Kinetic Analysis of Successive Reactions Catalyzed by Bovine Cytochrome $P450_{17\alpha,lyase}$[†]

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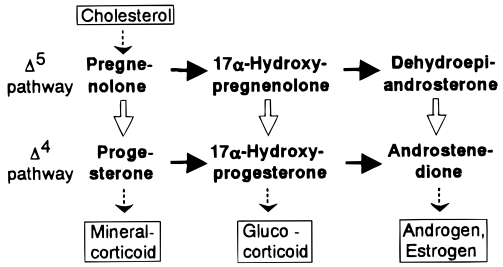
ABSTRACT: Bovine $P450_{17\alpha,lyase}$ containing an additional four histidine residues at the COOH terminus was expressed in *Escherichia coli* and purified by one-step column chromatography using Ni-chelate resin. The membrane enzyme was incorporated into liposome membranes having similar lipid composition to that of the endoplasmic reticulum. In the presence of excess substrate, the P450-proteoliposomes metabolize pregnenolone (Δ^5-steroid) to 17α-hydroxypregnenolone and further to dehydroepiandrosterone. The enzyme catalyzed 17α-hydroxylation of progesterone (Δ^4-steroid) but did not form androstenedione from progesterone, although the proteoliposomes could catalyze the conversion of 17α-hydroxyprogesterone to androstenedione. The kinetic analysis of rapid quenching experiments showed that about 20% of the pregnenolone consumed was converted successively to dehydroepiandrosterone via a fraction of 17α-hydroxypregnenolone that did not dissociate from the enzyme. The rapid quenching experiments for progesterone metabolism by the proteoliposomes revealed that the dissociation rate of 17α-hydroxyprogesterone was 10 times faster than that of 17α-hydroxypregnenolone. The release of the intermediate metabolite of Δ^4-steroid is sufficiently faster than the lyase reaction to prevent further reaction by the P450. It is concluded that the dissociation rates of the first hydroxylation metabolites regulate the successive reactions of $P450_{17\alpha,lyase}$.

$P450_{17\alpha,lyase}$[1] catalyzes 17α-hydroxylation of pregnenolone and progesterone and the C17−C20 bond cleavage reaction of the 17α-hydroxylated steroids to form androgens in adrenal, testis, ovary, stomach, and brain (*1*−*6*). In adrenal glands, the principal glucocorticoid, cortisol, is synthesized from 17α-hydroxylated steroids produced by the catalysis of $P450_{17\alpha,lyase}$. Pregnenolone can be converted to progesterone and further to mineral corticoid without the catalysis of $P450_{17\alpha,lyase}$ (Scheme 1). The reactions of $P450_{17\alpha,lyase}$ are located at the branching point of steroid hormone synthesis and might be regulated independently (*1*, *2*). The $P450_{17\alpha,lyase}$ is one of the key enzymes in the regulation of steroid hormone synthesis.

Since the intracellular concentrations of progesterone and pregnenolone are much higher than those of the 17α-hydroxylated steroids in adrenal cells, the metabolism of 17α-hydroxylated steroids to androgens must be strongly inhibited by progesterone or pregnenolone (*7*, *8*). Double-substrate double-label experiments and kinetic studies showed that the 17α-hydroxy steroids released during the metabolism of progesterone and pregnenolone were not converted to androgens in guinea pig adrenal microsomes, rat ovarian microsomes, cultured bovine adrenal cells, $P450_{17\alpha,lyase}$ proteoliposomes, and rat testis (*8*−*14*). Prasad and Lieberman (*15*) suggested that 17α-hydroxy steroid and 17-keto steroid could be produced from a common transient radical intermediate through a one-step monooxygenase reaction. The actual intermediate for androgen formation is not detectable under steady-state reaction conditions, since the quantity of intermediate in the active site of P450 is extremely small. Using a rapid quenching device, Tagashira et al. (*16*) have showed that androstenedione was formed from progesterone by successive monooxygenations catalyzed by $P450_{17\alpha,lyase}$ without the intermediate, 17α-hydroxyprogesterone, leaving the enzyme. In that successive reaction, the production ratio of androstenedione to 17α-hydroxyprogesterone was controlled by the electron transfer to the $P450_{17\alpha,lyase}$, and the overall reaction rate was

Scheme 1: Reaction Pathway for Pregnenolone Metabolism in Adrenal and Gonads[a]



[a] Closed and open arrows indicate reactions of $P450_{17\alpha,lyase}$ and 3β-HSD/I, respectively. Corticoids are formed only in adrenal gland.

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[1] Abbreviations: $P450_{17\alpha,lyase}$, cytochrome P450XVIIA1; 3β-HSD/I, 3β-hydroxy-5-ene steroid dehydrogenase/Δ^5-Δ^4 isomerase; $P450_{C21}$, cytochrome P450XXIA1; $P450_{11\beta}$, cytochrome P450XIB1; SDS, sodium dodecyl sulfate; SD, standard deviation.

