Cytochrome b₅ Augments the 17,20-Lyase Activity of Human P450c17 without Direct Electron Transfer*

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In the biosynthesis of steroid hormones, P450c17 is the single enzyme that catalyzes both the 17α-hydroxylation of 21-carbon steroids and the 17,20-lyase activity that cleaves the C₁₇-C₂₀ bond to produce C₁₉ sex steroids. Cytochrome b₅ augments the 17,20-lyase activity of cytochrome P450c17 in vitro, but this has not been demonstrated in membranes, and the mechanism of this action is unknown. We expressed human P450c17, human P450-oxidoreductase (OR), and/or human cytochrome b₅ in Saccharomyces cerevisiae and analyzed the 17α-hydroxylase and 17,20-lyase activities of the resulting yeast microsomes. Yeast expressing only P450c17 have 17α-hydroxylase and 17,20-lyase activities toward both Δ⁴ and Δ⁵ steroids. Coexpression of human OR with P450c17 increases the Vₘₐₓ of both the 17α-hydroxylase and 17,20-lyase reactions 5-fold; coexpression of human b₅ with P450c17 also increases the Vₘₐₓ of the 17,20-lyase reactions but not of the 17α-hydroxylase reactions. Simultaneous expression of human b₅ with P450c17 and OR, or addition of purified human b₅ to microsomes from yeast coexpressing human P450c17 and OR, further increases the Vₘₐₓ of the 17,20-lyase reaction without altering 17α-hydroxylase activity. Genetically engineered yeast and mixing experiments demonstrate that OR is both necessary and sufficient for microsomal 17,20-lyase activity. Addition of purified human holo-b₅, apo-b₅, or cytochrome c to microsomes containing both human P450c17 and OR demonstrate that the stimulatory action of b₅ does not require electron transfer from b₅ to P450c17. These data suggest that human b₅ acts principally as an allosteric effector that interacts primarily with the P450c17-OR complex to stimulate 17,20-lyase activity.

Among the many chemical transformations catalyzed by cytochrome P450 enzymes, steroid hormone hydroxylations, and cleavages are of particular interest because of their mechanistic complexities and essential roles in physiology (1). P450c17 catalyzes both 17α-hydroxylase and 17,20-lyase activities (2) (for review see Ref. 3) and also has a modest degree of 16α-hydroxylation activity (4). In human beings, the 17α-hydroxylase reaction leads to the glucocorticoid, cortisol, and the subsequent 17,20-lyase reaction leads to precursors of sex steroids. As the sole pathway leading to biosynthesis of circulating sex steroids, the regulation of this 17,20-lyase activity is central to understanding the developmental regulation of dehydroepiandrosterone sulfate (DHEA)³ with adrenarche and aging, and to the pathogenesis of the polycystic ovary syndrome (3). The 17,20-lyase activity, involving the oxidative cleavage of a carbon-carbon bond, is regulated in a tissue-specific and developmentally programmed manner by factors such as the abundance of the electron donor flavoprotein P450-oxidoreductase (OR) (5, 6), the co-existence of 3β-hydroxysteroid dehydrogenase and P450c21 (7), and post-translational modification of P450c17 (8).

To perform catalysis, P450c17, like all other microsomal P450 oxidases, must receive two electrons from NADPH via OR. Cytochrome b₅ has also been implicated as a component of the 17,20-lyase reaction, as b₅ augments 17,20-lyase activity and occasionally 17α-hydroxylase activity of P450c17 in reconstituted systems (9, 10); however, our laboratory could not confirm this effect in transfected monkey kidney COS-1 cells (5). Inconsistencies in the animal species of P450c17, OR, and b₅ used in previous studies preclude extrapolation of the available biochemical data to human adrenal and gonadal physiology; furthermore, the mechanism(s) of these reported b₅-mediated increases in 17,20-lyase activity remain unknown.

Among the various systems developed to study mammalian cytochromes P450, transfection of genetically modified yeast cells provides the opportunity to study the activities of cytochrome P450 in the presence of various combinations of electron transfer proteins in the native microsomal environment (11). To clarify the function of cytochrome b₅ in 17,20-lyase activity, we systematically varied the abundance of putative electron transfer proteins in yeast microsomes containing human P450c17. We find that human, but not yeast cytochrome b₅, can selectively augment the rate of the 17,20-lyase reaction by more than 10-fold. However, this augmentation requires OR and occurs without electron transfer to or from cytochrome b₅.

