Identification of Outer Mitochondrial Membrane Cytochrome $b_5$ as a Modulator for Androgen Synthesis in Leydig Cells*

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Tadashi Ogishima, Jun-ya Kinoshita, Fumiko Mitani, Makoto Suematsu, and Akio Ito
From the Department of Chemistry, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan and the Departments of Biochemistry and Integrative Medical Biology, Keio University, Tokyo 180-8582, Japan

Outer mitochondrial membrane cytochrome $b_5$ is an isoform of microsomal membrane cytochrome $b_5$. In rat testes the outer mitochondrial membrane cytochrome $b_5$ is present in both mitochondria and microsomes, whereas microsomal membrane cytochrome $b_5$ is undetectable. Outer mitochondrial membrane cytochrome $b_5$ present in the testis was localized in Leydig cells with cytochrome P-450$_{17\alpha}$, which catalyzes androgenesis therein. We therefore analyzed the functions of outer mitochondrial membrane cytochrome $b_5$ in rat testis microsomes by using a proteoliposome system. In a low but physiological concentration of NADPH-cytochrome P-450 reductase and excess amount of progesterone, outer mitochondrial membrane cytochrome $b_5$ stimulated the cytochrome P-450$_{17\alpha}$-catalyzed reactions, 17α-hydroxylation and C17-C20 bond cleavage. These were different from those by microsomal membrane cytochrome $b_5$ as follows: preferential elevation of the 17α-hydroxylase activity by outer mitochondrial membrane cytochrome $b_5$ in an amount-dependent manner versus that of the lyase activity by microsomal membrane cytochrome $b_5$ at the low concentration, and the inhibition of both activities at the high concentration. At a low concentration of progesterone reflecting a physiological cholesterol supply, outer mitochondrial membrane cytochrome $b_5$ elevated primarily the production of 17α-hydroxyprogesterone and then facilitated the conversion of the released intermediate to androstenedione. Thus, we demonstrated that outer mitochondrial membrane cytochrome $b_5$ and not microsomal membrane cytochrome $b_5$ functions as an activator for androgenesis in rat Leydig cells.

Two isoforms of cytochrome $b_5$ are known to exist in a single cell. One is microsomal cytochrome $b_5$ ($b_5$) in the endoplasmic reticulum (ER), and the other is outer mitochondrial membrane cytochrome $b_5$ (OMb) (1–3). They consist of the following three domains: (a) the amino-terminal hydrophilic, (b) medial hydrophobic, and (c) carboxyl-terminal hydrophilic domains. A protomer is bound to the first domain, which is highly conserved between $b_5$ and OMb with 70% identity (4, 5). The hydrophobic domain, consisting of about 20 amino acid residues, is embedded in the lipid bilayer and functions for the insertion of proteins into the membranes as tail-anchored proteins (6). The carboxyl-terminal 10 amino acid residues of $b_5$ are exposed to the luminal side of the ER cisterna (7, 8) and are required for the targeting of the cytochrome to the organelle (9). The visible spectroscopic properties of the reduced forms are characteristic of the $b_5$-type hemoproteins. OMb and $b_5$ are spectrographically indistinguishable from each other due to the highly conserved heme-binding portion (1, 2). OMb and $b_5$ have a similar mobility on SDS-PAGE and are co-purified unless specific precaution was taken in the purification procedures. Antibodies directed against $b_5$ that had been purified without the removal step of OMb cross-react with OMb. In some case, cross-reactions are observed even if a highly purified form of OMb or $b_5$ was immunized in rabbits. These facts made the discrimination between OMb and $b_5$ difficult. The most convincing way of the discrimination is use of the specific antibodies. We have such antibodies in a large collection of anti-OMb and anti-$b_5$ antibodies.

Because $b_5$ is a hemoprotein with sixth co-ordination, it is incapable of activating an oxygen molecule as cytochrome P-450 does. One of its physiological functions is an electron transfer to terminal oxidases such as stearyl-CoA desaturase (10). Some evidence also suggests that $b_5$ functions as a modifier for some cytochrome P-450-catalyzed reactions although its mechanism is not clear (11, 12). For example, $b_5$ stimulates the 6β-hydroxylation of testosterone and nifedipine oxidation by recombinant CYP3A4 (13). It also augments C17-C20 lyase activity of pig, guinea pig, and human P-450$_{17\alpha}$ leading to predominant formation of androgens (androstenedione and dehydroepiandrosterone) over 17α-hydroxylated steroids (progesterone and pregnenolone) (14–16). Without involvement of added $b_5$, the lyase activity of the P-450$_{17\alpha}$ is too low to account for physiological production of androgens in vivo.

