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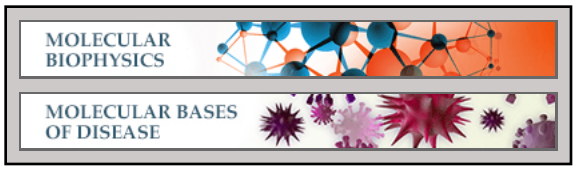

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Substrate-modulated Cytochrome P450 17A1 and Cytochrome b_5 Interactions Revealed by NMR*

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Background: Steroidogenic cytochrome P450 17A1 (CYP17A1) performs hydroxylase and lyase reactions, with only the latter facilitated by cytochrome b_5.
Results: NMR mapping confirms the CYP17A1/b_5 interface and reveals substrate modulation of the interaction.
Conclusion: Allosteric communication exists between the buried CYP17A1 active site and its peripheral b_5 binding site.
Significance: The CYP17A1 reaction mechanism may be governed by proximal conformational control.

The membrane heme protein cytochrome b_5 (b_5) can enhance, inhibit, or have no effect on cytochrome P450 (P450) catalysis, depending on the specific P450, substrate, and reaction conditions, but the structural basis remains unclear. Here the interactions between the soluble domain of microsomal b_5 and the catalytic domain of the bifunctional steroidogenic cytochrome P450 17A1 (CYP17A1) were investigated. CYP17A1 performs both steroid hydroxylation, which is unaffected by b_5, and an androgen-forming lyase reaction that is facilitated 10-fold by b_5. NMR chemical shift mapping of b_5 titrations with CYP17A1 indicates that the interaction occurs in an intermediate exchange regime and identifies charged surface residues involved in the protein/protein interface. The role of these residues is confirmed by disruption of the complex upon mutagenesis of either the anionic b_5 residues (Glu-48 or Glu-49) or the corresponding cationic CYP17A1 residues (Arg-347, Arg-358, or Arg-449). Cytochrome b_5 binding to CYP17A1 is also mutually exclusive with binding of NADPH-cytochrome P450 reductase. To probe the differential effects of b_5 on the two CYP17A1-mediated reactions and, thus, communication between the superficial b_5 binding site and the buried CYP17A1 active site, CYP17A1/b_5 complex formation was characterized with either hydroxylase or lyase substrates bound to CYP17A1. Significantly, the CYP17A1/b_5 interaction is stronger when the hydroxylase substrate pregnenolone is present in the CYP17A1 active site than when the lyase substrate 17α-hydroxypregnenolone is in the active site. These findings form the basis for a clearer understanding of this important interaction by directly measuring the reversible binding of the two proteins, providing evidence of communication between the CYP17A1 active site and the superficial proximal b_5 binding site.

Microsomal cytochrome b_5 (b_5)[2] is a small, ubiquitous membrane-bound heme protein important for electron transport in a variety of cellular processes (1, 2). Although b_5 is not essential for catalysis by the cytochrome P450 (P450) monooxygenases, b_5 often significantly modulates the function of these enzymes, thereby impacting the metabolism of a broad range of xenobiotic and endogenous compounds. Cytochrome b_5 has been reported to enhance, inhibit, or have no effect on rates of P450 catalysis, with some reliance on either the particular P450 studied or, in some cases, on the particular substrates being metabolized (2–6) or the reaction conditions (7). The underlying mechanism for how b_5 is able to have such differential effects on P450 catalysis is poorly understood. It is generally well accepted that P450 requires the sequential input of two electrons to accomplish catalysis and that at least the first must be delivered by NADPH-cytochrome P450 reductase (CPR). One suggestion in the literature has been that, although CPR can donate both required electrons in the catalytic cycle, b_5 is an alternate donor for the second electron. However, this role was brought into question by studies in which b_5 without the heme (*apo*-b_5) and unable to donate an electron was also shown to affect catalysis, at least for some P450 enzymes (8, 9). Single turnover experiments (4) suggest that at low concentrations of b_5, catalysis is stimulated because of decreased uncoupling, whereas at high relative concentrations, b_5 protein outcompetes reductase binding, thus inhibiting catalysis. Evidence, primarily from mutagenesis experiments, suggests that b_5 and reductase share a partially overlapping binding site (4, 6, 10). How b_5 decreases uncoupling is unknown, but one common suggestion has been that b_5 binding to a P450 might elicit a conformational change in the P450 enzyme that could alter catalysis (8, 11).

One particularly intriguing example of b_5 effects on P450 function is found in the monooxygenase system of cytochrome P450 17A1 (CYP17A1). This P450 plays an essential role in the production of steroid androgens by mediating two subsequent steps in the steroidogenic pathway. First, pregnenolone is hydroxylated to form 17α-hydroxypregnenolone via the well known catalytic cycle involving an iron(IV) oxo intermediate. However, within the same active site, CYP17A1 can also perform an unusual carbon-carbon bond cleavage to convert the C21 steroid 17α-hydroxypregnenolone to the C19 androgen dehydroepiandrosterone (1, 12). The 17,20-lyase activity, which is proposed to occur via a different catalytic mechanism (1, 13), is enhanced 10-fold by the presence of cytochrome b_5,

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[2] The abbreviations used are: b_5, cytochrome b_5; P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; CV, column volume; 2D, two-dimensional; HSQC, heteronuclear single quantum coherence.

but there is little or no effect on 17α-hydroxylation (5, 11, 14). This suggests that P450/b_5 interaction can vary, even with the same protein. The critical developmental effects of this interaction are eminently apparent at adrenarche when the CYP17A1-mediated lyase reaction is required to produce androgenic precursors to hormones such as testosterone and dihydrotestosterone. Such prepubertal androgen increases are driven primarily by increasing cytochrome b_5 levels rather than by changes in CYP17A1 expression (15). Defects in either gene can result in the absence of normal sexual development and fertility (15–18).

