Kinetic Studies of Cytochrome P-450$_{17\alpha,lyase}$ Dependent Androstenedione Formation from Progesterone†

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ABSTRACT: The reaction mechanism of androstenedione formation from progesterone was analyzed in a membrane reconstituted system consisting of P-450$_{17\alpha,lyase}$ and NADPH−cytochrome P-450 reductase using a rapid quenching device at 10 °C. In these rapid quenching experiments, only the metabolites of [^{3}H]progesterone bound to P-450$_{17\alpha,lyase}$ at the initial stage were detectable during the limited cycles of the P-450$_{17\alpha,lyase}$ reactions (1−120 s). The level of 17α-hydroxy[^{3}H]progesterone increased rapidly in a short period (1−5 s) and then decreased to about half. That of [^{3}H]androstenedione increased gradually from 2 s, which exactly corresponded to the decrease in 17α-hydroxy[^{3}H]progesterone. 17α-Hydroxyprogesterone was conclusively the actual intermediate steroid which did not dissociate from P-450$_{17\alpha,lyase}$ during the successive hydroxylation reaction into androstenedione. A kinetic model can clearly describe the successive reaction catalyzed by P-450$_{17\alpha,lyase}$, in which progesterone is converted successively into androstenedione via 17α-hydroxyprogesterone, some of which dissociates from the active site of P-450$_{17\alpha,lyase}$ and is never metabolized into androstenedione. We analyzed the effects of pH and the amount of NADPH−cytochrome P-450 reductase on the successive reaction and proved that the reaction was regulated by the rate of electron transfer for the conversion of the bound 17α-hydroxyprogesterone to androstenedione. Furthermore, we found that the product dissociation from P-450$_{17\alpha,lyase}$ is the rate-limiting process in the steady-state metabolism of progesterone by P-450$_{17\alpha,lyase}$.

P-450$_{17\alpha,lyase}$[1] in the membrane of endoplasmic reticulum has been highly purified from steroidogenic organs such as guinea pig adrenals (Kominami *et al.*, 1982a), neonatal pig testes (Nakajin & Hall, 1981a), and adult pig adrenals (Nakajin *et al.*, 1984). Some kinetic studies demonstrated that a single molecular species of P-450$_{17\alpha,lyase}$ catalyzes both 17α-hydroxylation of progesterone and pregnenolone and the C17−C20 bond cleavage reaction of the 17α-hydroxylated steroids to form androgens (Nakajin *et al.*, 1981; Shinzawa *et al.*, 1985). Conclusive evidence for the dual function of P-450$_{17\alpha,lyase}$ was provided by genetic expression in COS 1 cells (Zuber *et al.*, 1986). 17α-Hydroxylated steroids were reasonably assumed to be the obligatory intermediates for androgen formation from progesterone or pregnenolone. Nakajin and Hall (1981b) showed that progesterone and pregnenolone were converted to the androgen via the 17α-hydroxylated steroids in the presence of a limited amount of substrates. Studies on the steroid binding to P-450$_{17\alpha,lyase}$ showed that the affinity of progesterone was not much different from that of 17α-hydroxyprogesterone (Kominami *et al.*, 1986). Since the intracellular concentration of progesterone is much higher than that of 17α-hydroxyprogesterone in adrenal cells (Nishikawa & Strott, 1984), the metabolism of 17α-hydroxyprogesterone to androstenedione must be strongly inhibited competitively by the progesterone (Higuchi *et al.*, 1991). In order to clarify P-450$_{17\alpha,lyase}$-mediating pathway of androgen formation, double-substrate, double-label experiments were conducted with ovarian microsomes (Yamazaki *et al.*, 1992a) and cultured adrenal cells (Yamazaki *et al.*, 1992b) in which [^{14}C]-progesterone or [^{14}C]pregnenolone was metabolized in the presence of almost equal amounts of ^{3}H-labeled 17α-hydroxylated steroids. Androgens produced under such experimental conditions retained predominantly the same isotope as the original progesterone or pregnenolone. These results show that the released 17α-hydroxylated steroids from P-450$_{17\alpha,lyase}$ cannot be the intermediate of androgen production from progesterone or pregnenolone. If the reaction intermediates would leave P-450$_{17\alpha,lyase}$ during catalysis, the active site of P-450$_{17\alpha,lyase}$ must be occupied immediately by the alternative substrates which are present in a large amount in the reaction solution. Kinetic studies on androstenedione formation from progesterone also showed in P-450$_{17\alpha,lyase}$ proteoliposomes and adrenal microsomes that the dissociated 17α-hydroxyprogesterone from P-450$_{17\alpha,lyase}$ cannot be the intermediate for the androstenedione production (Kominami *et al.*, 1989; Higuchi *et al.*, 1991). These results mean that androgens must be produced from C_{21} steroids such as progesterone or pregnenolone by the mechanism where the intermediate steroid does not leave P-450$_{17\alpha,lyase}$ or by one-step monooxygenase reaction without any stable intermediates. Prasad and Lieberman (1990) suggested that 17α-hydroxysteroid and 17-ketosteroid could be produced from a common transient radical intermediate through one-step monooxygenase reaction. Shimizu (1978) showed the possibility that C-19 steroid might not be produced via 17α-

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[1] *Abbreviations*: P-450$_{17\alpha,lyase}$, cytochrome P-450 having steroid 17α-hydroxylase and C17,C20-lyase activities (P-450 17A1); P-450$_{C21}$, cytochrome P-450 having steroid 21-hydroxylase activity (P-450 21A1); P-450$_{11\beta}$, cytochrome P-450 having steroid 11β-hydroxylase activity (P-450 11B1); P-450$_{scc}$, cytochrome P-450 having cholesterol desmolase activity (P-450 11A1); P-450$_{arom}$, cytochrome P-450 having steroid aromatization activity (P-450 19).