The physiological functions of OMb are not well understood. The sole experimental evidence for the function is that specific antibodies against rat OMb inhibited semidehydroascorbate reductase, which catalyzes regeneration of half-oxidized ascorbic acid, in outer mitochondrial membrane fractions of rat liver cells (17, 18). In a study on the mechanism for subcellular localization of OMb, we have recently observed that it moves from the outer mitochondrial membrane to the ER membrane in a few hours after administration of dexamethasone, pregnenolone-16α-carbonitrile, or phenobarbital to rats.2 In tissues other than liver, such as kidney and adrenal glands of rats,
OMb was present in ER in addition to mitochondria. These results indicate that OMb has the potential to be localized either in outer mitochondrial membranes or in ER membranes, although the localization signal of OMb seems to reside in the carboxy-terminal 10 amino acid residues favorable for mitochondrial targeting (19). In guinea pig, OMb is abundant in adrenal glands, where its localization is exclusively ER. Surprisingly, b5 was occasionally detected on testicular cells. OMb is distributed almost equally between the outer mitochondrial membranes and ER membranes in considerably high amounts. This suggests that OMb and not b5 could regulate testicular androgen synthesis by modifying the lyase activity of P-450_17α. The indistinguishable properties between OMb and b5, as described above propose a possibility that the stimulatory effects on P-450 that have been believed to be done by b5 are exerted by OMb. Thus, in this study we analyzed the effects of OMb on rat P-450_17α, and we found that this cytochrome is a genuine modulator for testicular androgen synthesis in rats.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats weighing 120–150 g and male Hartley guinea pigs weighing 450–500 g were purchased from Kyudo Co., Ltd., (Osaka, Japan). All surgically prepared animals were approved and conducted in accordance with the policies of Kyushu University’s Animal Care and Use Committee.

Cellular Fractionation and Detection of OMb and b5—Male Sprague-Dawley rats weighing 120–150 g were intraperitoneally injected with sodium phenobarbital at a dose of 80 mg/kg and killed after 24 h. Liver and testes were removed and homogenized using a Teflon glass homogenizer in ice-cold 10 mM Tris-HCl buffer (pH 7.6) containing 0.25 mM sucrose, 0.1 mM EDTA, 2 μg/ml leupeptin, and 2 μg/ml peptide A. After the homogenates were centrifuged at 6000 × g for 10 min, the resultant supernatants were re-centrifuged at 60,000 × g for 10 min to obtain the mitochondrial fractions. Microsomal fractions were obtained by centrifugation of the post-mitochondrial supernatants at 105,000 × g for 60 min. All procedures were done at 4 °C. For analysis of the distribution of OMb and b5 in the tissues, the subcellular fractions form the tissues and were separated by Tricine/SDS-PAGE (20) and subsequently transferred to polyvinylidene difluoride membranes. The membrane was incubated first with specific anti-rat OMb or anti-rat b5 IgG and second with goat anti-rabbit IgG-horseradish peroxidase complex, and the immunoreactive bands were finally visualized by chemiluminescence using Western LightingTM (PerkinElmer Life Sciences).

Enzyme Preparations—All enzyme preparations except as described otherwise were conducted with K-P buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Rat liver microsomes were obtained from rats treated with 1000 x g for 15 min. After sonication the suspension was centrifuged at 177,100 × g for 1 h. The pellets were solubilized in 10 mM Hepes-KOH (pH 7.5) buffer containing 20% glycerol and 2% Triton X-100 for 1 h at 4 °C. After centrifugation at 177,100 × g for 1 h, the resultant supernatant was applied to a Ni2+-preloaded HiTrap column (1 ml, Amersham Biosciences, Piscataway, NJ) equilibrated and eluted with buffer containing 20% glycerol, 0.1% Triton X-100, 10 mM imidazole, and 0.5 mM NaCl. After washed with the equilibrating buffer, the column was eluted with the same buffer except containing 0.5 mM imidazole. Because effects of the recombinant OMb on the P-450_17α-catalyzed reaction were indistinguishable from those of the native forms that were purified from testes and livers, the recombinant OMb was mainly used in this study.

Measurement of 17α-Hydroxylase and C17-20 Lyase Activities of P-450_17α—P-450_17α, containing proteoliposomes were preincubated with P-450 reductase and either OMb or b5 in a volume of 0.05 ml of 50 mM K-P buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM diethiothreitol for 60 min at 30 °C. After diluting 10-fold with 20 mM Hepes-KOH (pH 7.4), progesterone in propylene glycol was added to the reaction mixture. Reaction was started with addition of NADPH at a final concentration of 1 mM and proceeded for 10–20 min at 30 °C. The reaction was stopped by addition of 2 ml of n-hexane, and the products were extracted twice with the solvent. After drying under a stream of N2 gas, the steroids were separated by high pressure liquid chromatography using a COSMOSIL SSL-II column (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan). The eluate was eluted with a solvent (n-hexane:2-propanol:acetic acid = 95:5:1). Androstenedione (lyase activity) and 17α-hydroxyprogesterone (17α-hydroxylase activity) were quantified by measuring their absorbance at 240 nm. In some experiments, 17α-hydroxyprogesterone was used as the substrate.