Some insight toward understanding b_5 modulation of CYP17A1 activity may be gained upon examination of the putative interface between these two proteins. A subset of patients found to have isolated 17,20-lyase deficiency had mutations in two conserved arginines in CYP17A1, R347H and R358Q (19). Subsequent studies implicated these mutations in disruption of CYP17A1-mediated lyase activity, whereas the hydroxylase activity of the enzyme was maintained (20). Recently, the first structure of CYP17A1 (21) revealed that these arginines form part of a surface-exposed, concave surface on the proximal face of CYP17A1. Conversely, previously determined structures of b_5 show that its heme-binding domain consists of three short helices containing numerous conserved anionic residues framing the partially solvent-exposed heme (22). Mutation of b_5 residues Glu-48 and Glu-49 ablated nearly almost all b_5 effects on *in vitro* 17,20-lyase activity (23), reaffirming the role of electrostatics. Electrostatics have been reported to mediate b_5 binding to other cytochrome P450s (24, 25) but may bind different P450 enzymes using distinct surfaces (26). Analysis of cross-linking studies implicate the same negatively charged region of b_5 (residues Glu-48 and Glu-49) in forming a complex with CYP3A4 (24). However, a recent functional analysis of b_5 mutants instead implicates b_5 residues Asp-58 and Asp-65 in interactions with CYP2E1 and CYP2C19 (25, 27).

High-resolution mapping of P450/b_5 interaction(s) has thus far been largely elusive. No crystal structures are available of complexes of P450 enzymes with either b_5 or CPR, likely because the electrostatic nature of the interaction (28) is disrupted in crystallization solutions, which often contain high salt concentrations. Other direct biophysical evidence of the interactions has been limited, primarily to the cross-linking studies discussed above. Therefore, here the interaction between the soluble domains of microsomal b_5 and CYP17A1 were probed using solution NMR.

A number of studies have suggested that the soluble domain of b_5 alone is unable to form a complex with P450 enzymes (29–31). However, here the binding between the soluble domains of CYP17A1 and b_5 in solution was observed on an intermediate exchange NMR time scale and employing the same anionic Glu-48 and Glu-49 residues implicated earlier in studies of the full-length b_5 (23). Results demonstrate that this CYP17A1-b_5 complex can be disrupted by addition of rat CPR, providing direct biophysical evidence that b_5 and CPR compete for binding surface to CYP17A1. Finally, the relative affinity of CYP17A1 for b_5 *versus* CPR was evaluated when CYP17A1 was in a variety of ligand-bound states. The data suggest that the presence of the 17α-hydroxylase substrate pregnenolone in the CYP17A1 active site results in stronger relative affinity for b_5 *versus* CPR than occurs when the 17,20-lyase substrate 17α-hydroxypregnenolone is present in the CYP17A1 active site. Significantly, this suggests a mechanism whereby CYP17A1 metabolism in the active site and the superficial b_5 binding surface communicate by allostery.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The soluble domain of human microsomal cytochrome b_5, represented by residues 1–107, was constructed as a synthetic gene (GenScript) with a poly-histidine tag at the C terminus. The construct was designed after other b_5 constructs studied previously by NMR spectroscopy (32). *Escherichia coli* BL21(DE3) cells were transformed with a pET-15b plasmid containing the b_5 gene and grown in M9 (50 mM Na_2HPO_4, 20 mM KH_2PO_4, 10 mM NaCl, 18.7 mM ^{15}N NH_4Cl) minimal medium until log phase (0.6 optical density) minimal medium made with ^{15}N ammonium chloride until log phase (0.6 optical density). At this point isopropyl 1-thio-β-D-galactopyranoside and δ-aminolevulinic acid were added to induce b_5 expression and provide for heme synthesis, respectively. Cells were grown at 37 °C with shaking (220 rpm.) for 20 h. Cells were harvested, resuspended in lysis buffer (50 mM Tris, 0.3 M NaCl (pH 8.0)), lysed via freeze-thaw, and sonicated in 30-s alternating bursts for a total of 3 min. Cell lysate was recovered by centrifugation (27,200 $\times$ *g*, 15 min.) and applied to a nickel-nitrilotriacetic acid column (GE Healthcare) equilibrated with loading buffer (50 mM Tris, 0.3 M NaCl (pH 7.4)). The column was washed using 2 column volumes (CV) of lysis buffer and 6 CV of wash buffer (50 mM Tris, 0.3 M NaCl, 100 mM glycine (pH 7.4)) and then eluted with 4 CV of elution buffer (50 mM Tris, 0.3 M NaCl, 100 mM glycine, 100 mM histidine (pH 7.4)). The purity of the eluted sample was evaluated by SDS-PAGE and UV-visible spectroscopy ($A_{412}/A_{280} > 4.0$). NMR samples were prepared by transferring purified b_5 into 50 mM potassium phosphate, 50 mM NaCl (pH 6.5), concentrating to 0.1–0.15 mM, and augmenting to 10% D_2O.

A form of CYP17A1 with a truncation of the N-terminal transmembrane helix and a C-terminal histidine tag was expressed and purified as described previously (21, 33). Briefly, *E. coli* JM109 cells transformed with the CYP17A1 gene in a pCWori plasmid were grown in Terrific broth until log phase (~0.6 optical density) and then induced using isopropyl 1-thio-β-D-galactopyranoside and ALA γ-aminolevulinic acid. Expression was allowed to continue for 72 h, with gentle shaking (140 rpm), at 28 °C. Upon harvesting, cells were lysed using freeze-thaw and sonication for 30-s bursts for a total of 3 min. Lysed cells were centrifuged at 6800 $\times$ *g* for 1 min, and the lysate was subjected to detergent extraction using 1% (v/v) Emulgen 913 with stirring for 1 h. Following ultracentrifugation, the lysate was applied to a pre-equilibrated nickel-nitrilotriacetic acid column (GE Healthcare). The column was washed using 2 CV of lysis buffer (50 mM Tris, 0.3 M NaCl, 20% (v/v) glycerol, 0.2% Emulgen 913) and 6 CV of wash buffer (50 mM Tris, 0.3 M NaCl, 100 mM glycine, 20% glycerol, 0.2% Emulgen 913). CYP17A1 was eluted using 4 CV of elution buffer (50 mM Tris, 0.3 M NaCl, 100 mM glycine, 20% glycerol, 0.2% Emulgen 913). Eluted fractions were pooled and diluted 4-fold into a low

ionic strength buffer (50 mM Tris, 0.3 M NaCl, 20% glycerol) and loaded on a pre-equilibrated carboxymethyl cellulose Fast-Flow column (GE Healthcare), washed using 5 CV of the low ionic strength buffer, and eluted using a high-salt buffer (50 mM Tris, 0.3 M NaCl, 20% glycerol). CYP17A1 purity was evaluated by SDS-PAGE and UV-visible spectroscopy ($A_{417}/A_{280} > 1.0$). Purified CYP17A1 was transferred to b_5 NMR buffer containing 50 μM ligand as required for individual experiments. Abiraterone-bound samples were coexpressed in the presence of this inhibitor. Ligand binding was verified by monitoring changes in the Soret signal using UV-visible spectroscopy.

