Tight binding of cytochrome $b_5$ to cytochrome P450 17A1 is a critical feature of stimulation of C21 steroid lyase activity and androgen synthesis

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It has been recognized for >50 years that cytochrome $b_5$ ($b_5$) stimulates some cytochrome P450 (P450)–catalyzed oxidations, but the basis of this function is still not understood well. The strongest stimulation of catalytic activity by $b_5$ is in the P450 17A1 lyase reaction, an essential step in androgen synthesis from 21-carbon (C21) steroids, making this an excellent model system to interrogate $b_5$ function. One of the issues in studying $b_5$–P450 interactions has been the limited solution assay methods. We constructed a fluorescently labeled variant of human $b_5$ that can be used in titrations. The labeled $b_5$ bound to WT P450 17A1 with a $K_d$ of 2.5 nM and rapid kinetics, on the order of 1 s$^{-1}$. Only weak binding was observed with the clinical P450 17A1 variants E305G, R347H, and R358Q; these mutants are deficient in lyase activity, which has been hypothesized to be due to attenuated $b_5$ binding. $K_d$ values were not affected by the presence of P450 17A1 substrates. A peptide containing the P450 17A1 Arg-347/Arg-358 region attenuated Alexa 488-T70C-$b_5$ fluorescence at higher concentrations. The addition of NADPH–P450 reductase (POR) to an Alexa 488-T70C-$b_5$:P450 17A1 complex resulted in a concentration-dependent partial restoration of $b_5$ fluorescence, indicative of a ternary P450:$b_5$:POR complex, which was also supported by gel filtration experiments. Overall, these results are interpreted in the context of a dynamic and tight P450 17A1:$b_5$ complex that also binds POR to form a catalytically competent ternary complex, and variants that disrupt this interaction have low catalytic activity.

Cytochrome P450 (P450, CYP) enzymes are the major catalysts involved in the oxidation of chemicals (1). In addition to playing major roles in areas as diverse as drug metabolism and the biosynthesis of natural products, P450s are the major catalysts involved in steroid metabolism and catalyze most of the oxidations beginning with the side-chain cleavage of cholesterol to form pregnenolone (2). P450 17A1 plays a critical role in the synthesis of androgens as well as in the generation of 17α-hydroxy (17α-OH) steroids and in the synthesis of mineralocorticoids and glucocorticoids (Fig. 1). More than 125 clinically deficient variants of P450 17A1 have been identified (3–5). Some of the variants show normal 17α-hydroxylation activity but are deficient in the second, so-called “lyase,” reaction (Fig. 1), attenuating androgen production and subsequently estrogen levels. Conversely, prostate cancers are dependent on androgens (6), and the lyase reaction is an important drug target (7–17).

Aside from its clinical relevance, P450 17A1 is of inherent biochemical interest in the context of the two reactions it catalyzes. The first reaction (Fig. 1) is a relatively straightforward P450 hydroxylation involving compound I (FeO$^{3+}$) chemistry, but the mechanism of the second, the lyase step, is not without controversy (15, 18, 19). Although a number of mammalian P450 enzymes are stimulated by another hemeprotein, cytochrome $b_5$ (CYB5A, $b_5$), the P450 17A1 lyase reaction is the one stimulated the most (20–23). This effect has been shown to be important biologically (i.e., the interaction can be demonstrated using fluorescence resonance energy transfer in cells (24)), and deficient variants show low androgen levels in vivo (25, 26).

However, a phosphorylation event postulated to be at Ser-258 of P450 17A1 has also been proposed to facilitate the lyase reaction (27–30), and that charge (negative) is opposite to that of the arginine residues (Arg-347 and Arg-358) proposed to be involved in binding $b_5$ (3, 5, 31–36). Zebrafish P450 17A1 lyase activity is only slightly enhanced by $b_5$, and the related zebrafish P450 17A2 enzyme catalyzes only 17α-hydroxylation, not lyase activity, with or without $b_5$ (37, 38). Mutation of residues to arginine in this region of zebrafish P450 17A2 did not lead to the acquisition of lyase activity (39).

One of the experimental deficiencies in this research field has been useful assay for $b_5$ binding. We considered several approaches with fluorescence, in order to utilize sensitive and solution-based methods, and were able to use labeling of a previously described mutant with a dye (40, 41). Fluorescence attenuation assays allowed for analysis with submicromolar concentrations of proteins, with estimates of binding constants and rates of association and dissociation. In contrast to previous proposals about this system, we provide evidence that P450–$b_5$ interactions are not tightly linked to substrate identity and occupancy and that a ternary P450 17A1–$b_5$–NADPH–P450 reductase (POR) complex is preferred to a model in which $b_5$ and POR shuttle at a single site.
**P450 17A1 variants and cytochrome b<sub>5</sub>**

![Chemical structures and reactions](image_url)

**Figure 1. Reactions catalyzed by P450 17A1.** The first reaction in each sequence is a 17α-hydroxylation, and the second reaction is a 17α, 20-cleavage reaction of the 21-carbon steroid (lyase reaction). DHEA, dehydroepiandrosterone.

**Results**

**Expression and purification of P450 17A1 variant enzymes**

Plasmids for WT P450 17A1 and five clinically observed variants were constructed in a pCW expression vector and expressed in *Escherichia coli* JM109 cells (Figs. 2 and S1). WT P450 17A1 showed an expression level of ~450 nmol per liter culture, and the expression levels of the E305G, R347H, and R358Q variants were 400, 100, and 200 nmol per liter, respectively (Fig. S1). However, no P450 holoenzyme spectra were detected for the R347C and P428L variants (Fig. S1, C and F). Purified WT P450 17A1 and three variant proteins (E305G, R347H, and R358Q) were prepared by Ni<sup>2+</sup>–nitrilotriacetate (NTA) affinity column chromatography. The spectra of these purified variant proteins showed very little of peaks corresponding to inactive cytochrome P420 (Fig. 2, B–D). All subsequent assays were based on the amount of spectrally detectable P450 heme for WT and variant P450 17A1 enzymes.

