Biochimica et Biophysica Acta, 984 (1989) 50–56
Elsevier

BBAMEM 74525

Adrenal cytochrome $P\text{-}450_{11\beta}$-proteoliposomes catalyzing aldosterone synthesis: preparation and characterization

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(Received 20 February 1989)

Key words: Cytochrome $P\text{-}450_{11\beta}$; Aldosterone synthesizing enzyme; Liposome; Steroidogenesis; (Bovine adrenocortical mitochondria)

Purified cytochrome $P\text{-}450_{11\beta}$ from bovine adrenocortical mitochondria was successfully incorporated into the liposome membranes composed of phosphatidylcholine, phosphatidylethanolamine and cardiolipin at a molar ratio of 2:2:1. The incorporation of $P\text{-}450_{11\beta}$ into the liposome membranes was ascertained by the Ficoll density gradient centrifugation and the protein refractoriness to trypsin digestion. The prepared proteoliposomes containing $P\text{-}450_{11\beta}$ and phospholipid at a molar ratio of 1:3000 were unilamellar vesicles of about 40 nm in average diameter. The $P\text{-}450_{11\beta}$ embedded in the liposome membranes was found to be more stable than the detergent-solubilized form. The reconstituted system containing the $P\text{-}450_{11\beta}$-proteoliposomes, adrenodoxin and NADPH-adrenodoxin reductase showed catalytic activities not only for the hydroxylation of 11-deoxycorticosterone at 11β- and 18-positions but also for its conversion into aldosterone with a turnover number of 2.3 nmol/min per nmol of $P\text{-}450_{11\beta}$. A successive reaction without the intermediates leaving from the enzyme was suggested for the $P\text{-}450_{11\beta}$-mediated conversion of 11-deoxycorticosterone to aldosterone following the result that the formation of aldosterone was linear with respect to time without the lag phase; this was confirmed by the result that radioactivity in aldosterone from ^{3}H-labeled 11-deoxycorticosterone was scarcely decreased by the addition of unlabeled intermediates to the reactions system.

Introduction

Adrenal cortex mitochondria contain at least two types of cytochrome *P*-450: $P\text{-}450_{SCC}$ catalyzing the side-chain cleavage of cholesterol and $P\text{-}450_{11\beta}$ catalyzing hydroxylations of steroids at various positions. These species of cytochrome *P*-450 as well as the electron transfer components have been highly purified and the steroidogenic electron transfer system has been investigated in detail using the purified proteins [1]. Purified $P\text{-}450_{11\beta}$ from bovine adrenocortical mitochondria has been shown to catalyze both 11β- and 18-hydroxylations of 11-deoxycorticosterone, both 11β- and 19-hydroxylations of 18-hydroxy-11-deoxycorticosterone, and 18-hydroxylation of corticosterone [2–4].

It has been indicated that a cytochrome *P*-450 plays a role in the final step of the biosynthetic pathway of the most potent mineralocorticoid, aldosterone, but the nature of aldosterone-synthesizing enzyme has not been fully elucidated [5]. Wada et al. [6–8] have demonstrated that formation of aldosterone from corticosterone or from 18-hydroxycorticosterone could be catalyzed by purified $P\text{-}450_{11\beta}$ in the presence of phospholipids. Although immunochemically indistinguishable $P\text{-}450_{11\beta}$ is distributed in all three zones of adrenal cortex [9,10], aldosterone is exclusively secreted from glomerulosa cells but not from fasciculata and reticularis cells in adrenal cortex of most mammalian [11]. Therefore, the control mechanism of aldosterone-synthesizing activity of $P\text{-}450_{11\beta}$ in adrenal cortex, which may be related to the zone specificity for aldosterone biosynthesis, is attracting much attention not only among biochemists but also among endocrinologists.

