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THE ROLE OF CYTOCHROME b_5 IN ADRENAL MICROSOMAL STEROIDOGENESIS

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Summary—The role of cytochrome b_5 in adrenal microsomal steroidogenesis was studied in guinea pig adrenal microsomes and also in the liposomal system containing purified cytochrome P-450s and NADPH–cytochrome P-450 reductase. Preincubation of the microsomes with anti-cytochrome b_5 immunoglobulin decreased both 17α- and 21-hydroxylase activity in the microsomes. In liposomes containing NADPH–cytochrome P-450 reductase and P-450_{C21} or P-$450_{17\alpha,lyase}$, addition of a small amount of cytochrome b_5 stimulated the hydroxylase activity while a large amount of cytochrome b_5 suppressed the hydroxylase activity. The effect of cytochrome b_5 on the rates of the first electron transfer to P-450_{C21} in liposome membranes was determined from stopped flow measurements and that of the second electron transfer was estimated from the oxygenated difference spectra in the steady state. It was indicated that a small amount of cytochrome b_5 activated the hydroxylase activity by supplying additional second electrons to oxygenated P-450_{C21} in the liposomes while a large amount of cytochrome b_5 might suppress the activity through the interferences in the interaction between the reductase and P-450_{C21}.

INTRODUCTION

Adrenocortical steroid hormones are synthesized from cholesterol through the actions of various cytochrome P-450s and 3β-hydroxysteroid dehydrogenase-isomerase in adrenal glands [1]. Cholesterol is converted to pregnenolone by P-450_{scc} in the mitochondria and is subsequently metabolized to progesterone in the endoplasmic reticulum. The activity of P-450_{C21} relative to that of P-$450_{17\alpha,lyase}$ in adrenal microsome has a close relation with the amounts of steroid hormones secreted from the gland. Should nearly all progesterone be metabolized to 11-deoxycorticosterone by P-450_{C21}, neither cortisol nor androgens would be synthesized in the gland. Assuming P-450_{C21} to be deficient, corticosterone and aldosterone would not be secreted from the gland, resulting in the overproduction of androgens [2, 3].

The role of cytochrome b_5 in hepatic microsomal cytochrome P-450-mediated monooxygenase reactions has been extensively investigated [4–7]. Cytochrome b_5 is abundantly distributed within adrenal and testicular microsomes [8] but its physiological role in cytochrome P-450 dependent reactions related to steroidogenesis has not been completely understood. In testicular microsomes, cytochrome b_5 has been reported to stimulate and also to suppress the cytochrome P-450 reactions, progesterone 17α-hydroxylation and C17–C20 lyase reaction for 17α-hydroxyprogesterone [9, 10]. For adrenal P-$450_{17\alpha,lyase}$, it was found that cytochrome b_5 had greater effect on lyase activity than that of 17α-hydroxylase in reconstituted systems [11]. Results, contradicting with each other however, have been reported for the effect of cytochrome b_5 on the 21-hydroxylase activity of adrenal P-450_{C21} [12, 13].

In this study, the role of cytochrome b_5 in adrenal microsomal steroidogenesis was investigated using anti-cytochrome b_5 immunoglobulin (IgG). Anti-cytochrome b_5 IgG was prepared

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Trivial names and abbreviations: pregnenolone, 3β-hydroxy-5-pregnen-20-one; 11-deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; P-450_{C21}, cytochrome P-450 having steroid 21-hydroxylase activity (P-450XXIA1); P-$450_{17\alpha,lyase}$, cytochrome P-450 having steroid 17α-hydroxylase and C17–C20 lyase activities (P-450XVIIA1); P-450_{scc}, cytochrome P-450 having cholesterol desmolase activity (P-450XIA1); IgG, immunoglobulin G; HPLC, high performance liquid chromatography. The names of gene families are represented in parentheses.

against purified cytochrome b_5 from guinea pig adrenal microsomes. The effects of cytochrome b_5 were confirmed in a liposomal system containing NADPH–cytochrome P-450 reductase and P-450_{C21} or P-$450_{17\alpha,lyase}$. Kinetic studies were conducted to obtain some clarification of the stimulative and suppressive effects of cytochrome b_5 in such a system.