EXPERIMENTAL PROCEDURES

Yeast Strains and Expression Vectors—Wild type yeast strains W303A (Y150WT) (lea2-3, 112; his3-11, 15; trp1-1; ade2-1; ura3-1; mat a) and W303B (JC104) (trp1-1; ura3-1; ade2-1; can1-100; mat a) were generous gifts of Drs. Gregory Petsko and Ira Herskowitz. Engineered yeast strains, W(B), Wh(R), and W(BΔ), generated by targeted disruption of the yeast CPR1 or YCY b₅ loci (11–14) and the yeast expression vectors V10 and V60 (11) were generous gifts of Dr. Denis Pompon (CNRS, Gif-sur-Yvette, France). Human P450c17 cDNA (15) was PCR amplified with Pfu polymerase (Stratagene, La Jolla, CA) using primers 1 The abbreviations used are: DHEA, dehydroepiandrosterone; OR, P450-oxidoreductase; b₅, cytochrome b₅; V₁₀, vector pYeDP10; V₆₀, vector pYeDP60; cD2, vector pYeCD-2; PCR, polymerase chain reaction.

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How Cytochrome b5 Stimulates 17,20-Lyase Activity

TABLE I

| Template      | Name         | Sequence (5’ to 3’)             |
|---------------|--------------|---------------------------------|
| pECE-c17      | Sense        | c17-S-1                         |
| Sense         | c17-AS-1     |                                 |
| pECE-OR       | Sense        | OR-S-1                          |
| Sense         | OR-AS-1      |                                 |
| b5 cDNA       | Sense        | b5-S-1                          |
| Antisense     | b5-AS-1      |                                 |

Restriction sites (BamHI or EcoRI) are underlined, and ATG start codons are in bold type. Silent base pair changes to eliminate hairpin loop formation in OR-S-1 are underlined and in bold.

Cytosolic P450 and cytochrome b5 have been observed in intact cell and microsome preparations (5). The development of “humanized” yeast strains (12) enabled us to dissect this problem without detergent solubilization of individual components.

To study the effects of human OR and b5 on human P450c17 activities in yeast microsomes, parental yeast strain W303B was doubly transfected with vector V10 expressing human P450c17 and with vector cDE2 expressing either the cDNA for human OR or b5 (empty vector). Microsomes from these transfecants were characterized and used for kinetic studies; microsomes were also prepared from yeast strains W303A and W303B transfected with the V10-c17 plus cDE2 (for expressing an electron donor) vector. The resulting material was confirmed by difference spectroscopy. Kinetic behavior was approached as a Michaelis-Menten system for data analysis, and all error bars show standard deviations.

RESULTS

Yeast Transfection and Microsome Characterization—The capacity of b5 to increase the 17,20-lyase activity of P450c17 has been shown by several laboratories using purified, reconstituted protein systems (9, 25), but this phenomenon has not been observed in intact cell and microsome preparations (5). The development of “humanized” yeast strains (12) that express both P450c17 and selected electron donor proteins has enabled us to dissect this problem without detergent solubilization of individual components.

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Microsomal proteins were quantitated colorimetrically. Immunoblotting on polyvinylidene difluoro membranes (Millipore, Bedford, MA) was performed with rabbit antisera to human P450c17 (5) or to human OR (generously provided by Prof. C. Roland Wolf, Imperial Cancer Institute, Dundee, United Kingdom) using secondary antibody-peroxidase conjugate and ECL reagents (Amersham, Arlington Heights, IL) and with goat antisera to human b5 (Oxford Biomedical, Rochester Hills, MI) using secondary antibody-peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagents. Microsomal P450 and cytochrome b5 contents were measured spectroscopically (21) using either a Cary 3E or a Shimadzu UV160 spectrophotometer. P450 oxoreductase activity was measured as described (22). P450c17 Enzyme Assay—Microsomes were assayed under initial rate kinetics by preincubation in 50 mM potassium phosphate buffer (pH 7.4) with 0.5–5 μM steroid (added in 4 μl of ethanol) in 200 μl total volume at 37 °C for 2 min before the addition of 1 μl NADPH to start the reaction. Each reaction contained either 20,000 cpm of [3H]pregnenolone, [3H]17α-hydroxypregnenolone (NEN Life Science Products Inc., Boston, MA), or [3H]17α-hydroxyprogesterone (Amersham) or 10,000 cpm of [14C]progesterone (NEN Life Science Products). Steroids were extracted with 400 μl of ethyl acetate/isooctane (1:1), concentrated under nitrogen, separated by thin layer chromatography (Whatman PE 100 silica gel plates, Maidstone, Kent, UK) using 3:1 chloroform/ethyl acetate, and quantitated as described (23). Purified recombinant human cytochrome b5 (Pan Vera, Madison, WI), apo-human cytochrome b5, or horse heart cytochrome c (Sigma) were included in incubations as indicated. Apo-cytochrome b5 was prepared from the Pan Vera holocytochrome b5 as described (24), and absent electron transfer properties of the resulting material was confirmed by difference spectroscopy.
total cytochrome \(b_5\) content were similar among the three microsome preparations from co-transfected W303B yeast (Table II). Essentially all of the P450s is from P450c17, whereas cytochrome c reductase activity and total cytochrome \(b_5\) content were similar in all transfectants, indicating that endogenous yeast OR and \(b_5\) are the predominant electron transfer proteins in these microsomes. Comparable expression of P450c17 was demonstrated in all samples by Western blotting, and human OR and \(b_5\) were detected only in samples from yeast containing their respective cDNAs, as expected (Fig. 1).

