The Rate-determining Step in P450 C21-catalyzing Reactions in a Membrane-reconstituted System*

Received for publication, July 10, 2000, and in revised form, December 22, 2000
Published, JBC Papers in Press, January 11, 2001, DOI 10.1074/jbc.M006043200

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Adrenal cytochrome P450 C21 in a membrane-reconstituted system catalyzed 21-hydroxylation of 17α-hydroxyprogesterone at a rate higher than that for progesterone in the steady state at 37 °C. The rate of product formation in the steady state increased with the concentration of the complex between P450 C21 and the reductase in the membranes. The complex formation was independent of the volume of the reaction, showing that the effective concentrations of the membrane proteins should be defined with the volume of the lipid phase. The rates of conversion of progesterone and 17α-hydroxyprogesterone to the product in a single cycle of the P450 C21 reaction were measured with a reaction rapid quenching device. The first-order rate constant for the conversion of progesterone by P450 C21 was 4.3 ± 0.7 s⁻¹, and that for 17α-hydroxyprogesterone was 1.8 ± 0.5 s⁻¹ at 37 °C. It was found from the analysis of kinetic data that the rate-determining step in 21-hydroxylation of progesterone in the steady state was the dissociation of product from P450 C21, whereas the conversion to deoxycortisol was the rate-determining step in the reaction of 17α-hydroxyprogesterone. The difference in the rate-determining steps in the reactions for the two substrates was clearly demonstrated in the pre-steady-state kinetics.

Microsomal cytochrome P450 isozymes are integral membrane proteins responsible for the metabolism of various exogenous and endogenous compounds, including steroid hormones. The monoxygenase reaction of P450 requires two electrons and an oxygen molecule. NADPH-cytochrome P450 reductase in the microsomal membranes supplies the first and the second electrons to P450s in the same membrane. Although the supply of the second electron to P450 is often the rate-limiting step in P450 reactions, results of recent experiments have revealed that some P450 reactions have rate-determining steps other than in the electron supply (1–5). Bell and Guengerich (1) clearly showed in the oxidation of ethanol catalyzed by P450 2E1 that the kinetic deuterium isotope effect on $k_{cat}$ with no effect on $k_{on}$ was attributed to the rate-limiting product release. P450 2E1 catalyzes the oxidation of ethanol to acetic acid via acetaldehyde, in which about 90% of the intermediate acetaldehyde is directly converted to acetic acid without dissociation from the active site of the enzyme (2). They showed for the first time in P450 reactions that the reactions for two substrates catalyzed by one molecular species of P450 could have different rate-determining steps (1). We demonstrated in studies on the successive reactions of P450 11β and P450 17α that the rates of product formation in the steady state were regulated by the rates of product release from the enzymes (3–5). P450 17α and P450 11β catalyze multistep reactions for the formation of androgens and aldosterone, respectively, where the final products are formed from fractions of the intermediates that do not dissociate from the enzymes (3–5). The same mechanism for diminishing the rate of dissociation of final products from P450 2E1, P450 11β, and P450 17α might decrease the dissociation rates of the intermediates, facilitating the successive monoxygenase reactions in these P450s without the intermediate metabolites leaving the enzyme. It is of interest to examine whether the product release can be the rate-determining step for P450 that does not catalyze multistep reactions.

The interaction between NADPH-cytochrome P450 reductase and P450 has been investigated by several laboratories in the systems reconstituted with detergents (6, 7) and diisopropylphosphatidylcholine (8–13). Miwa et al. (14, 15), using a phospholipid vesicular membrane system, found that the active species for the P450-catalyzing monoxygenase reaction in the steady state was the binary complex consisting of P450 and the reductase. Kawato and co-workers (16–20) have extensively studied the interaction between P450 and the reductase in phospholipid vesicles by measuring the rotational diffusion of P450s in the membranes, and they suggested that the mode of interaction depends on the individual P450. Rotational diffusion measurement of P450 C21 in liposomal membranes revealed a complex formation with the reductase (21). In this study, we obtained experimental evidence that the effective concentrations of P450 C21 and NADPH-P450 reductase for the complex formation should be defined with the volume of the lipid phase of the membranes rather than the total volume of the reaction solution.