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hydroxylated intermediates on the basis of an analysis of [17α-^{2}H]pregnenolone metabolism by boar testicular microsomes.

It is also possible that androgens might be produced from progesterone or pregnenolone through some non-dissociating intermediates, which is known as the successive reaction (Takemori *et al.*, 1993). The actual intermediate during successive reaction is undetectable under the steady-state conditions. Several kinetic studies about the reaction mechanism for androgen synthesis have been carried out under the steady state using microsomal preparations, in which it was assumed that androgens were synthesized through 17α-hydroxylated steroids as the intermediate (Swinny & Mak, 1994; Kuhn-Velten *et al.*, 1991). Under the steady-state condition, we can only detect the steroid metabolites which were eliminated from P-450$_{17\alpha,lyase}$. Such free molecules of the steroid metabolites are not the actual intermediate for the androgen formation. Since the bound steroids in the enzyme active site must be an extremely small quantity under the steady-state analysis, it is impossible to detect the actual intermediate in the reaction of androgen production.

The first aim of this study was to clarify the reaction mechanism for androstenedione formation from progesterone using a rapid quenching device. Since there is more P-450$_{17\alpha,lyase}$ than [^{3}H]progesterone in the rapid quenching experiments, only the substrate bound to P-450$_{17\alpha,lyase}$ at the initial stage can be metabolized during the limited cycles of reactions. Furthermore, the regulation mechanism that controls the ratio of the activity of the production of 17α-hydroxyprogesterone to that of androstenedione was analyzed in detail. This ratio is the key factor which must determine the relative activity for cortisol and androgen production in adrenal steroidogenesis.

EXPERIMENTAL PROCEDURES

Chemicals. Materials were obtained from the following sources: progesterone, 17α-hydroxyprogesterone, and 11-deoxycorticosterone were from Fluka Chemie AG, Buchs; androstenedione and L-α-phosphatidylcholine (egg yolk, type III-E), from Sigma Chemical Co., St. Louis, MO; L-α-phosphatidylserine (bovine spinal cord, grade 1) and L-α-phosphatidylethanolamine (egg yolk, grade 1), from Lipid Products, Nutfields, Surrey; NADPH, from Boehringer-Yamanouchi, Tokyo; and [^{14}C]dipalmitoylphosphatidylcholine, [1,2-^{3}H]progesterone, 17α-[1,2-^{3}H]hydroxyprogesterone, 17α-[4-^{14}C]hydroxyprogesterone, [4-^{14}C]progesterone, and [4-^{14}C]androstenedione, from DuPont-NEN, Boston, MA. All other chemicals were of the best commercially available grade.

Preparation of P-450$_{17\alpha,lyase}$ Proteoliposomes Containing NADPH−Cytochrome P-450 Reductase. P-450$_{17\alpha,lyase}$ from guinea pig adrenal microsomes and NADPH−cytochrome P-450 reductase from bovine liver microsomes were purified as described (Kominami *et al.*, 1982a,b). P-450$_{17\alpha,lyase}$ proteoliposomes were prepared by the cholate dialysis method using purified P-450$_{17\alpha,lyase}$ and a phospholipid mixture composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a molar ratio of 5:3:1 (Kominami *et al.*, 1989). The molar ratio of P-450$_{17\alpha,lyase}$ to phospholipids in all proteoliposomes ranged between 1:1200 and 1:2000. The purified reductase was incorporated into membranes by incubation with preformed P-450$_{17\alpha,lyase}$ proteoliposomes for 1 h at 0 °C. The ratio of P-450$_{17\alpha,lyase}$ to the reductase in the proteoliposomes was 1:2 in most experiments except for experiments of a variation in the amount of the reductase.

Rapid Quenching Device. A rapid quenching device (0JI 3 system) was constructed according to the design of Kanazawa *et al.* (1970) with some modifications. The three micropipettes connected to the three syringes were placed in the reaction vessel. The syringes were driven by N_2 gas pressure controlled by electrovalves. The solutions in the pipettes were thus added to the reaction vessel at defined times. The signals at 0 and 5 mV for opening and closing the electrovalves, respectively, were generated from a personal computer (PC-9800F, NEC, Tokyo) and were amplified by a hand-made interface.

To test our rapid quenching device, the amount of alkaline hydrolysis of 2,4-dinitrophenyl acetate was measured (Cash *et al.*, 1981). The measurements showed that the alkaline hydrolysis followed pseudo-first-order kinetics (data not shown). We confirmed that this device was useful for following the reaction at intervals of 0.1 s.