**Kinetics**—To determine how the presence of human OR and/or \(b_5\) alters the activities of human P450c17 in yeast microsomes, we measured apparent \(K_m\) and \(V_{\text{max}}\) values for both 17α-hydroxylase and 17,20-lyase reactions for \(\Delta^5\) and \(\Delta^4\) substrates (Table III). Lineweaver-Burk plots (Fig. 2) show that yeast transfected with human P450c17 alone perform both 17α-hydroxylase and 17,20-lyase reactions despite the absence of human electron transfer proteins, indicating that the endogenous yeast OR can couple with human P450c17, as has been shown for bovine P450c17 (26, 27). In this system, however, \(\Delta^4\) substrates are used, human P450c17 efficiently catalyzed the conversion of progesterone to 17α-hydroxyprogesterone, but the conversion of 17α-hydroxyprogesterone to androstenedione was much less efficient than the corresponding conversion of 17α-hydroxyprogrenenolone to DHEA (Table III). The slow turnover of 17α-hydroxyprogesterone by human P450c17 explains why circulating androgens in humans derive principally from the isomerization and reduction of DHEA rather than by cleavage of 17α-hydroxyprogesterone to androstenedione, the predominant pathway in rodents. Guinea pig P450c17, for example, preferentially converts progesterone to DHEA formed by microsomes containing both human P450c17 and OR is metabolized further to a more polar compound, possibly 16α-hydroxy-DHEA, the major DHEA metabolite of a bovine P450c17/rat OR fusion protein (29). Human OR markedly stimulated both activities without a significant change in total cytochrome c reductase activity, indicating that yeast OR is an inefficient electron donor for P450c17 and does not significantly interfere with catalysis in the presence of human OR.

When \(\Delta^4\) substrates are used, human P450c17 efficiently catalyzed the conversion of progesterone to 17α-hydroxyprogesterone, but the conversion of 17α-hydroxyprogesterone to androstenedione was much less efficient than the corresponding conversion of 17α-hydroxyprogrenenolone to DHEA (Table III). The slow turnover of 17α-hydroxyprogesterone by human P450c17 explains why circulating androgens in humans derive principally from the isomerization and reduction of DHEA rather than by cleavage of 17α-hydroxyprogesterone to androstenedione, the predominant pathway in rodents. Guinea pig P450c17, for example, preferentially converts progesterone to

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androstenedione, some of which is sequentially metabolized without dissociation of the intermediate 17α-hydroxyprogesterone from the active site (30, 31). Co-expression of human OR similarly increases the $V_{\text{max}}$ of both activities toward Δ4 steroids but without a significant change in apparent $K_m$ values (Table III). A second, more polar product, presumably 16α-hydroxyprogesterone (4, 5, 32), constitutes ~20–25% of the products when Δ4-progesterone is the substrate with all microsomes tested.