EXPERIMENTAL

Preparation of microsomes and proteins

Male guinea pigs (Dunkin–Hartley, 500–600 g) were decapitated and their adrenal glands collected immediately. Whole glands were homogenized with 0.25 M sucrose and microsomes prepared at 4°C from the homogenates by differential centrifugations at 700 g for 10 min, 10,000 g for 20 min and 105,000 g for 1 h. After washing the microsomal pellets with 0.25 M KCl, the microsomes were suspended in 20 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM EDTA and stored at −80°C. The microsomes contained 1.7 ± 0.4 nmol and 3.1 ± 0.6 nmol of cytochrome P-450 and cytochrome b_5 per mg of microsomal protein, respectively.

P-450_{C21} and NADPH–cytochrome P-450 reductase were purified from bovine adrenal microsomes as described previously [14, 15]. P-$450_{17\alpha,lyase}$ and detergent solubilized cytochrome b_5 were prepared from guinea pig adrenal microsomes as shown elsewhere [11, 16]. Concentration of cytochrome P-450 in the sample was determined using difference absorption coefficient of $\Delta\epsilon$ (450–490 nm) = 91 $mM^{-1}cm^{-1}$ for the CO-dithionite reduced difference spectrum [17]. That of cytochrome b_5 was estimated from reduced–oxidized difference spectrum using $\Delta\epsilon$ (424–409 nm) = 185 $mM^{-1}cm^{-1}$ [17], and that of the reductase from the cytochrome c reduction activity in comparison with that of purified reductase. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

The antibody against purified cytochrome b_5 from detergent solubilized guinea pig adrenal microsomes was elicited in male white rabbits and anti-cytochrome b_5 IgG was prepared from the anti-sera by ammonium sulfate precipitations, a column chromatography on DEAE cellulose (Whatman Ltd, Maidstone, Kent) and a gel-filtration on Sephadex G-200 (Pharmacia, Uppsala) [18]. Control IgG was prepared in the same methods from preimmune rabbit sera. Anti-cytochrome b_5 IgG was further purified with cytochrome b_5-immobilized Sepharose 4B where BrCN activated Sepharose 4B was cross-linked with cytochrome b_5 [18, 19]. The specific reactivity of the purified IgG to cytochrome b_5 in guinea pig microsomes was confirmed by Western blotting.

Preparation of proteoliposomes

The incorporation of P-450_{C21} and P-$450_{17\alpha,lyase}$ into liposome membranes was performed by the cholate dialysis method [20] using a phospholipid mixture containing phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at a molar ratio of 5:3:1 [21]. The molar ratio of cytochrome P-450 to phospholipids in all proteoliposomes ranged between 1:1500 and 1:2000 while other protein content varied according to the experiment. NADPH–cytochrome P-450 reductase and cytochrome b_5 were incorporated into the membranes by incubation with preformed vesicles at 4°C for 2 h [22, 23].

Enzyme assay

The steroid metabolizing activity of microsomes and proteoliposomes was measured aerobically at 37°C in 100 mM potassium phosphate buffer, pH 7.3, containing 0.1 mM EDTA, the standard buffer in this study. The reaction system in the standard condition contained 10 nmol of substrate steroid and 10 pmol of cytochrome P-450 or 10 μg of microsomes in 1 ml of the buffer. The reaction was initiated with addition of 10 nmol NADPH and was continued for 20 min. Guinea pig adrenal microsomes were preincubated with anti-cytochrome b_5 IgG or control IgG at 4°C for 1 h. Steroid metabolites were separated by HPLC (HLC 803 and UV-8, TOSOH Inc, Tokyo) using a silica gel column (0.46 × 15 cm, Cosmosil 5SL, Nacalai Tesque Inc, Kyoto) with a solvent system of n-hexane–isopropanol–acetic acid (93:7:1, by vol) and were quantified by the absorption at 250 nm [24]. Data accumulation and digital calculations were performed with an OBA-3 system comprised of an auto sampler (AS-80, TOSOH Inc.), a personal computer (PC-9800, NEC Inc, Tokyo) and HPLC system.