We used the rapid quenching device for the reaction of P-450$_{17\alpha,lyase}$ from progesterone to androstenedione. The reaction mixture contained 50 pmol of [^{3}H]progesterone (0.5 μCi), as well as proteolipsomes containing 250 pmol of P-450$_{17\alpha,lyase}$ and 500 pmol of NADPH−cytochrome P-450 reductase in 0.5 mL of 50 mM potassium phosphate buffer at various pHs. After incubation for 2 min at 10 °C, the reaction was initiated by adding 100 nmol of NADPH (20 μL) from the first of three micropipettes. At 0.5 s after the initiation of the reaction, 20 nmol of nonlabeled progesterone (20 μL) was added as the chaser to the reaction mixture from the second micropipette. At various periods after the initiation of the reaction, 50 μL of 1 M HCl was expelled into the reaction mixture from the third micropipette to quench the reaction. The steroids were extracted with 1.5 mL of chloroform containing [^{14}C]progesterone, 17α-[^{14}C]-hydroxyprogesterone, and [^{14}C]androstenedione to estimate recoveries throughout the entire procedure. The extracted steroids were separated by an HPLC system as described previously (Higuchi *et al.*, 1991). In some experiments, radioactivity in the eluate from HPLC was monitored by a Beckman 171 radioisotope detector (Beckman Instruments Inc., Fillerton, CA).

Other Methods. Progesterone metabolizing activity of the proteoliposomes in the steady state was measured aerobically at 10 °C in a reaction mixture containing 20 pmol of P-450$_{17\alpha,lyase}$, 40 pmol of NADPH−cytochrome P-450 reductase, and 50 nmol of [^{3}H]progesterone (1.0 μCi) in 1 mL of 50 mM potassium phosphate buffer at various pHs (5.4−8.1) containing 0.1 mM EDTA (Higuchi *et al.*, 1991).

RESULTS

pH Dependence of the Activity of P-450$_{17\alpha,lyase}$ in the Steady State. P-450$_{17\alpha,lyase}$ can metabolize progesterone into 17α-hydroxyprogesterone and androstenedione. The activity of formation of 17α-hydroxyprogesterone and androstenedione in the steady state varied with the pH of the assay system as shown in Figure 1A. The 17α-hydroxyprogesterone liberated from P-450$_{17\alpha,lyase}$ could not be further metabolized to androstenedione under the assay conditions, since there was a lot of progesterone and little of the released 17α-hydroxyprogesterone in the reaction mixture. The molar

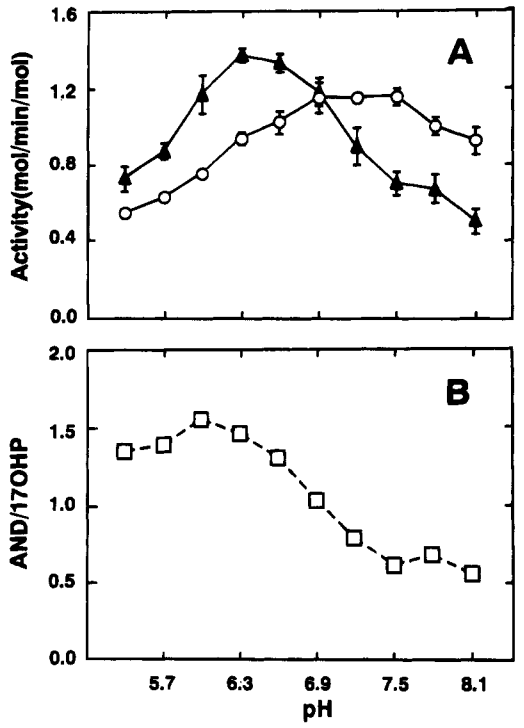


FIGURE 1: Effects of pH on the activity of P-450$_{17\alpha,lyase}$ in the steady state. The enzyme assay was performed as described under Experimental Procedures. Panel A shows the effects of pH on the production of 17α-hydroxyprogesterone (○) and androstenedione (▲) in the steady state. Data are means for six separate determinations, and vertical bars denote the mean ± SD. Panel B indicates the molar ratio of androstenedione to 17α-hydroxyprogesterone produced from progesterone as shown in panel A.

ratio of androstenedione to 17α-hydroxyprogesterone produced from progesterone was maximal at pH 6.3 and minimal at pH 7.5 (Figure 1B). We analyzed the effect of pH on the stability of P-450$_{17\alpha,lyase}$ and NADPH−cytochrome P-450 reductase in liposomal membranes. Incubation of the enzymes in the pH range from 5.4 to 8.5 at 10 °C for 1 h did not have any effect on steroid metabolizing activity. All experiments in this study were performed at 10 °C.

Determination of Intermediates in Androstenedione Production from Progesterone. To identify the actual intermediate in androstenedione production from progesterone, the reaction product of P-450$_{17\alpha,lyase}$ from ^{3}H-labeled progesterone at pH 6.3 was analyzed a short time after initiation using the rapid quenching device. We confirmed by optical titration that more than 90% of the substrate was bound to P-450$_{17\alpha,lyase}$ before the reaction (data not shown). At 0.5 s after the reaction initiation, 20 nmol of nonradioactive progesterone was added as the chaser. The radioactive substrate bound to the reduced P-450$_{17\alpha,lyase}$ cannot be replaced by the chaser progesterone, because the reduced P-450$_{17\alpha,lyase}$ has much higher affinity for the substrate than the oxidized enzyme (Sligar *et al.*, 1976; Hely *et al.*, 1986). The quencher, 0.1 M HCl (50 μL), was added to the assay mixture at various times to terminate the reaction, and the radioactive metabolites were analyzed. It should be noted that, in these rapid quenching experiments, the metabolites of only [^{3}H]progesterone bound to P-450$_{17\alpha,lyase}$ at the initial stage are detectable, and the radioactive steroids which dissociate from the active site cannot be further metabolized by the enzyme in the presence of excess nonradioactive progesterone. When the metabolites were submitted to