Most of the studies on purified $P\text{-}450_{11\beta}$ have been carried out in the detergent-solubilized system, but the instability of the solubilized form has hindered the detailed investigation of enzyme activity. Cytochromes *P*-450 are not so labile in the membranes of mitochondria and endoplasmic reticula in adrenal cortex

Abbreviations: $P\text{-}450_{SCC}$, cytochrome *P*-450 having cholesterol desmolase activity; $P\text{-}450_{11\beta}$, cytochrome *P*-450 having steroid 11β-hydroxylase activity; $P\text{-}450_{C21}$, cytochrome *P*-450 having steroid 21-hydroxylase activity, DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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cells, but the solubilization with detergents from the organelle membranes might make the cytochromes unstable in the micelle systems. It has been shown in cytochromes *P*-450 from adrenal microsomes that the enzymes were much more stabilized in liposome membranes than in the detergent micelle system [12,13]. Detailed kinetic studies have been performed for *P*-450_{SCC} incorporated into liposome membranes by Lambeth et al. [14–16]. With respect to *P*-$450_{11\beta}$, Lombardo et al. [17] have reported some structural properties of *P*-$450_{11\beta}$ located in liposome membranes. These reports suggest that incorporation of purified *P*-$450_{11\beta}$ into liposome membranes might provide a stable system for the investigation on the mechanism of *P*-$450_{11\beta}$-dependent aldosterone synthesis.

In the present investigation, purified *P*-$450_{11\beta}$ from bovine adrenal mitochondria has been incorporated into phospholipid vesicles. The prepared *P*-$450_{11\beta}$-proteoliposomes have been characterized with respect to the aldosterone-synthesizing activity as a first step towards the elucidation of the control mechanism of aldosterone biosynthesis.

Materials and Methods

Preparation of enzymes

Purification of *P*-$450_{11\beta}$ from bovine adrenocortical mitochondria of zonae fasciculata-reticularis was performed according to the method of Takemori et al. [18] with some modifications. 50 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM EDTA, 0.1 mM DTT and 10 μM 11-deoxycorticosterone was used as the basal buffer throughout the purification. After the *P*-$450_{11\beta}$-rich fraction (0–35% saturation of ammonium sulfate) had been treated with alumina C_γ and dialyzed, the resulting precipitate containing *P*-$450_{11\beta}$ was dissolved in the basal buffer containing 200 mM KCl and 0.7% (w/v) sodium cholate and subsequently applied to ω-amino-*n*-octyl-Sepharose 4B column (1.5 × 4.5 cm). The *P*-$450_{11\beta}$ was eluted with the basal buffer containing 500 mM KCl, 0.7% sodium cholate and 0.7% (w/v) *n*-octyl β-D-glucoside and the fractions showing a high-spin type spectrum were pooled. The eluted *P*-$450_{11\beta}$ fraction was dialyzed against the basal buffer containing 0.5% sodium cholate and applied to the second ω-amino-*n*-octyl-Sepharose 4B column (1.0 × 4.0 cm). The elution of *P*-$450_{11\beta}$ was performed with the basal buffer containing 500 mM KCl, 0.7% sodium cholate and 0.05% (w/v) phosphatidylcholine. The final preparation was dialyzed against the basal buffer containing 0.5% sodium cholate, 500 mM NaCl and 20% (v/v) glycerol, and stored at −80 °C. The purified preparation had a specific content of 13.7 nmol *P*-450/mg protein and the catalytic activity of 100 nmol corticosterone/min per nmol *P*-450 for 11β-hydroxylation of 11-deoxycorticosterone, values which were comparable to those reported by Takemori et al. [18]. As judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and steroid hydroxylase activity, the preparation did not contain any species other than *P*-$450_{11\beta}$. Adrenodoxin and NADPH-adrenodoxin reductase were prepared from bovine adrenocortical mitochondria according to the methods of Suhara et al. [19,20].