Other methods

Stopped flow measurements were made at 37°C in the standard buffer containing 50 mM

glucose, 200 U/ml of glucose oxidase and 1200 U/ml of catalase with a dual wavelength (450 and 490 nm) stopped flow device (Unisoku Co., Hirakata). The reaction was initiated by the rapid mixing of liposomes containing P-450_{C21} and the reductase with 250 μM of NADPH in the presence of CO gas. The digital data stored in personal computer (PC-9801 VM, NEC) were analyzed as the combination of two first order reactions by a non-linear least square method. Optical absorption spectra and difference spectra were obtained with a Beckman DU-7 spectrophotometer at 37°C in the standard buffer.

Chemicals

Progesterone, 11-deoxycorticosterone, pregnenolone and acetic acid (HPLC grade) were obtained from Nacalai Tesque Inc., Kyoto. Sodium cholate, 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione, 11-deoxycortisol, spironolactone, L-α-phosphatidylcholine from egg yolk (Type III), L-α-phosphatidylethanolamine from egg york and L-αphosphatidylserine from bovine brain were from Sigma Chemical Co. (St Louis, MO). NADPH, dithiothreitol, β-glucose, glucose oxidase, and catalase were from Boehringer Mannheim, GmbH (Fed. Rep. Germany) and 17α-hydroxyprogesterone from Fluka Chemie AG, Buchs. All other chemicals were of the best grade commercially available.

RESULTS

Anti-cytochrome b_5 IgG treatment of adrenal microsomes

To examine the role of cytochrome b_5 in steroid metabolism in adrenal microsomes, guinea pig adrenal microsomes were preincubated with various amounts of anti-cytochrome b_5 IgG or control IgG. Figure 1 shows the effects of anti-cytochrome b_5 IgG on progesterone metabolism of the microsomes. P-$450_{17\alpha,\text{lyase}}$ in the microsomes metabolized progesterone to 17α-hydroxyprogesterone and to androstenedione. P-450_{C21} converted progesterone to 11-deoxycorticosterone and the produced 17α-hydroxyprogesterone to 11-deoxycortisol whose amount was much less than that of any other products. Control IgG did not make any effect on the progesterone hydroxylase activity in the range of Fig. 1. Activity for the production of 17α-hydroxyprogesterone and

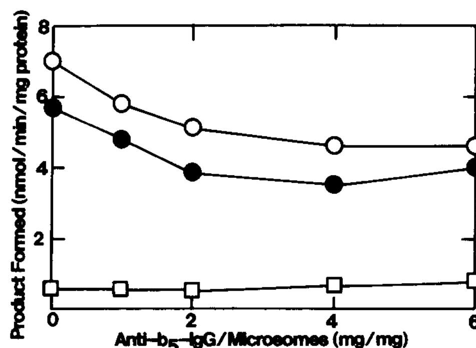


Fig. 1. Effect of anti-cytochrome b_5 IgG on the progesterone metabolism of guinea pig adrenal microsomes. Open and closed circles and open squares represent activity for the formation of 17α-hydroxyprogesterone, 11-deoxycorticosterone, and androstenedione, respectively. Horizontal axis shows mg of the IgG preincubated per mg protein of microsomes. Activity was measured at 37°C in the standard buffer. The points in the figure show the average of at least five experimental data and the standard deviations are smaller than the size of the symbols. Details are described in Experimental.

11-deoxycorticosterone apparently decreased with the addition of anti-cytochrome b_5 IgG. Above an IgG to microsomes ratio exceeding 4 in protein weight, the activity remained constant. The decrease was also noted in the metabolism of 17α-hydroxyprogesterone to 11-deoxycortisol and to androstenedione by the IgG treatment. The effect of the IgG on pregnenolone metabolism of microsomes was essentially the same as that on progesterone metabolism. Androstenedione production from progesterone, however, increased somewhat by the IgG treatment.