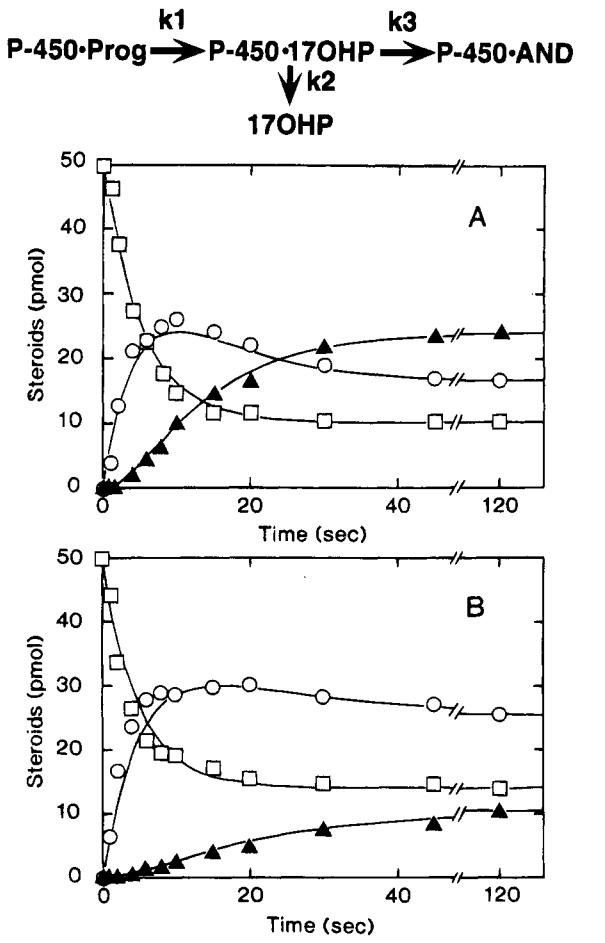


FIGURE 2: Time course of progesterone metabolism catalyzed by P-450$_{17\alpha,lyase}$. The scheme shows the successive reaction of androstenedione production from progesterone, in which P-450, Prog, 17OHP, and AND represent P-450$_{17\alpha,lyase}$, progesterone, 17α-hydroxyprogesterone, and androstenedione, respectively. The reactions using the rapid quenching device were carried out at pH 6.3 (panel A) and 7.5 (panel B) as described under Experimental Procedures. The symbols represent progesterone (□), 17α-hydroxyprogesterone (○), and androstenedione (▲). The solid lines represent the theoretical curves obtained using rate constants of k_1 = 0.184, k_2 = 0.044, and k_3 = 0.064 for pH 6.3 and k_1 = 0.200, k_2 = 0.044, and k_3 = 0.027 for pH 7.5. These were obtained by the theoretical fits to the data (see Appendix). The profile was reproducible in three individual experiments.

HPLC, progesterone was the only steroid detectable at zero time. A new peak having a relative retention time corresponding to 17α-hydroxyprogesterone appeared 2 s after the initiation. Androstenedione as well as 17α-hydroxyprogesterone appeared after 10 s. After 120 s, the androstenedione peak exceeded that of 17α-hydroxyprogesterone. No other metabolites were detectable during the reaction.

Figure 2A shows the time course of progesterone metabolism at pH 6.3 when the reaction was analyzed using the rapid quenching device. The amount of progesterone decreased sharply and led to a constant value within 20 s. The production of 17α-hydroxyprogesterone increased until 10 s after the reaction initiation and then decreased by about half. The production of androstenedione increased with a decrease in the amount of 17α-hydroxyprogesterone. After 30 s, the amount of androstenedione exceeded that of 17α-hydroxyprogesterone. After 60 s, the levels of 17α-hydroxyprogesterone and androstenedione reached a constant value. We concluded from the results shown in Figure 2A that the