Preparation of P-$450_{11\beta}$-proteoliposomes

A phospholipid mixture composed of phosphatidylcholine, phosphatidylethanolamine and cardiolipin at a molar ratio of 2 : 2 : 1 (5 mg) was dispersed at 4 °C in 0.4 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 500 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 100 μM 11-deoxycorticosterone, 20% glycerol and 1% sodium cholate by means of a Vortex mixer and were ultrasonicated by a sonicator until the suspension became clear. Purified *P*-$450_{11\beta}$ (5 nmol) was added to this phospholipid solution and incubated at 0 °C for 4 h. The mixture was dialyzed at 4 °C for 12 h against 50 mM potassium phosphate buffer (pH 7.2) containing 500 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol. The dialyzed solution was centrifuged at 24000 × *g* for 1 h and the supernatant was applied to a HPLC TOSOH TSK gel PWH column (0.75 × 7.5 cm). The liposome fractions were detected by ultraviolet scattering at 300 nm and were pooled. The *P*-$450_{11\beta}$ concentration in the liposomes was determined from the CO-dithionite-reduced difference spectrum or metyrapone-induced difference spectrum using $\Delta\varepsilon$ (423–390 nm) = 90 $mM^{-1} \cdot cm^{-1}$. The lipid concentration of the liposomes was estimated from the recovery of the radioactivity of [^{14}C]dipalmitoylphosphatidylcholine, which had been previously added to the lipid mixture. Spectra of the proteoliposomes were recorded on a Beckman DU-7HS spectrophotometer which was equipped with a stable light source and operated in a single-beam mode with computer control.

Enzyme assay

For the assay of *P*-$450_{11\beta}$ mediating metabolism of 11-deoxycorticosterone, the reaction mixture contained 50 nmol 11-deoxy[^{3}H]corticosterone (1 μCi), 5 nmol adrenodoxin, 0.5 nmol NADPH-adrenodoxin reductase and proteoliposomes containing 25 pmol *P*-$450_{11\beta}$ in 0.5 ml of 30 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 100 nmol NADPH at 37 °C and terminated by the addition of 1.6 ml of a mixture of chloroform/methanol (1 : 1, v/v) containing the expected steroid products (2 nmol) and [^{14}C]androstenedione (5 pCi) as internal standard. The organic layer was evaporated and the residue was chromatographed on a TSK gel ODS 80T_M column (4.6 × 250 mm) in a TOSOH HPLC system (CCMP and UV8000) employing 60% aqueous methanol. The elu-

tion of steroids was detected by absorbance at 254 nm. The fractions of each peak corresponding to the expected products were collected and assayed for radioactivity. The amounts of steroids produced were calculated from the data with correction of the recovery in the whole procedure using the ^{14}C-labeled internal standards.

Materials

The materials used in this study were obtained from the following sources: 11-deoxy[1,2-^{3}H]corticosterone, [1,2,3,6-^{3}H]corticosterone and 18-hydroxy[1,2-^{3}H]-corticosterone were from Amersham International, Amersham, U.K.; [4-^{14}C]androstenedione and L-α-dipalmitoyl-[2-*palmitoyl*-1-^{14}C]phosphatidylcholine were from New England Nuclear, Boston, MA; 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone and aldosterone were from Makor Chemical, Israel; 11-deoxycorticosterone was from Nakarai Chemicals, Kyoto, Japan; corticosterone, androstenedione, L-α-phosphatidylcholine (egg yolk, type III-E), L-α-phosphatidylethanolamine (egg yolk, type III), cardiolipin (bovine heart) and trypsin (bovine pancreas, type III) were from Sigma Chemical, St. Louis, MO; metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was from Aldrich Chemical, Milwaukee, WI; *n*-octyl β-D-glucoside was from Dojindo Laboratories, Kumamoto, Japan; NADPH was from Boehringer Mannheim, Mannheim, F.R.G. ω-Amino-*n*-octyl Sepharose 4B was prepared according to the method of Cuatrecasas [21]. All other chemicals used were of the best grade commercially available.

