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ROLE OF CYTOCHROME b_5 IN THE CYTOCHROME P-450-MEDIATED C_{21}-STEROID 17,20-LYASE REACTION

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Summary: The cytochrome P-450 (P-450_{sccII}) and its reductase, NADPH-cytochrome reductase [EC 1.6.2.4], associated with conversion of progesterone to 4-androstene-3,17-dione, were extensively purified from pig testis microsomes. Higher lyase activity (turnover number of 15 mol of the product formed/min/mol of P-450) could be restored by mixing the P-450_{sccII}, its reductase, pig liver cytochrome b_5 and cytochrome b_5-reductase [EC 1.6.2.2], and phospholipid in the presence of NADPH, NADH, and O_2. Omission of either cytochrome b_5 or NADH resulted in a significant loss of the lyase activity indicating actual participation of cytochrome b_5 in this P-450-mediated steroidogenic system in the testis.

INTRODUCTION

At least two main important roles of mammalian P-450 are known, one, on the catabolism of a variety of exogenous or excreting substrates, and the other, on the biosynthetic metabolism of specific steroids of physiological importance such as hydroxylative activation of corticosteroids and D_3-vitamins, and biogenic conversions of cholesterol (C_{27}) to bile acid (C_{24}), cholesterol to corticosteroids (C_{21}), corticosteroids to androgens (C_{19}), and androgens to estrogens (C_{18}) (1). However, despite there is considerable literature on the involvement of b_5 in the P-450-mediated xenobiotic metabolism or catabolism (2-5), knowledge about requirement for b_5 in the steroid metabolism is meager.

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Abbreviations used: androstenedione, 4-androstene-3,17-dione; b_5, cytochrome b_5; FpD, cytochrome b_5 reductase [EC 1.6.2.2]; FpT, NADPH-cytochrome reductase [EC 1.6.2.4]; P-450, cytochrome P-450; P-450_{sccII}, C_{21}-steroid 17α-hydroxylating, 17,20-side-chain cleaving P-450; and SDS, sodium dodecyl sulfate.

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It is fairly recently that the effect of NADH and antibody against FpD was examined on the unpurified steroid 17,20-lyase from testis microsomes (6). The results of these experiments suggest possible participation of b_5 in the testicular P-450 system. We have, therefore, decided to ascertain further whether b_5 is involved in the steroid 17,20-lyase reaction using systems reconstituted with the purified constituents, which are now available (7). The present study provides the first direct evidence that b_5 is involved in the reconstituted progesterone 17,20-lyase system isolated from pig testicular microsomes whose protein constituents are highly purified P-$450_{scc\,II}$ and its reductase, FpT.

METHODS

Assays The steroid 17,20-lyase activity was measured by detecting the amount of radioactive androstenedione produced by the P-$450_{scc\,II}$-catalyzed cleavage of radioactive 17α-hydroxyprogesterone. The standard reaction mixture contained 20 pmol of P-$450_{scc\,II}$, 0.13 unit of FpT, 20 pmol of b_5, 0.2 unit of FpD, 80 nmol (about 1×10^4 cpm) of [7(n)-^{3}H]-17α-hydroxyprogesterone (Amersham International), 50 nmol of NADPH, 50 nmol of NADH, and 5 nmol of dilauroylphosphatidylcholine (Sigma), in 500 µl of 30 mM potassium phosphate buffer, pH 7.2. The reaction was initiated at 37° by the addition of NADPH. After 30-min incubation, the reaction mixture was combined with 1.6 ml of chloroform : methanol (1 : 1, v/v) and then appropriate amounts of [4-^{14}C]-androstenedione was added. After centrifugation, the extract was measured on separation of the radioactive androstenedione (Rf = 0.72) from the substrate (Rf = 0.53) by chromatography on thin layer plates (Merck silica gel 60 F-254) using chloroform : acetone (9 : 1, v/v) system. The radioactivity of the formed androstenedione was detected as described previously for other steroids (8). Alterations are given in figure and table.