The QuikChange site-directed mutagenesis approach (Stratagene) was used to generate all mutations. The b_5 E48Q, E49Q, and V50S mutants were purified as described for wild-type b_5, and their overall structure was verified by acquisition of a 2D ^{15}N-heteronuclear single quantum coherence (HSQC) spectrum. The CYP17A1 R347H, R358Q, and R449L mutants were purified as described above, except that 10 μM abiraterone was included in all buffers.

Rat CPR was generated by expressing the full-length protein in HMS174 (DE3) *E. coli* and inducing with isopropyl 1-thio-β-D-galactopyranoside at log phase. The cells were harvested (2200 × *g*) after a 48-hour growth period at 30 °C. CPR was then purified according to the published protocol (34).

Titration of ^{15}N-b_5 with Unlabeled CYP17A1 and CPR—Stepwise additions of CYP17A1 to the ^{15}N-b_5 NMR sample were carried out using a constant b_5 concentration of 0.1 mM. To avoid loss of the CYP17A1 sample because of precipitation at high concentrations, all protein concentrations were kept low during binding experiments. An initial ^{15}N-HSQC spectrum of b_5 was collected in the presence of ligand alone to verify that there were no significant perturbations or broadening of the b_5 spectrum because of the CYP17A1 ligand itself. Subsequently, the ^{15}N-b_5 sample was concentrated and combined with an equal molar (1:1) amount of unlabeled wild-type CYP17A1 so that the final ^{15}N-b_5 concentration remained constant, and the data were reacquired using the same acquisition parameters. Binding experiments using mutants of either b_5 or CYP17A1 were carried out identically. Titrations of the CYP17A1-b_5 complex with full-length rat CPR was carried out by repeating data acquisition for free ^{15}N-b_5 (1:0) and then 1:1 CYP17A1/^{15}N-b_5 (1:1), followed by addition of concentrated CPR corresponding to 0.5, 1.0, and 1.5 molar equivalents.

NMR Spectroscopy—NMR data were acquired at 25 °C using a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe. Processing of raw data was carried out using NMRPipe (35), and the data were analyzed using NMR-ViewJ (36). Backbone assignments corresponding to the b_5 soluble domain were obtained from the Biological Magnetic Resonance Data Bank, accession number 6921. Typical 2D ^{15}N-HSQC experiments were carried out using 32 scans and 128 increments, with the exception of CPR competition experiments, which were extended to 64 scans because of dilution of the labeled protein upon addition of CPR.

RESULTS

Chemical Shift Mapping of the CYP17A1/b_5 Interface—The 2D ^{15}N-HSQC spectrum and the backbone chemical shift assignments of the human b_5 soluble domain (residues 1–107) have been reported previously (PDB code 2I96) (32). The corresponding ^{15}N-HSQC spectrum collected for this work very closely resembled the reported data (not shown), thereby allowing use of the previously determined amide chemical shift assignments in this analysis. Overall, this b_5 spectrum is narrowly dispersed for amide resonances corresponding to the 22-residue linker region at the C-terminal end of the construct and well dispersed between 6.5 and 11 ppm for signals corresponding to the heme-binding domain. To characterize the base-line protein interaction of the two proteins, ^{15}N-b_5 was first titrated with unlabeled, substrate-free CYP17A1 using 1:0.25, 1:0.5, and 1:1 molar ratios. This stepwise addition of CYP17A1 induced a combination of modest chemical shift perturbations and significant line broadening of specific resonances in the ^{15}N-HSQC spectra, characteristic of binding on an intermediate exchange time scale (Fig. 1*A*). Although the signal intensity slightly decreased for all b_5 residues in a stepwise fashion over the titration, a subset of sequential amide signals was affected disproportionally. Although most residues show little decrease in intensity, ^{47}GEEV^{50} were significantly line-broadened in the b_5-CYP17A1 complex, thereby suggesting a role for these residues in the interaction. The Gly-47 and Val-50 resonances are the first to disappear, being completely absent upon addition of 0.5 equivalents of CYP17A1, whereas Glu-48 is absent by 1 equivalent and Glu-49 is reduced substantially (Fig. 1*A*). This disproportionate effect is clearly demonstrated by plotting the ratio of all residue intensities with increasing CYP17A1 concentrations (Fig. 1*B*). This ^{47}GEEV^{50} four-residue sequence maps to the b_5 anionic α2 helix (Fig. 1*C*, *red*). Additionally, more minor perturbations were observed for other residues across b_5 (Fig. 1*C*, *yellow*), including His-44 and His-68, which coordinate the b_5 heme cofactor on either side of the heme, suggesting either a secondary protein-protein contact site or a change in heme positioning upon binding to CYP17A1.

Residues Glu-48 and Glu-49 of b_5 and Arg-347, Arg-358, and Arg-449 of CYP17A1 are Required for b_5/CYP17A1 Binding—To validate the b_5 residues suggested by the titration data as being involved in CYP17A1 binding, complex formation was evaluated for ^{15}N-b_5 in which the residues Glu-48, Glu-49, or Val-50 were mutated. Significantly, Glu-48 and Glu-49 have been reported to be critical for b_5 enhancement of CYP17A1 17,20-lyase activity (23). The ^{15}N-b_5 mutants E48Q and E49Q were generated to neutralize the charges while minimizing steric differences. V50S was largely a steric change. A 2D ^{15}N-HSQC spectrum of each mutant b_5 protein alone confirmed that the amino acid substitutions did not disrupt the fold of the b_5 molecule (data not shown). In contrast to the wild-type b_5 (Fig. 1*A*), when the ^{15}N-b_5 mutants E48Q or E49Q were titrated with an equal molar equivalent of unlabeled CYP17A1, no significant chemical shift perturbations or line broadening of the respective resonances were observed (Fig. 2*A*, *top* and *center panels*), suggesting that the protein-protein complex was not formed. In contrast, ^{15}N-b_5 with mutation of the adjacent valine residue showed significant line broadening for both Glu-48 and Glu-49 in the b_5/CYP17A1 1:1 molar conditions similar to that observed with wild-type b_5, suggesting that this V50S mutant