Two types of cytochrome P450 function in the endoplasmic reticulum of the adrenal cortex (22, 23): P450 C21, catalyzing steroid 21-hydroxylation (24, 25), and P450 17α, catalyzing steroid 17α-hydroxylation and androgen formation (26, 27).

21-hydroxylation activity; P450 11b, cytochrome P450 11b-hydroxylase activity; P450 17a, cytochrome P450 having steroid 17α-hydroxylase activity; HPLC, high performance liquid chromatography.
Some of the progesterone originally produced from cholesterol is hydroxylated to 17α-hydroxyprogesterone, a part of which is further metabolized to androstenedione without dissociation from P450 17α (5). Progesterone and 17α-hydroxyprogesterone are the physiological substrates for P450 C21 and are converted to deoxycorticosterone and deoxycorticisol, respectively. The activity of P450 C21 for the reaction of 17α-hydroxyprogesterone is higher than that for progesterone in bovine and guinea pig microsomes and also in the reconstituted systems (23, 24). The difference in the activities of P450 C21 in the reactions of the two substrates has been investigated by various methods. The rate of first electron transfer from the reductase to P450 C21 in the presence of progesterone was not much different from that in the presence of 17α-hydroxyprogesterone, where the high spin content of the P450 C21–17α-hydroxyprogesterone complex was higher than that of the P450 C21-progesterone complex (28). The rates of substrate binding to P450 C21 in liposomal membranes do not differ much between the two substrates (29).

To elucidate why the activity of P450 C21 in the steady state is higher for 17α-hydroxyprogesterone than for progesterone, it is necessary to determine the rate-determining step in 21-hydroxylation reactions of progesterone and 17α-hydroxyprogesterone. We performed kinetic studies on P450 C21-catalyzing hydroxylation reactions of progesterone and 17α-hydroxyprogesterone in the steady state, in single turnover experimental conditions, and in the pre-steady state.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteoliposomes—Cytochrome P450 C21 and NADPH-cytochrome P450 reductase were purified from bovine adrenocortical and hepatic microsomes, respectively, according to methods described of 21-Hydroxylase Activity in the Steady State**

The reaction system of P450 C21 was reconstituted in liposomal membranes. The incorporation of P450 C21 and NADPH-P450 reductase in the liposome membranes was confirmed by density gradient centrifugation (31, 32). Almost all of the P450 C21 in the proteoliposomes is reduced upon the addition of the reductase and NADPH, suggesting that the heme domain of P450 C21 interacts with the functional domain of the reductase on the outer surface of the vesicles. Fig. 1a shows the time courses of 21-hydroxylation of progesterone and 17α-hydroxyprogesterone catalyzed by P450 C21 at 37 °C in the presence of NADPH-P450 reductase at an equimolar amount of P450 C21 (10 pmol) in the liposome membranes (15 μg of phospholipids in 0.5 ml of reaction solution). The amounts of deoxycorticisol and deoxycorticosterone, which were the 21-hydroxylated products from 17α-hydroxyprogesterone and progesterone, respectively, increased linearly with the reaction time. The rates of product formation by P450 C21, which were calculated from the slopes of the lines, were 0.33 ± 0.04 and 0.17 ± 0.03 nmol/min for 17α-hydroxyprogesterone and progesterone, respectively. The rates of product formation both from progesterone and 17α-hydroxyprogesterone were not altered by change in the volume of the reaction solution as long as the total amounts of P450 C21, the reductase, and phospholipids were kept constant in the reaction solution, as shown in Fig. 1b.