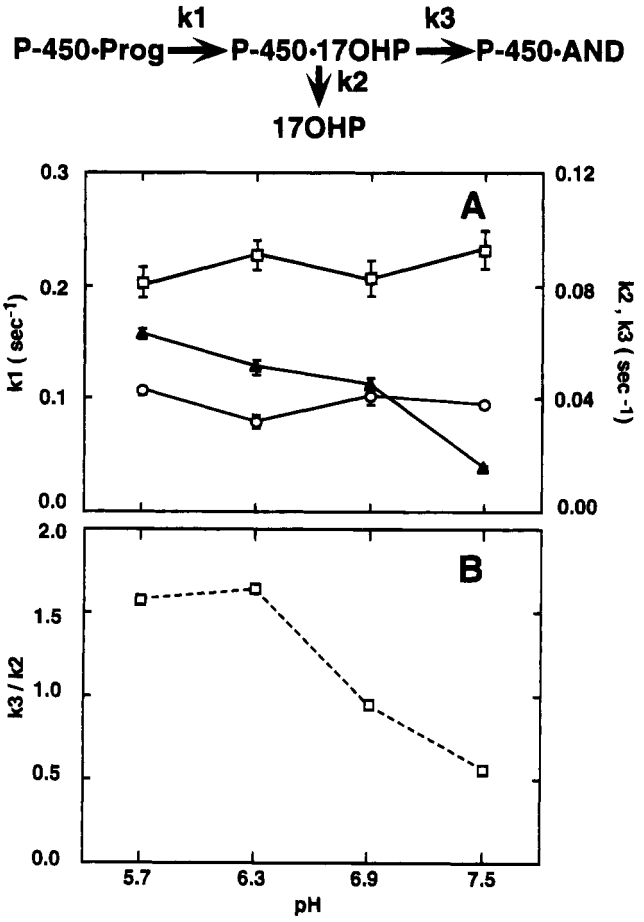


FIGURE 3: Effects of pH on the reaction rate constant of the P-450$_{17\alpha,lyase}$ reaction. Reactions using the rapid quenching device were carried out as described under Experimental Procedures. The rate constants were obtained by curve fittings to the data as described in the Appendix. The symbols in panel A represent k_1 (□), k_2 (○), and k_3 (▲), respectively. Data are means for the three separate determinations, and vertical bars denote the mean ± SD. Panel B shows the ratio of k_3 to k_2.

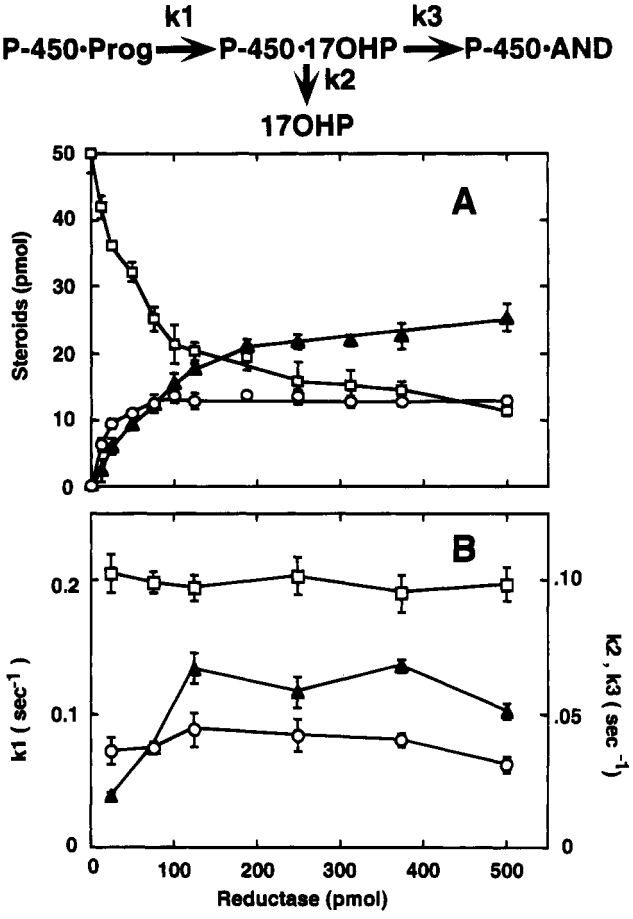


FIGURE 4: Effects of the amount of NADPH−cytochrome P-450 reductase on progesterone metabolism catalyzed by P-450$_{17\alpha,lyase}$. Reactions using the rapid quenching device were performed as described under Experimental Procedures. The symbols in panel A represent the amount of metabolites produced 120 s after the initiation of the reaction: progesterone (□), 17α-hydroxyprogesterone (○), and androstenedione (▲). Panel B shows the effects of the amount of NADPH−cytochrome P-450 reductase on the kinetic parameters. The reaction rate constants were obtained by curve fittings to the data described in the Appendix. The symbols represent k_1 (□), k_2 (○), and k_3 (▲), respectively. Data are means for three separate determinations, and vertical bars denote the mean ± SD.

metabolic intermediate in the successive androstenedione production from progesterone is 17α-hydroxyprogesterone, which remains bound to P-450$_{17\alpha,lyase}$ during the reaction.

The lines shown in Figure 2A were drawn theoretically using the equations described in the Appendix. The rate constants for 17α-hydroxylation (k_1), C17−C20 bond cleavage (k_3), and intermediate dissociation (k_2) (Scheme 1, Appendix) were estimated to be 0.184, 0.044, and 0.064 s^{-1}, respectively. If androstenedione were formed through the one-step monooxygenase reaction proposed by Prasad and Lieberman (1990), the time course of androstenedione formation would not show a lag and the production of 17α-hydroxyprogesterone would not decrease under these reaction conditions.

Effect of pH on the Successive Reaction. In the steady-state reaction, androstenedione production was lower than 17α-hydroxyprogesterone production at high pH. Figure 2B shows the time course of product formation when [^{3}H]-progesterone metabolism by P-450$_{17\alpha,lyase}$ at pH 7.5 was analyzed using a rapid quenching device. The rate constants k_1, k_2, and k_3 were 0.200, 0.044, and 0.027 s^{-1}, respectively. Only the value of k_3 was significantly different from that at pH 6.3. Figure 3A shows the pH dependence of the rate constants. k_1 and k_2 did not vary within the pH range from 5.7 to 7.5, but k_3 decreased with an increase of pH. As shown in the Appendix, the ratio of the production of androstenedione to that of 17α-hydroxyprogesterone in the steady-state reaction can be expressed by k_3/k_2, which were obtained from the rapid quenching experiments. The pH dependence of the ratio in the steady-state reactions (Figure 1A) follows well that of k_3/k_2 shown in Figure 3B. We concluded that the pH dependence in the steady-state reaction shown in Figure 1A is due to the decrease of k_3 with the increase of pH in the reaction mixture.