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determined by the dissociation rate of androstenedione from the enzyme.

Androstenedione, a precursor for testosterone and estrogens, is formed from pregnenolone by reactions of two enzymes, $P450_{17\alpha,lyase}$ and 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase (3β-HSD/I), as shown in Scheme 1. There are two possible reaction pathways for androstenedione formation. Pregnenolone is converted to dehydroepiandrosterone by the successive reaction of $P450_{17\alpha,lyase}$ (Δ^5 pathway) and then metabolized to androstenedione by 3β-HSD/I, or androstenedione is formed by the P450 from progesterone in the Δ^4 pathway after conversion of pregnenolone to progesterone by 3β-HSD/I. In the case of bovine and human, the androgen is formed only by the Δ^5 pathway, since $P450_{17\alpha,lyase}$ in these species does not catalyze androstenedione formation from progesterone (*2*, *17*). $P450_{17\alpha,lyase}$ in rodents converts progesterone to androstenedione, thus these species synthesize the androgen through the Δ^4 pathway (*18*). It is still not clear why $P450_{17\alpha,lyase}$ from some animals cannot catalyze the successive reaction of progesterone but can for pregnenolone.

In this study, we analyzed the reactions of bovine $P450_{17\alpha,lyase}$, a Δ^5 pathway enzyme, in the presence of excess amount of substrates and also with the rapid quenching method. The $P450_{17\alpha,lyase}$ was purified from an expression system with *Escherichia coli* established by Waterman and colleagues and was incorporated into liposome membranes (*19*, *20*). Kinetic analysis indicated that the dissociation rate of metabolites played a key role in the multistep reactions of $P450_{17\alpha,lyase}$.

MATERIALS AND METHODS

Materials. Plasmid pAα1 containing bovine $P450_{17\alpha,lyase}$ cDNA was prepared as described (*21*). The expression plasmid pCW was kindly donated by Dr. Dahlquist, University of Oregon (*22*). Chemicals were obtained from the following sources: steroids from Fluka Chemie AG, Buchs, or Sigma Chemical Co., St. Louis, MO; and [1,2-^{3}H]-pregnenolone, [1,2-^{3}H]progesterone, 17α-[1,2-^{3}H]hydroxypregnenolone, 17α-[1,2-^{3}H]hydroxyprogesterone, [4-^{14}C]-pregnenolone, [4-^{14}C]dehydroepiandrosterone, [4-^{14}C]progesterone, and [4-^{14}C]androstenedione, from Dupont-NEN, Boston, MA, or Amersham, Amersham, U.K. 17α-[4-^{14}C]-Hydroxypregnenolone and 17α-[4-^{14}C]hydroxyprogesterone were produced from [4-^{14}C]pregnenolone and [4-^{14}C]progesterone, respectively, by the enzymatic reaction of $P450_{17\alpha,lyase}$ proteoliposomes and purified by HPLC (*8*). All other chemicals were of the best commercially available grade.

Expression of Bovine $P450_{17\alpha,lyase}$ in E. coli. Plasmids pCW17mod and pCW17modHis, expression plasmids of bovine $P450_{17\alpha,lyase}$, were constructed according to the methods of Barnes et al. (*19*) and Jenkins and Waterman (*23*) respectively. Briefly, a 272 bp fragment encoding amino acids 1−91 of $P450_{17\alpha,lyase}$ was constructed by PCR mutagenesis, which introduced an *Nde*I site and some modifications at the NH_2-terminal region of the enzyme for the expression in *E. coli*. pAα1 was digested by *Eco*RI/*Hin*dIII and a 1257 bp fragment was inserted with the PCR fragment into the *Nde*I and *Hin*dIII site of pCW to construct a plasmid, pCW17mod. The pCW17mod was further recombined to form pCW17modHis at *Sac*I and *Hin*dIII site with a PCR product that codes for 449−509 amino acids and an extra 4 histidine residues at the COOH-terminus of the P450. The sequences of PCR fragments of pCW17mod and pCW17modHis were confirmed by ABI373 DNA sequencer using a dye-terminator sequencing kit (Perkin-Elmer, Foster City, CA).

The pCW17mod and pCW17modHis were transfected into *E. coli* JM109. These bacteria were cultured in 6 mL of Luria−Bertani medium and then inoculated into 2 L of Terrific broth. After the *E. coli* were grown at 37 °C to OD = 0.5−1, 1 mM isopropyl β-D-thiogalactopyranoside was added into the medium which was further incubated for 48 h at 32 °C with shaking at 160 rpm. The cells were harvested by centrifugation at 8000*g* for 10 min at 4 °C and kept frozen at −80 °C until used.