Results

Incorporation of P-450$_{11\beta}$ into the liposome membranes was accomplished by a combination of the cholate dialysis method and HPLC gel filtration. The purified P-450$_{11\beta}$ was suspended with the phospholipid mixture containing phosphatidylcholine, phosphatidylethanolamine and cardiolipin at the molar ratio of 2:2:1. After dialysis, the sample began to show a little turbidity, which was removed by centrifugation at 24000 × g for 1 h. The precipitates also contained P-450$_{11\beta}$ which might be an aggregated form of P-450$_{11\beta}$ molecule not incorporated into the membranes. The supernatants containing the liposome preparations still had 0.06% (w/v) cholate as detected by the radioactivity of ^{3}H-labeled cholate. By the subsequent HPLC gel filtration step, the cholate concentration of the final preparation was decreased to less than 0.002%. The dialysis and HPLC processes also decreased the concentration of 11-deoxycorticosterone added during the preparation as a stabilizer, and most of the P-450$_{11\beta}$ in the final preparation was optically in the low-spin form (Fig. 1A). The addition of 11-deoxycorticosterone to the P-450$_{11\beta}$-proteoliposomes converted the optical absorption spectrum into the high-spin form and produced a typical type I difference spectrum (Fig. 1B). Using $\Delta\epsilon$ (390–420 nm) = 69 mM^{-1} · cm^{-1} for the 11-deoxycorticosterone-induced spectral change, estimated in the purified P-450$_{11\beta}$ [23], more than 90% of the P-450$_{11\beta}$ was in the substrate-free form in the liposome membrane. Table I summarizes the recoveries of P-450$_{11\beta}$ and phospholipids at each step during the preparation of the proteoliposomes. Nearly 20% of the original P-450$_{11\beta}$ was incorporated into the liposomes at a molar ratio of P-450$_{11\beta}$ to phospholipid of about 1:3000. The prepared proteoliposomes were found to be unilamellar vesicles of about 40 nm in average diameter, as revealed by electron microscopic observation. When P-450$_{11\beta}$-proteoliposomes were solubilized with Tween 20, the P-450$_{11\beta}$ with the electron transfer components revealed almost the same 11β-hydroxylase activity for 11-deoxycorticosterone as did purified P-450$_{11\beta}$ (90–100 nmol corticosterone formed/min per nmol P-450$_{11\beta}$) under the same conditions, suggesting that the preparation procedure of the proteoliposomes might not change the characteristics of P-450$_{11\beta}$.

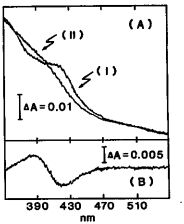

Fig. 1. Absorption spectra of P-450$_{11\beta}$-proteoliposomes. The measurements were performed in 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA and 200 mM NaCl at 25°C. (A), absolute absorption spectra in the absence (curve I) and in the presence (curve II) of 100 μM 11-deoxycorticosterone. (B), a difference spectrum between curves I and II in (A). The concentrations of P-450$_{11\beta}$ and phospholipids were 0.2 μM and 0.47 mg/ml, respectively.

TABLE I

Preparation of P-450$_{11\beta}$-proteoliposomes

The percentage of yield at each stage is shown in parentheses.

Preparation stage	Phospholipid (nmol)	P-450$_{11\beta}$ (nmol)	P-450$_{11\beta}$/Phospholipid (mol/mol)
Before dialysis	8690 (100)	6.5 (100)	1:1340
Supernatant after centrifugation	7170 (83)	2.2 (34)	1:3260
Final P-450$_{11\beta}$-proteoliposomes	3660 (42)	1.1 (17)	1:3330

In order to ascertain the incorporation of $P\text{-}450_{11\beta}$ into the liposome membranes, the prepared proteoliposomes were analyzed by Ficoll density gradient centrifugation. As can be seen in the sedimentation profile of Fig. 2, a peak of $P\text{-}450_{11\beta}$ was observed at almost the same position of phospholipids at a density of approx. 1.022 mg/cm^3. In contrast, when detergent-solubilized $P\text{-}450_{11\beta}$ was analyzed under the same methods, the $P\text{-}450_{11\beta}$ was found at the bottom of the centrifuge tube. The molar ratio of $P\text{-}450_{11\beta}$ to phospholipids of the proteoliposomes in the peak fraction was almost the same as that of the sample applied for the density gradient centrifugation, indicating that the prepared sample does not contain any unincorporated $P\text{-}450_{11\beta}$. A similar centrifugation pattern was observed when the prepared proteoliposomes were analyzed in the higher concentration of NaCl (200 mM), suggesting that the interaction of $P\text{-}450_{11\beta}$ with liposome membranes does not mainly depend on the electrostatic force.