Expression and Purification of OMb—The construct pET-hisOMb was cultured for 40 h at 25 °C and immediately fixed in 4% paraformaldehyde solution buffered with 10 mM phosphate, pH 7.4, at 4 °C overnight. Fixed tissues were then dehydrated and embedded in paraffin. Three-micron sections were deparaffinized and hydrated using graded alcohol concentrations for standard indirect peroxidase immunohistochemistry. In brief, the prepatterned sections were treated first with 0.5% hydrogen peroxide for 15 min. After blocking with 5% normal goat serum in PBS, the sections were incubated for 2 h at room temperature with the P-450_17α monoclonal antibody (EnzymoPreps, Inc., Aurora, OH). The immunoreactive proteins were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat Fab’ fragment to rabbit immunoglobulin G (ICN Pharmaceuticals, Inc., Aurora, OH). The immunoreactive proteins were visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide as described before (27). Nuclear counterstaining was performed using methyl green (Lab Vision Corp., Fremont, CA). The endogenous peroxidases and then with 5% of normal goat serum in PBS. The sections were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat Fab’ fragment to rabbit immunoglobulin G (ICN Pharmaceuticals, Inc., Aurora, OH). The immunoreactive proteins were visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide as described before (27). Nuclear counterstaining was performed using methyl green (Lab Vision Corp., Fremont, CA). The endogenous peroxidases and then with 5% of normal goat serum in PBS.
RESULTS

Localization of OMb in Rat Testis Cells—The antibodies used in this study are very specific. No cross-reaction between OMb and anti-b5 IgG nor between b5 and anti-OMb IgG was observed if appropriate application of the samples and appropriate dilution of the antibodies were employed. Fig. 1 shows that OMb was distributed between mitochondrial and microsomal fractions almost at a ratio of 1:1 on a protein concentration basis in rat testicular cells, where b5 was not detected. A large amount of the sample loading on the SDS-PAGE scarcely detected the b5 band, which is less than 1/100 that of liver cells. Amounts of OMb present in testes were comparable with those in liver per protein. Dual localization (mitochondria and microsomes) of OMb was also observed in the kidneys and adrenal glands (data not shown). Although localization of OMb in the liver cells had been believed to be strictly the outer mitochondrial membrane, translocation of OMb from there to the microsomal membranes was observed at 12 h after administration of phenobarbital to rats. Because this reagent induces not only P-450 reductase in rat liver microsomes but also P-450 enzymes of the CYP2A but also CYP3A families, the amount of CYP3A2 was increased in the microsomal fraction. Translocation of OMb to the microsomes was reproduced with other P-450-inducible agents such as dexamethasone, and pregnenolone-16α-carbinolactone (data not shown). Rat OMb thus has a capability to reside in both the outer mitochondrial and microsomal membranes. In the testicular cells, translocation of OMb as manifested in the liver cells was not observed as tested so far by administration of phenobarbital or dexamethasone. In contrast to OMb, b5 did not change its localization in liver and other cells. Precise results and discussion about the translocation of OMb in liver cells are to be published elsewhere.2

Purification of Enzymes Used in This Study—Fig. 2A shows the purity of the enzyme preparations used in this study. We purified P-45017α, b5, and OMb from rat testis microsomes and both b5 and P-450 reductase from rat liver microsomes. We mainly used a recombinant OMb because this form had the same effects on rat P-45017α with the native form that was purified from the testis microsomes. Use of the recombinant form allowed us to perform the experiments with a constant and sufficient supply of OMb. The data presented herein were collected by using the recombinant OMb instead of the purified OMb. Guinea pig enzymes were purified from the adrenal glands because the P-45017α, b5, and OMb were probably most abundant in the tissue. The specific content of P-45017α in rat testis microsomes was 0.1 nmol/mg protein as determined from the CO difference spectrum. Contribution of P-450scc, a mitochondrial protein, to the spectrum is negligible for the microsomal preparation as verified from immunoblotting with anti-P-450 reductase-IgG and goat anti-rabbit IgG-horseradish peroxidase complex. The chemiluminescence of the immunoreactive bands was calibrated with those of standard bands (lanes 1, 2, and 9–11; 0.25 0.125, 0.0125, 0.025, and 0.1 pmol, respectively). The integration was performed with CS Analyzer (version 2.0, Atto Instruments).

