μM of 17α-hydroxyprogesterone at 37°C in the presence of CO. The ratio of *P*-450$_{C21}$ to the phospholipids was in the range of 1/2500–1/3000. Inset: a typical time course of the absorbance increase at 450 nm.

Progesterone hydroxylase activities of P-450s in the liposomes

As can be seen in Fig. 2, progesterone hydroxylase activity of *P*-450$_{C21}$-proteoliposomes in the steady state increased with increase of the molar ratio of the reductase to *P*-450$_{C21}$ in the liposomes. The activity became 60 nmol deoxycorticosterone produced per min per nmol of *P*-450$_{C21}$ at the ratio of the reductase to *P*-450$_{C21}$ of 10:1 (data not shown). The hydroxylase activity of *P*-450$_{17\alpha,lyase}$-proteoliposomes increased with the reductase content up to the ratio of 0.5:1, and

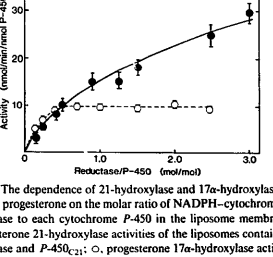

Fig. 2. The dependence of 21-hydroxylase and 17α-hydroxylase activities for progesterone on the molar ratio of NADPH–cytochrome-*P*-450 reductase to each cytochrome *P*-450 in the liposome membranes. ●, progesterone 21-hydroxylase activities of the liposomes containing the reductase and *P*-450$_{C21}$; ○, progesterone 17α-hydroxylase activities of the liposomes containing the reductase and *P*-450$_{17\alpha,lyase}$. The sum of 17α-hydroxyprogesterone and androstenedione produced from progesterone was measured for the 17α-hydroxylase activity. The ratio of each cytochrome *P*-450 to the phospholipids was in the range of 1/2500–1/3000. All the reactions were carried out at 37°C in the basal buffer with 30 μM of initial concentration of progesterone.

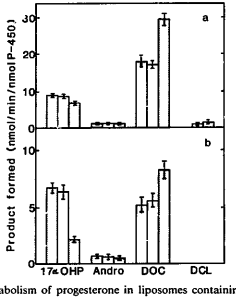

Fig. 3. Metabolism of progesterone in liposomes containing NADPH–cytochrome-*P*-450 reductase, *P*-450_{C21}, and/or *P*-$450_{17\alpha,lyase}$: (a) The steroid-producing activities of the liposomes containing the reductase, *P*-450_{C21} and *P*-$450_{17\alpha,lyase}$ at the molar ratio of 3 : 1 : 1 (bars with dots) and of the combined liposomal system (open bars) consisted of equal amount of liposomes, one containing the reductase and *P*-450_{C21} (1.5 : 1) and another containing the reductase and *P*-$450_{17\alpha,lyase}$ (1.5 : 1). Bars with horizontal and vertical shadows were the activities of the liposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ (1.5 : 1) and of the liposomes containing the reductase and *P*-450_{C21} (1.5 : 1), respectively. (b) The activities of the liposomes containing the reductase, *P*-450_{C21} and *P*-$450_{17\alpha,lyase}$ at the molar ratio of 0.44 : 1 : 1 (bars with dots) and of the combined liposomes consisted of equal amounts of liposomes, one containing the reductase and *P*-450_{C21} (0.22 : 1) and another containing the reductase and *P*-$450_{17\alpha,lyase}$ (0.22 : 1) (open bars). The bars with vertical and horizontal shadows show the activities in the liposomes containing the reductase and *P*-450_{C21} (0.22 : 1) and in the liposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ (0.22 : 1), respectively. Progesterone (30 μM) was metabolized in the steady state in reaction systems containing 10 pmol of each cytochrome *P*-450 for 20 min at 37 °C in the basal buffer. For the activities of 17α-hydroxyprogesterone, deoxycortisol and androstenedione production, the produced amounts were divided by the contents of *P*-$450_{17\alpha,lyase}$ (10 pmol) and 20 min. For the activities of deoxycorticosterone, the produced amounts were divided by the contents of *P*-450_{C21} (10 pmol) and 20 min. 17αOHP, Andro, DOC, and DCL in the figure represent 17α-hydroxyprogesterone, androstenedione, deoxycorticosterone and deoxycortisol, respectively. The ratio of each cytochrome *P*-450 to the phospholipids was in the range of 1/2500–1/3000.

beyond that the activity stayed at a constant level of 10 nmol of 17α-hydroxylation products per min per nmol of *P*-$450_{17\alpha,lyase}$. The sum of 17α-hydroxyprogesterone and androstenedione was regarded to be the 17α-hydroxylation products at this point.