**Dependence of the Rate of Product Formation on the Amount of the Reductase in Liposome Membranes in the Steady State**

The rate of product formation in the steady state increased hyperbolically with the amount of the reductase in the membranes as shown in Fig. 2, where the rate for 17α-hydroxyprogesterone was almost twofold that for progesterone in the concentration range observed. An increase in the rate of product formation by various P450s has been observed with the increase in the amount of the reductase in the reaction solution and has been attributed to formation of an active complex between P450 and the reductase (14, 15). The dissociation constant of the active P450 C21-reductase complex (1:1, mol/m
homogeneously in the reaction solution. These assumptions that P450 C21 and the reductase are distributed parent dissociation constants were calculated under the as-

hydroxyprogesterone and progesterone, respectively. The ap-

obtained 16

6

membranes.

amount of NADPH-cytochrome P450 reductase in liposome

(open circles

were measured at 37 °C in the

hydroxyprogesterone

(open circles

lines

are theoretical curves drawn using the apparent dissociation

constants for the P450 C21-reductase complex, respective,

presence of 10 pmol of progesterone and 17-

a

dissociation constants of 15 and 16 nM for the P450 C21-reductase complex in the

rate-determining Step in P450 C21 Reactions

are a little smaller than those reported for other P450 systems

(14, 38). Below, we discuss the calculation of dissociation con-

stants of the complex using effective concentrations of the enzymes defined with the volume of lipid phase of the mem-

branes. V_{max}, which is the rate in the presence of an excess amount of the reductase with 0.01 nmol of P450 C21, was 0.78 ± 0.06 nmol of deoxycorticisol produced per min for the reaction of 17α-hydroxyprogesterone and 0.48 ± 0.06 nmol of deoxycorticosterol produced per min for progesterone. The lines in Fig. 2 are the theoretical curves drawn with the above values. It was remarkable that $V_{max}$ was about 2 times higher for the 17α-hydroxyprogesterone reaction than for the progesterone reaction but that $K_{d}$ was about 2 times higher for the 17α-hydroxyprogesterone than for the progesterone complexes. Since the 3H-substrate and 3H-products once released from the enzyme do not rebind to P450 C21 in the presence of an excess amount of unlabeled substrate, only the 3H-substrate in the P450 C21-reductase complex at the initiation of the reaction is observable under these con-

Single Turnover Experiments for P450 C21 Reactions—To obtain the rate of conversion of the substrate to the 21-hydroxy-

rated product in the single turnover reaction, a solution contain-

ing a P450-reductase-3H-substrate ternary complex was mixed rapidly with excess amounts of NADPH and the unla-

abeled substrate. Since the 3H-substrate and 3H-products once released from the enzyme do not rebind to P450 C21 in the presence of an excess amount of unlabeled substrate, only the 3H-substrate in the P450 C21-reductase complex at the initiation of the reaction is observable under these con-

Fig. 3. Single turnover experiments for the conversion of pro-

gesterone (open circles) to deoxycorticosterone (closed circles) and of 17α-hydroxyprogesterone (open squares) to deoxycortic-

sol (closed squares), catalyzed by P450 C21-proteoliposomes. The experiments were performed at 37 °C with a rapid quenching device (UNISOKU MX-200) in the presence of 50 pmol each of P450 C21 and the reductase and 2.5 pmol (0.25 μCi) of the 3H-labeled steroid in the membranes (0.077 mg of phospholipids). The lines were drawn using the first-order rate constants of 4.3 s^{-1} and 1.8 s^{-1} in a and b, respectively, which were obtained using the simulation software, Kaleida graph (Version 3.0.5, Albelck Software). The details are described under “Results.”
increase in [3H]deoxycortisol were apparently slower than those for progesterone. The first-order rate constants for reactions of progesterone and 17α-hydroxyprogesterone, which were obtained by fitting the observed data to single exponential curves, were 4.3 and 1.8 s⁻¹, respectively. It is concluded that the higher activity of P450 C21 for the reaction of 17α-hydroxyprogesterone in the steady state is due to the higher conversion rate of 17α-hydroxyprogesterone than that of progesterone. The time courses for reactions of progesterone in the single turnover conditions were also dependent on the amount of the reductase in the reaction solution as shown in Fig. 4. The amount of [3H]progesterone that was converted to [3H]deoxycorticosterone in the single turnover reaction increased with the amount of the reductase in the membranes, but the rate of the conversion did not vary, remaining 4.0 ± 0.5 s⁻¹. The increase in the amount of product in the single turnover condition could be attributed to the increase in the amount of active complex in the reaction solution.