The orientation of $P\text{-}450_{11\beta}$ embedded in the liposome membranes was examined with its reducibility by the external addition of electron transfer components and also with its susceptibility to the proteinase digestion. When sufficient amounts of adrenodoxin, NADPH-adrenodoxin reductase and NADPH were added to the prepared proteoliposomes in the presence of CO, more than 90% of the $P\text{-}450_{11\beta}$ in the liposome membranes was converted into P-450-CO complex within 20 min at 25°C. After a long-time digestion of $P\text{-}450_{11\beta}$ proteoliposomes with trypsin, the molecular mass of $P\text{-}450_{11\beta}$ was decreased from 50 to 34 kDa, as detected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while no protein bands could be detected for the detergent-solubilized $P\text{-}450_{11\beta}$ in the same treatment. These data indicate that most of the $P\text{-}450_{11\beta}$ are located at the outer side of the liposomes

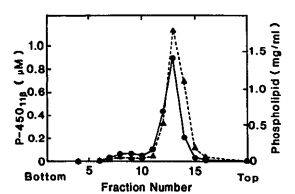

Fig. 2. Ficoll density gradient analysis of $P\text{-}450_{11\beta}$-proteoliposomes. $P\text{-}450_{11\beta}$-proteoliposomes containing 0.55 nmol $P\text{-}450_{11\beta}$ and 1.21 mg phospholipids were centrifuged on a Ficoll density gradient (3–10%, w/v) in 50 mM Tris-HCl buffer (ph 7.2) containing 50 mM NaCl for 12 h at 190000 × g with a Hitachi 70P72 centrifuge using a RPS 50-2 rotor. The full line with closed circles and the broken line with closed triangles show the concentrations of $P\text{-}450_{11\beta}$ and phospholipid, respectively.

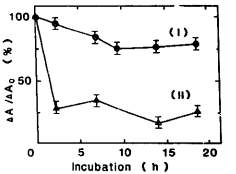

Fig. 3. Stability of $P\text{-}450_{11\beta}$ embedded in the liposome membranes. $P\text{-}450_{11\beta}$-proteoliposomes containing 0.1 nmol $P\text{-}450_{11\beta}$ and 0.24 mg phospholipids were incubated at 0°C without (curve I) or with (curve II) 1% sodium cholate in 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA and 200 mM NaCl. At the times indicated, an aliquot was withdrawn and the substrate binding ability was estimated from the magnitude of 11-deoxycorticosterone-induced difference spectrum (390–420 nm). ΔA_0 and ΔA are the magnitudes of difference spectrum at zero time and at the indicated times, respectively. ΔA_0 absolute value is 0.037 absorbance units.

and at least 34 kDa protein fragment of the $P\text{-}450_{11\beta}$ molecule might be embedded in the membranes.

The purified $P\text{-}450_{11\beta}$ in a detergent-solubilized form has been reported to be quite unstable in the absence of steroid substrate and 11-deoxycorticosterone has been used as the stabilizer during the purification procedure [18]. The stability of $P\text{-}450_{11\beta}$ in the liposomal membranes was examined in the absence of the substrate, as illustrated in Fig. 3. When $P\text{-}450_{11\beta}$-proteoliposomes were solubilized with the addition of 1% cholate, the $P\text{-}450_{11\beta}$ lost most of the binding ability for 11-deoxycorticosterone within a few hours at 0°C as detected with the magnitude of steroid-induced difference spectrum. The liposomal $P\text{-}450_{11\beta}$, however, still retained about 80% of the original binding ability after storage of the sample at 0°C for 20 h. The stability of liposomal $P\text{-}450_{11\beta}$ was also confirmed by the amplitude of the CO-reduced difference spectrum and by the remaining 11β-hydroxylase activity for 11-deoxycorticosterone.

After 11-[^{3}H]deoxycorticosterone (5 nmol) had been incubated at 37°C for 30 min with the reconstituted enzyme system containing adrenodoxin, NADPH-adrenodoxin reductase and $P\text{-}450_{11\beta}$-proteoliposomes, the products were analyzed by a reverse-phase HPLC column with the solvent system of 60% methanol. Almost all the 11-deoxycorticosterone was converted into several steroid metabolites whose retention times were coincident with those of authentic steroids, such as corticosterone, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone and aldosterone. The identification of the metabolites was also confirmed using a normal-phase HPLC column (TOSOH TSK gel OH-120) with the solvent system of n-hexane/butanol/ethanol (85 : 11 : 4, v/v). The amounts of the metabolites were determined from their radioactivities and that of pro-