FpT, FpD, and b_5 were determined by the methods of Yasukochi and Masters (9), Mihara and Sato (10), and Strittmatter et al. (11), respectively. P-450 was determined by the reduced CO-difference spectrum (12).

Preparative procedures Pig testes obtained at castration at the age of about 1-month were decapsulated and microsomes were prepared by the method of Shikita and Tamaoki for rat testis (13). After washing twice with 7 volumes of 150 mM KCl, the final pellet of microsomes was suspended in 250 mM sucrose. All the buffer (buffer A) used for the subsequent purification of P-$450_{scc\,II}$ contained indicated concentrations of potassium phosphate at pH 7.4, 100 µM dithiothreitol, 100 µM EDTA, 5 µM pregnenolone, and 20% (v/v) glycerol. The suspension (1.3 g of microsomal protein) was mixed with 150 mM buffer A, supplemented with Emulgen 913 (gift from Kao-Atlas) and sodium cholate. The final concentrations of microsomal protein, sucrose, buffer A, Emulgen 913, and cholate were 15 mg/ml, 100 mM, 100 mM, 1%, and 0.4%, respectively. After 30 min of exposure to the above solution at 0°, the unsolubilized

protein was removed by centrifugation at 136 × kg for 90 min. The solubilized preparation was purified for P-450$_{sccII}$ by the following chromatographic steps: hydroxylapatite (3.5 × 10 cm column, eluted with a gradient linear between 80 mM and 500 mM buffer A); aniline-Sepharose 4B (8) (1.9 × 11 cm column, eluted with a gradient linear between 0.2% and 1.5% Emulgen 913 in 20 mM buffer A, supplemented with 0.2% cholate); and CM-Sephadex C-50 (1.6 × 5 cm column, eluted with a gradient linear between 20 mM and 300 mM buffer A, supplemented with 0.2% Emulgen 913). The final preparation was stored in 150 mM buffer A, supplemented with 0.2% Emulgen 913. The purified protein of the P-450 was homogeneous as judged by electrophoresis on SDS-disc polyacrylamide gel according to the method of Laemmli (14). The preparation was practically free from FpT, FpD, and b_5.

For the preparation of pig testicular FpT, microsomes (1.5 g of microsomal protein) prepared as described above were solubilized for 60 min at 0° in 100 ml of 100 mM buffer B (the modified buffer A, in which pregnenolone was replaced by 1 µM FAD and 1 µM FMN), supplemented with 1% cholate. After removal of the unsolubilized protein by centrifugation at 136 × kg for 60 min, the supernatant fluid was fractionated with ammonium sulfate (between 0.3 and 0.6 saturations). The FpT fraction was further purified by the following successive steps: Ultrogel A 34 (3.7 × 60 cm column, eluted with 20 mM buffer B, supplemented with 0.1% cholate, 0.5% Emulgen 913, and 100 mM KCl); DEAE-Sephacel (1.3 × 15 cm column, eluted with a gradient linear between 0.1% cholate-100 mM KCl and 0.5% cholate-500 mM KCl in 20 mM buffer B); and 2',5'-ADP-Sepharose 4B (1.2 × 3.5 cm column, eluted with 20 mM buffer B, supplemented with 0.1% cholate and 500 µM $NADP^+$). The final preparation was dialyzed against 20 mM buffer B, supplemented with 0.05 % cholate, and was stored in the same buffer. This preparation was essentially homogeneous as judged by electrophoresis on SDS-disc polyacrylamide gel according to the method of Laemmli (14) and was practically free from P-450, FpD, and b_5.

FpD and b_5 were prepared from pig liver according to modification of established procedures (10, 11), respectively.