As in Fig. 3a, liposomes containing the reductase, *P*-450_{C21} and *P*-$450_{17\alpha,lyase}$ at the molar ratio of 3 : 1 : 1 showed 21-hydroxylase activity of about 30 nmol/min per nmol of *P*-450_{C21} which was almost the same as that of the liposomes containing the reductase and *P*-450_{C21} at the molar ratio of 3 : 1 (see Fig. 2). This means that in the time scale of hydroxylation reaction, *P*-450_{C21} is interacting freely with all of the reductase in the membranes even in the co-presence of *P*-$450_{17\alpha,lyase}$ in the same vesicles. 17α-Hydroxylase activity of the liposomes containing the reductase and both cytochrome *P*-450s at the molar ratio of 3 : 1 : 1 looked a little smaller than the 17α-hydroxylase activity of the liposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ at the molar ratio of 3 : 1, but the producing activity of the sum of 17α-hydroxyprogesterone, androstenedione and deoxycortisol, in which the latter two might be produced from 17α-hydroxyprogesterone, was about the same as that of the liposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ (3 : 1). The empty bars in Fig. 3a show the producing activities of steroids in the combined system consisted of proteoliposomes containing the reductase and *P*-450_{C21} (1.5 : 1) and proteoliposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ (1.5 : 1). The 21-hydroxylase activity in this combined system was about the same as those observed in the separate vesicles (vertically hatched bar) but significantly lower than that at the liposomes containing the reductase and both cytochrome *P*-450s (3 : 1 : 1) (dotted bar), although the total amount of each enzyme in the reaction solution was the same between the two systems. This is one of the indications that the proteins in liposome membranes can not readily transfer between vesicles. A significant amount of deoxycortisol was detected in the combined liposomal system, meaning that 17α-hydroxyprogesterone produced from progesterone in *P*-$450_{17\alpha,lyase}$-proteoliposomes was transferred into *P*-450_{C21}-proteoliposomes. The deoxycortisol-producing activity in the combined liposomal system was similar to that of the liposomes containing both cytochrome *P*-450s, indicating that the transfer of the intermediate 17α-hydroxyprogesterone must be quite rapid compared with the time scale of hydroxylation reaction.

The proteoliposomes containing the reductase and both cytochrome *P*-450s at the molar ratio of 0.44 : 1 : 1 showed a 17α-hydroxylase activity of about 2, as in Fig. 3b, which corresponds to the activity of liposomes containing the reductase and *P*-$450_{17\alpha,lyase}$ at the ratio of 0.1 : 1 in Fig. 2, indicating that only one-fourth of the reductase in the liposomes containing both *P*-450s seemed to be effective for 17α-hydroxylase activity. 21-Hydroxylase activity of the liposomes containing both *P*-450s and the reductase at the molar ratio of 0.44 : 1 : 1 showed the same activity as that of the liposomes containing the reductase and *P*-450_{C21} at the molar ratio of 0.44 : 1. These data suggest that a preferential electron transfer from the reductase to *P*-450_{C21} is occurring in the liposomes containing both cytochrome *P*-450s.

Effects of progesterone concentration on deoxycortisol production

The effect of progesterone concentration on the production of deoxycortisol was examined as in Fig. 4,

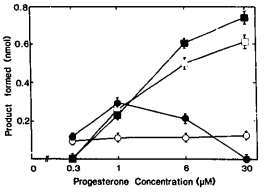

Fig. 4. Effect of progesterone concentration on the steroid metabolism in the combined liposomal system consisted of equal amounts of liposomes, one containing NADPH–cytochrome-*P*-450 reductase and *P*-450_{C21} (0.1 : 1) and another containing the reductase and *P*-$450_{17\alpha,lyase}$ (0.1 : 1). The vertical axis shows the produced amount of each steroid in the system containing 10 pmol of each liposomal cytochrome *P*-450 in the basal buffer at 37 °C after 20 min incubation. The horizontal axis shows the progresterone concentration initially present in the reaction solution. The closed and open circles in the figure show the amounts of produced deoxycortisol and androstenedione, respectively, and the closed and open squares show those of 17α-hydroxyprogesterone and deoxycorticosterone, respectively.