**Steady-state kinetics of P450 17A1 variants**

Steady-state kinetic parameters for all (four) hydroxylation and lyase reactions (Fig. 1) of WT P450 17A1 and the three purified variants (E305G, R347H, and R358Q) were measured. For the 17α-hydroxylation of progesterone, all three variants formed the 17α-OH product, but their activities (specificity constants, k<sub>cat</sub>/K<sub>m</sub>) were attenuated to 3 to 11% of that of WT P450 17A1 (Fig. 3A and Table 1). The 17α-OH progesterone

![Graphs and data](image_url)

**Figure 2. Fe<sup>2+</sup>–CO versus Fe<sup>2+</sup> binding spectra of purified WT P450 17A1 and variant enzymes.** A, WT; B, E305G; C, R347H; and D, R358Q. The P450 concentrations in these particular assays were 3.1, 0.41, 0.82, and 0.34 μM, respectively. These spectra are presented to demonstrate the lack of (inactive) cytochrome P420 and do not reflect the ratio of bound heme in the individual proteins.
lyase activities of the R347H and R358Q variants were reduced more dramatically (to <1%) (Fig. 3B and Table 1). However, the E305G variant differed in that progesterone 17α-hydroxylation activity was decreased (to 8%) more than the lyase reaction activity (28%) (Fig. 3A and Table 1).

The R347H and R358Q variants showed similar levels of pregnenolone 17α-hydroxylation (slight reduction) but only very low levels of 17α-OH pregnenolone lyase activity (Fig. 3, C and D and Table 1). The E305G variant differed in that progesterone 17α-hydroxylation activity was decreased (to 8%) more than the lyase reaction activity (28%) (Fig. 3A and Table 1).

The R347H and R358Q variants showed similar levels of pregnenolone 17α-hydroxylation (slight reduction) but only very low levels of 17α-OH pregnenolone lyase activity (Fig. 3, C and D and Table 1). The E305G variant displayed highly reduced pregnenolone 17α-hydroxylation and 17α-OH pregnenolone lyase activities (Fig. 3, C and D and Table 1). One previous study reported a similar result (42), but another study did not (43). These results indicated that the 17,20-lyase activity in all three variants was impaired even when excess b5 was present.

Substrate-binding affinities of P450 17A1 mutants

Substrate binding of purified P450 17A1 (WT and three variants) was analyzed in titrations with all four major substrates. WT P450 17A1 and all variant enzymes showed a typical “type I” substrate-binding spectral titration change (increase at 388 nm, decrease at 423 nm, corresponding to a shift in the iron spin state from low to high (44)) (Figs. S2 and S3). The calculated $K_d$ values of WT P450 17A1 with progesterone were ~0.065 μM, although even with quadratic analysis, there is considerable error in such a low value (Table 2). The $K_d$ values of the three variants were somewhat increased, for example, from 0.17 to 0.26 μM (Table 2). The $K_d$ values of variants for 17α-OH progesterone, pregnenolone, and 17α-OH pregnenolone were similar to or slightly higher than measured with WT P450 17A1 (Table 2). These results are interpreted to mean that changes in the substrate-binding affinities of the three variants are not primarily responsible for the decreased enzymatic activities, which were observed even at high substrate concentrations (Fig. 3).

b5 dependence of catalytic activities of P450 17A1

The lyase catalytic activities of P450 17A1 are strongly stimulated by $b_5$ (20–23). $b_5$ did not significantly stimulate the progesterone and pregnenolone 17α-hydroxylation reactions by WT P450 17A1 or the three variants (Fig. 4). WT P450 17A1 lyase activities were stimulated by $b_5$ in a concentration-dependent manner (Fig. 4). However, the R347H and R358Q variants did not display $b_5$ concentration-dependent stimulation in either of the lyase reactions. The E305G variant displayed weak $b_5$ stimulation of the 17α-OH progesterone lyase reaction but not the 17α-OH pregnenolone lyase reaction (Fig. 4, C and D).

Attenuation of Alexa 488-T70C-b5 fluorescence by WT P450 17A1 and variants

WT $b_5$ has no cysteine residues, and the T70C substitution in $b_5$ was chosen based on previous studies of fluorescent labeling with an acrylodan derivative and interactions of that

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![Figure 3](https://example.com/fig3.jpg)

**Figure 3.** Steady-state kinetics of enzyme reactions catalyzed by WT P450 17A1 and variants. A, WT (blue); B, E305G (green); C, R347H (orange); and D, R358Q (purple). Each point is a mean of duplicate assays, shown as a mean ± range. The kinetic parameters ($k_{cat}$, $K_m$, and $k_{cat}/K_m$) are indicated in Table 1.
modified protein with bacterial and other heme proteins (40, 41). Preliminary labeling studies were done with NanoTemper reagents designed for microthermophoresis work (45). The proprietary “Red Reagent” was unsatisfactory for fluorescence titrations because of low sensitivity. The “Blue Reagent” provided much more sensitivity, and the resulting Blue-T70C reported fluorescence values were never reached. The calculated Kd value of POR (for the WT P450 17A1:Alexa 488-T70C complex) was 0.11 μM, suggesting much lower affinity than b5 with P450 17A1 (Fig. 7).