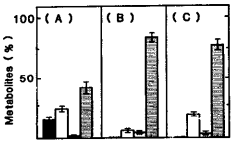

Fig. 4. Metabolites from 11-deoxycorticosterone in reaction systems containing $P\text{-}450_{11\beta}$-proteoliposomes (A), detergent-solubilized $P\text{-}450_{11\beta}$ (B) and mitochondria from bovine adrenal fasciculata-reticularis cells (C). In (A) and (B), the reaction mixtures contained 5 nmol 11-deoxy[^{3}H]corticosterone (1 μCi), 10 nmol adrenodoxin, 1 nmol NADPH-adrenodoxin reductase and 50 pmol $P\text{-}450_{11\beta}$ in liposomes (A) or 50 pmol $P\text{-}450_{11\beta}$ solubilized with 0.05% Tween 20 (B) in 0.5 ml of 30 mM potassium phosphate buffer (pH 7.0). In (C), the reaction mixture contained 5 nmol 11-deoxy[^{3}H]corticosterone and mitochondria (176 μg protein, 251 pmol P-450) in 0.5 ml of 30 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 600 nmol NADPH (A and B) or 10 μmol malate (C) and continued for 30 min at 37°C. The reaction products were analyzed as described in Materials and Methods. The amounts of aldosterone, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone and corticosterone are represented by closed, open, shaded and hatched bars, respectively.

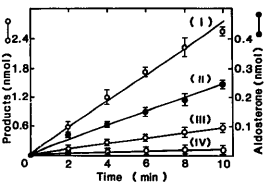

Fig. 5. Time-course of the product formation from 11-deoxycorticosterone in the reconstituted system containing $P\text{-}450_{11\beta}$-proteoliposomes. 50 nmol 11-deoxy[^{3}H]corticosterone (1 μCi) was incubated with the enzyme assay system containing 10 pmol $P\text{-}450_{11\beta}$-proteoliposomes, 5 nmol adrenodoxin and 0.5 nmol NADPH-adrenodoxin reductase in 0.5 ml of 30 mM potassium phosphate buffer (pH 7.0) at 37°C. Each of the lines indicates the amount of corticosterone (I), aldosterone (II), 18-hydroxycorticosterone (III) and 18-hydroxydeoxycorticosterone (IV), respectively.

duced aldosterone was reconfirmed using radioimmunoassay with anti-aldosterone IgG. As can be seen in Fig. 4A, about 15% of the added substrate, 11-deoxycorticosterone, was converted into aldosterone. This result was quite interesting because the $P\text{-}450_{11\beta}$ used for the preparation of the proteoliposomes was purified from mitochondria of zonae fasciculata-reticularis, which did not produce any aldosterone, as shown in Fig. 4C. In the presence of 0.05% Tween 20, the reconstituted enzyme system containing $P\text{-}450_{11\beta}$, adrenodoxin and NADPH-adrenodoxin reductase produced predominantly corticosterone but could not produce any aldosterone (Fig. 4B). Further incubation of these assay system did not change the product patterns so much.

Fig. 5 shows the time-course of the steady-state metabolism of 11-deoxycorticosterone in the assay system containing $P\text{-}450_{11\beta}$-proteoliposomes, where all the amounts of steroid metabolites increased linearly with time at least up to 10 min. The molecular activities (nmol/min per nmol $P\text{-}450_{11\beta}$) of the metabolite production were 26.1, 1.2, 5.7 and 2.3 for corticosterone, 18-hydroxydeoxycorticosterone, 18-hydroxycorticosterone, and aldosterone, respectively. Since aldosterone has been considered to be produced via corticosterone and 18-hydroxycorticosterone, it seems a little strange that the production of aldosterone increased linearly from the initial stage of the reaction. The molecular activities of the formation of aldosterone were determined to be 0.7 from corticosterone and 0.3 from 18-hydroxycorticosterone, under the same conditions as in Fig. 5, indicating that these expected intermediates are poorer substrates than 11-deoxycorticosterone.