Efficient Electron Transfer Enhances Lyase Activity of P-45017α—By using excess amounts of progesterone as the substrate, we can measure both 17α-hydroxylase and lyase activities simultaneously because P-45017α first converts the steroid to the 17α-hydroxylated intermediate, 17α-hydroxyprogesterone, and then the second P-450-catalyzed reaction cleaves the C17–C20 bond to give androstenedione without the 17β-hydroxyprogestosterone formation from pregnenolone by bovine P-45017α. Proceeded successively (28, 29). In the successive reaction, rates of release of the intermediate from the substrate-binding pocket in the enzyme and the C–C bond cleavage together with release of the

![Fig. 1: Distribution and translocation of OMb and b5 in rat liver and testis](image1)

![Fig. 2: Purities of enzymes used in this study and quantification of P-450 reductase in rat testis microsomes](image2)
mediate. The lyase activity was about 1/10th the 17β-hydroxylation activity without affecting the release of the intermediate and C-C bond cleavage reaction. This assumption is valid as shown in Fig. 3.  

Effects of OMb and b5 on 17β-hydroxyprogesterone.  

Addition of OMb to the P-450 17β-hydroxylase activity at a ratio of P-450 reductase:rat P-45017β of 2.5 times the rat P-45017β activity continued to increase up to a ratio of 0.5, where the activity was 6-fold and that of the 17β-hydroxylase activity about 3- and 4.5-fold, respectively (Fig. 4B). It stimulated the lyase activity to an extent similar to that of OMb (3-fold) at ratios of b5 to P-45017β of 0.2–0.5, whereas it enhanced the 17α-hydroxylase activity about twice at the most. At the high ratio, the 17α-hydroxylase and lyase activities dropped to the same value and one-third of the original (without b5) activity, respectively (Fig. 4B), and further decreased above the ratio of 1.0 (data not shown). The differences in the stimulatory effects between OMb and b5 were apparent when the 17α-hydroxylase:lyase activity ratio was plotted in the y axis (Fig. 4C). OMb had a tendency to elevate preferentially the 17α-hydroxylase, whereas b5 stimulated the lyase preferentially. A rise of the 17α-hydroxylase:lyase at a ratio of b5 to P-45017β of 1.0 was due to the strong inhibitory effect on the lyase in a high concentration of b5 relative to P-45017β.

Effects of OMb and b5 on 17α-Hydroxylase and Lyase of Guinea Pig P45017β—In general, P-45017β of most animals has strong 17α-hydroxylase and weak lyase activities in the absence of b5 except murine P-45017β. In the guinea pig P45017β-catalyzed reaction, b5 has been reported to stimulate the lyase activity without significant elevation of the 17α-hydroxylase activity (30). We purified b5 from microsomes of guinea pig adrenals and analyzed the effects of these proteins toward the P-45017β that had also been purified from the same membrane preparations and embedded in proteoliposomes. Rat b5 greatly stimulated the lyase activity up to a ratio of b5 to guinea pig P45017β of 0.5 together with slight activation of the 17α-hydroxylase. However, it reduced both activities at the high ratio (Fig. 5B). The stimulatory profile of rat b5 on guinea pig P45017β was essentially the same on rat P-45017β except that activation of the lyase was more dominant than that of the 17α-hydroxylase and that the maximum stimulation of both activities occurred at lower ratios: b5:P-45017β of 0.05–0.1. At a ratio of rat b5 to guinea pig P45017β of 0.5, the increase in the lyase activity was about 6-fold and that of the 17α-hydroxylase was 1.5-fold. Guinea pig b5 also greatly stimulated the lyase activity up to the ratio of 0.5, where the activity was 6-fold and the effect reached a plateau (Fig. 5E). The 17α-hydroxylase activity continued to increase up to a ratio of guinea pig b5 to guinea pig P45017β of 1.0. In contrast, the stimulatory effect of rat OMb on guinea pig P45017β was not observed over the range measured (Fig. 5A). Only activation of the 17α-hydroxylase was observed in an OMb-dependent manner up to a ratio of rat OMb to guinea pig P45017β of 0.5, where stimulation was about 3.5-fold. The effect of guinea pig OMb on guinea pig P45017β was intermediate between those of rat OMb and guinea pig b5 (Fig. 5D). Guinea pig OMb stimulated the 17α-hydroxylase and lyase activities about 3- and 4.5-fold, respec-ted at a low ratio of OMb to P-45017β when the electron transfer was slow due to a low concentration of P-450 reductase (compare the profiles in Fig. 3, B–D). Because the amount of the reductase is very low in the testis microsomes as revealed by immunoblotting with anti-P-450 reductase IgG (Fig. 2B), we hereafter studied the effects of OMb at a ratio of P-450 reductase:P-45017β of 1:50 unless otherwise mentioned. OMb stimulated the 17α-hydroxylase activity linearly at ratios of OMb to P-45017β below 0.1 and continued to increase it gradually up to the ratio of 1.0 to an extent of 7–8-fold (Fig. 4A). Stimulation of the lyase activity was also observed but reached a plateau at a ratio of OMb to P-45017β of about 0.1. Above the molar ratio of OMb:P-45017β of 1.0, the lyase activity was almost constant up to the ratio of 2.0, whereas the 17α-hydroxylase activity continued to increase gradually (data not shown). Maximum stimulation of the lyase activity by OMb was about 3-fold. Addition of b5 to the reaction system containing P45017β and P450 reductase also augmented both lyase and 17α-hydroxylase activities but with a manner different than that of OMb (Fig. 4B). It stimulated the lyase activity to an extent similar to that of OMb (3-fold) at ratios of b5 to P45017β of 0.2–0.5, whereas it enhanced the 17α-hydroxylase activity about twice at the most. At the high ratio, the 17α-hydroxylase and lyase activities dropped to the same value and one-third of the original (without b5) activity, respectively (Fig. 4B), and further decreased above the ratio of 1.0 (data not shown). The differences in the stimulatory effects between OMb and b5 were apparent when the 17α-hydroxylase:lyase activity ratio was plotted in the y axis (Fig. 4C). OMb had a tendency to elevate preferentially the 17α-hydroxylase, whereas b5 stimulated the lyase preferentially. A rise of the 17α-hydroxylase:lyase at a ratio of b5 to P45017β of 1.0 was due to the strong inhibitory effect on the lyase in a high concentration of b5 relative to P45017β.  