**Table 2**

| Substrate | Kd values of P450 17A1 substrate complexes |
|-----------|------------------------------------------|
| P450 17A1 variant | 17α-hydroxylation | 17α-hydroxylation |
| | kcat, min⁻¹ | Km, μM | kcat/Km | % WT (kcat/Km) | kcat, min⁻¹ | Km, μM | kcat/Km | % WT (kcat/Km) |
| WT | 6.4 ± 0.5 | 7.8 ± 1.4 | 0.82 ± 0.16 | 100 | 0.29 ± 0.02 | 5.5 ± 0.8 | 0.17 ± 0.015 | 100 |
| E305Q | 0.19 ± 0.01 | 3.1 ± 0.4 | 0.06 ± 0.01 | 8 | 0.002 ± 0.001 | 6.1 ± 1.2 | 0.003 ± 0.002 | 0.6 |
| R347H | 0.13 ± 0.02 | 8.1 ± 2.2 | 0.02 ± 0.01 | 4 | 0.006 ± 0.002 | 15 ± 9 | 0.004 ± 0.003 | 0.8 |
| R358Q | 0.13 ± 0.02 | 4.6 ± 0.7 | 0.09 ± 0.02 | 11 | 0.056 ± 0.013 | 10 ± 5 | 0.006 ± 0.013 | 0.6 |

DHEA, dehydroepiandrosterone.

four substrates (present at 10 μM), not substantially altered from the binding of Alexa 488-T70C-b5 in the absence of substrate. The variants did not show binding in the presence or absence of substrates in the titrations (Fig. S6).

**Competition of POR with b5 for P450 17A1**

The loss of Alexa 488-T70C-b5 fluorescence induced by binding of P450 17A1 was partially reversed by titration with POR (Fig. 7). The P450 interaction of POR appeared to be competitive with Alexa 488-T70C-b5, but the original fluorescence values were never reached. The calculated Kd value of POR (for the WT P450 17A1:Alexa 488-T70C-b5 complex) was 0.11 μM, suggesting much lower affinity than b5 with P450 17A1 (Fig. 7).

**Binding rates for interaction of Alexa 488-T70C-b5 and P450 17A1**

The rate of binding of Alexa 488-T70C-b5 to (WT) P450 17A1 was measured by observing attenuation of fluorescence upon mixing the two proteins in a stopped-flow fluorimeter. When concentrations of 0.50 μM Alexa 488-T70C-b5 and 0.50 μM P450 17A1 were mixed, the first-order kobs value was 0.7 s⁻¹ (Fig. 8, A and B). The loss of Alexa 488-T70C-b5 fluorescence induced by binding of P450 17A1 was reversed by adding unmarked b5 protein (data not presented), indicating that the unmarked b5 replaces Alexa 488-T70C-b5 in interacting with P450 17A1. When the kinetics were measured, the reaction occurred at a rate of 1.9 s⁻¹ (Fig. 8, C and D). This value is considered to be the first-order koff rate.

In principle, for binding reactions in a freely reversible system, kobs = kcat + koff (46), but the apparent koff rate here was higher than the kobs (on) rate. Thus, the true koff rate cannot be calculated from these measurements. However, the fluorescence traces clearly show that the binding and dissociation events are both occurring with rates of ~1 s⁻¹.

**Interaction of peptides with Alexa 488-T70C-b5 and P450 17A1**

Peptides were obtained corresponding to the putative binding sites of P450 17A1 and b5 (see Experimental procedures section and Fig. S7). The peptide corresponding to the putative b5 binding region of P450 17A1 (residues
347–358, “P450 peptide”) attenuated the fluorescence of Alexa 488-T70C-\(b_5\), although high concentrations were required (Fig. 9). The “\(b_5\) peptide,” however, did not result in a gain of fluorescence when added to an Alexa 488-T70C-\(b_5\):P450 17A1 complex, even at a concentration of 160 \(\mu\)M (data not presented).

When added to reconstituted P450 17A1 steroid oxidation systems (Fig. 10), the P450 17A1 peptide was more inhibitory to the lyase reaction (17\(\alpha\)-OH pregnenolone) than the \(b_5\) peptide, which may appear to be surprising in that \(b_5\) was present in the reaction at a concentration 10-fold higher than the P450. This peptide might be expected to bind to POR and block its interaction with P450 17A1, although that should have also inhibited the 17\(\alpha\)-hydroxylation reaction. Inhibition was only observed at the very highest concentration.

Demonstration of a ternary P450 17A1:POR:\(b_5\) ternary complex using size-exclusion chromatography

The fluorescence titration results (Fig. 7) suggested that P450 17A1, POR, and \(b_5\) form a ternary complex. Accordingly, we tested this hypothesis further using a different approach, that is, gel filtration (Fig. 11A). In a previous work with P450 17A1 (47), we reported that about one-half of the protein migrated as a monomer, but in this case, all the P450 17A1 eluted as a single monomeric peak on a Superose 12 column, at an elution volume between those of ovalbumin (45 kDa) and bovine serum albumin (67 kDa, monomer) (Fig. S8). \(b_5\) eluted later, as might be expected, and POR eluted as a multimer near the void volume of the column (Fig. 11A). (The identity of the second peak in the POR sample is unknown and presumed to be a small molecule, in that no proteins were visualized upon SDS-gel electrophoresis and Coomassie Blue staining; Fig. 11B).

A complex of P450 17A1 and \(b_5\) yielded peaks in the monomeric P450 17A1 and \(b_5\) regions plus a larger complex eluting earlier, as verified with gel electrophoresis (Fig. 11, A and B). A mixture of POR, P450 17A1, and \(b_5\) had most of the 280 nm-absorbing material (protein) in a large peak eluting later than free POR but earlier than the P450 17A1–\(b_5\) complex, as validated by gel electrophoresis (Fig. 11, A, C, and D). The presence of all three proteins in the ternary complex peak fractions (Fig. 11, A and D) is documented in Figure 11C.