RESULTS AND DISCUSSION

Like its frequently found effect on the drug metabolism in liver microsomal P-450 systems, b_5 increased the rate of P-450$_{sccII}$-catalyzed steroid 17,20-lyase activity by the reconstituted testicular system. From the data in Table 1, it is clear that the maximum turnover (about 15 mol of product formed/min/mol of P-450) is obtained in the complete system in which all the components as specified in Methods section are present. The data also indicate that b_5 itself or in combination with NADH and FpD causes a synergistic effect on the rate of the lyase reaction. Comparative results were found when the effect of varying concentrations of b_5 and NADH was examined on the P-450$_{sccII}$-mediated lyase reaction (Fig. 1). As in Fig. 1A, b_5 stimulated the rate of the

Table 1. Effect of b_5, FpD, and NADH on the P-450$_{sccII}$-dependent formation of androstenedione from 17α-hydroxyprogesterone. The composition of the complete reaction mixture and assay are as described in Methods section.

Alterations	Apparent turnover number[1] mol/min/mol of P-450
None	14.7
b_5 boiled[2]	5.6
FpD boiled[2]	12.5
-NADH	10.1
-NADPH	0.9

[1]Individual values are avarage of at least two determinations.
[2]Allowed to stand for 20 min at 100° before the addition.

lyase reaction either in the presence of NADH or in its absence. In contrast, the stimulatory effect of NADH (Fig. 1B) was seen only in the presence of b_5. Therefore, whatever the mechanism and physiological significance, these results obtained from our reconstituted system are in general agreement with those frequently seen with liver microsomal drug-metabolizing systems in which b_5 has high affinity toward both FpT and FpD, and the second of

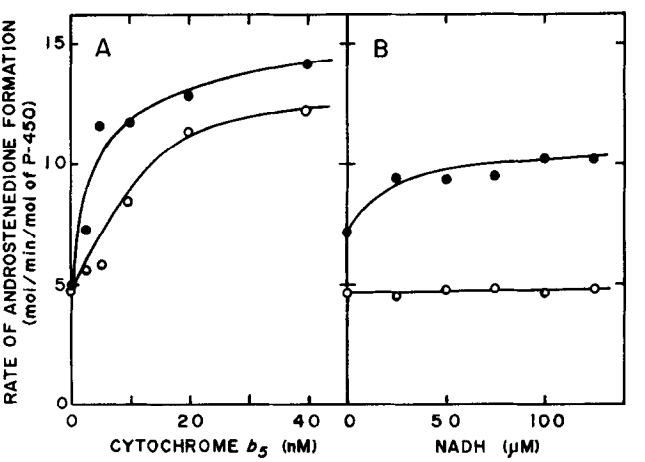

Fig. 1. Effect of varying concentrations of b_5 (A) and NADH (B) on the activity of the P-450$_{sccII}$-dependent 17α-hydroxyprogesterone 17,20-lyase reaction. In (A), boiled b_5 (100°, 20 min) was supplemented to hold the total (native plus boiled) concentrations of b_5 constant, and the formation of androstenedione was assayed with (•) or without (o) added NADH. In (B), the concentration of NADPH was 50 μM, and the formation of androstenedione was assayed in the presence of either native (•) or 20-min-boiled (o) b_5. Other conditions and assay are as described in Methods section.

the two electrons required for the P-450-mediated monooxygenase cycle may be supplied via either FpT or b_5 (2-5).

Of additional interest is the fact that, in the unpurified microsomal system of adrenal cortex which catalyzes 21-hydroxylation of corticosteroids, NADH supplemented with $NADP^+$ can serve as electron donor whereas NADH alone cannot (15). Although this effect of NADH has been considered to be simply due to contamination of $NAD(P)^+$ transhydrogenase, our preliminary experiments with a cholate-solubilized microsomal preparation from bovine adrenal cortex showed that b_5 markedly stimulated the rate of the P-450-mediated formation of 11-deoxycortisol from 17α-hydroxyprogesterone.

Thus, the results presented in this communication provide a prototype for studying possible effect of b_5 on the P-450-mediated steroid metabolism in various endocrine tissues, many of which have been shown to contain fairly large amounts of b_5 (16-18). Work is in progress to extend these studies and to examine the interactions between the components in the testicular steroid 17,20-lyase system.

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