Co-expression of human $b_5$ with human P450c17 increases $V_{\text{max}}$ 10-fold for the 17,20-lyase reaction but not for the 17α-hydroxylase reaction with both Δ5 and Δ4 substrates and does not change the apparent $K_m$ for any substrate tested (Table III). Although human OR improves the catalytic efficiency of P450c17 in yeast microsomes, both by lowering the $K_m$ of Δ5 substrates and increasing the $V_{\text{max}}$ for all reactions, the sole effect of human $b_5$ is to augment the $V_{\text{max}}$ for the 17,20-lyase reactions. Our results generally agree with those obtained with recombinant human P450c17 and rat OR, except that rat $b_5$ approximately doubles the rate of hydroxylation of Δ5-pregnenolone but not of Δ4-progesterone (25). Differences in species of origin of the OR and $b_5$ used may explain some differences in the results obtained in the two systems, as well as subtle differences in the activities of microsomal and detergent-solubilized proteins.

Activities in the Absence of Yeast OR or Yeast Cytochrome $b_5$—The experiments described above were performed in the presence of endogenous yeast OR and $b_5$ in the microsome preparations. To determine whether the yeast electron donors influence human P450c17 activities, we expressed human P450c17, with human OR or $b_5$, in engineered yeast strains lacking the endogenous yeast OR or $b_5$ genes. When human P450c17 and OR were coexpressed in yeast strain W(BΔ), which lacks the yeast homolog of the human $b_5$ gene (13), the resulting microsomes contained 85% of the 17α-hydroxylase activity and 73% of the 17,20-lyase activity of microsomes from W303B yeast (Fig. 3A). The 17,20-lyase activity was minimally affected by the absence of yeast $b_5$, demonstrating that OR is both necessary and sufficient to confer both 17α-hydroxylase and 17,20-lyase activity to human P450c17 in yeast microsomes.

To confirm that OR was required for catalysis, we expressed human P450c17 in strain W(B), in which the endogenous yeast OR locus is replaced by the human $b_5$ cDNA under the control of the inducible Gal10/Cyc1 promoter (12). No 17α-hydroxylase or 17,20-lyase activity is present in microsomes prepared from W(B) yeast transfected with human P450c17 and empty cDE2 vector, but both activities are restored by cotransfection of human OR (Fig. 3B). When W(B) yeast, transfected with both human P450c17 and OR, were grown in galactose to induce expression of human $b_5$ as well, the presence of human $b_5$ increased the $V_{\text{max}}$ of the 17,20-lyase reaction using 17α-hydroxyprogrenenolone from 0.14 min⁻¹ to 1.1 min⁻¹ but did not change the apparent $K_m$ (0.3 μM) (Fig. 3, B and C). This induc-

### Table III
**Kinetic constants**

| Human proteins | Pregnenolone | 17α-Hydroxy pregnenolone | Progesterone | 17α-Hydroxy progesterone |
|----------------|--------------|--------------------------|--------------|--------------------------|
|                | $K_m$ (μM) | $V_{\text{max}}$ (min⁻¹) | $K_m$ (μM) | $V_{\text{max}}$ (min⁻¹) | $K_m$ (μM) | $V_{\text{max}}$ (min⁻¹) |
| P450c17 alone  | 0.79        | 0.66                     | 0.83        | 0.022                    | 0.73        | 0.66                     |
| P450c17 + OR   | 0.40        | 3.0                      | 0.40        | 0.10                     | 1.1         | 6.6                      |
| P450c17 + $b_5$| 0.61        | 0.76                     | 1.0         | 0.29                     | 0.78        | 0.59                     |

*$^a$ ND, activity too low to determine accurately.

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**Fig. 3. Human P450c17 activities in engineered yeast strains.** Panel A, 17α-hydroxylase (open bars) and 17,20-lyase (hatched bars) activities in microsomes prepared from yeast strains W(BΔ), lacking yeast $b_5$, or from W303B yeast using 1 μM steroid. Note the 10-fold difference in the scales of hydroxylase activity (left) and lyase activity (right). Error bars (± S.D.) are too tight to be seen in three of the four bars. Panel B, P450c17 activities in microsomes from W(B) yeast (lacking yeast OR) co-transfected with V10-c17 and empty cDE2 vector or cDE2-OR. Clones were grown in glucose, producing trace (lo) amounts of human $b_5$, or in galactose, inducing high (hi) amounts of $b_5$. Incubations contained 1 μM steroid and 25 or 125 μg of microsomal protein to assay 17α-hydroxylase activity (1 h incubation, lanes 1–4) or 17,20-lyase activity (4 h incubation, lanes 5–8), respectively. Panel C, Lineweaver-Burk plot of 17,20-lyase activity in microsomes from W(B) yeast co-transfected with pYeSF2-c17 and cDE2-OR. Microsomes were prepared from the same yeast clone expressing trace human $b_5$ (grown in glucose, squares), or expressing high $b_5$ (grown in galactose, circles). Apparent $K_m$ and $V_{\text{max}}$ values were derived from least-squares fits to the data.
tion of human $b_5$ did not significantly change $17\alpha$-hydroxylase activity, reflected by comparable pregnenolone consumption (Fig. 3B, lanes 3 and 4), but the $17\alpha$-hydroxyprogrenenolone formed in the presence of high amounts of human $b_5$ was rapidly converted to DHEA, so that little $17\alpha$-hydroxyprogrenenolone accumulated (Fig. 3B, lane 4).