Pre-steady-state Kinetics of P450 C21 Reactions—We measured the time courses of the 21-hydroxylation reactions at 37 °C under the pre-steady-state reaction conditions using a rapid mixing device, UNISOKU MX-200. A solution (100 µl) containing equimolar amounts of P450 C21 and the reductase (5 pmol) and 100 pmol of radioactive substrate was mixed rapidly with an equal volume of NADPH solution, and the conversions of substrates were measured in the range of 0–3 s. Fig. 5, a and b, show the conversions of progesterone and 17α-hydroxyprogesterone to the products, respectively, in which the amount of deoxycorticosterone increased rapidly in the initial 300 ms with a slower linear increase after that, while the amount of deoxycortisol increased almost linearly up to 3 s. The burst increase of deoxycorticosterone in the pre-steady state showed that the rate of conversion from progesterone to deoxycorticosterone must be significantly faster than the rate of dissociation of product deoxycorticosterone from the enzyme (39). The lines in Figs. 5 are the theoretical curves drawn using the computer software HopKINSIM (36, 37).

DISCUSSION

If P450 C21 and the reductase were distributed homogeneously in the reaction solution, the effective concentrations of P450 C21 and the reductase would be decreased to one-tenth by a 10-fold increase in the volume of the reaction solution. Fig. 1b shows, however, that there was almost no change in the rate of product formation with an increase in the volume of the reaction solution, suggesting that the formation of the active complex does not depend on the volume of the reaction solution. The concentrations of P450 C21 and the reductase in the lipid phase of the membranes are not affected by change in the volume of the reaction solution as long as the absolute amounts of P450 C21, the reductase, and the phospholipids are kept constant. P450 C21 proteoliposomes contain 1 mol of P450 C21 in 2000 × 770 g of phospholipids, where 770 is the average molecular weight of the phospholipids. The concentration of P450 C21 was 0.65 mM in the lipid phase under the assumption that 1 g of phospholipids occupies 1 ml of the volume in the membranes (34).

The apparent dissociation constants, $K_d^{\text{(app)}}$, for P450 C21-reductase complex were calculated from the reductase dependence of the rate of product formation under the assumption of a homogeneous distribution of enzymes in the reaction solution using the equation $K_d^{\text{(app)}} = (P/R)(V/PR)/V$, where $P$, $R$, and $PR$ represent the absolute amounts (mol) of free P450C21, free reductase, and P450 C21-reductase complex, respectively, and $V$ is the volume of the reaction solution (0.0005 liters in Fig. 2). The amount of P450 C21-reductase complex can be calculated using the equation $PR = (\text{observed rate of product formation})/V \times (\text{total amount of P450 C21})$, where $V$ is the rate when all of the P450 C21 is in the form of a complex with the reductase. The concentration of P450 C21 in the lipid phase can be defined as $P/Lip$, where $Lip$ represents the volume in liters of the lipid phase of the membranes. The
dissociation constant of the complex defined in the lipid phase can be written as 
\[ K_d(lip) = \frac{([P]_{lip}R_{lip}E_{app})([PR]_{lip})}{([E]_{app})} = K_d(app) \times (Vl/lip) \]
(29). In the experiments for Fig. 2 using 10 pmol of P450 C21, the volume of the lipid phase can be calculated as 
\[ 10 \times 10^{-12} \times 2000 \times 770 \text{ ml}, \text{ and } V \text{ is } 0.5 \text{ ml.} \]
The value for Vl/lip is 3.2 \times 10^{-4}, and the value for K_d(lip) becomes 520 \pm 120 \mu s for P450 C21-reductase complex, which must be the real dissociation constant for the P450 C21-reductase complex in the liposome membranes. A similar K_d(lip) value, about 0.5 mm, is calculated for a hepatic P450-reductase complex in egg yolk liposomes from the data of Miwa and Lu (14).