Discussion

The development of a sensitive fluorescence-based assay for \(b_5\) binding, based on a literature precedent with an unrelated system (40, 41), enabled assays that could address several issues in the field of P450 17A1. A major finding was the very tight binding of \(b_5\) to P450 17A1, with an estimated \(K_d\) of
binding phenomena we studied are attributed to the ionic charges in P450 17A1 and b\textsubscript{5}, in that all binding assays were done in the absence of phospholipid vesicles.

Stopped-flow measurements with P450 17A1 showed that both the binding of b\textsubscript{5} and its dissociation are rapid processes. The rates are on the order of 1 s\textsuperscript{-1}, although the k\textsubscript{obs} value for binding (Fig. 8A) is probably not accurate in that it should be greater than the (first-order) dissociation rate (Fig. 8C). The k\textsubscript{obs} value for binding is probably a reflection of a faster “on” rate followed by a conformational change to a complex in which the fluorescence is decreased (46). The k\textsubscript{obs} values for binding and dissociation are fast enough not to be rate limiting in the overall P450 17A1 oxidation reactions (Table 1). However, the results can be used to argue against a model in which POR and b\textsubscript{5} are “switching” at the same position on P450 17A1 during catalysis, unless this would be facilitated in the phospholipid vesicles. That is, the (substrate-bound) P450 Fe\textsuperscript{3+}-O\textsubscript{2} complex needs to receive an electron, thus converting it to the “compound 0” form, Fe\textsuperscript{3+-O\textsubscript{2}}. If POR donated the electron, then it would have to leave and allow the b\textsubscript{5} to bind (k ∼ 1 s\textsuperscript{-1}) (48) and induce a conformational change to permit the lyase reaction. It is unlikely that compound 0 would have enough stability for this to occur (i.e., a k\textsubscript{obs} of 1 s\textsuperscript{-1} is
equivalent to a $t_{1/2}$ of 0.7 s). The bulk of the evidence is that $b_5$ does not donate the second electron in the reaction (24, 33, 47, 49, 50), even if it can to some extent in the results of Duggal et al. (51, 52). Furthermore, lyase activity was stimulated in mammalian cells in which apo-$b_5$ was expressed, demonstrating the biological relevance of these findings (53). In the event that $b_5$ were donating the second electron, the Fe$^{2+}$O$_2$ complex would have to be stable enough for the binding to occur after POR left the P450. We conclude that our proposal of a ternary P450:$b_5$:POR complex (Figs. 7, 11, and 12) describes the mechanism more accurately than does a shuttle system.

The fluorescence attenuation titrations (Figs. 5 and 6) were critical in development of the conclusions about the tight binding of $b_5$ to (WT) P450 17A1. The attenuation of fluorescence in the presence of P450 17A1 cannot be attributed to an inner filter effect, because of the low absorbance, the reversal of the decrease by $b_5$ (Fig. 8) or a second protein (POR), and the lack of attenuation by the three P450 17A1 variants, even at the highest P450 concentrations. The $K_d$ values (Table 3) should be considered estimates and have some uncertainty associated with them, for several reasons. With WT P450 17A1, the value is very low, and even with a low concentration of Alexa 488-T70C-$b_5$ (50 nM) and a quadratic equation, there is probably error in the $K_d$ value (2.5 nM, i.e., 20-fold less than the enzyme concentration) (Table 2). With regard to the variants, they clearly did not attenuate the Alexa 488-T70C-$b_5$ fluorescence much (Fig. 5), but any $K_d$ calculations have the caveat that we do not know exactly what the titration endpoint should be, that is, a complex formed with Alexa 488-T70C-$b_5$ and one of the variants may have the $b_5$ (and the Alexa 488 dye) in a position in which it does not show as much loss of fluorescence.

A molecular description of the fluorescence of the Alexa 488 derivative of $b_5$ is beyond the scope of this investigation. Glu-48 and Glu-49 are on the edge of $b_5$ (Protein Data Bank: 2I96), and the heme is between these residues and Thr-70. Stayton et al. (40, 41) attached the fluorescent dye acrylodan, which has a very different structure than Alexa 488, to Cys-65, engineered to replace Thr-65 in rat $b_5$ (corresponding to human Thr-70). The fluorescence of this derivative increased upon binding to metmyoglobin, cytochrome $c$, or (bacterial) P450$_{cam}$, with estimates of $K_d$ of $\sim$1 μM for each. Blue shifts in the emission spectrum were also observed (40), which is known to be very sensitive to changes in dielectric constants. We have not prepared the acrylodan derivative of T70C $b_5$ for use with P450 17A1. In other work (not shown), the fluorescence of Alexa 488-T70C-$b_5$ was attenuated by several other human P450 enzymes (e.g., 2C9, 2E1, 3A4), but the $K_d$ values were not as low as with P450 17A1. Apparently, the heme of P450 (17A1) is not absolutely required for fluorescence attenuation, in that the P450 17A1 peptide was able to reduce the fluorescence (Fig. 9), albeit with weaker affinity. Preliminary screening studies have also identified several small

### Table 3

| P450 17A1 variant | No substrate, nM | Progesterone, nM | 17-OH progesterone, nM | Pregnenolone, nM | 17-OH pregnenolone, nM |
|------------------|------------------|-----------------|------------------------|-----------------|------------------------|
| WT               | 2.5 ± 0.6        | 5.8 ± 2.3       | 6.0 ± 1.0              | 12 ± 5          | 2.2 ± 1.0              |