Effect of the Amount of NADPH−Cytochrome P-450 Reductase on the Successive Reaction. The amounts of metabolites at 120 s after the initiation in the rapid quenching experiments at pH 6.3 were dependent on the amount of the reductase. As shown in Figure 4A, the amount of residual progesterone sharply dropped with the increase of the reductase amount from 12.5 to 125 pmol, at which amount the ratio of P-450$_{17\alpha,lyase}$ to reductase was 2:1. The amount of 17α-hydroxyprogesterone produced was increased sharply by increasing the amount of the reductase to about 50 pmol, whereas androstenedione formation was increased gradually

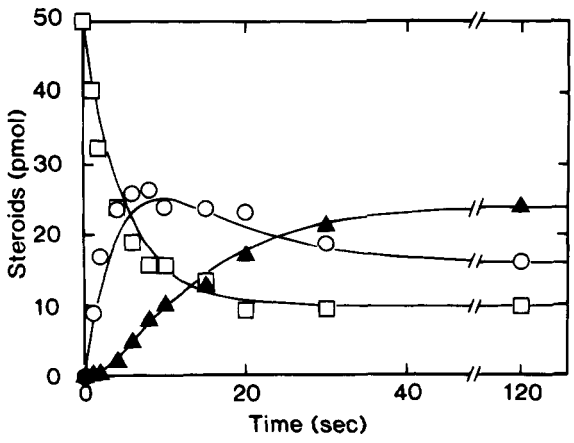


FIGURE 5: Time course of progesterone metabolism at pH 6.3 when NADPH−cytochrome P-450 reductase was reduced with NADPH prior to adding the substrate. The proteoliposomes containing P-450$_{17\alpha,lyase}$ and NADPH−cytochrome P-450 reductase were incubated with NADPH at 10 °C for 2 min before initiation of the reaction. The reaction was initiated by adding progesterone. Rapid quenching of the reaction and analysis of the metabolites were performed as described under Experimental Procedures. The symbols represent progesterone (□), 17α-hydroxyprogesterone (○), and androstenedione (▲). The lines represent the theoretical fits obtained using the equations described in the Appendix. The profile was reproducible in three individual experiments.

by increasing the reductase amount. It is remarkable that there was more 17α-hydroxyprogesterone at the low reductase amount than androstenedione even at pH 6.3. The effects of the amount of the reductase on the kinetic parameters are shown in Figure 4B. Rate constants k_1 and k_2 remained constant, but k_3 was changed from about 0.02 to 0.06 s^{-1} by increasing the reductase amount from 25 to 125 pmol.

Effect of the Reduced State of NADPH−Cytochrome P-450 Reductase. The value of k_1 was apparently much larger than that of k_3. In the rapid quenching experiments in this study, the successive reaction was initiated by adding NADPH to the proteoliposomes containing P-450$_{17\alpha,lyase}$ and the reductase. Under these conditions, the first electron for the first monooxygenase reaction might be delivered to P-450$_{17\alpha,lyase}$ from the fully reduced reductase, which might reach the three electron reduced state (air-stable semiquinone). The first electron for the second monooxygenase reaction could come from the reductase in three or two electron reduced state. There is a possibility that the reduced state of the reductase might cause the difference in the values of k_1 and k_3. To examine this possibility, we performed the following experiment. The proteoliposomes containing P-450$_{17\alpha,lyase}$ and the reductase were incubated with NADPH at 10 °C for 2 min before initiating the reaction where the reductase reaches the air-stable semiquinone state (three electron reduced state) (Iyanagi *et al.*, 1973). The reaction was then started by adding the ^{3}H-labeled substrate. The reaction products were analyzed as described above. As shown in Figure 5, the rate constants remained unaltered from those in Figure 2A. The reduced state of the reductase did not affect the rapid quenching kinetics of the P-450$_{17\alpha,lyase}$ reaction in progesterone metabolism.

DISCUSSION

We concluded from the results shown in Figure 2 that 17α-hydroxyprogesterone is the actual intermediate in the successive monooxygenase reactions of androstenedione formation from progesterone without dissociating from P-450$_{17\alpha,lyase}$. In the successive reaction, a defined portion of 17α-hydroxyprogesterone which is produced from progesterone by the first monooxygenase reaction leaves P-450$_{17\alpha,lyase}$, while the remainder is subsequently metabolized to androstenedione by the second monooxygenase reaction. We applied a kinetic model of the successive reaction catalyzed by P-450$_{17\alpha,lyase}$ (see Appendix). The model fits quite well to the data of the rapid quenching measurement, and the values of k_1, k_2, and k_3 in Scheme 1 (Appendix) can be calculated. The final ratio of 17α-hydroxyprogesterone to androstenedione produced from progesterone corresponded to the ratio of k_2 to k_3 (see Appendix). Although the value of k_3 decreased with pH, the rates of progesterone metabolism (k_1) and intermediate release from P-450$_{17\alpha,lyase}$ (k_2) were not affected by pH (Figure 3). If the electron transfer for the second monooxygenase reaction occurred prior to the release of the intermediate 17α-hydroxyprogesterone from P-450$_{17\alpha,lyase}$, the 17α-hydroxyprogesterone would be converted to androstenedione. We analyzed the effect of the amount of the reductase on the successive reaction by means of rapid quenching measurements. As shown in Figure 4A, the low amount of the reductase suppressed the successive androstenedione formation more than that of 17α-hydroxyprogesterone. Although the rate constants of progesterone metabolism ($k_1 = 0.2$ s^{-1}) and the dissociation of intermediate from P-450$_{17\alpha,lyase}$ ($k_2 = 0.04$ s^{-1}) were not affected by the amount of the reductase, the value of the rate constant of the second monooxygenase reaction (k_3) increased with the reductase as shown in Figure 4B. The variation of the final ratio of androstenedione to 17α-hydroxyprogesterone by pH and by the amount of the reductase in the rapid quenching experiments is attributable to a variation in k_3 and not to either k_1 or k_2. Figure 4A shows that the total amount of progesterone metabolism depends on the amount of the reductase in the reaction mixture, suggesting that only a ternary complex of P-450$_{17\alpha,lyase}$ with radioactive progesterone and the reductase at the initial stage is the active species in the rapid quenching study.