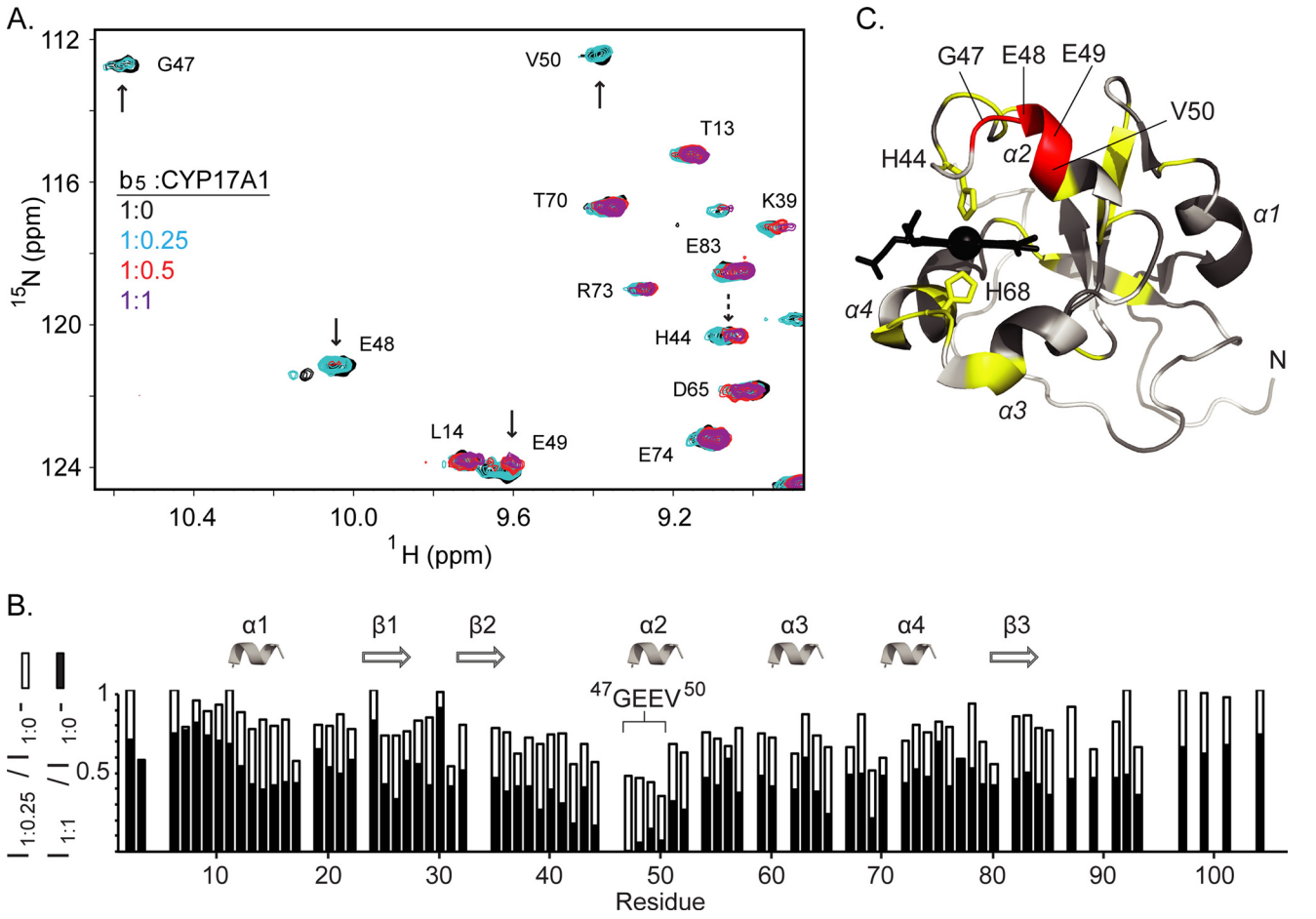

FIGURE 1. **Chemical shift mapping of cytochrome b_5/CYP17A1 interaction shows binding on an intermediate exchange time scale.** *A*, HSQC spectra of ^{15}N-b_5 titrated with unlabeled CYP17A1 show a combination of chemical shift perturbations and line broadening. Residues indicated by *solid arrows* showed substantial line broadening so that a signal was no longer detectible or very weak at a half-molar equivalent of CYP17A1, whereas small shifts were observed in other residues (*dashed arrows*). *B*, peak intensity for two titration points (b_5:CYP17A1 1:0.25 in *open bars* and 1:1 in *black bars*) plotted as a fraction of the free b_5 intensity (b_5:CYP17A1 1:0 molar ratio) revealed consistent and disproportionate line broadening for residues ^{47}GEEV^{50} throughout the titration. *C*, these broadened residues (*red*) occur in the b_5 α2 helix and its preceding loop. Other residues with small shifts (*yellow*) include His-44 and His-68 on both sides of the b_5 heme, suggesting a change in the heme position. Residues shown in *gray* have little or no perturbation, whereas those shown in *white* were not evaluated because of either spectral overlap or the presence of a proline.

retained the ability to bind CYP17A1 (Fig. 2*A*, *bottom panel*). The inability of the b_5 Glu-48 and Glu-49 mutants to form a complex with CYP17A1 is consistent with functional results showing that these mutants were unable to facilitate the lyase reaction (23).

To probe residues of CYP17A1 that might be involved in the protein/protein interface with b_5, similar experiments were performed with CYP17A1 protein harboring mutations of Arg-347, Arg-358, or Arg-449. Mutations of each of these cationic residues have been implicated previously in 17,20-lyase deficiency (20, 37), and are all located in what is considered to be the putative b_5 binding site (21). Titration experiments showed that the HSQC spectra of wild-type ^{15}N-b_5 in a 1:1 molar ratio with the CYP17A1 mutants R347H, R358Q, or R449L were not consistent with b_5-CYP17A1 complex formation. In the case of the single CYP17A1 mutants R347H or R449L, the b_5 spectra with and without CYP17A1 addition are nearly identical, with no real broadening of Glu-48 or Glu-49 resonances (Fig. 2*B*, *top* and *center panels*). The CYP17A1 R358Q mutation did alter the HSQC spectrum of ^{15}N-b_5 by inducing chemical shift perturbations for various amides, but the signature of complex formation (line broadening for the ^{47}GEEV^{50} sequence) did not occur (Fig. 2*B*, *bottom panel*). Therefore, the CYP17A1 R358Q mutant may interact with b_5 but clearly not in a way that corresponds to wild-type CYP17A1 binding of b_5. As a whole, these results provide direct biophysical confirmation that the inability of b_5 to facilitate the lyase reaction for these CYP17A1 mutants is most likely due to disruption of the b_5/CYP17A1 binding interaction.