Table II summarizes the recoveries of radioactivity in produced metabolites when ^{3}H-labeled 11-deoxycorti-

TABLE II

The effect of unlabeled steroid on the recovery of radioactivity in the metabolites from ^{3}H-labeled 11-deoxycorticosterone in the reconstituted system containing $P\text{-}450_{11\beta}$-proteoliposomes

50 nmol 11-deoxy[^{3}H]corticosterone (1 μCi) was incubated with an assay system containing 25 pmol $P\text{-}450_{11\beta}$-proteoliposomes, 5 nmol adrenodoxin and 0.5 nmol NADPH-adrenodoxin reductase in 0.5 ml of 30 mM potassium phosphate buffer (pH 7.0) at 37°C for 10 min in the absence or presence of 50 nmol unlabeled steroids. The percentage of the radioactivity recovery with respect to that without unlabeled steroids is shown in parentheses.

Unlabeled steroid	^{3}H-labeled metabolites produced (nmol)			
	aldosterone	18-hydroxy-corticosterone	18-hydroxydeoxy-corticosterone	corticosterone
None	0.50±0.02 (100)	1.05±0.24 (100)	0.22±0.04 (100)	4.68±0.04 (100)
Corticosterone	0.46±0.04 (92)	0.92±0.04 (88)	0.21±0.03 (95)	4.21±0.06 (90)
18-Hydroxydeoxycorticosterone	0.48±0.02 (96)	0.93±0.03 (89)	0.35±0.02 (160)	4.04±0.13 (86)
18-Hydroxycorticosterone	0.45±0.02 (90)	0.92±0.02 (88)	0.16±0.03 (73)	4.42±0.13 (94)

costerone (100 μM) was incubated with the assay system in the presence of the same concentration of unlabeled intermediates. If all the intermediates dissociated from $P\text{-}450_{11\beta}$ and some of them converted into aldosterone after their accumulation, the addition of unlabeled intermediates, such as corticosterone or 18-hydroxycorticosterone to the assay system, would decrease the recovery of the radioactivity in aldosterone to much less than 50%. No significant dilution of the radioactivity was observed for the formation of each metabolite in the presence of non-radioactive intermediates such as corticosterone, 18-hydroxydeoxycorticosterone and 18-hydroxycorticosterone. These data show that the aldosterone production is not inhibited competitively by the intermediates and indicate that aldosterone is produced in the successive hydroxylation reaction without the intermediates leaving from $P\text{-}450_{11\beta}$. This reaction mechanism explains well the linear increase of aldosterone production from 11-deoxycorticosterone as shown in Fig. 5.

Discussion

In a standard purification procedure of $P\text{-}450_{11\beta}$, Tween 20, a nonionic detergent, has been used to solubilize the protein [18], but the presence of such a nonionic detergent having a low critical micelle concentration prohibits the formation of liposome vesicles. In order to remove the nonionic detergent used in the purification of adrenal microsomal $P\text{-}450_{C21}$, Kominami et al. [12] washed out the detergent by adsorbing $P\text{-}450_{C21}$ onto a hydroxylapatite column and eluted $P\text{-}450_{C21}$ with a buffer containing phosphatidylcholine and sodium cholate. In the present study, the purification of $P\text{-}450_{11\beta}$ has been performed with a buffer containing phosphatidylcholine, *n*-octylglucoside and sodium cholate, and the highly purified $P\text{-}450_{11\beta}$ was successfully incorporated into the liposome membranes composed of phosphatidylcholine, phosphatidylethanolamine and cardiolipin at a molar ratio of 2 : 2 : 1, which was comparable with the phospholipid composition of the inner membranes of bovine adrenal mitochondria [22]. Judging from the density gradient centrifugation analysis (Fig. 2), the susceptibility to proteinase digestion and the stability of liposomal $P\text{-}450_{11\beta}$ (Fig. 3), it is concluded that $P\text{-}450_{11\beta}$ is undoubtedly incorporated into the liposome membranes. It is of great interest that liposomal $P\text{-}450_{11\beta}$ was stable without steroid substrate, although the detergent-solubilized $P\text{-}450_{11\beta}$ was quite labile. Since about 90% of $P\text{-}450_{11\beta}$ in adrenal mitochondria exists in the substrate-free form which is not so labile as in the detergent-solubilized form [23], liposome vesicles appear to provide $P\text{-}450_{11\beta}$ membrane environments which are similar to those of naturally occurring membranes.