E. coli cells were suspended with 200 mL of 0.1 M Tris−acetate, pH 7.8, containing 0.5 mM EDTA, 0.5 M sucrose, and 0.1 mM dithiothreitol and treated with 1 mg/mL lysozyme on ice for 30 min. The spheroplasts were precipitated by centrifugation at 12000*g* for 10 min and resuspended with 50 mL of buffer A (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl, 50 μM progesterone, 0.1 mM dithiothreitol, and 20% glycerol). Cells were broken by ultrasonication (1 min) five times with Heat-system W-225 (Farmingdale, NY). The cell lysates were ultracentrifuged at 158000*g* for 1 h to precipitate the membrane fraction.

Purification of $P450_{17\alpha,lyase}(His)_4$ and Proteoliposome Preparation. The membrane fraction of *E. coli* was suspended in buffer A containing 0.5% Triton X-100 by brief ultrasonication and then ultracentrifuged at 146000*g* for 15 min. Solubilized $P450_{17\alpha,lyase}(His)_4$ was applied to a Ni-NTP−agarose column (Qiagen, Hilden, Germany) that had been equilibrated with buffer A containing 0.5% Triton X-100. After the column was washed with 0.2% sodium cholate in buffer A, the P450 was eluted with buffer A containing 20 mM histidine and 0.2% sodium cholate and then concentrated using a CF-25 centrifugal concentrator (Amicon, Danvers, MA).

Proteoliposomes were prepared by the cholate dialysis method with the purified $P450_{17\alpha,lyase}(His)_4$ and a phospholipid mixture composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1 (*20*). The molar ratio of the P450 to phospholipids was about 1:1500 in the proteoliposomes. Bovine liver NADPH−cytochrome P450 oxidoreductase was purified as described elsewhere (*24*). The reductase was incorporated into the membrane by incubation with the preformed proteoliposomes for 1 h at 0 °C. The ratio of P450 to the reductase was 1:2 in the experiments throughout this study.

Reactions in the Presence of Excess Amount of Substrates. Pregnenolone and progesterone metabolisms were measured aerobically with the *E. coli* membrane fraction (50 pmol of P450 with externally added 100 pmol of reductase) or the P450 proteoliposomes (20 pmol of P450 and 40 pmol of reductase) at 37 or 10 °C in 0.2 mL of 50 mM potassium phosphate buffer, pH 7.5, containing 20 nmol of ^{3}H-labeled substrate (1 μCi) and 0.1 mM EDTA. The reaction was started by adding 100 nmol of NADPH. Steroid metabolites were extracted with 1 mL of chloroform containing 2 nCi each of [^{14}C]-17α-hydroxypregnenolone and [^{14}C]dehydroepiandrosterone for Δ^5 pathway assay or [^{14}C]-17α-hydroxyprogesterone and [^{14}C]androstenedione for Δ^4 pathway assay,

respectively, for estimation of the recovery of the entire procedure. Extracted steroids were separated by HPLC and quantified from the radioactivity as described (*8, 10*). Lyase activity of 17α-hydroxysteroids was measured by a method similar to that described above except [^{3}H]-17α-hydroxypregnenolone or [^{3}H]-17α-hydroxyprogesterone was used as a substrate.

Rapid Quenching Experiment. Rapid quenching experiments were conducted at 10 °C as described (*16*). The apparatus places three micropipets just above the reaction vessel. The solutions in the pipets were added into the vessel at the defined times by N_2 gas pressure. For analysis of the Δ^5 pathway, reaction vessel contained 200 pmol of P450 proteoliposomes with 400 pmol of reductase, 8 pmol of [^{3}H]-pregnenolone or [^{3}H]-17α-hydroxypregnenolone (1 μCi), and 0.1 mM EDTA in 0.1 mL of 50 mM potassium phosphate buffer, pH 7.5. The reaction was started by adding 80 nmol of NADPH from a micropipet. Four nanomoles of nonlabeled pregnenolone or 17α-hydroxypregnenolone was added 0.5 s after initiation of the reaction from the other micropipet to prevent rebinding of the released [^{3}H]-labeled steroids to the P450. It was confirmed that changes in the timing of the addition of the chaser steroid after the initiation of the reaction (0.1−0.7 s) did not show any differences in the kinetics. The reaction was quenched at the appropriate time by the addition of 50 μL of 0.1 M HCl from the third pipet. This apparatus can follow the reaction at an accuracy of less than 0.1 s. The steroid metabolites were extracted and analyzed by HPLC as described above. Progesterone metabolism was analyzed in the same system except that 50 pmol of P450 proteoliposomes, 100 pmol of reductase and 8 pmol of [^{3}H]progesterone (1 μCi) were used.

Other Methods. P450 concentration was determined from the CO difference spectrum using an extinction coefficient of 91 mM^{-1} for the difference in the absorbance at 490 and 448 nm (*25*). Staining of the polyacrylamide gel after electrophoresis containing SDS was performed with a silver-staining kit (Daiichi Pure Chemicals, Tokyo).