Using porcine adrenal and testicular P-450$_{17\alpha,lyase}$, Yanagibashi and Hall (1986) investigated why the side-chain cleavage activity is low in adrenal microsomes as compared with that in testicular microsomes. They found that the increasing concentrations of the reductase to P-450$_{17\alpha,lyase}$ caused progressive enhancement of the side-chain cleavage activity on 17α-hydroxyprogesterone relative to 17α-hydroxylase activity on progesterone. Grogan *et al.* (1993) reported that limiting the electron flux from NADPH−cytochrome P-450 reductase to P-450$_{arom}$ increased minor metabolite formation relative to that of estrogen. They also observed a change in the metabolite ratio with pH. Their experimental results are consistent with the enhancement of the P-450$_{17\alpha,lyase}$-dependent successive reaction by the increase in the reductase amount. The functional role of cytochrome b_5 in the P-450$_{17\alpha,lyase}$-dependent monooxygenase reaction has also been investigated, and it was proposed that cytochrome b_5 plays some role in controlling the dual activities of P-450$_{17\alpha,lyase}$ (Katagiri *et al.*, 1982; Onoda *et al.*, 1982; Shinzawa *et al.*, 1985). We measured the time course of P-450$_{17\alpha,lyase}$-dependent androstenedione production from progesterone in the presence of cytochrome b_5 by the rapid quenching method, but the data obtained did not fit the

Table 1: Reaction Rate Constants (s^{-1}) for Reaction of P-$450_{17\alpha,lyase}$[a]

rate constant	value at pH 6.3	value at pH 7.5
k_1	0.184 ± 0.010	0.200 ± 0.002
k_2	0.044 ± 0.006	0.044 ± 0.002
k_3	0.064 ± 0.006	0.027 ± 0.001
k_4(17OHP)	0.063 ± 0.008	0.018 ± 0.002
k_4(AND)	0.052 ± 0.012	0.026 ± 0.002

[a] The values of k_1, k_2, and k_3 were obtained by rapid quenching. The values of k_4(17OHP) and k_4(AND) were calculated independently from eqs 7 and 8 in the Appendix for 17α-hydroxyprogesterone and androstenedione formation in the steady state using the above values for k_1, k_2, and k_3.

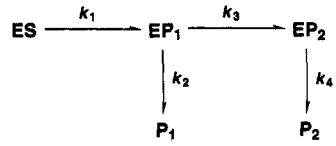
theoretical curves, suggesting that the catalytic reaction of P-$450_{17\alpha,lyase}$ in the presence of cytochrome b_5 might proceed through a more complex mechanism.

The value of k_1, representing the rate of the first monooxygenase reaction, is much higher than that of k_3, which corresponds to the second monooxygenase reaction. The high rate constant of k_1 was not due to the reduced state of the reductase. The fully reduced and semiquinone forms of the reductase had similar kinetics (Figures 2A and 5). This result is in agreement with the report of Oprian *et al.* (1979). In the C17−C20 bond cleavage of the successive reaction, it may be necessary for the intermediate 17α-hydroxyprogesterone to change its orientation in the active site of P-$450_{17\alpha,lyase}$ to facilitate the second monooxygenase reaction. It is reasonable that the rate of the second monooxygenase reaction ($k_3 = 0.064\ s^{-1}$) was slower than that of the first monooxygenase reaction ($k_1 = 0.184\ s^{-1}$) in this successive reaction.