C17 ketosteroid determine yields of both the 17α-hydroxyl and 17-ketosteroids. Based on this assumption, efficient supply of electrons from P-450 reductase to P-45017β should facilitate the cleavage reaction. This assumption is valid as shown in Fig. 3. Production of androstenedione was increased in a linear fashion with the increase of P-450 reductase added (Fig. 3A). The amount of 17α-hydroxyprogesterone produced, however, was constant in the range tested. These indicate that the first hydroxylation step is relatively fast and that the following steps (i.e. the release of the intermediate and C-C bond cleavage) are rate-limiting as shown previously (28). The increase of the electron transfer should enhance the second P-450-catalyzed reaction (i.e. C17-C20 bond cleavage) leading to androstenedione formation without affecting the release of the intermediate. The lyase activity was about 1/10th the 17α-hydroxylase activity at a low ratio of P-450 reductase to P-45017β (reductase:P-45017β of 1:100), and it was more than 2.5 times the 17α-hydroxylase activity at a ratio of P-450 reductase to P-45017β of 0.5. Obviously, rat P-45017β has a lyase activity that is higher than the 17α-hydroxylase activity compared with the guinea pig enzyme (see Refs. 28 and 30 and see the text below).

Effects of OMb and b5 on 17α-Hydroxylase and Lyase of Rat P-45017β—Addition of OMb to the P-45017β-dependent reaction system enhanced both the 17α-hydroxylase and lyase activities over the range of ratios of P-450 reductase to P-45017β tested. The stimulatory effects, however, tended to be satu-
were quantified as described under "Experimental Procedures." androstenedione; \( \bullet \), 17\( \alpha \)-hydroxyprogesterone. The product ratio of 17\( \alpha \)-hydroxyprogesterone:androstenedione was plotted (C). 17OHP, 17\( \alpha \)-hydroxyprogesterone; AND, androstenedione. The values in the presence of OMB (\( \square \)) and \( b_5 \) (\( \triangle \)) are shown.

**Fig. 4. Effects of rat OMB and \( b_5 \) on rat P-450\(_{17\alpha} \)-catalyzed reactions.** The P-450\(_{17\alpha} \)-containing proteoliposomes were incubated with P-450 reductase (0.02 eq) and various amounts of rat OMB (A), rat \( b_5 \) (B), guinea pig OMB (D), and guinea pig \( b_5 \) (E) for 60 min at 0 °C. The enzyme reaction was performed, and the products were quantified as described under "Experimental Procedures." \( \square \), androstenedione; \( \bullet \), 17\( \alpha \)-hydroxyprogesterone. The product ratio of 17\( \alpha \)-hydroxyprogesterone:androstenedione was plotted for the rat enzymes (C) and guinea pig enzymes (F). 17OHP, 17\( \alpha \)-hydroxyprogesterone; AND, androstenedione. The values in the presence of OMB (\( \square \)) and \( b_5 \) (\( \triangle \)) are shown.