*From Figure S6. See raw data for titrations with the variants (E305G, R347H, and R358Q).*

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**Figure 7.** Partial recovery of Alexa 488-T70C-$b_5$ fluorescence of an Alexa 488-T70C-$b_5$P450 17A1 complex by titration with POR. A, the fluorescence of Alexa 488-T70C-$b_5$ (50 nM) is shown with a red trace. Addition of P450 17A1 (50 nM) caused a drop in the fluorescence, to give the blue trace. The intensity of the fluorescence spectra increased upon adding increasing concentrations of POR (to 2.6 μM), in the direction shown with the arrow. B, recovery of fluorescence in the Alexa 488-T70C-$b_5$P450 17A1 complex as a function of POR concentration. The estimated $K_d$ was 0.11 μM.
Figure 8. Rates of binding and dissociation of Alexa 488-T70C-b5 and P450 17A1. A, stopped-flow analysis of binding of Alexa 488-T70C-b5 to P450 17A1 (1 μM concentrations of each protein in each syringe, excitation at 488 nm, emission >530 nm, and 23 °C). The decrease in fluorescence was fit to a single exponential using the OLIS GlobalWorks program (red line, 0.7 ± 0.1 s⁻¹). B, residuals analysis for part A. C, stopped-flow analysis of binding of dissociation of Alexa 488-T70C-b5:P450 17A1. One syringe contained 100 nM Alexa 488-T70C-b5:P450 17A1, and the other contained 30 μM b5 (excitation at 488 nm and emission >530 nm). (When Alexa 488-T70C-b5 dissociated, it was replaced by b5.) The increase in fluorescence was fit to a single exponential using the OLIS GlobalWorks program (red line, 1.9 ± 0.2 s⁻¹).
Increasing concentrations of the peptide were added. The inset shows the peptide corresponding to the putative binding region of P450 17A1.

Figure 9. Attenuation of the fluorescence of Alexa 488-T70C-5 by a peptide corresponding to the putative binding region of P450 17A1. Increasing concentrations of the peptide were added. The inset shows the fluorescence at 513 nm as a function of concentration. The concentration of Alexa 488-T70C-5 was 50 nM.

Figure 10. Inhibition of P450 17A1 reactions by peptides corresponding to the putative binding regions of P450 17A1 and b5. A, progesterone 17α-hydroxylase; B, 17α-OH pregnenolone lyase activity. The “b5 peptide” corresponds to residues 43 to 52 of b5, and the “17A1 peptide” corresponds to residues 347 to 358 of P450 17A1 (see Experimental procedures section and Fig. S7).

molecules (at 10 μM concentration) that can attenuate the fluorescence of Alexa 488-T70C-b5 (results not presented). Whether the heme of b5 is critical for the attenuation of fluorescence by small molecules of proteins is unknown, in that we have not studied the apo-b5 version of the conjugate. Another issue is any conformational changes that occur upon P450 17A1–b5 binding. The stepwise addition of P450 17A1 to 15N-labeled b5 induced some NMR shift perturbations, which may be indicative of conformational changes (in b5), although the Thr-70 signal did not appear to be changed (36). Exactly how a conformational change, if it occurs, would effect the fluorescence is unknown in the absence of more information.

Our fluorescence results are in agreement with earlier reports on the roles of Arg-347 and Arg-358 in b5 binding and the basis of loss of lyase activity (3, 32–36, 42). We also agree with Ershov et al. (54) that binding of b5 to P450 17A1 is relatively tight. However, our results lead to several different conclusions about the interaction of P450 17A1 and b5, primarily because of the sensitivity of the assays (Figs. 5 and 6), which involve the components in solution.

The Kd values for binding of substrates to P450 17A1 (Table 2) are all lower than previously reported from this laboratory (23). The main reason is that the previous results were based on assays involving endpoints of stopped-flow measurements of binding rates, and the values reported here were obtained in steady-state assays with lower P450 concentrations (Fig. S3 and Table 2). Although the Kd values for substrate binding were somewhat higher with some of the variants (Table 2), attenuated substrate binding is not the major reason for low activity, and activities were low even at high concentrations of substrate (Fig. 3). The results of the work with E305G are not consistent with some of the conclusions of Sherbet et al. (43), who used an indirect approach to characterizing substrate binding. As discussed later, the basis of loss of activity of the E305G variant is more complex than the other variants.

In contrast to the reports of Estrada et al. (36, 55), who used NMR measurements at very high enzyme concentrations, we did not find a major effect of any substrate on b5 binding (Figs. S2 and S3 and Table 2). Our results are consonant with our published work indicating that the presence of b5 did not alter rates of substrate binding or dissociation (23, 47).

The surface plasmon resonance (SPR) results of Ershov et al. (54) also show relatively low Kd values for b5 binding, but these values are an order of magnitude higher than our own (Table 3). The SPR method involves immobilization of a protein on a chip, and a major deficiency of the approach is “mass transfer,” a term used to describe the diffusion of the ligand from the solution through the matrix to reach the receptor (P450 17A1 in this case) (46). The reported on-rate constants (54) are extremely low (i.e., 360–5000 M−1 s−1) for the binding of a substrate to an enzyme, as is usually the case in SPR work, and are not in line with our own on- and off-rates (Fig. 8), even if our on-rates may not be completely accurate.

A key finding of our study is that POR only partially disrupted the Alexa 488-T70C-b5:P450 17A1 complex (Fig. 7). If all the Alexa 488-T70C-b5 had become displaced by POR and
completely free, the fluorescence would have reverted to the original value. The hyperbolic nature of the binding (Fig. 7B) could be used to calculate a $K_D$ with multiple equilibria if there were complete displacement, but if a ternary complex is formed, then the level of residual fluorescence may not be estimated. If there is a ternary complex, then the observed $K_D$ for binding the POR (110 nM) is valid (~25 times the $K_D$ for $b_5$). Mixing POR with Alexa 488-T70C-$b_5$ did not decrease its fluorescence, although interaction must exist between those two proteins in that POR reduces $b_5$ (56).