Cytochrome b_5 and CPR Bind CYP17A1 in a Mutually Exclusive Manner—Functional evidence in the literature is generally consistent with the concept of partially overlapping binding sites on P450 enzymes for b_5 and CPR (4, 24). For example, high concentrations of b_5 have been shown to inhibit electron transfer from CPR to CYP2B4, presumably by blocking CPR binding (6). On the basis of a functional analysis of 17,20-lyase deficiency, a similar partially overlapping and mutually exclusive binding relationship can be hypothesized for CYP17A1 (19). To test this hypothesis, the unique features of the HSQC spectrum for ^{15}N-b_5 bound to CYP17A1 were used to monitor the ability of full-length rat CPR to outcompete b_5 for CYP17A1 binding. Beginning with the ^{15}N-b_5-CYP17A1 complex in a 1:1 ratio,

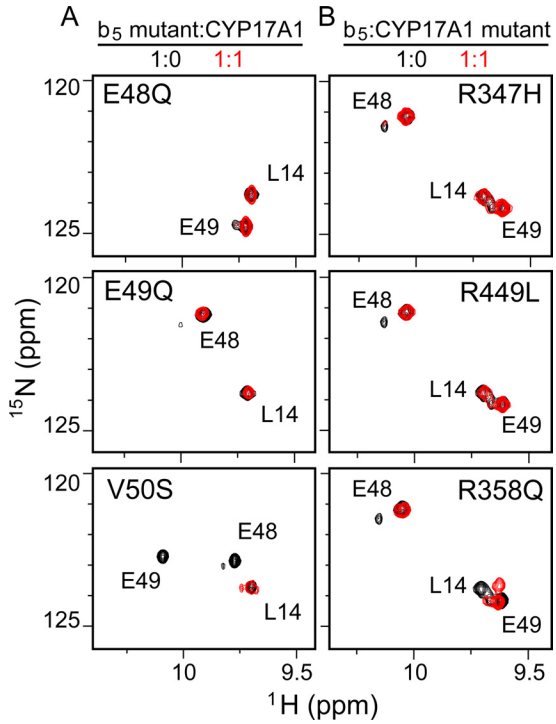

FIGURE 2. **Conserved anionic (b_5) and cationic (CYP17A1) residues are required to form the b_5-CYP17A1 complex.** Shown are 2D ^{15}N-HSQC spectra of b_5 (1:0, *black*) overlaid with the same samples containing an equal molar equivalent (1:1, *red*) of CYP17A1. *A*, effects of b_5 mutation. Broadening of Glu-48 or Glu-49 was not observed in the b_5 E48Q and E49Q mutants (*top* and *center panels*, respectively), suggesting that the *b5*/CYP17A1 complex was not formed. However, the b_5 V50S mutant did show broadening of both resonances (*bottom panel*), suggesting *b5*-CYP17A1 complex formation similar to wild-type b_5. *B*, effects of CYP17A1 mutation. None of the CYP17A1 R347H, R358Q, and R449L mutant proteins induced line broadening in either Glu-48 or Glu-49, suggesting that the *b5*-CYP17A1 complex was not formed.

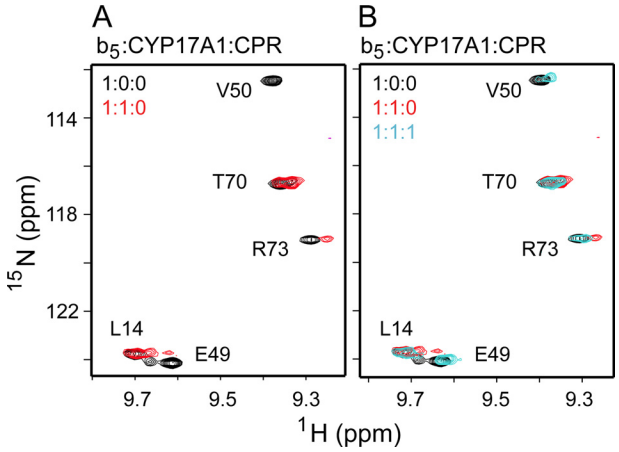

FIGURE 3. **Addition of P450 reductase disrupts the b_5-CYP17A1 complex.** Representative segments of the overlaid 2D ^{15}N-HSQC spectra demonstrated that *b5*/CYP17A1 binding (shown in the absence of CPR in *A* in *red*) can be disrupted by adding an equal molar amount of full-length rat CPR, as indicated by the return of the liberated b_5 spectrum (*B*, *blue*).

unlabeled rat CPR was added in half-molar and then full-molar equivalents. Peaks in the initial b_5 spectrum (Fig. 3*A*, *black*) were perturbed in the usual way upon addition of CYP17A1 (*red*). The subsequent addition of half-molar CPR (b_5/CYP17A1/CPR 1:1:0.5) resulted in partial recovery of the free ^{15}N-b_5 NMR signals (not shown). However, increased CPR concentration in the 1:1:1 data set (Fig. 3*B*, *blue*) caused the resonances for residues Glu-48, Glu-49, and Val-50 to reappear, thereby signaling the liberation of free b_5 as a direct response to the addition of full-length rat CPR. An important ancillary observation was that ^{15}N-b_5 peaks that had been slightly perturbed upon binding to CYP17A1 (*e.g.* Arg-73 in Fig. 3), return to the original chemical shift value corresponding to free b_5. Taken together, the regeneration of the original spectrum strongly suggests that CPR was not interacting with b_5 in the experiment and further indicated the absence of a ternary complex. These experiments provide direct biophysical confirmation that b_5 and CPR bind CYP17A1 in a mutually exclusive manner, most likely by competing for a similar binding site on the CYP17A1 proximal surface.