**Effect of Exogenous Soluble $b_5$ on $17\alpha$-Hydroxylase and $17\alpha$-Hydroxyprogesterone—**Exogenously added soluble $b_5$ can influence other P450 reactions in yeast microsomes (11); therefore, we added purified human $b_5$ to yeast microsomes containing human P450c17. Although $17\alpha$-hydroxylase activity against $\Delta^2$-pregnenolone or $\Delta^4$-pregesterone was not changed (Fig. 4, A and C), $17\alpha$-lyase activity against $17\alpha$-hydroxyprogrenenolone was increased up to 10-fold in microsomes that did not already contain human $b_5$ (Fig. 4B). Purified $b_5$ also increased $17\alpha$-lyase activity toward $17\alpha$-hydroxyprogesterone, but only about 2-fold (Fig. 4D). These data demonstrate that yeast $b_5$ can neither support nor stimulate human P450c17 activities, as found for other human P450s (12). Furthermore, our results show that the only effect of human $b_5$, either added in solution or coexpressed into microsomes, is to increase the rate of the $17\alpha$-lyase reactions, and that this action of $b_5$ requires the presence of yeast or human OR.

The results described above do not exclude a contribution of human $b_5$ as the donor of the second of the two electrons in the P450 catalytic cycle, as has been suggested (33, 34). If $b_5$ functions as the donor of the second electron, $b_5$ should support catalysis by transporting electrons either from a reducing agent (sodium dithionite) or from NADPH-reduced OR to microsomes containing P450c17 that has already been reduced with the first electron. Dithionite, which can provide one electron to either P450c17 or $b_5$, does not support catalysis in microsomes containing both human P450c17 and $b_5$, but dithionite does not abolish catalysis when the second electron is provided to P450c17 from NADPH via OR (Fig. 5A). To confirm this observation, we attempted to reconstitute $17\alpha$-lyase activity by transferring electrons from NADPH to one pool of microsomes containing human OR (and no P450c17), then to soluble human $b_5$, as an electron conduit, and finally to human P450c17 in another pool of microsomes lacking OR. Soluble $b_5$ was first reduced with NADPH by microsomes containing OR (35), and then added to microsomes lacking yeast OR (strain W(B)) but containing human P450c17 alone (lane 1), human P450c17 and OR (lane 2), or human P450c17 and $b_5$ (but no OR, lane 3), all of which had been preincubated with $17\alpha$-hydroxyprogrenenolone and dithionite to provide the first electron to P450c17. Microsomes lacking human OR converted only a trace of $17\alpha$-hydroxyprogrenenolone to DHEA under these conditions, but microsomes containing both human P450c17 and OR could use the added NADPH to convert substrate to DHEA (Fig. 5B). These results confirm that $b_5$, reduced either by dithionite or OR, cannot provide sufficient electron transfer to P450c17 to support significant $17\alpha$-lyase activity. Therefore, these data suggest that the mechanism by which $b_5$ enhances $17\alpha$-lyase activity does not involve electron transfer.

**How Does Human Cytochrome $b_5$ Augment $17\alpha$-Lyase Activity?—**To explore the mechanism by which $b_5$ increases $17\alpha$-lyase activity, we assayed the $17\alpha$-lyase activity of microsomes containing constant, high amounts of P450c17 and OR and varying amounts of $b_5$. A sharp increase in $17\alpha$-lyase activity was observed when the molar ratio of $b_5$ to P450c17 approached 1:1 (Fig. 6A). Activity reached a maximum at ratios