RESULTS

Expression of P450$_{17\alpha,lyase}$ in E. coli. The *E. coli* transfected with plasmids pCW17mod and pCW17modHis expressed P450$_{17\alpha,lyase}$ and P450$_{17\alpha,lyase}$(His)$_4$, respectively, at about 450 nmol/L of culture medium after 48 h of cultivation. This expression level is higher than the original report of Barnes et al. (*19*) but similar to that of Imai et al. (*26*) for expression of human P450$_{17\alpha,lyase}$. Both the P450s were modified at the second amino acid (Trp to Ala) for the expression in the bacterial system (*19*). P450$_{17\alpha,lyase}$(His)$_4$ possesses four extra histidine residues at the COOH terminus.

Enzymatic activities of these P450s in bacterial membranes were measured in the presence of excess amount of substrates with externally added bovine liver NADPH−cytochrome P450 oxidoreductase (Table 1). The activity of P450$_{17\alpha,lyase}$(His)$_4$ was higher than that of P450$_{17\alpha,lyase}$ for both pregnenolone and progesterone, although no effect of the histidine tag was reported on reactions of human P450$_{17\alpha,lyase}$ (*26*). The activities are much less than those reported for P450$_{17\alpha,lyase}$ in bovine adrenal microsomes and rat testis microsomes (*3, 11*). The bacterial membranes might have some inhibitory effect on the reactions. It must be noted that the production ratio of 17α-hydroxypregnenolone to dehydroepiandrosterone is almost the same in both the membranes and that neither P450$_{17\alpha,lyase}$ nor P450$_{17\alpha,lyase}$(His)$_4$ produced androstenedione from progesterone as previously reported (*17, 19*).

Table 1: Activities of P450$_{17\alpha,lyase}$ in *E. coli* Membrane Fractions[a]

reactions	expressed P450 activity [nmol of steroids formed min^{-1} (nmol of P450)$^{-1}$]	
	P450$_{17\alpha,lyase}$	P450$_{17\alpha,lyase}$(His)$_4$
Preg → 17OH-Preg	0.36 ± 0.14	0.81 ± 0.14
Preg → DHEA	0.063 ± 0.008	0.12 ± 0.01
(17OH-Preg/DHEA)[b]	(5.7)[b]	(6.8)[b]
Prog → 17OH-Prog	1.6 ± 0.2	4.2 ± 0.1
Prog → AND	ND[c]	ND[c]

[a] The metabolism of pregnenolone and progesterone was measured for membrane fractions of *E. coli* that expressed P450s. Reactions were carried out in the presence of excess amount of substrates at 37 °C as described in Materials and Methods. Preg, pregnenolone, 17OH-Preg, 17α-hydroxypregnenolone; DHEA, dehydroepiandrosterone; Prog, progesterone; 17OH-Prog, 17α-hydroxyprogesterone; AND, androstenedione. Values are the mean ± SD of triplicate determinations. [b] Ratio of the formation of 17α-hydroxypregnenolone to that of dehydroepiandrosterone from pregnenolone. [c] Not detected.

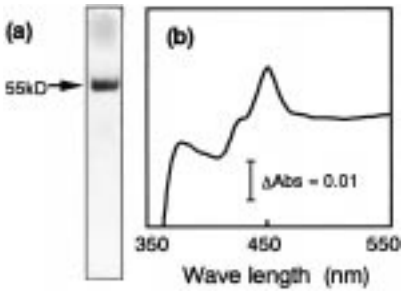

FIGURE 1: Profile on SDS−polyacrylamide gel electrophoresis (a) and CO difference spectrum (b) of purified P450$_{17\alpha,lyase}$(His)$_4$.

Purification of Bovine P450$_{17\alpha,lyase}$ and Construction of Proteoliposomes. Several trials have been unable to purify P450$_{17\alpha,lyase}$ without histidine tag, because of its instability during purification. P450$_{17\alpha,lyase}$(His)$_4$ could be purified by one-step column chromatography with chelated Ni^{2+}. The P450 was solubilized from the membrane fraction of *E. coli* with Triton X-100 and adsorbed on a Ni-NTP−agarose column with the histidine tag. The P450 was eluted from the column with sodium cholate and histidine in very high yield (more than 95%). Overall the purification from *E. coli* lysate took about 24 h and the recovery of the P450 was about 45%, where most of the loss of the enzyme occurred during solubilization. The purified P450 shows one band on polyacrylamide gel electrophoresis containing SDS and showed a sharp peak at 448 nm with a faint shoulder at 420 nm in the CO difference spectrum (Figure 1). The P450$_{17\alpha,lyase}$ was very unstable under detergent-solubilized conditions in the absence of progesterone. The CO difference spectrum of the P450 in solubilized form with 1% sodium cholate decreased to 30% within 10 min and was not detectable after 24 h at 25 °C (data not shown). The enzyme was greatly stabilized in liposome membranes even without progesterone, in which the P450 sustained 70% of the CO difference spectrum after 24 h at 25 °C. The stabilization of membrane proteins in liposome membranes has been reported for guinea