where various amounts of progesterone were incubated at 37 °C for 20 min with the combined liposomal system. Deoxycortisol was a major product at progesterone concentrations of 0.3 and 1 μM, but was not detected at 30 μM. The amounts of produced 17α-hydroxyprogesterone and deoxycorticosterone increased with the increase in progesterone concentration but that of androstenedione did not change much. It was separately confirmed that the produced deoxycorticosterone did not make any inhibitory effects on the hydroxylation reaction in Fig. 4. These results lead us to the conclusion that a high concentration of progesterone inhibits

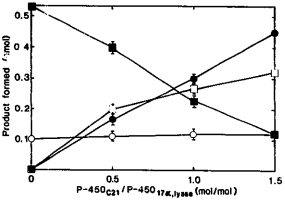

Fig. 5. Effect of liposomal *P*-450_{C21} on the production of androstenedione from progesterone by liposomal *P*-$450_{17\alpha,lyase}$. The vertical axis shows the produced amount of each steroid in the combined liposome system consisted of liposomes containing 1 pmol of the reductase and 10 pmol of *P*-$450_{17\alpha,lyase}$ and various amount of liposomes containing the reductase and *P*-450_{C21} (0.1 : 1). One nanomole of progesterone was incubated in the combined liposomal system at 37 °C for 20 min. Symbols in the figure have the same meanings as those in Fig. 4.

the *P*-450_{C21}-catalyzed conversion of 17α-hydroxyprogesterone into deoxycortisol.

Androstenedione production from progesterone by P-$450_{17\alpha,lyase}$

Androstenedione production from progesterone by *P*-$450_{17\alpha,lyase}$ was not inhibited by the presence of a high concentration of progesterone, as shown in Fig. 4, although 17α-hydroxyprogesterone might be competing with progesterone at the substrate binding site of *P*-$450_{17\alpha,lyase}$. This result indicates that androstenedione might not be formed from 17α-hydroxyprogesterone released from *P*-$450_{17\alpha,lyase}$ into the medium under this reaction condition. The co-presence of *P*-450_{C21}-proteoliposomes with *P*-$450_{17\alpha,lyase}$-proteoliposomes could decrease the amount of 17α-hydroxyprogesterone in the medium by the conversion into deoxycortisol, which should reduce androstenedione production from progesterone in the case that androstenedione were to be produced from 17α-hydroxyprogesterone in the medium. As can be seen in Fig. 5, 17α-hydroxyprogesterone was decreased with the increase in the amount of *P*-450_{C21}-proteoliposomes in which the decrease correlated well with the increase in deoxycortisol. Androstenedione production was not decreased by the decrease of 17α-hydroxyprogesterone in the medium. This result definitely shows that androstenedione is not formed much from the 17α-hydroxyprogesterone released in the medium.

Discussion

Interaction of the reductase with P-450s in liposomal membranes

For some time it has been discussed how NADPH–cytochrome-*P*-450 reductase supplies electrons from NADPH to several species of cytochrome *P*-450 in the microsomal membranes. Peterson et al. [21] proposed a cluster model for electron transfer in microsomes. Taniguchi et al. [22] showed experimental results that cytochrome *P*-450 was accepting electrons from the reductase through random collisions between two proteins in the liposomal membranes. The random collision model for the electron transfer has been supported by Ingelman-Sundberg and Johansson [23] and also by Archakov et al. [24]. Active complex for the hydroxylation reaction composed of equimolar of cytochrome *P*-450 and the reductase was proposed by Miwa et al. [10] and the equimolar complex has been found not only in a system with detergent [11] but also in a system without detergents [13]. The complex formation was also supported by the measurements of mobilities of cytochrome *P*-450s in membrane systems [25,26]. The reductase dependence of the reduction rate of liposomal *P*-450_{C21} in this study supports the random collision model for the electron transfer. In the proteoliposomes