Effects of cytochrome b_5 in steroid metabolism in liposomal systems

Figure 2 shows the effects of cytochrome b_5 in liposomes containing P-450_{C21} and the reductase. With or without cytochrome b_5, 21-hydroxylase activity for progesterone was higher in liposomes containing the reductase at a higher molar ratio to P-450_{C21} than in liposomes containing the reductase at a low molar ratio. Increase in cytochrome b_5 in liposome membranes containing constant amounts of the reductase and cytochrome P-450 stimulated hydroxylase activity up to a cytochrome b_5 to cytochrome P-450 molar ratio of about 0.3, followed by gradual decrease in the activity. Maximum stimulation was higher at lower reductase content in liposomes, being 130, 140, and 150% that of the original activity of liposomes containing the reductase at the molar ratios to P-450_{C21} of 1, 0.5, and 0.25, respectively.

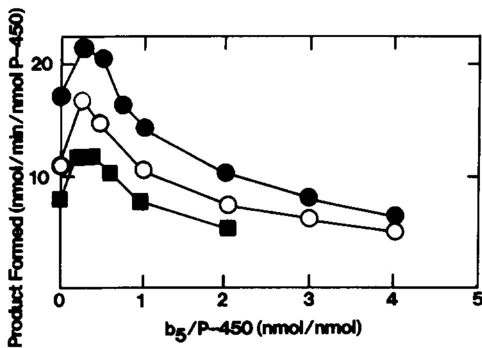


Fig. 2. Effect of cytochrome b_5 on the progesterone metabolism of liposomes containing P-450$_{C21}$, NADPH–cytochrome P-450 reductase, and cytochrome b_5. Activity of liposomes containing P-450$_{C21}$ and the reductase at molar ratios of 1:1, 1:0.5, and 1:0.25 is shown by closed and open circles and closed squares, respectively. Molar ratios of cytochrome b_5 to P-450$_{C21}$ appear on the horizontal axis. The points in the figure show the average of at least five experimental data and the standard deviations are smaller than the size of the symbols. Assay conditions are described in Experimental.

The effect of cytochrome b_5 on activity of P-450$_{17\alpha,\text{lyase}}$ for progesterone metabolism was also assessed in a liposomal system as shown in Fig. 3. With increase in cytochrome b_5 in liposomes containing P-450$_{17\alpha,\text{lyase}}$ and the reductase at a molar ratio of 1:0.25, progesterone 17α-hydroxylase activity was stimulated up to cytochrome b_5/cytochrome P-450 of 0.3 mol/mol, at which maximum stimulation was about 1.5. Androstenedione production from progesterone showed similar dependence on the molar ratio of cytochrome b_5. Stimulation by cytochrome b_5 was greatest at the C17–C20 lyase activity of P-450$_{17\alpha,\text{lyase}}$ for 17α-hydroxyprogesterone, where maximum activation was 400% that without cytochrome b_5. The effect of cytochrome b_5 on liposomal P-450$_{17\alpha,\text{lyase}}$ for pregnenolone metabolism was basically the same to that on progesterone metabolism. It should be pointed out that the binding of pregnenolone to liposomal P-450$_{17\alpha,\text{lyase}}$ failed to bring about any significant spectral changes while pregnenolone was metabolized at about the same rate as progesterone. The high spin state of P-450$_{17\alpha,\text{lyase}}$ may thus not necessarily be required for cytochrome P-450 activity [25, 26].

Effects of cytochrome b_5 on electron transfer to liposomal P-450$_{C21}$

Interaction between cytochrome b_5 and NADPH–cytochrome P-450 reductase may affect that between the reductase and cytochrome P-450 [27]. The rate of the first electron transfer from the reductase to cytochrome P-450 can be measured by observing the increase in absorption at 450 nm in the presence of CO as shown in the insert of Fig. 4. This electron transfer proceeds as two first-order kinetics, fast and slow phases [28]. For the slow phase, the rate of cytochrome P-450 reduction, $0.03 \pm 0.005\ \text{s}^{-1}$, and its amount, 30–40% of the total, did not change much on adding cytochrome b_5 to liposomes, as evidenced from Fig. 4 (insert). The rate of cytochrome P-450 reduction in the slow phase was much less than that of the hydroxylase reaction and will not be