Table 2: Activity of $P450_{17\alpha,lyase}(His)_4$ Proteoliposomes in the Presence of Excess Substrate at 37 °C[a]

reactions	activity [nmol of steroids formed min^{-1} (nmol of P450)$^{-1}$
Preg → 17OH−Preg	4.3 ± 0.5
Preg → DHEA	0.71 ± 0.02
(17OH−Preg/DHEA)[b]	(6.1)[b]
17OH−Preg → DHEA	1.8 ± 0.1
Prog → 17OH−Prog	46 ± 5
Prog → AND	ND[c]
17OH−Prog → AND	0.17 ± 0.04

[a] The metabolism of pregnenolone, 17α-hydroxypregnenolone, progesterone, and 17α-hydroxyprogesterone was measured for the P450 proteoliposomes at 37 °C as described in Materials and Methods. Abbreviations are as in Table 1. Values are the mean ± SD of triplicate determinations. [b] Ratio of the formation of 17α-hydroxypregnenolone to that of dehydroepiandrosterone from pregnenolone. [c] Not detected.

Table 3: Activity of $P450_{17\alpha,lyase}(His)_4$ Proteoliposomes in the Presence of Excess Substrate at 10 °C[a]

reactions	activity [nmol of steroids formed min^{-1} (nmol of P450)$^{-1}$]
Preg → 17OH−Preg	1.1 ± 0.2
Preg → DHEA	0.18 ± 0.01
(17OH−Preg/DHEA)[b]	(6.1)[b]
17OH−Preg → DHEA	0.35 ± 0.04
Prog → 17OH−Prog	5.0 ± 0.4
Prog → AND	ND[c]
17OH−Prog → AND	ND[c]

[a] The metabolism of pregnenolone, 17α-hydroxypregnenolone, progesterone, and 17α-hydroxyprogesterone was measured for the P450 proteoliposomes at 10 °C as described in Materials and Methods. Abbreviations are as in Table 1. Values are the mean ± SD of triplicate determinations. [b] Ratio of the formation of 17α-hydroxypregnenolone to that of dehydroepiandrosterone from pregnenolone. [c] Not detected.

pig $P450_{17\alpha,lyase}$, bovine $P450_{11\beta}$, $P450_{c21}$, and 3β-HSD/I (*20*, *27*−*29*).

Reactions in the Presence of Excess Amounts of Substrates. Pregnenolone metabolism in the $P450_{17\alpha,lyase}$ proteoliposomes was measured in the presence of excess amount of substrates at 37 and 10 °C (Tables 2 and 3). In the Δ^5 pathway, the enzyme can metabolize pregnenolone to 17α-hydroxypregnenolone and dehydroepiandrosterone at both temperatures. Although the enzyme can catalyze the formation of dehydroepiandrosterone from 17α-hydroxypregnenolone, almost all the androgen was formed from pregnenolone directly by the successive reaction without a fraction of the intermediate leaving the P450 in the presence of excess amount of pregnenolone (*10*). The enzyme cannot metabolize the 17α-hydroxypregnenolone dissociated from the active site, since the high concentration of substrate pregnenolone prevents rebinding of the released 17α-hydroxypregnenolone to the P450. When the reaction temperature was reduced to 10 °C, reaction rates decreased to a quarter but the ratio of the rates of 17α-hydroxylation to successive androgen formation was not changed from that at 37 °C. It is also interesting that the production ratio of 17α-hydroxypregnenolone to DHEA in the proteoliposomes was about the same as that in the *E. coli* membranes, suggesting the regulation of the reaction is independent from membrane composition (Tables 1−3).

In the Δ^4 pathway, the rate of 17α-hydroxylation of progesterone by the P450 proteoliposomes was 10-times higher than that in the Δ^5 pathway at 37 °C (Table 2). Although the enzyme can metabolize 17α-hydroxyprogesterone to androstenedione, which is about 10 times less than that for 17α-hydroxypregnenolone, no androstenedione formation from progesterone was detected even at 37 °C. Since the rate of 17α-hydroxylation for progesterone was 10 times higher than that for pregnenolone, it seems possible that a similar amount of androstenedione to that of dehydroepiandrosterone could be formed by the reaction of bovine $P450_{17\alpha,lyase}$. There must be some specific mechanism that prevents the formation of androstenedione from progesterone. The reaction rate of the 17α-hydroxylation at 10 °C decreased to one-ninth that at 37 °C, and androstenedione formation from 17α-hydroxyprogesterone might be too slow to be detected at 10 °C (Table 3).