![Graph showing effects of rat OMB and \( b_5 \) on rat P-450\(_{17\alpha} \)-catalyzed reactions.](image)

**Fig. 5. Effects of OMB and \( b_5 \) on guinea pig P-450\(_{17\alpha} \)-catalyzed reactions.** The guinea pig P-450\(_{17\alpha} \)-containing proteoliposomes were incubated with P-450 reductase (0.02 eq) and various amounts of rat OMB (A), rat \( b_5 \) (B), guinea pig OMB (D), and guinea pig \( b_5 \) (E) for 60 min at 0 °C. The enzyme reaction was performed, and the products were quantified as described under "Experimental Procedures." \( \square \), androstenedione; \( \bullet \), 17\( \alpha \)-hydroxyprogesterone. The product ratio of 17\( \alpha \)-hydroxyprogesterone:androstenedione was plotted (C). 17OHP, 17\( \alpha \)-hydroxyprogesterone; AND, androstenedione. The values in the presence of OMB (\( \square \)) and \( b_5 \) (\( \triangle \)) are shown.

![Graph showing effects of OMB and \( b_5 \) on guinea pig P-450\(_{17\alpha} \)-catalyzed reactions.](image)

activity as the ratio to rat P-450\(_{17\alpha} \) increased, and the activation reached 5-fold at the ratio of 1.0. As shown in the successive reaction with an excess amount of progesterone (Fig. 5A), the inability of rat OMB to exert a stimulatory effect on the lyase activity of guinea pig P-450\(_{17\alpha} \) toward 17\( \alpha \)-hydroxyprogesterone was reproduced.

**Effect of OMB on P-450\(_{17\alpha} \)-catalyzed Reaction at a Low Concentration of Progesterone**—The experiments on the effects of OMB and \( b_5 \), which were described above, were performed with a saturating concentration of progesterone (0.1 mM). Under this condition, we measured initial velocities of both 17\( \alpha \)-hydroxylase activity and successive 17\( \alpha \)-hydroxyprogesterone; androstenedione was plotted (C). 17OHP, 17\( \alpha \)-hydroxyprogesterone; AND, androstenedione. The values in the presence of OMB (\( \square \)) and \( b_5 \) (\( \triangle \)) are shown.

Effects of OMB and \( b_5 \) on Lyase Activity toward 17\( \alpha \)-Hydroxyprogesterone—We then directly analyzed the C17-C20 lyase activities toward 17\( \alpha \)-hydroxyprogesterone using the steroid as the substrate (Fig. 6). Rat \( b_5 \) augmented the lyase activity of rat P-450\(_{17\alpha} \) up to a ratio of \( b_5 \) to P-450\(_{17\alpha} \), of 0.5 about 4-fold, but the activity drastically dropped at the ratio of 1:0. Such a stimulatory profile was essentially the same on guinea pig P-450\(_{17\alpha} \) by rat \( b_5 \). Rat OMB stimulated the lyase activity as the ratio to rat P-450\(_{17\alpha} \) increased, and the activation reached 5-fold at the ratio of 1.0. As shown in the successive reaction with an excess amount of progesterone (Fig. 5A), the inability of rat OMB to exert a stimulatory effect on the lyase activity of guinea pig P-450\(_{17\alpha} \) toward 17\( \alpha \)-hydroxyprogesterone was reproduced.
its C17-C20 cleavage. At 1.5 μM progesterone, rat P-45017α alone produced a small amount of androstenedione together with a little more 17α-hydroxyprogesterone (Fig. 7). In the presence of rat OMb (0.5 eq), P-45017α rapidly converted progesterone mainly to 17α-hydroxyprogesterone and secondly to androstenedione reflecting the product ratio under an excess amount of progesterone as shown in Fig. 4A. The amount of 17α-hydroxyprogesterone was constant between 30 and 60 min, during which period the input and output were equilibrated and then started to decline, and the intermediate was finally almost consumed. The rate of decrease of progesterone became slow after 60 min and seemed to have stopped at 120 min. On the other hand, production of androstenedione accelerated after 30 min and persisted up to 120 min, at which time point most of the substrate and intermediate were consumed. Addition of rat b5 to the P-45017α-catalyzed reaction system stimulated productions of 17α-hydroxyprogesterone as much as that of OMb during the first 30-min reaction and facilitated a rapid consumption of the intermediate after 30 min. It enhanced androstenedione formation more than that of OMb at the first 30-min reaction, and the formation accelerated to 60 min and had almost stopped by that time probably because of exhaustion of usable substrates (progesterone and 17α-hydroxyprogesterone). The reason for a significant amount of progesterone remaining even after 180 min of incubation could be attributed to an inhibitory effect of androstenedione on the P-45017α-catalyzed reaction. Although androstenedione formation facilitated by b5 was faster than that by OMb, amounts of the product at 120 min were almost equal. After the reaction for 180 min, the androstenedione formation stimulated by OMb was more than five times that by P-45017α alone. Effects of rat OMb and b5 on guinea pig P-45017α were also examined in the reactions at a low concentration of progesterone. Rat b5 stimulated androstenedione formation by guinea pig P-45017α in a fashion similar to that by rat P-45017α. In contrast, rat OMb did not exert any stimulatory effects on the production despite a significant activation of 17α-hydroxyprogesterone production (data not shown).