containing the reductase and both cytochrome *P*-450 at the molar ratios of 3 : 1 : 1 and 0.44 : 1 : 1, the reductase could supply electrons to *P*-450_{C21} almost equally to that in the liposomes containing the reductase and *P*-450_{C21} at the molar ratios of 3 : 1 and 0.44 : 1, respectively, and, furthermore, the reductase donates significant amounts of electrons to *P*-$450_{17\alpha,lyase}$ in the same membranes. These hydroxylase activities cannot be explained by the equimolar active complex in the strict meaning that in the time scale of hydroxylation reaction one molecule of the reductase was complexed with one molecule of cytochrome *P*-450, and that the electron transfer was carried out only in the complex. The saturation tendency of the hydroxylation activity of liposomal *P*-$450_{17\alpha,lyase}$ could be explained as that at the low reductase content in the membranes, the electron transfer might determine the rate of hydroxylation, but that at a higher content of the reductase, other steps might become the rate-limiting step [27]. Maximum benzpyrene hydroxylase activity has also been observed in hepatic microsomes at the molar ratio of the reductase to *P*-448 around 0.2 : 1 [28]. The results in this study could be explained by the random collision model for the electron transfer in the membranes. The preference of the electron transfer between two cytochrome *P*-450s suggests that the rate of electron transfer might not be determined only by the frequency of the collisions in the membranes. There is, however, the problem that the reductase and *P*-$450_{17\alpha,lyase}$ were not purified from same sources, and it might be possible that the enzymes from different sources could not interact efficiently.

Steroid transfer between liposomes

When progesterone was incubated with the combined system consisting of *P*-450_{C21}-proteoliposomes and *P*-$450_{17\alpha,lyase}$-proteoliposomes, the intermediate 17α-hydroxyprogesterone was shown to transfer rapidly from the latter liposomes to the former liposomes. A rapid transfer of steroids between vesicles had been shown in a stopped-flow experiment [16]. Almgren et al. [29] showed that the rate of transfer of hydrophobic molecules between liposomes was predominantly determined by the rate of release of the molecules from the mother vesicles, and was almost proportional to the inverse value of the partition coefficient of the molecules between lipid and aqueous phases. All the steroids involved in adrenal steroidogenesis except for cholesterol have partition coefficients of less than 10^4 [19,30], which suggests that the release of the steroids from the membranes takes less than 10^{-2} s. This is quite fast compared with the rate of steroid hydroxylation which is slower than 1 s^{-1}.

Inhibition of steroidogenesis by progesterone

Both of progesterone and 17α-hydroxyprogesterone are substrates for *P*-450_{C21} and the competition between them had already been reported in the binding to *P*-450_{C21} in a detergent system [31]. The apparent concentration of progesterone in guinea pig adrenals can be calculated to be around 10 μM under the rough assumption that the total volume of the tissue could be regarded as being occupied by water [32]. The inhibition of 21-hydroxylation reaction of 21-deoxycortisol by 50 μM of progesterone has been reported in cultured bovine adrenocortical cells by Hornsby [33]. It might be possible that the concentration of progesterone in vivo might have some important role in the regulation of deoxycortisol production.

Successive hydroxylation reactions in androstenedione production

Successive hydroxylation reactions play important roles in steroidogenesis. The side-chain cleavage of cholesterol catalyzed by *P*-450_{SCC} [34], aldosterone formation from deoxycorticosterone by *P*-$450_{11\beta}$ [8], and aromatization of steroids by *P*-450_{arom} [35] has been shown to be mainly carried out by successive hydroxylation reactions without the intermediates leaving from the cytochrome *P*-450 [36]. The reaction mechanism is usually deduced from the result of a little recovery of the radioactivity in the final product from the additionally supplied radioisotope-labeled intermediates [4,37, 38]. By the co-presence of *P*-450_{C21} proteoliposomes with *P*-$450_{17\alpha,lyase}$-proteoliposomes, we could decrease 17α-hydroxyprogesterone which had been released from *P*-$450_{17\alpha,lyase}$ in the metabolism of progesterone, but the decrease did not affect the production of androstenedione. This is the direct proof that 17α-hydroxyprogesterone in the medium is not much involved in the production of androstenedione from progesterone in that condition. This does not mean that androstenedione is never produced from 17α-hydroxyprogesterone in the medium. If 21-hydroxylase activity is low and 17α-hydroxyprogesterone is accumulated in the reaction system, androstenedione must be produced from 17α-hydroxyprogesterone. Detailed kinetic studies are now in progress for androstenedione production with respect to how much is formed directly from progesterone and from the released 17α-hydroxyprogesterone in various conditions.

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