Cytochrome b_5 Binding to CYP17A1 Is Modulated by CYP17A1 Substrate—Cytochrome b_5 enhances the 17,20-lyase activity of CYP17A1 but not its hydroxylase activity, which occurs in the same active site (21, 38). To probe the mechanism of this observation, b_5-CYP17A1 complex formation was evaluated with various ligands present in the CYP17A1 active site. Ligands included the 17α-hydroxylase substrate pregnenolone, the 17,20-lyase substrate 17α-hydroxypregnenolone, and the inhibitor abiraterone.

When either pregnenolone or 17α-hydroxypregnenolone was bound within the CYP17A1 titrated into ^{15}N-b_5, not only was b_5/CYP17A1 complex formation detected via line broadening for the ^{47}GEEV^{50} sequence, as observed for the unliganded protein (Fig. 4*A*, *left* (*pregnenolone*) and *center* (*17α-hydroxypregnenolone*) *panels*, *solid arrows*), but additional residues located near this sequence were also broadened (*left* and *center panels*, *dashed arrows*). For example, in the spectral window shown in Fig. 4, the resonances corresponding to the backbone amides of Lys-39 and His-44 are no longer detectable in the b_5:CYP17A1 1:1 spectrum. These differences in signal intensity as a function of CYP17A1 ligand are further illustrated by the intensity ratio plots in Fig. 4*C*, in which b_5 binding to substrate-bound CYP17A1 resulted in expansion of the broadened ^{47}GEEV^{50} sequence to include adjoining residues near the b_5 binding site. Mapping these residues onto the b_5 structure reveals that they share a surface with ^{47}GEEV^{50} (Fig. 4*C*, *right panel*). This apparent enhancement in line broadening, resulting in the largest impact on residues clustered near Glu-48 and Glu-49, is strongly suggestive of a paramagnetically enhanced relaxation of NMR signal for sites located near the binding site. To confirm that the CYP17A1 heme, instead of the b_5 heme, is the source of paramagnetism, we observed that the b_5 heme-coordinating residue His-68 is observed in all the free and bound spectra. Given its position, this amide signal would be broadened significantly (hence, not detected) in the case of a paramagnetic b_5 heme, thus confirming the CYP17A1 heme as the likely paramagnetic source. Additionally, this paramagnetic effect would be in agreement with the current understanding of the P450 catalytic cycle because substrate binding is known to displace a water molecule from the heme iron, hence inducing a change from the low-spin, six-coordinate ferric iron to the high-spin, pentacoordinate ferric species (1). To probe this hypothesis, the study was expanded to include evaluation of b_5-CYP17A1 complex formation when the prostate cancer drug abiraterone was bound within the CYP17A1 active site.