Localization OMb in Leydig Cells of Rat Testis—Because testicular cells are heterogeneous, the following question arises: Is OMb really present in the steroidogenic cells, i.e., Leydig cells? To solve this, we conducted immunohistochemical analysis using anti-rat OMb IgG on the rat testis. OMb as well as P-45017α was only detected in Leydig cells; no positive reactions were observed in Sertoli cells, spermatogonia, spermatocytes, spermatids, and spermatzoa (Fig. 8). In contrast, cells were not significantly stained with anti-rat b5 IgG (data not shown).

Localization of OMb in Leydig cells was also confirmed with the guinea pig testis (data not shown).

Discussion
Although the roles of OMb have not been known, those of b5 are considerably well known. They are as follows: (a) transfer of electrons from NADH to desaturases such as stearoyl-CoA desaturase (10) and Δ7-sterol 5-desaturase (31); (b) NADH-dependent reduction of methemoglobin to regenerate hemoglobin (32), and (c) stimulation of some P-450-dependent oxygenation. The intracellular localization of b5 is absolutely ER, whereas that of OMb is variable between outer mitochondrial and ER membranes from cells to cells or animals to animals. Furthermore, OMb changes its localization at least in hepatic cells under different conditions (Fig. 1). In Leydig cells of rat testes, OMb is distributed between outer mitochondrial and ER membranes. In contrast, b5, which is believed to be responsible for stimulation of the P-45017α, of several animals other than rats in efficient production of androgen, is scarcely detectable in rat testicular cells. In the present study, we analyzed the effects of rat OMb on P-45017α purified from rat testis microsomes using a reconstituted system with proteoliposomes. This system is believed to provide membrane proteins with a reaction field comparable with the biomembrane environment and allowed us to perform quantitative analyses in such a way as controlling the enzyme, modulator, and substrate concentrations. Androgen syntheses from progesterone by guinea pig P-45017α (28) and from pregnenolone by bovine P-45017α, (29) are proven to proceed by a successive reaction in the presence of excess amounts of progesterone, in which efficient electron supplies from P-450 reductase to P-450 facilitate the C17-C20 cleavage of the enzyme, modulator, and substrate concentrations. 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could be attributed to some interaction of $b_5$ with the reductase or even with P-450$_{17\alpha}$ in a complicated manner. Rat OMB also showed stimulatory effects on rat P-450$_{17\alpha}$ with some different manners. The lyase activity was increased linearly to the amount of OMB at low ratios of OMB to P-450$_{17\alpha}$ and reached a constant around the ratio of 0.2 without a significant decrease at the high ratios. Increase in the 17$\alpha$-hydroxylase activity was larger than that in the lyase activity, and the increase curve also tended to saturate around the ratio of 0.2 but proceeded gradually to the ratio of 1.0 and more. Differences of the stimulation profiles for P-450$_{17\alpha}$ between $b_5$ and OMB was more obvious when they were evaluated for effects on guinea pig P-450$_{17\alpha}$, which has a very high 17$\alpha$-hydroxylase activity relative to the lyase activity. The effects of rat $b_5$ were essentially the same for rat P-450$_{17\alpha}$, whereas rat OMB only activated the 17$\alpha$-hydroxylase activity without elevation of the lyase activity. Although a reaction system of guinea pig P-450$_{17\alpha}$ with rat $b_5$ or rat OMB is non-physiological, it disclosed differences of the effects of two similar hemoproteins clearly.

In rat testicular cells, OMB is present nearly equally in both the mitochondria and microsomes with a concentration comparable with that in liver mitochondria. By immunohistochemical analyses, the rat testicular OMB was localized in Leydig cells, where P-450$_{17\alpha}$ coexisted. In contrast, we scarcely detected $b_5$ in the rat testis microsomes, not to mention its presence in the mitochondria, by cell fractionation. Immunohistochemical analysis using anti-rat $b_5$ IgG did not detect significant staining of $b_5$ in Leydig cells.