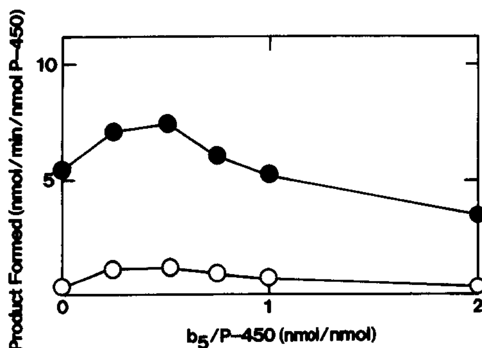


Fig. 3. Effect of cytochrome b_5 on the progesterone metabolism of liposomes containing P-450$_{17\alpha,\text{lyase}}$, NADPH–cytochrome P-450 reductase and cytochrome b_5. The molar ratio of the reductase to P-450$_{17\alpha,\text{lyase}}$ is 0.25 and that of cytochrome b_5 to P-450$_{17\alpha,\text{lyase}}$ is indicated on the horizontal axis. Closed and open circles show activity of 17α-hydroxyprogesterone and androstenedione production. The points in the figure show the average of at least five experimental data and the standard deviations are smaller than the size of the symbols. Reaction conditions are specified in Experimental.

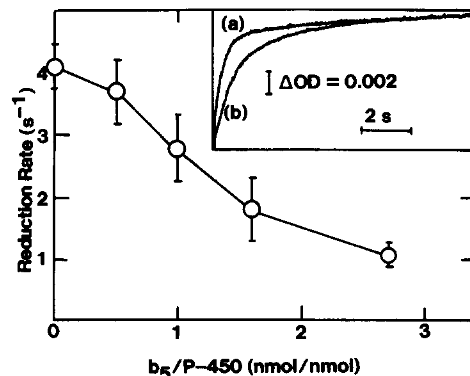


Fig. 4. Effect of cytochrome b_5 on the reduction rate of P-450$_{C21}$ in liposomes containing P-450$_{C21}$ and NADPH–cytochrome P-450 reductase at a molar ratio of 1:0.25. Vertical axis represents rate of reduction of P-450$_{C21}$ in the fast phase. Molar ratio of cytochrome b_5 to P-450$_{C21}$ is shown on the horizontal axis. Details of measurements and data analysis were presented in the text. Insert: Traces, (a) and (b), are increases in absorbance at 450 nm after mixing with NADPH in liposomes containing P-450$_{C21}$, the reductase and cytochrome b_5 at molar ratios of 1:0.25:0 and 1:0.25:2.7, respectively.

discussed here in detail. The effect of cytochrome b_5 on the rate of the first electron transfer in the fast phase in a liposomal system containing P-450_{C21} and the reductase at a molar ratio of 1:0.25 is shown in Fig. 4, where the rate can be seen to decrease with an increase in cytochrome b_5 content in liposomes.

The effect of cytochrome b_5 on the second electron transfer was also examined. Figure 5(a) shows the difference spectra of liposomal P-450_{C21} obtained by subtracting the oxidized spectrum before initiation of the hydroxylation reaction from that during the progesterone hydroxylation reaction, this being designated as the oxygenated difference spectrum hereafter. The oxygenated difference spectrum without cytochrome b_5 showed a peak at 435 nm immediately following addition of NADPH, remained at the same level for several minutes and then decayed after about 20 min. The difference spectrum in the system containing cytochrome b_5 showed a peak around 425 nm, attributable primarily to difference spectra of cytochrome b_5 between reduced and oxidized forms. The oxygenated difference spectrum significantly decreased by the presence of cytochrome b_5. This decrease could be quantified from difference absorption between at 450 nm and at 490 nm where the contribution of the absorption of cytochrome b_5 was negligible. The effect of cytochrome b_5 on oxygenated difference spectra is shown in Fig. 5(b).