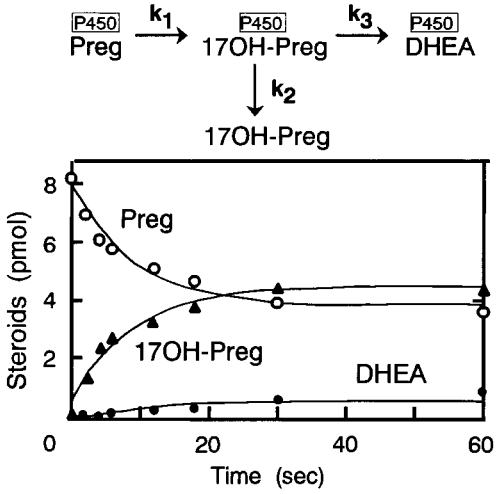

FIGURE 2: Rapid quenching measurements of pregnenolone metabolism by bovine $P450_{17\alpha,lyase}$ proteoliposomes. Reaction was carried out at 10 °C as described in Materials and Methods. Pregnenolone, ○; 17α-hydroxypregnenolone, ▲; dehydroepiandrosterone, ●. P450 represents $P450_{17\alpha,lyase}$ and the other abbreviations are as in Table 1. The solid lines represent the theoretical curves obtained using $k_1 = 6.6$, $k_2 = 2.5$, and $k_3 = 0.48$ min^{-1}. The profile was reproducible in three individual experiments.

Kinetic Analysis with Rapid Quenching Experiments. The multistep reactions of $P450_{17\alpha,lyase}$ could hardly be analyzed in the presence of excess amount of substrates, since intermediate steroids bound in the P450 were present in extremely small quantities during the reactions. Kinetic constants of each step of the reaction could be obtained from the rapid quenching experiments as described (*16*). In this system, the reactions proceed synchronously, since most of the [^{3}H]-labeled substrates are at the binding site of P450 before the start of the reaction. The [^{3}H]-labeled products and intermediates were formed only from the bound [^{3}H]-labeled substrates, since rebinding of the dissociated [^{3}H]-labeled products to the P450 was prevented by the addition of an excess amount of nonlabeled chaser steroids. Diffusion of the chaser steroids into the lipid bilayer is very fast compared with the reaction rates of the P450 in the liposomal system (*30*, *31*). The dissociated [^{3}H]-labeled products should be diffused away from the P450 immediately, since the lateral diffusion of lipophilic small molecules in biological membrane is faster than 10^{-9} cm^2 s^{-1} (*32*).

The reactions were carried out at 10 °C, because 17α-hydroxylation of progesterone was too fast for precise analysis at 37 °C, where the reaction completes almost in a

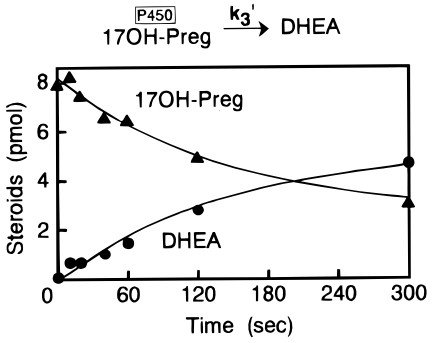

FIGURE 3: Rapid quenching measurements of 17α-hydroxypregnenolone metabolism by bovine $P450_{17\alpha,lyase}$ proteoliposomes. Reaction was carried out at 10 °C as described in Materials and Methods. 17α-Hydroxypregnenolone, ▲; dehydroepiandrosterone, ●. Abbreviations are as in Table 1 and Figure 2. The solid lines represent the theoretical curves obtained using $k_3' = 0.42$ min^{-1}. The profile was reproducible in two individual experiments.

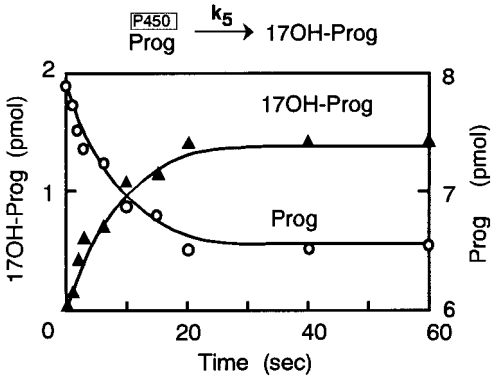

FIGURE 4: Rapid quenching measurements of progesterone metabolism by bovine $P450_{17\alpha,lyase}$ proteoliposomes. Reaction was carried out at 10 °C as described in Materials and Methods. Progesterone, ○; 17α-hydroxyprogesterone, ▲. No androstenedione was detected throughout the reaction. Abbreviations are as in Table 1 and Figure 2. The solid lines represent the theoretical curves obtained using $k_5 = 6.0$ min^{-1}. The profile was reproducible in three individual experiments.

second. The time course of pregnenolone metabolism detected with the rapid quenching method is shown in Figure 2. The amount of 17α-hydroxypregnenolone increased immediately at the onset of the reaction and plateaued after 30 s. The increase was concomitant with the decrease of pregnenolone. The formation of dehydroepiandrosterone shows a lag time of a few seconds after the start of the reaction (Figure 2). These results indicate that the androgen was formed by the subsequent reaction of a fraction of 17α-hydroxypregnenolone that did not dissociate from the active site of the P450. A similar conclusion was obtained from the kinetic analysis of progesterone metabolism with guinea pig adrenal $P450_{17\alpha,lyase}$ proteoliposomes (*16*).