The gel filtration results provide more evidence for the existence of a ternary P450 17A1–POR–$b_5$ complex (Fig. 11). The elution profile of a 17A1–$b_5$ complex was shifted relative to either monomer and the complex of a 1:1:1 M mixture of all three proteins yielded a new peak that contained all three proteins (Fig. 11, C and D). These results, along with the fluorescence results presented in Figure 7, are consistent with a ternary complex (Fig. 12). The complex is dynamic, as shown by the kinetic assays of P450 17A1–$b_5$ binding (Fig. 8) but stable enough to persist during migration in a gel filtration column (Fig. 11). This model of a ternary complex (Fig. 12) differs from a shuttle mechanism developed on the basis of NMR measurements (36, 57). Our results and our model are also probably not consistent with the model of Holien et al. (58), in which $b_5$ and POR bind to the far ends of a P450 17A1 dimer (in a ternary complex) but are not in contact with each other. However, it is conceivable that, in such a model, the binding of POR on one end of a P450 dimer could transduce a fluorescence increase of the $b_5$ on the other end. A shuttle mechanism with POR and $b_5$ alternately binding transiently to P450 has also been proposed for (rabbit) P450 2B4 (59, 60) and human P450 3A4 (61), but at this time, we cannot speculate on the general application of this model (Fig. 12) to other P450s that use $b_5$.

The P450 17A1 E305G variant is common that, in our assays, it lost most of the catalytic activities, with the exception of only partial loss of 17α-OH progesterone conversion to androstenedione (Fig. 3B). These results contrast with a previous report using a different system (heterologous expression in yeast and use of yeast microsomes), in which the lower lyase activity was attributed to attenuated substrate binding (43). We did not observe altered substrate binding (Table 2 and Figs. S2 and S3). In addition, the E305G variant did not quench the fluorescence of Alexa 488-T70C-$b_5$, which we interpret as lack of binding of $b_5$, even though Glu-305 is not at the face of P450 17A1 thought to bind directly to $b_5$. Our current hypothesis is that the E305G substitution leads to a conformational change in P450 17A1 that disrupts $b_5$ binding (Figs. 4, B and D and 5B).

Although we have provided new information about the nature of the interaction of P450 17A1 and $b_5$, a number of issues and questions still remain. A sensitive interaction assay is described and applied, but there may be other dyes that could provide even greater sensitivity. As mentioned previously, the E305G variant is more complex than previously thought, and the charge distant from the putative $b_5$ binding site has an effect on binding. Also, this variant retained some lyase activity with 17α-OH progesterone but not 17α-OH pregnenolone (Figs. 3 and 4), even though $b_5$ binding was poor (Figs. 5B and 6). Finally, if our conclusion about a ternary complex (of P450 17A1, $b_5$, and POR) is correct, we do not know the spatial relationship of the proteins.

As mentioned earlier, a phosphorylation event postulated to be at Ser-258 of P450 17A1 has been proposed to facilitate the lyase reaction (27–30), and that charge (negative) is opposite to that of the arginine residues. What has not been clear from that work is whether the phosphorylation is sufficient in itself to stimulate the lyase reaction or whether the phosphorylation enhances the functional binding of $b_5$ to produce the enhancement. Resolving the issue would require the purification of a specifically phosphorylated P450 17A1 or the analysis of activity in a system (cells?) with a $b_5$− background.

In teleost fish, there are two P450 17A enzymes, 17A1 and 17A2 (62). Fish P450 17A1 enzymes resemble human P450 17A1 and catalyze both 17α-hydroxylation and lyase activities, but P450 17A2 only catalyzes the former reaction. Zebrafish P450 17A1 showed only a twofold stimulation of the lyase activity by human or zebrafish $b_5$, and P450 17A2 shows no activity with or without $b_5$ (37). The exact basis of the lyase deficiency in fish P450 17A2 remains unknown, and the X-ray crystal structures of the two proteins are very similar (37). In contrast to fish, Xenopous laevis (frog) appears to have only P450 17A1, not 17A2 (UniProt search). Although recombinant X. laevis P450 17A1 androgen biosynthesis has been suggested to be independent of $b_5$ (38), a closer examination of that report and calculation of $K_{cat}/K_m$ values indicates that lyase activity was stimulated 12-fold by X. laevis $b_5$ in the case of 17α-OH pregnenolone and 1.5-fold in the case of 17α-OH progesterone. The extent of $b_5$ stimulation is difficult to reach conclusions about in such systems where P450 17A1 and POR are present in microsomal membranes and soluble $b_5$ is added, in that the $b_5$ may not have the same access to the P450 as seen in a more homogeneous or even a vesicular system, although stimulations are qualitatively seen in systems with $b_5$ added to yeast microsomes (3, 38).

The results with the peptides (Fig. 10) showed weak inhibition of the lyase activity by the P450 17A1 peptide but inhibition of progesterone 17α-hydroxylation only at the very highest concentration of that peptide. In principle, it might be possible to develop drugs that block binding of $b_5$ to P450 17A1 to discover selective inhibitors of the lyase reactions, an unmet need in treating prostate cancer (14, 15, 17).