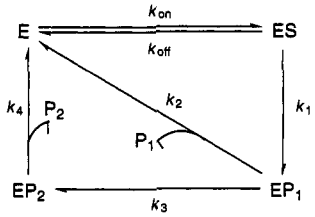
Kinetic equations for the steady-state reaction catalyzed by P-$450_{17\alpha,lyase}$ were derived by the King and Altman method (Segel, 1975) (see Appendix). The ratio of the formation of androstenedione to that of 17α-hydroxyprogesterone corresponded to the ratio of k_3 to k_2. The ratio of androstenedione and 17α-hydroxyprogesterone production in the steady state is shown in Figure 1B, which is quite similar to the k_3/k_2 ratio obtained by rapid quenching, showing the validity of the kinetic derivations. The rate constant k_4, which represents the dissociation of the final metabolite androstenedione from P-$450_{17\alpha,lyase}$, was calculated independently from two equations for 17α-hydroxyprogesterone and androstenedione formation in the steady state using the values of k_1, k_2, and k_3 obtained by rapid quenching experiments (Table 1). Two independently calculated values of k_4 were similar, showing the accuracy of the data both in the steady-state and the rapid quenching studies. The value of k_4 was the lowest among those rate constants. The rate of product formation catalyzed by P-$450_{17\alpha,lyase}$ in the steady state may be greatly regulated by the rate of product dissociation from P-$450_{17\alpha,lyase}$. In a fused enzyme consisting of P-450_{1A1} and NADPH−cytochrome P-450 reductase, the electron transfer from flavin to heme iron is quite fast, and the rate-limiting step of the reaction has been shown to be the release of product (Sakaki *et al.*, 1994).

The biosynthesis of steroid hormones involves a series of both simple and complex cytochrome P-450-catalyzed monooxygenase reactions: one is a simple monooxygenase reaction, and the other is a successive reaction in which the intermediate products of the preceding reaction are the

Scheme 1



Scheme 2



substrates for the subsequent reaction without the intermediates dissociating from cytochrome P-450 (Takemori *et al.*, 1993). For example, the side-chain cleavage of cholesterol by P-450_{scc} (Hume *et al.*, 1984; Burnstein *et al.*, 1976), aldosterone formation from 11-deoxycorticosterone by P-$450_{11\beta}$ (Ikushiro *et al.*, 1989, 1992), and aromatization of androgen to estrogen by P-450_{arom} (Thompson & Siiteri, 1974; Kelly *et al.*, 1977; Fishman & Goto, 1981) are thought to require successive monooxygenase reactions. In this kinetic study, we proved that P-$450_{17\alpha,lyase}$ also catalyzes successive androstenedione production from progesterone. Only P-450_{C21} cannot successively metabolize steroids among the steroidogenic cytochrome P-450s. The successive reaction processes in the steroidogenic pathway may facilitate the formation of steroid hormones from cholesterol by preventing the escape of membrane-permeable intermediates from cells.

APPENDIX

Kinetics of Successive Reaction in a Rapid Quenching Experiment. In Scheme 1, E, S, P_1, and P_2 represent P-$450_{17\alpha,lyase}$, progesterone, 17α-hydroxyprogesterone, and androstenedione, which are ^3H-labeled steroids. Before a large amount of nonradioactive progesterone is added, almost all [^3H]progesterone can be assumed to bind to P-$450_{17\alpha,lyase}$ under these reaction conditions, because the enzyme is present in excess. The observed values of the 17α-hydroxyprogesterone and androstenedione concentrations in the rapid quenching measurement are $[EP_1] + [P_1]$ and $[EP_2] + [P_2]$, respectively, which can be solved as the following equations:

$$[EP_1] + [P_1] = (A(k_1 - k_2)/\alpha)\exp(-k_1 t) - (Ak_1k_3/\alpha\beta)\exp(-\beta t) + Ak_2/\beta \quad (1)$$

$$[EP_2] + [P_2] = (-Ak_3/\alpha)\exp(-k_1 t) + (Ak_1k_3/\alpha\beta)\exp(-\beta t) + Ak_3/\beta \quad (2)$$

where $\alpha = -k_1 + k_2 + k_3$ and $\beta = k_2 + k_3$. The final ratio of androstenedione and 17α-hydroxyprogesterone can be expressed as

$$([EP_2] + [P_2])/([EP_1] + [P_1]) = k_3/k_2 \quad (3)$$

The values for k_1, k_2, and k_3 can be estimated from fitting to the data of the rapid quenching experiments using Kaleidagraph (Abelbeck Software).

Kinetics of the Successive Reaction in the Steady State. We used the schematic method of King and Altman (Segel, 1975) to derive the kinetic equations for the successive reaction in the steady state shown in Scheme 2. The symbols have the same meaning as those in Scheme 1. For P_1 and P_2

$$d[P_1]/dt = k_2[EP_1] = k_{on}k_1k_2k_4[S][E_t]/\Sigma \quad (4)$$

$$d[P_2]/dt = k_4[EP_2] = k_{on}k_1k_3k_4[S][E_t]/\Sigma \quad (5)$$

$$\Sigma/[S] = k_4(k_{off} + k_1)(k_2 + k_3)/[S] + k_{on}k_4(k_2 + k_3) + k_{on}k_1k_4 + k_{on}k_1k_3 \quad (6)$$

where $\Sigma = k_4(k_{off} + k_1)(k_2 + k_3) + k_{on}(k_2 + k_3)k_4[S] + k_{on}k_1k_4[S] + k_{on}k_1k_3[S]$. Under the steady state, $k_4(k_{off} + k_1)(k_2 + k_3)/[S]$ can be assumed to be zero, because there is a lot of substrate in the reaction mixture.

$$d[P_1]/dt = k_1k_2k_4[E_t]/((k_1 + k_2 + k_3)k_4 + k_1k_3) \quad (7)$$

$$d[P_2]/dt = k_1k_3k_4[E_t]/((k_1 + k_2 + k_3)k_4 + k_1k_3) \quad (8)$$

Equations 7 and 8 give the ratio of activity for the production of androstenedione to that of 17α-hydroxyprogesterone as k_3/k_2.

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