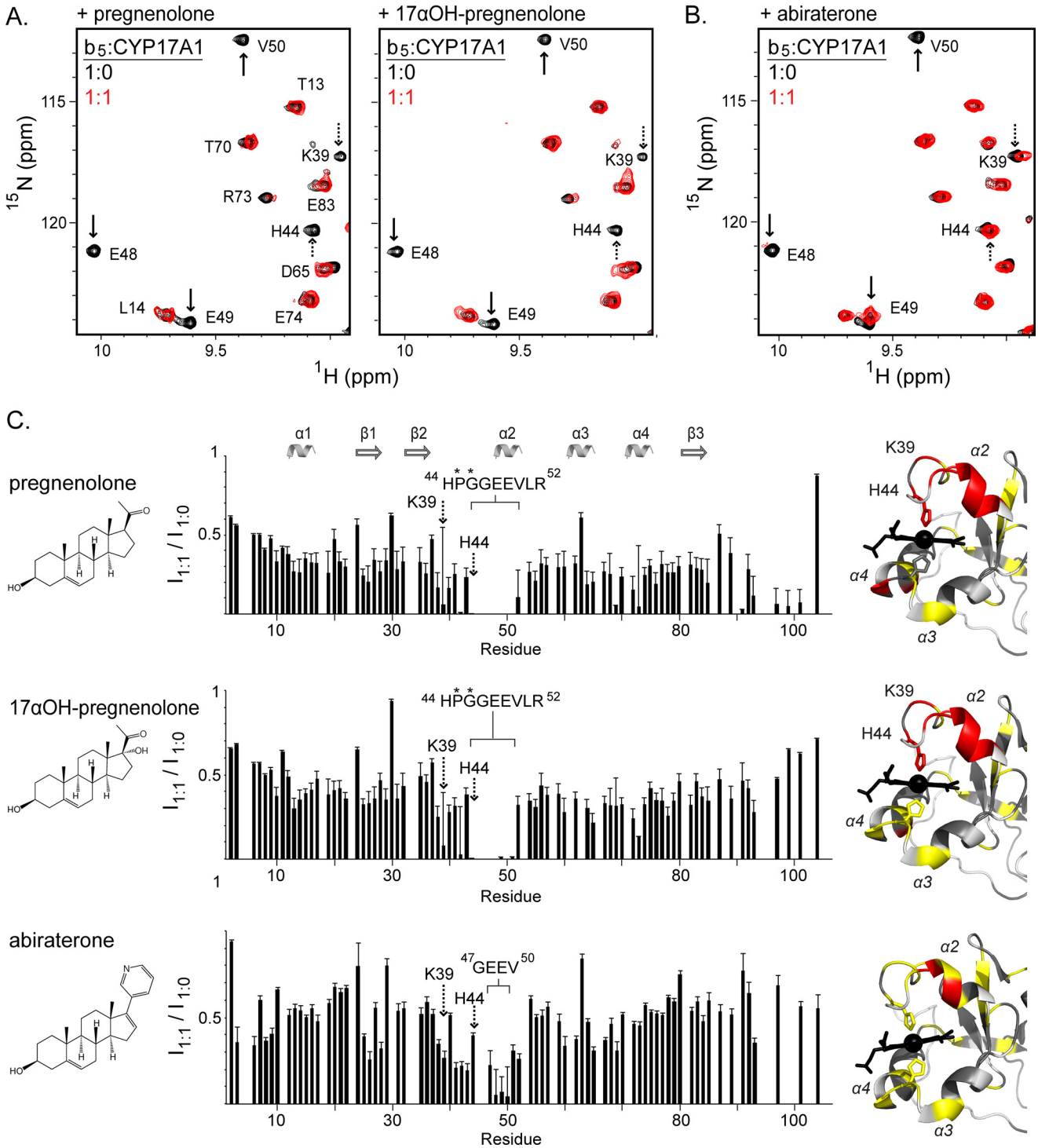

FIGURE 4. **Alterations in b_5-CYP17A1 complex formation in the presence of CYP17A1 ligands including the 17α-hydroxylase substrate pregnenolone, the 17,20-lyase substrate 17α-hydroxypregnenolone, and the heme-coordinating inhibitor abiraterone.** *A*, representative HSQC spectra of ^{15}N-b_5 alone (*1:0*) and in the presence of an equimolar concentration of CYP17A1 (*b5/CYP17A1 1:1*) were collected in the presence of each substrate, showing that in addition to broadening of ^{47}GEEV^{50} observed during b_5 binding to unliganded CYP17A1 (*solid arrows*), enhanced line broadening also occurred for specific residues near the binding interface (*dashed arrows*). *B*, the steroidal inhibitor abiraterone coordinates the CYP17A1 heme iron via its pyridine ring, resulting in a low-spin system that is less strongly paramagnetic, allowing distinction of paramagnetic effects on residues like His-44, Lys-39, and Glu-49 (*dashed arrows*) from intermediate exchange effects on Glu-48 and Val-50 because of complex formation (*solid arrows*). *C*, plots of the relative intensities for all b_5 resonances in the ligand bound *versus* free state illustrate substrate-induced reductions in intensity because of line broadening in regions adjoining the ^{47}GEEV^{50} sequence for substrates but not for the inhibitor abiraterone. Residues marked with an *asterisk* are located near the binding site but were either not assigned or consist of a proline. For each liganded condition, residues line-broadened below 15% of the original signal are mapped onto the structure in *red* in the *right panel*.

Abiraterone directly coordinates to the heme iron via its pyridine ring (21). This hexacoordinate system provides a low-spin ligand-CYP17A1 complex with which to examine b_5 complex formation. A corresponding region of the 2D HSQC spectra for b_5 and for *b5*/CYP17A1 1:1 with abiraterone revealed that resonances corresponding to residues Lys-39, Glu-42, His-44, and

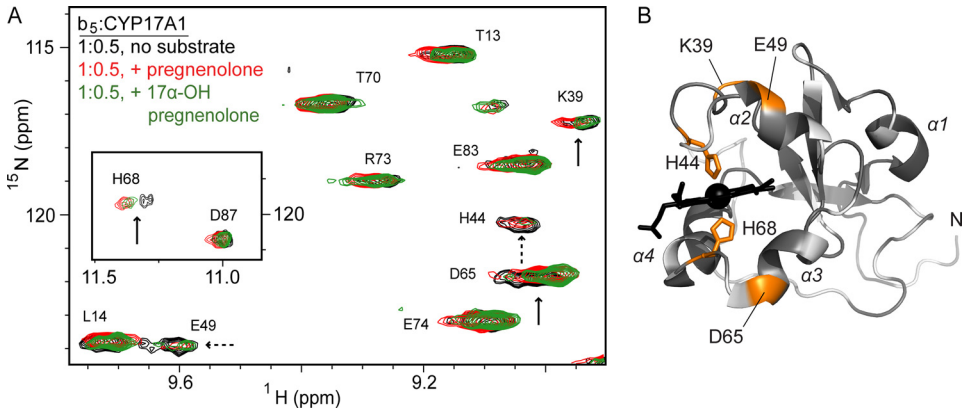

FIGURE 5. **CYP17A1 substrate differentially affects CYP17A1/b_5 interaction.** *A*, representative spectra of b_5 in the presence of a half-molar equivalent of CYP17A1:substrate. At a reduced substrate-bound CYP17A1 concentration, b_5 line broadening is less severe. As a result, minor differences can be observed in the binding to unliganded (*black*), pregnenolone bound (*red*), and 17α-hydroxypregnenolone bound (*green*) CYP17A1. In particular, Lys-39, Asp-65, and His-68 are perturbed differently for each liganded state (*black arrows*), whereas His-44 and Glu-49 (*dashed arrows*) are both perturbed as well as line-broadened to a different extent. *B*, the differentially affected residues (*orange*) map onto both sides of b_5, suggestive of a substrate-influenced change in the b_5 heme.

Glu-49 were retained (Fig. 4*B*, *dashed arrows*), whereas line broadening only occurred for residues Glu-48 and Val-50 (*solid arrows*). This stark difference is readily apparent in the peak intensity plots for abiraterone (Fig. 4*C*, *bottom panel*) as compared with the substrate-bound plots (*top* and *center panels*). This suggests that the alterations in Glu-48 and Val-50 are most likely due to intermediate exchange effects, whereas those on Lys-39, His-44, and Glu-49 are likely due to paramagnetic-related relaxation. In other words, Glu-48 and Val-50 are altered by the interaction of b_5 with CYP17A1 even in the absence of the paramagnetic effects of substrate-bound CYP17A1. Ultimately, this provided an additional tool with which to confirm the orientation of b_5 relative to the CYP17A1 active site.

To gain further insight into the interaction between b_5 and the CYP17A1 with substrate complexes, data were collected on an intermediate titration point corresponding to a 1:0.5 molar equivalent of b_5 and CYP17A1:substrate, respectively. At this half-molar equivalent, line broadening from complex formation is less severe, allowing observation of amide resonances for several of the residues near the binding site. Compared with the same half-molar (b_5:CYP17A1, 1:0.5) data set collected in the absence of CYP17A1 substrate (Fig. 5*A*, *black*), chemical shifts were observed for the b_5 heme-coordinating residue His-68 and the conserved anionic residue Asp-65 (Fig. 5*A*, *red* and *green spectra*). These residues are both located on b_5 opposite Glu-48/Glu-49 (Fig. 5*B*). This result could be explained by a two-point interaction between CYP17A1 and b_5 and/or a difference in the b_5 heme orientation when b_5 binding occurs to substrate-bound CYP17A1. Furthermore, this experiment also suggested a difference between the b_5-CYP17A1-pregnenolone complex and that of b_5-CYP17A1–17α-hydroxypregnenolone. For example, the resonances for the charged residues Lys-39 and Glu-49, although still somewhat broad in the half-molar titration points, are nonetheless perturbed differently with the lyase substrate 17α-hydroxypregnenolone in the CYP17A1 active site *versus* the hydroxylase substrate pregnenolone (Fig. 5*A*, *red* and *green spectra*). In addition, the heme-coordinating histidine His-44 is line-broadened to a greater extent with 17α-hydroxypregnenolone than with pregnenolone. Collectively, these differences suggest a modulation of the b_5/CYP17A1 interaction that depends on the liganded state of CYP17A1.