The rapid quenching data were analyzed on the basis of the scheme in Figure 2 as described elsewhere (*16*). The observed concentrations of 17α-hydroxypregnenolone and dehydroepiandrosterone in rapid quenching measurements are [P450−17OH-Preg] + [17OH-Preg] and [P450−DHEA] + [DHEA], respectively, which can be expressed as (*16*)

$$[\text{P450}-\text{17OH-Preg}] + [\text{17OH}-\text{Preg}]$$

$$= (A(k_1 - k_2)/\alpha)\exp(-k_1 t) - (Ak_1k_3/\alpha\beta)\exp(-\beta t) + Ak_2/\beta$$

$$[\text{P450}-\text{DHEA}] + [\text{DHEA}] = (-Ak_3/\alpha)\exp(-k_1 t) - (Ak_1k_3/\alpha\beta)\exp(-\beta t) + Ak_3/\beta$$

where $\alpha = -k_1 + k_2 + k_3$ and $\beta = k_2 + k_3$. [P450−steroid] and A indicate concentrations of $P450_{17\alpha,lyase}$−steroid complexes and metabolized pregnenolone, respectively. The other abbreviations are explained in Table 1. The kinetic constants, k_1, k_2, and k_3, were estimated from fitting the observed data to the above equations using KaleidaGraph (Abelbeck Software). Solid lines in Figure 2 are the theoretical curves with the estimated values of k_1, k_2, and k_3. The fitting of the lines to the observed data indicates the validity of the analysis.

A similar kinetic experiment was carried out using 17α-hydroxypregnenolone as a substrate (Figure 3). Since the formation of DHEA by the C17−C20 bond cleavage reaction was far slower than 17α-hydroxylation of pregnenolone, the reaction continued 5 times longer than Figure 2. The data fit well to

$$[\text{DHEA}] = B(1 - \exp(-k_3' t))$$

where B indicates concentration of metabolized 17α-hydroxypregnenolone. The estimated rate constant, k_3', is 0.42 min^{-1}, which is almost the same as that of k_3 in Figure 2 (0.48 min^{-1}). The good correlation of the two set of kinetic experiments indicates reliability of the analysis.

Figure 4 shows the metabolism of progesterone with the P450 proteoliposomes. The bovine enzyme produced 17α-hydroxyprogesterone but not androstenedione from progesterone. The data were analyzed by the similar method as indicated in Figure 3 using

$$[\text{17OH-Prog}] = C(1 - \exp(-k_5 t))$$

where C indicates concentration of metabolized progesterone. The solid lines in Figure 4 are the theoretical curves with the estimated value of k_5.

DISCUSSION

It is well-known that androgens are formed through two distinct pathways, Δ^4 and Δ^5, depending on the species of the animal (*18*). We have analyzed in considerable detail the reaction of $P450_{17\alpha,lyase}$ from guinea pig, an animal having the Δ^4 pathway for androgen formation (*16*). To clarify why the enzyme for the Δ^5 pathway cannot produce androstenedione from progesterone, we analyzed the activities of Δ^5 enzyme and compared the kinetic constants with those of guinea pig enzyme. At first, we tried to purify the $P450_{17\alpha,lyase}$ from bovine adrenals, but it was difficult to obtain the unstable enzyme with ordinary methods under detergent-solubilized conditions. Finally, enough P450 containing His tag was purified from the bacterial expression system by one-step column chromatography. Although the $P450_{17\alpha,lyase}(His)_4$ from *E. coli* had slight modifications at the NH_2- and COOH-terminal regions, the activities were higher than those of purified $P450_{17\alpha,lyase}$ from calf testis and the less modified enzyme (*19, 23, 33*). $P450_{17\alpha,lyase}(His)_4$ was greatly activated and stabilized by incorporation into liposomal membranes having a lipid composition similar to that of adrenal endoplasmic reticulum (Tables 1 and 2). This