It has been indicated that the biosynthetic pathway of aldosterone from corticosterone and 18-hydroxycorticosterone is mediated by a cytochrome *P*-450 using mitochondria from bovine adrenal glomerulosa cells [5]. Recently, it has been revealed that bovine adrenal $P\text{-}450_{11\beta}$ in the reconstituted enzyme system containing phospholipids is capable of synthesizing aldosterone [6–8]. In the present study, the $P\text{-}450_{11\beta}$ incorporated into the liposome membranes could catalyze the conversion of 11-deoxycorticosterone to aldosterone with a molecular activity of 2.3 nmol aldosterone formed/min per nmol of $P\text{-}450_{11\beta}$, in which the production of aldosterone increased linearly with time from the initial stage of the reaction and no lag was observed (Fig. 5). The linear increase of the aldosterone production together with scarcely any decrease in radioactivity in aldosterone by the addition of unlabeled intermediates indicate that aldosterone might be formed through a successive hydroxylation without the intermediates leaving from $P\text{-}450_{11\beta}$. Since the aldosterone-synthesizing activities of $P\text{-}450_{11\beta}$-proteoliposome from the possible intermediates, such as corticosterone or 18-hydroxycorticosterone, are lower than that from 11-deoxycorticosterone, the aldosterone formation from 11-deoxycorticosterone cannot be explained by the simple sequential mechanism in which the intermediates dissociate from the active site and then compete with the original substrate. Detergent-solubilized $P\text{-}450_{11\beta}$ showed a quite high activity for 11β-hydroxylation of 11-deoxycorticosterone (100 nmol corticosterone/min per nmol $P\text{-}450_{11\beta}$) compared with that in liposomal $P\text{-}450_{11\beta}$ (26 nmol corticosterone/min per nmol $P\text{-}450_{11\beta}$) but could not catalyze the synthesis of aldosterone. This phenomenon might be related to the ability for dissociation of the intermediate, corticosterone, from $P\text{-}450_{11\beta}$ and this dissociation might interfere with the successive hydroxylation of $P\text{-}450_{11\beta}$-catalyzed aldosterone synthesis.

It is well-established that aldosterone is produced exclusively from zona glomerulosa cells in bovine adrenal cortex, although $P\text{-}450_{11\beta}$ is distributed in all three zones [9,11]. In bovine adrenal cortex, no immunochemical and no enzymological differences of $P\text{-}450_{11\beta}$ can be detected between those of zona glomerulosa and zonae fasciculata-reticularis [10,24]. In the case of rat adrenal, it has been reported that a 49 kDa protein, which was immunoreactive with bovine $P\text{-}450_{11\beta}$ antibody and which showed a correlation with aldosterone production, was localized only in zona glomerulosa mitochondria, although a 51 kDa protein of $P\text{-}450_{11\beta}$ was present in mitochondria from all three zones [25]. In the present study, $P\text{-}450_{11\beta}$ was purified from mitochondria of zonae fasciculata-reticularis in bovine adrenal cortex and exhibited aldosterone-synthesizing activity in the liposomal system. The cholate extracts from mitochondria of zonae fasciculata-reti-

cularis as well as zona glomerulosa in bovine adrenal cortex have been reported to be capable of producing aldosterone [26,27]. Taken together with these results, it is suggested that $P\text{-}450_{11\beta}$ in zonae fasciculata-reticularis of bovine adrenal cortex might have the intrinsic ability to convert 11-deoxycorticosterone to aldosterone. There must be some suppression mechanism for the aldosterone-synthesizing activity of $P\text{-}450_{11\beta}$ in the zonae fasciculata-reticularis mitochondria. The aldosterone synthesis from 11-deoxycorticosterone could be suppressed by those factors that could accelerate the dissociation of the intermediates from $P\text{-}450_{11\beta}$. Ohnishi et al. [28] have suggested that adrenocortical calmodulin might inhibit the aldosterone-synthesizing activity of $P\text{-}450_{11\beta}$ purified from bovine adrenal cortex. The distribution of those factors in adrenal cortex cells could explain the zone specificity of aldosterone biosynthesis. The stable $P\text{-}450_{11\beta}$-proteoliposomes can be expected to provide a good system for the investigation of the control mechanism of $P\text{-}450_{11\beta}$-mediated aldosterone synthesis at the molecular level.

Acknowledgment

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

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