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**Fig. 4. Effect of exogenously added human $b_5$ on P450c17 activities.** Microsomes were prepared from yeast strain W303B expressing human P450c17 and co-transfected with cDE2 vector expressing no protein, OR, or $b_5$ as indicated. The indicated steroid for each panel (5 μM) was incubated with (+) and without (−) 1 molar equivalent of exogenously added purified human $b_5$, per molar equivalent of P450c17. Incubations contained $\Delta^2$ pregnenolone (panel A), $\Delta^4$ $17\alpha$-hydroxyprogrenenolone (panel B), $\Delta^4$ progesterone (panel C), and $\Delta^4$ $17\alpha$-hydroxyprogesterone (panel D). Note the different scales in each panel.
of $b_5$ to P450c17 between 10:1 and 30:1; however, further addition of human $b_5$ progressively inhibited 17,20-lyase activity in both yeast and human adrenal microsomes. If human $b_5$ was acting as the preferred electron donor, 17,20-lyase activity should saturate and remain constant rather than fall at high $b_5$/P450c17 ratios. Similarly, when we examined the influence of $b_5$ on the 17,20-lyase activity of microsomes containing very small amounts of human OR and no yeast OR (strain W(hR) transfected with P450c17 grown to high density in glucose), maximal stimulation occurred at a $b_5$/P450c17 ratio between 1:1 and 3:1, and higher ratios were again inhibitory (Fig. 6A).

Thus, the influence of human $b_5$ changes dramatically as the abundance of human OR and the $b_5$/P450c17 ratio are varied. These data suggest that $b_5$ does not function as an electron donor, but instead exerts some other action, perhaps facilitating electron transfer from OR to P450c17 or improving coupling efficiency, as has been suggested for other P450 reactions stimulated by $b_5$ (24, 36).

Inhibition of enzymatic activity at high $b_5$/P450c17 ratios, a phenomenon also observed in guinea pig adrenal microsomes (10), could result from a second, inhibitory $b_5$-binding site on P450c17 or from a competition between $b_5$ and P450c17 for electrons from limiting amounts of OR. Cytochrome c, which is also a substrate for reduction by OR (37), also inhibits 17,20-lyase activity at molar ratios above 10:1, the same molar ratios at which $b_5$ becomes inhibitory (Fig. 6C). Inhibition by equivalent molar ratios of cytochrome c to P450c17 is consistent with $b_5$ competing with P450c17 for reduction when OR is limiting, but “reverse” electron transfer from P450c17 to $b_5$ (38) may also contribute to the inhibition observed at higher $b_5$/P450c17 ratios. These data suggest that electron transfer from OR to $b_5$ is actually detrimental to 17,20-lyase activity. Therefore, we determined whether human apo-$b_5$, which lacks the heme and hence cannot participate in electron transfer, modulates 17,20-lyase activity differently than human holo-$b_5$. In microsomes containing either low or high amounts of human OR, molar ratios of apo-$b_5$ to P450c17 between 1:1 and 10:1 augment 17,20-lyase activity (Fig. 6B). Unlike the data with holo-$b_5$, the stimulatory effect of apo-$b_5$ remains constant rather than falling at higher $b_5$/P450c17 ratios. These results exclude direct electron transfer from $b_5$ as the principal means by which $b_5$ augments 17,20-lyase activity and suggest that $b_5$ exerts a saturable, allosteric effect on the P450c17-OR complex.

**DISCUSSION**

The 17,20-lyase/17α-hydroxylase ratio in the human adrenal rises dramatically with the onset of adrenarche at age 8–10, reaches maximal values at age 25–35, and then falls progressively with aging (39); as these phenomena occur only in human beings and great apes (40), their study is difficult. These selective, physiologic, developmentally programmed changes in human adrenal 17,20-lyase activity imply regulatory mechanisms beyond transcription of P450c17 or OR (3, 8). Most P450 enzymes catalyze multiple reactions, but the ratio of their activities remains fixed. The developmentally and possibly hor-