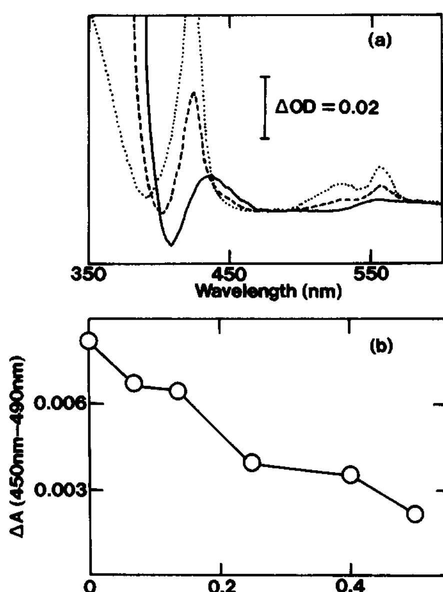


Fig. 5. Effect of cytochrome b_5 on oxygenated difference spectra of liposomal P-450_{C21}. 0.44 μM liposomal P-450_{C21} with 0.11 μM of the reductase and various amounts of cytochrome b_5 in the standard buffer was mixed with 10 μM progesterone and 170 μM NADPH aerobically. (a) Difference spectra were obtained by subtracting the oxidized spectra from those during progesterone hydroxylation in the aerobic steady state. The liposomes contained P-450_{C21}, the reductase and cytochrome b_5 at molar ratios of 1:0.25:0 (full line), 1:0.25:0.25 (broken line) and 1:0.25:0.5 (dotted line). (b) Absorbance difference between at 450 nm and at 490 nm was plotted against the molar ratio of cytochrome b_5 to P-450_{C21} in the liposomes.

DISCUSSION

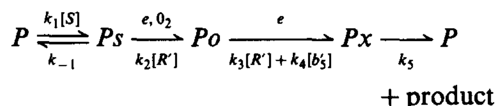
Figure 1 indicates that cytochrome b_5 might be functioning in guinea pig adrenal microsomes as an activator of 21- and 17α-hydroxylase reactions of progesterone and also of the lyase reaction of 17α-hydroxyprogesterone but not of androstenedione formation from progesterone. Special attention should be directed to androstenedione formation from progesterone, since the main pathway for androstenedione production from progesterone in the steady state was shown to be through successive monooxygenase reactions without the intermediate leaving P-$450_{17\alpha,lyase}$ in the previous paper [24, 29, 30]. As described in the text, that anti-cytochrome b_5 IgG suppressed lyase activity for 17α-hydroxyprogesterone but rather activated androstenedione formation from progesterone (Fig. 1) may be one of the indications that androstenedione is formed from progesterone by successive reactions rather than by lyase reaction of 17α-hydroxyprogesterone released in the medium.

The stimulative effect of cytochrome b_5 on P-450_{C21} reaction has been discussed briefly by Katagiri *et al.* [12]. Hiwatashi *et al.* [13], however, could detect no such effect. Figure 2 shows that the stimulative effects become weaker with an increase in the molar ratio of the reductase to P-450_{C21} in liposomes and the effect of cytochrome b_5 to be virtually absent in liposomes containing the reductase and P-450_{C21} at a molar ratio of 8:1 (data not shown). The reconstituted system of Hiwatashi *et al.* [13] contained the reductase at 8 times the molar concentration of P-450_{C21} and their results could be explained on the basis of high reductase content in the system. The molar ratio of the reductase to P-450_{C21} in guinea pig microsomes was found to be <0.3 and cytochrome b_5 actually has stimulative effects in microsomes.

Hildebrandt and Estabrook [31] observed a transient spectrum in hepatic microsomes during drug metabolism in an aerobic steady state and noted the absorption maximum to be around 440 nm. They attributed this transient

spectrum to oxygenated cytochrome P-450 during the reaction. They also found reduced cytochrome b_5 to provide electrons to oxygenated cytochrome P-450. Noshiro *et al.* [32] observed that, in hepatic microsomes, amplitudes of oxygenated difference spectra during hydroxylation increased with the addition of anti-cytochrome b_5 IgG, accompanied by a decrease in hydroxylase activity. Ingelman-Sundberg and Johansson [33], using reconstituted phospholipid vesicles containing NADPH–cytochrome P-450 reductase and P-450_{LM2}, found an increase in product formation mediated by cytochrome b_5 to be correlated with a decrease in H_2O_2 or O_2^- production. Stimulation of reactions by cytochrome b_5 has been attributed to electron transfer from reduced cytochrome b_5 to oxygenated cytochrome P-450s [5]. Suppressive effects of cytochrome b_5 have also been observed in the hepatic microsomal system but have not been explained in the molecular basis [7].