In the experiments for Fig. 4, the amount of P450 C21 was kept constant (50 pmol in 0.077 mg of phospholipids), and the amount of reductase was increased from 50 to 100 and then to 300 pmol. The amount of [3H]progesterone hydroxylated by P450 C21 in the single turnover reaction increased with the amount of the reductase in the liposome membranes, but the rate of conversion of [3H]progesterone did not change.

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V_{max}</th>
<th>k_2</th>
<th>k_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>0.48 ± 0.06</td>
<td>4.3 ± 0.7</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>17\alpha-Hydroxyprogesterone</td>
<td>0.78 ± 0.06</td>
<td>1.8 ± 0.5</td>
<td>4.5 ± 0.8</td>
</tr>
</tbody>
</table>

This table represents kinetic parameters for P450 C21 reactions in the membrane reconstituted system. V_{max} is the rate of product formation by 10 pmol of P450 C21 in the presence of an excess amount of the reductase in the membranes. k_2 is the rate constant for the conversion of the substrate to the product, and k_3 is that for the product dissociation from P450 C21-reductase complex in the scheme shown in Fig. 6. Values are means ± S.D. of at least triplicate determinations. The details of the calculation are described under “Discussion.”

The difference in the rate-determining steps in P450 C21 reactions must affect the pre-steady-state kinetics of the reactions of progesterone and 17\alpha-hydroxyprogesterone (1). The time course of products formation from progesterone and 17\alpha-hydroxyprogesterone in the range of 0–3 s are shown in Fig. 5, a and b, respectively. As discussed above, the rate of conversion of progesterone to the product in the active complex is about 4 times faster than the dissociation of deoxycorticosterone from the active complex, which is reflected by the burst and the subsequent slow linear increase of the product. On the other hand, the conversion of 17\alpha-hydroxyprogesterone to deoxycorticisol is the slowest step in the reaction cycle, and we did not observe any burst formation of deoxycorticisol. The linear lines in Fig. 5 are the simulated curves obtained using the simulation program HopKINSIM version 1.7.2 with the rate constants in Table I, where the value of k_3 was selected to be 0.02 (nmol s^{-1}) for the binding of both progesterone and 17\alpha-hydroxyprogesterone (29, 36, 37). The simulation study shows that the burst increase in the product formation is due to the fast conversion of the substrate to the product with subsequent slow dissociation of the product from the enzyme.

The successive reactions catalyzed by P450 2E1, P450 11\beta, and P450 17\alpha had as rate-determining steps the dissociation of the products, with slow dissociation of intermediate products facilitating the further monooxygenation of intermediates without dissociation from the enzyme active sites (2–5). The rate-determining step in the hydroxylation reaction of progesterone catalyzed by P450 C21 is the product dissociation, and P450 C21 does not catalyze a multistep reaction. This means that the rate-determining product release from the enzyme was not restricted to the successive reactions. The rate-determining product release might not be a special phenomenon in P450 reactions. White and Coon (41) had speculated some 20
years ago that the difference in the activity of one species of P450 for different substrates might be explained by the difference in the product dissociation rate. The difference in the rates of product release might be due to the difference in the hydrophobicities of the products (42, 43). Deoxycortisol has two hydroxyl groups, but deoxycorticosterone has only one. It is not surprising that deoxycorticosterone dissociates faster from P450 C21 than the more hydrophobic deoxycorticosterone (29). Since it could be the rate-determining step in the P450 reaction, much more attention should be paid to the product dissociation from P450s.

REFERENCES

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doi: 10.1074/jbc.M006043200 originally published online January 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M006043200

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