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**FIG. 5.** Reconstitution of human P450c17 activities using sodium dithionite and cytochrome $b_5$. Panel A, 17α-hydroxylase and 17,20-lyase activities in microsomes from W(B) yeast (lacking yeast OR) co-transfected with P450c17 and cDE2-b5 (0.1 mg of protein containing 9 pmol of P450 and 42 pmol of $b_5$). Microsomes were incubated with either 1 μM pregnenolone (lanes 1 and 3) or 1 μM 17α-hydroxyprogrenenolone (lanes 2 and 4) and a saturating portion of solid sodium dithionite (21). Incubations for lanes 3 and 4 also contained NADPH and additional microsomes (16 μg of protein) containing human P450c17 (1 pmol) and OR. Panel B, 17,20-lyase activity in microsomes containing 5 pmol of human P450c17 alone (lane 1), with human OR (lane 2), or with human $b_5$ (lane 3). Microsomes were preincubated with 1 μM 17α-hydroxyprogrenenolone and solid sodium dithionite in 100 μl, and the reactions were started by adding an equal volume of a second incubation containing soluble human $b_5$ (50 pmol) plus microsomes containing human OR but no P450c17 (50 μg of protein, cytochrome c reductase activity, 104 ± 15 nmol/min/mg protein) and NADPH (2 mM) to reduce the soluble $b_5$. Migrations of Δ5 steroids are indicated (S, 17α-hydroxyprogrenenolone standard).

**FIG. 6.** Activation and inhibition of 17,20-lyase activity by cytochromes $b_5$ and c. Conversion of 0.5 μM 17α-hydroxyprogrenenolone to DHEA by human adrenal microsomes (triangles), yeast microsomes with high amounts of human OR (squares), or yeast microsomes with low amounts of human OR (circles) plus the indicated molar ratios of human holo-$b_5$ (panel A), human apo-$b_5$ (panel B), or horse heart cytochrome c (panel C). Activity is expressed as the percent of conversion by the microsomes alone. The yeast microsomes were prepared from strain W(hR) either co-transfected with pYeSF2-c17 plus cDE2-OR and grown in galactose, yielding the microsomes with high amounts of human OR, or co-transfected with pYeSF2-c17 plus empty cDE2 vector and grown in glucose, yielding the microsomes with low amounts of human OR (cytochrome c reductase activities of 223 and 12 nmol/min/mg protein, respectively).
monally programmed changes in the ratio of 17,20-lyase to 17α-hydroxylase activities of human P450c17 provide a unique system for studying the differential regulation of two reactions catalyzed by a single P450 enzyme.

An augmentation of the 17,20-lyase activity of P450c17 by b₅ has been observed in vitro (9, 25) but was not seen in transfected COS-1 cells (5), possibly because the endogenous b₅ in those cells was sufficient to stimulate 17,20-lyase activity maximally. Thus, it has not been clear how or if b₅ regulates human P450c17 activities in vivo. The use of microsomes from yeast engineered to express human P450c17, OR, or b₅ from inducible promoters permits the quantitative manipulation of each protein in a membrane environment that should simulate events in vivo. This permits greater experimental flexibility than the use of bicistronic plasmids (41), fusion proteins (29), or viral vectors (42), and obviates concerns about the relevance of data from detergent-solubilized systems to in vivo systems.

Titration experiments with purified human holo-b₅, apo-b₅, and cytochrome c showed that the stimulatory effect of b₅ on 17,20-lyase activity is not mediated by electron transfer from b₅ and suggest that b₅ exerts an allosteric effect on the P450c17-OR complex. This proposed mechanism could explain three observations from other laboratories. First, b₅ facilitates electron transfer from OR to P450c17 3A4 only when all three proteins are premixed before adding NADPH and substrate, but not when b₅ is premixed with P450c17 3A4 and added to OR, NADPH, and substrate in stop-flow experiments (24). These data suggested that the stimulatory action of b₅ on testosterone 6β-hydroxylation by P450c17 3A4 was an allosteric effect and was not mediated by an action of b₅ as an alternate electron donor (24). Second, b₅ is a more potent stimulator of 17,20-lyase activity when the abundance of OR is low, and this stimulation is quite sensitive to small changes in these low amounts of OR (10). Our results corroborate these studies and suggest that b₅ interacts primarily with the P450c17-OR complex and not with P450c17 alone. Third, the redox-active core 1 segment of porcine b₅ alone cannot augment the 17,20-lyase activity of human P450c17 (43), consistent with our findings that electron transfer from human b₅ is not required to stimulate 17,20-lyase activity.

Three conclusions about human physiology emerge from our analysis of the kinetics of human P450c17. First, human androgen biosynthesis proceeds predominantly through the pathway 17α-hydroxyprogrenolone → DHEA → androstenedione, rather than through the pathway 17α-hydroxyprogrenolone → 17α-hydroxyprogesterone → androstenedione. The pathway via DHEA predominates because the apparent Kₘ for Δ⁵ 17α-


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