The kinetics of the first and second electron transfer to P-450_{C21} in the liposomes can be written simply as follows:



where P, Ps, Po, and Px are the oxidized, substrate-complexed, oxygenated, and other intermediate forms of P-450_{C21}, and $[S]$, $[R']$ and $[b_5']$, effective concentrations of the substrate, the reductase and cytochrome b_5, respectively. In the presence of excess O_2, the rate of Po formation may be considered equal to that of the reduction of P-450_{C21}. In a liposomal system, the reductase has been shown to transfer electrons to cytochrome P-450 by second-order kinetics through random collisions in membranes [24, 34]. The first and second electron transfer rates may thus be expressed as $k_2[R'][Ps]$ and $k_3[R'][Po]+k_4[b_5'][Po]$, respectively. In the steady state, the ratio of $[Po]/[Pt]$, $[Pt]$ being the total concentration of cytochrome P-450 in liposomes, can be written as,

$$[Po]/[Pt]=\frac{1/(k_3[R']+k_4[b_5'])}{(k_{-1}+k_2[R'])/(k_1[S]k_2[R'])+1/(k_2[R'])+1/(k_3[R']+k_4[b_5'])+1/k_5} \quad (1)$$

In the case of $[S]\gg[Pt]$ and $[b_5']=0$, the ratio is simplified as

$$[Po]/[Pt]=\{1/(k_3[R'])\}/\{1/(k_2[R'])+1/(k_3[R'])+1/k_5\} \quad (2)$$

The concentration of oxygenated cytochrome P-450, $[Po]$, was roughly estimated as about 2/3 the total concentration of cytochrome P-450 based on the amplitude of oxygenated spectrum of P-450_{scc} [35]. From this value, $1/(k_3[R'])$ was found to be the largest term in the denominator of equation (2). The rate of steady state hydroxylation activity is expressed as $[Pt]/\{1/(k_2[R'])+1/(k_3[R'])+1/k_5\}$, showing that, in the absence of cytochrome b_5, the rate limiting step in hydroxylation is the second electron transfer from the reductase to oxygenated cytochrome P-450. Additional second electron transfer from cytochrome b_5 will certainly stimulate hydroxylation in this case.

In the presence of cytochrome b_5, hydroxylation activity is expressed as

$$[Pt]/\{1/(k_2[R'])+1/(k_3[R']+k_4[b_5'])+1/k_5\} \quad (3)$$

and at a molar ratio of cytochrome b_5 to cytochrome P-450 of 0.5:1, oxygenated cytochrome P-450 during the reaction was found to be about 1/6 the total cytochrome P-450 as in Fig. 5(b). Thus, the second electron transfer ceases to be the rate limiting step in hydroxylation. On such a case, the observed rate of hydroxylation reaction is about 11 nmol/min/nmol cytochrome P-450 and that of the first electron transfer about 180 min^{-1}. It is thus apparent that the first electron transfer cannot be the rate limiting step but rather the last step in which the rate constant k_5 must surely have this function. In the presence of cytochrome b_5 and cytochrome P-450 at a molar ratio exceeding 0.5, cytochrome b_5 showed suppressive rather than stimulative effect. The inhibitory effect on hydroxylation was basically similar to that on the first electron transfer in Fig. 4. This may suggest that the last step of the hydroxylation cycle requires interaction between the reductase and cytochrome P-450 and the decrease in such interaction may lessen the rate of hydroxylation.

Increase in $[R']$ will certainly weaken the effect of cytochrome b_5 on the second electron

transfer, as apparent from equation (3). This would explain the decrease of stimulative effect of cytochrome b_5 on hydroxylation reaction activity with increase in the molar ratio of the reductase to $P\text{-}450_{C21}$ as shown in Fig. 2 and explain well the results of Hiwatashi *et al.* [13].

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