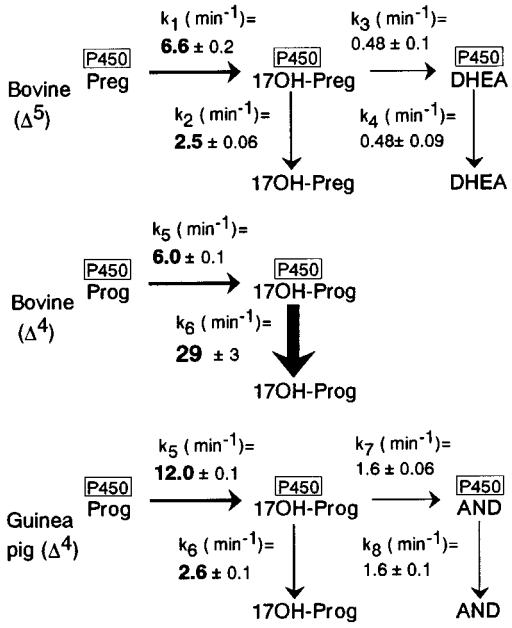

FIGURE 5: Kinetic parameters in pregnenolone and progesterone metabolism by bovine and guinea pig $P450_{17\alpha,lyase}$ proteoliposomes at 10 °C. Values for bovine enzyme were obtained from the analysis of rapid quenching experiments and reactions in the presence of excess amount of substrates in this study. Values for guinea pig enzyme were calculated from the data in ref *16*. Abbreviations are as in Table 1 and Figure 2. The values are expressed in units min^{-1} and are the mean and SD of three individual experiments.

indicates the importance of phospholipid membrane for the stability and the reactivity of membrane enzymes.

The kinetic constants for the bovine $P450_{17\alpha,lyase}$ proteoliposomes are summarized in Figure 5. The values for k_1, k_2, k_3 and k_5 have been estimated from rapid quenching experiments (Figures 2 and 4). The k_4 and k_6 values were obtained by analysis of reactions in the presence of excess amount of substrates at 10 °C (Table 3) using the values of k_1, k_2, k_3, and k_5. The production rate of metabolites in the reactions is expressed by (*16*)

$$\mathrm{d[17OH\text{-}Preg]/d}t = k_1k_2k_4[\mathrm{P450}]/((k_1 + k_2 + k_3)k_4 + k_1k_3)$$

$$\mathrm{d[DHEA]/d}t = k_1k_3k_4[\mathrm{P450}]/((k_1 + k_2 + k_3)k_4 + k_1k_3)$$

$$\mathrm{d[17OH\text{-}Prog]/dt} = k_5k_6[\mathrm{P450}]/(k_5 + k_6)$$

where [P450] is the total concentration of $P450_{17\alpha,lyase}$ in the reaction mixture. Two values could be estimated for k_4 from two turnover numbers for 17α-hydroxylation and DHEA formation from pregnenolone, which are 0.57 and 0.39 min^{-1}, respectively. The closeness of these values shows the plausibility of the reaction scheme. The average of these two values for k_4 and calculated k_6 are shown in Figure 5. In the presence of excess amount of substrates, the ratio of the rate of production of 17α-hydroxypregnenolone to that of dehydroepiandrosterone should be equal to the ratio of reaction rates, k_2/k_3 (see above equations). The calculated value for k_2/k_3 is 5.3, which is similar to the observed ratio in the presence of excess substrate, 6.1, in Table 3. The good correlation shows the reliability of the analysis. The dissociation rate for 17α-hydroxyprogesterone, k_6, is larger than the rate for 17α-hydroxylation of progesterone, k_5, in the Δ^4 pathway. The activity for production of 17α-hydroxyprogesterone in the presence of excess amount of substrates was 5 times higher than that for 17α-hydroxypregnenolone at 10 °C (Table 3), although k_1 and k_5 were almost the same. The higher 17α-hydroxylation activity for progesterone in the presence of excess substrate is attributable to the 10-fold faster dissociation of 17α-hydroxyprogesterone than 17α-hydroxypregnenolone from the enzyme.

From the comparison of the rate constants of guinea pig $P450_{17\alpha,lyase}$ with those of bovine enzyme, the rate of 17α-hydroxylation of progesterone, k_5, is 2-times higher in guinea pig than in bovine enzyme (Figure 5). In contrast, the rate of 17α-hydroxyprogesterone production in the presence of excess amount of substrates is a quarter of that for bovine P450 (1.2 vs 5 min^{-1}) (*16*). The high turnover number for the bovine enzyme in the production of 17α-hydroxyprogesterone in the presence of excess amount of substrates is attributable to the dissociation rate of 17α-hydroxyprogesterone, k_6, which is 10 times higher than that for guinea pig enzyme (Figure 5). The dissociation of the metabolites from $P450_{17\alpha,lyase}$ is remarkably slow compared with the other enzymatic reactions, at which dissociation rates of small molecules from enzymes are 10^3-10^6 min^{-1} (*34*).

The bovine $P450_{17\alpha,lyase}$ does not form androstenedione from progesterone, although the enzyme can catalyze androstenedione formation from 17α-hydroxyprogesterone. It can be concluded that the dissociation of the first hydroxylation metabolite, 17α-hydroxyprogesterone, from the enzyme prevents the successive reaction to form androstenedione.

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