In conclusion, we have developed a sensitive fluorescence assay that can be utilized to study $b_5$ interactions with P450 17A1. We confirm previous conclusions that Arg-347 and Arg-358 of P450 17A1 are important in $b_5$ binding. We also implicated loss of $b_5$ binding in the low activity of the P450 17A1 variant E305G. In contrast to previous work, we did not observe effects of P450 17A1 on its interactions with $b_5$, and we provide evidence for a functional P450 17A1–POR–$b_5$ ternary complex instead of a shuttle mechanism.
**Figure 11.** Gel filtration analysis of complexes of P450 17A1, b5, and POR. All analyses were done using a Superose 12 10/300 FPLC column. A, absorbance at 280 nm profiles of individual proteins (POR [green] and P450 17A1 [orange]) and a binary (blue) and a ternary mixture (red) are shown. Individual fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis, and densitometry was done of the Coomassie Blue–stained bands corresponding to the individual proteins. The POR preparation contained unknown 280 nm-absorbing material eluting near the position of free b5 but not showing any protein after electrophoresis and staining. B, densitometry traces of P450 17A1 (orange) and b5 (purple) eluted in a binary equimolar mixture of the two proteins. The migration positions of the individual proteins (P450 [17A1] and b5) are indicated. C, Coomassie Blue staining of the proteins in a ternary complex, as eluted from the column in Part A. The numbers on the left indicate M, values of markers relevant to the three proteins of interest, which have approximate Ms of 79 kDa (POR), 57 kDa (P450 17A1), and 17 kDa (b5). D, densitometry traces of P450 17A1 (orange), POR (green), and b5 (purple) eluted in a ternary equimolar mixture of the three proteins. The migration positions of the individual proteins (POR, P450 [17A1], and b5) are indicated.

**Figure 12.** Proposed scheme for interaction of P450 17A1, b5, and POR. The fluorescence of Alexa 488-T70C-b5 (F depicts the fluorophore Alexa 488) is decreased upon interaction with P450 17A1. POR binds to form a ternary complex and causes a shift of the fluorophore (Alexa 488) to a position in which the fluorescence is partially restored (Fig. 7).
**P450 17A1 variants and cytochrome b5**

**Experimental procedures**

**Chemicals and enzymes**

Progestosterone, pregnenolone, 17-OH progesterone, 17-OH pregnenolone, androstenedione, dehydroepiandrosterone, protease inhibitor cocktail, cholesterol oxidase, and 1,2-dilauroyl-sn-glycero-3-phosphocholine were purchased from Sigma–Aldrich. Ni\(^{2+}\)–NTA–agarose was purchased from Qiagen. 5-Aminolevulinic acid was purchased from Frontier Scientific. IPTG and CHAPS were purchased from Anatrace. Other chemicals were of the highest grade commercially available. Peptides were purchased from New England Peptides, with purity and identity analysis provided (Fig. S8):

"b5 peptide": 48 49 52 E H P G E E V L E E

"P450 17A1 peptide": 347 358 R N R L L L L E A T I R,

with the numbering of the residues shown from the primary sequence.

**Enzyme expression and purification**

_E. coli_ JM109 cells were purchased from Invitrogen. Recombinant rat POR and human _b5_ were expressed in _E. coli_ and purified as described previously (56, 63–65).

Expression and purification of the P450 17A1 enzymes and variants was carried out as previously described but with some modifications (66, 67). Briefly, _E. coli_ JM109 cells transformed using pCW (Ori+) vectors were inoculated into terrific broth medium containing 100 μg ml\(^{-1}\) ampicillin, 0.5 mM 5-aminolevulinic acid, and 1.0 mM IPTG. The expression cultures (500 ml) were grown at 37 °C for 3 h and then at 30 °C under conditions of shaking at 200 rpm for 28 h in 2.8-l Fernbach flasks. Soluble fractions containing P450 17A1 enzymes were prepared after ultracentrifugation (105,000 × g, 60 min). The soluble fraction was then loaded onto a Ni\(^{2+}\)–NTA column (Qiagen), and (after washing), the purified protein was eluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 M NaCl, 0.5% (w/v) CHAPS, 20% (v/v) glycerol, and 250 mM imidazole. The eluted fraction containing highly purified P450 17A1 was dialyzed at 4 °C against 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1 mM EDTA to remove CHAPS and NaCl.

Fe\(^{2+}\)–CO versus Fe\(^{3+}\) binding spectra were recorded using the method of Omura and Sato (68), using an extinction coefficient of 91,000 M\(^{-1}\) cm\(^{-1}\) (A\(_{450–490}\)). Protein concentrations were estimated from _A_\(_{280}\) measurements using a Nanodrop spectrophotometer (Thermo Scientific) and the amino acid compositions of human P450 17A1 and _b5_. The estimated specific content of WT P450 17A1 was 7.3 nmol P450 (mg protein\(^{-1}\))\(^{-1}\), which is somewhat low but within the historical range of 5.6 to 16.8 nmol P450 (mg protein\(^{-1}\))\(^{-1}\) for purified P450s expressed in _E. coli_ and purified in this laboratory (69–73). The estimated specific contents of the purified E305G, R437H, and R358Q variants were 5.5, 2.3, and 4.2 nmol P450 (mg protein\(^{-1}\)), respectively. We have observed heme contents as low as 10% of the WT values for expressed variants of other human P450s, for example, P450 21A2 (74). All catalytic and titration assays comparing the WT and variant P450 17A1 enzymes were done on the basis of spectrally detected P450 heme, though (as before (74)), in order to avoid differences because of heme content.

**Construction of Alexa 488-T70C-b5**

The expression plasmid for T70C-b5 was constructed using an Agilent quickChange site-directed mutagenesis kit according to the manufacturer’s instructions. The constructed mutant plasmid was verified with DNA nucleotide sequencing analysis and then transformed into _E. coli_ DH5a. The purification was performed with the same procedure for WT _b5_ previously described (56). The calculated specific content of _b5_ heme in T70C-b5 was 59.9 nmol (mg protein\(^{-1}\)), within experimental error of the theoretical amount for a protein of 16.9 kDa.