There have been numerous reports on stimulation of $b_5$ on P-450$_{17\alpha}$ except rat enzymes employing various assay systems (14–16, 33). Most of them confirmed the function as a stimulator for the lyase. There is no study, however, on involvement of OMB in the androgen synthesis except a recent one on human OMB (type 2 cyt-5) and P-450$_{17\alpha}$ both expressed transiently in HEK-293 cells (34). In the present study employing enzymatic and cell biological analyses, we were able to demonstrate the role of OMB and not $b_5$ as a physiological modulator for rat testicular P-450$_{17\alpha}$. Although rat OMB preferentially stimulates 17$\alpha$-hydroxypregesterone production from progesterone, it should be an activator for androgen synthesis by P-450$_{17\alpha}$. In an excess amount of progesterone, rat P-450$_{17\alpha}$ produces androstenedione by the successive reaction from the substrate. This situation, however, could not be feasible under physiological conditions. In steroidogenic cells, cholesterol, the precursor of steroidogenesis, is supplied from a pool in the outer mitochondrial membrane to the inner membrane where P-450$_{scc}$ is present through action of steroidogenic acute regulatory (StAR) protein (35). Activation of StAR protein is triggered by the stimulus of pituitary hormones such as luteinizing hormone and ACTH. The cholesterol is rapidly converted to pregnenolone by the rate-limiting enzyme, P-450$_{scc}$, and the pregnenolone is then oxidized by 3$\beta$-hydroxysteroid dehydrogenase to give a transient increase of progesterone. The produced progesterone is promptly consumed to the lower stream products because steroidogenetic enzyme systems in general have no rate-limiting step other then the side chain cleavage. Such a flow of steroids would be reflected by the reaction condition with a low amount of progesterone as conducted in the experiment of Fig. 7. At a low concentration of progesterone, OMB stimulates rat P-450$_{17\alpha}$ to produce both 17$\alpha$-hydroxyprogesterone and androstenedione, in which 17$\alpha$-hydroxyprogesterone production is preferential. As the result, 17$\alpha$-hydroxyprogesterone accumulates and then serves as the second but main substrate. The intermediate steroid is then converted to androstenedione, which process is also stimulated by OMB. Although the rate of androgen production stimulated by $b_5$ is about twice as fast as that by OMB, the amount at 120–180 min was nearly the same for both hemoproteins. The question then arises as to why OMB and not $b_5$ acts as the modulator for androgen production in the rat testis. One reason could be the inhibitory effects of $b_5$ on P-450$_{17\alpha}$ at a high ratio of $b_5$ per P-450$_{17\alpha}$. In this respect, OMB does not inhibit P-450$_{17\alpha}$ activity at the ratio of 1.0. The early study by Onoda and Hall (14) pointed out difference of the effects between pig liver and newborn testis $b_5$ on androgen production in adult pig testis microsomes. The testicular $b_5$ stimulated concentration-dependently the lyase activity, whereas the liver $b_5$ had an optimum concentration for the maximum stimulation. Rat OMB has a tendency to stimulate preferentially the 17$\alpha$-hydroxylase activity of P-450$_{17\alpha}$. In fact, rat P-450$_{17\alpha}$ has a week 17$\alpha$-hydroxylase activity relative to the P-450$_{17\alpha}$ of other animals. A high 17$\alpha$-hydroxylase activity is necessary for cortisol production by other animals than murines, which do not produce cortisol due to the absence of P-450$_{17\alpha}$ in the adrenal glands. Preferential stimulation of the 17$\alpha$-hydroxylase activity by rat OMB suggests that the considerably high 17$\alpha$-hydroxylase should be needed for an unknown reason in Leydig cells. Finally, the mobile property of OMB in rat liver cells (Fig. 1) could also have a physiological role as a modulator that transfers between mitochondria (inactive) and ER (active) in Leydig cells, although we have not analyzed translocation of OMB in rat testicular cells.

There are essentially two possible explanations for the stimulatory effects of $b_5$ on some P-450-catalyzed reactions. One is that $b_5$ is involved in the second electron transfer during the P-450 17$\alpha$-hydroxylase activity of $b_5$.
tion by CYP3A4, however, disapproved (16). A second explanation is to give P-450 conformational changes through complex formation between the P-450 and \(b_\text{p} \). Thus, the P-450 obtains a stimulated catalytic activity in the complex. The stimulatory profile of P-450\(_{17\alpha}\) activities in the presence of \(b_\text{p} \) thus suggests some interaction between these proteins but with complexity (Figs. 4B and 5B). The effect of the amount of P-450 reductase on the lyase activity of P-450\(_{17\alpha}\) in the present study (Fig. 3A) does not seem in conflict with the first hypothesis. The more efficiently the electrons are supplied, the more rapidly the second P-450-catalyzed reaction (C17-C20 cleavage) proceeds, and thereby production of androstenedione overtakes the release of androstenedione. Thus, the P-450 obtains a role as modulator for androgenesis (29). The effect of the amount of P-450 reductase on the lyase activity of P-450\(_{17\alpha}\) is to give P-450 conformational changes through complex formation between the P-450 and \(b_\text{p} \). The functions in other tissues as well as the mechanism and function of the translocation between mitochondria and ER \(OMb\). The functions in other tissues as well as the mechanism and function of the translocation between mitochondria and ER should be elucidated.

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