The purified protein was labeled using a NanoTemper Monolith Protein Labeling Kit BLUE NHS second Generation (NanoTemper Technologies), which reacts with sulphydryl groups in a protein sample to label the protein with a fluorescent dye (BLUE) (proprietary). The extent of labeling was determined by a modification of the manufacturer instructions.

Labeling of the T70C-b5 mutant was then performed using a maleimide conjugation reaction with Alexa Fluor 488 dye (Thermo Scientific). Briefly, the _b5_ T70C mutant (in 100 mM phosphate buffer, pH 7.4) was mixed with Alexa Fluor 488 maleimide (dissolved in dimethyl sulfoxide) at a 1:10 M ratio, and the conjugation reaction was allowed to proceed at room temperature for 20 h in the dark. The labeling mixture was then passed through a Zeba spin column (Thermo Scientific) to eliminate unreacted dye. The concentration of (labeled) Alexa 488-T70C-b5 was calculated by measuring the absorbance, using _ε_\(_{493}\) = 72,000 M\(^{-1}\) cm\(^{-1}\). The extent of modification was 74%.

**Substrate-binding analysis**

Binding titration analysis was carried out using purified WT P450 17A1 and variant enzymes to determine the binding affinity parameters of the substrate–enzyme complexes. The purified enzymes were diluted in 100 mM potassium phosphate buffer (pH 7.4) and divided in two 1.0-ml glass cuvettes. Spectra (350–500 nm) were recorded using an OLIS-DW2 spectrophotometer (On-Line Instrument Systems) with subsequent additions of the substrate. Differences in the absorbance between the wavelength maximum and minimum were plotted versus the substrate concentration (44). _K_d_ values were calculated using nonlinear regression analysis in GraphPad Prism software (GraphPad) and the quadratic equation:

\[
Y = B + \frac{A}{2E} \left( \frac{(K_d + E + X)}{\sqrt{(K_d + E + X)^2 - 4EX}} \right),
\]

set in Prism as: \(Y = B + (A/2) \times (1/E) \times ((K_d + E + X) - \sqrt{(K_d + E + X)^2 - 4EX})\).
**P450 catalytic activity analysis**

P450 catalytic activity assays to measure steroid hydroxylation and lyase reactions were carried out as described previously (23, 37, 64). P450 17A1 enzymes were reconstituted and preincubated in 500 μl of 100 mM potassium phosphate buffer (pH 7.4) containing POR, 1,2-di-αrωyl-sn-glycero-3-phosphocholine, and the substrate (progesterone, 17-OH progesterone, pregnenolone, or 17-OH pregnenolone). Typical enzyme concentrations used were P450 17A1 (0.1–0.2 μM), POR (0.4–0.8 μM), and b5 (2–4 μM) for hydroxylation/lyase reactions (e.g., Figs. 3 and 4 and Table 1).

The reactions were initiated by adding an NADPH-regenerating system (final concentrations of 15 mM glucose 6-phosphate, 1.5 mM NADP+, and 1 IU ml⁻¹ glucose 6-phosphate dehydrogenase) (75) and terminated by quenching and extracting with 2.0 ml CH₂Cl₂. Aliquots of the organic phases were dried and then dissolved in 100 μl of a CH₃CN/H₂O (1:1, v/v) mixture. The extracts from the pregnenolone and 17-OH pregnenolone reactions were dissolved in 50 μl of CH₃OH, mixed with 200 μl of cholesterol oxidase (0.5 units/reaction) in 100 mM phosphate buffer (pH 7.4), and incubated at 30°C with shaking at 150 rpm for 12 h. The extraction procedure was then repeated, and the (∆⁴) reaction products were analyzed in a Waters Acquity UPLC system using a BEH C18 octadecylsilane column (2.1 mm × 100 mm, 1.7 μm). The reaction products were resolved using a mobile phase composed of solvents A (70% CH₃CN/H₂O, v/v) and B (CH₃CN), at a gradient gel), with staining with Coomassie Blue and densitometry using GelAnalyzer software analysis of the experimental data, fitting to a single exponential (data from at least three replicates were averaged.)

**Size-exclusion chromatography**

Chromatography was done using a Supersorb 12 10/300 GL column (11 μm, 10 × 300 mm; GE Healthcare) with an NCG Quest 100 Plus Chromatography system (BioRad). The buffer was 50 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl, and the flow rate was 1.0 ml min⁻¹. The column was equilibrated for each run with one column volume (23.6 ml), and the injection volume was 3% of the column volume (10 nmol of each protein was injected, i.e., 100 μl of 100 μM solutions). Elution was with 1.5 column volumes of buffer. Absorbance was monitored at 280 nm. Fractions were collected (1.0 ml) and analyzed by SDS-polyacrylamide gel electrophoresis (4–15% gradient gel), with staining with Coomassie Blue and densitometry using GelAnalyzer software (www.gelanalyzer.com; Istvan Lazar and Istvan Lazar Jr). M₇ standards included ovalbumin and bovine serum albumin (Fig. S9).

**Data availability**

All data are contained within the article and the supporting information.

**Supporting information**—This article contains supporting information.

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P450 17A1 variants and cytochrome b5

Conflict of interest—The authors declare that they have no conflict of interest with the contents of this article.

Abbreviations—The abbreviations used are: b5, cytochrome b5; 17α-OH, 17α-hydroxy; NTA, nitritolactate; P450, cytochrome P450; POR, NADPH–P450 reductase; SPR, surface plasmon resonance.

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