CYP17A1 Substrates Affect the Relative Affinity for b_5 and CPR—The mutual exclusivity of the b_5 and CPR binding to CYP17A1 was further employed to probe differences in how b_5 binds CYP17A1-substrate complexes. As demonstrated previously, higher concentrations of rat CPR can outcompete b_5 for CYP17A1 binding, resulting in the liberation of b_5 and regeneration of the free ^{15}N-b_5 HSQC signal (Fig. 3). This relationship provides an orthogonal way to characterize substrate-specific protein-protein interactions. A series of 2D ^{15}N-HSQC experiments were conducted in which the b_5-CYP17A1 (1:1) complexes saturated with substrate were titrated with varying amounts of CPR. Representative portions of these spectra showing the resonances for Glu-48 and Glu-49, which broaden and disappear upon b_5/CYP17A1complex formation, are shown, along with the resonance for Leu-14, which is unaffected and serves as a control. As demonstrated previously, in the absence of CYP17A1 ligand, it takes an equal molar equivalent of CPR to outcompete b_5, as signaled by the reappearance of the Glu-48 and Glu-49 resonances (b_5/*CYP17A1*/*CPR 1:1:1*, Fig. 6*A*). However, when the same competition experiment is carried out with either substrate in the CYP17A1 active site, addition of an equimolar amount of CPR failed to regenerate the unbound b_5 signal (Fig. 6, *B* and *C*, b_5/*CYP17A1*/*CPR 1:1:1*). Adding additional CPR to a 1.5 molar equivalent resulted in only a slight recovery of the free b_5 Glu-48 signal from the b_5-CYP17A1-pregnenolone complex (Fig. 6*B*, *1:1:1.5*, *right panel*) but a near full recovery of the free b_5 Glu-48 and Glu-49 signals from the b_5/CYP17A1:17α-hydroxypregnenolone complex (*C*, 1:1:1.5, *right panel*). Notably, the difference in the ability of rat CPR to disrupt the b_5/CYP17A1 interaction appeared to be impacted both by the presence of substrate as well as by the particular substrate present, with pregnenolone promoting a relatively stronger interaction with b_5 than when 17α-hydroxypregnenolone is present. Therefore, these results provide additional evidence of a b_5/CYP17A1 binding interaction that is differentially affected by the liganded state of CYP17A1.

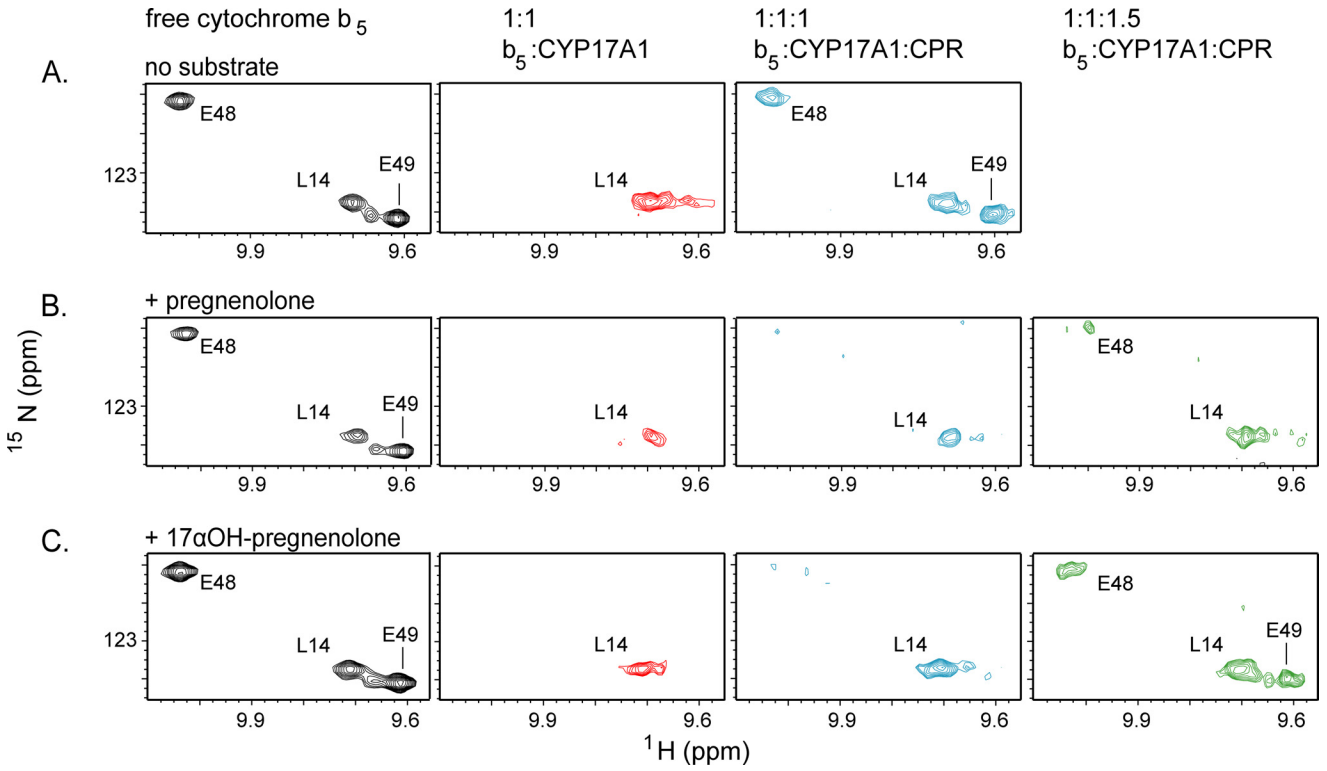

FIGURE 6. **CYP17A1 substrate differentially affects the ability of CPR to disrupt binding to b_5.** Samples containing ^{15}N-b_5 and CYP17A1 in 1:1 molar ratios were titrated with CPR, and HSQC spectra were reacquired. *A*, as demonstrated, in the absence of CYP17A1 substrate, an equal molar amount of CPR (*1:1:1*) was sufficient to observe the complete return of resonances for Glu-48 and Glu-49, indicating the liberation of free b_5. *B*, when pregnenolone is bound to CYP17A1, a greater molar equivalent of CPR was required to liberate free b_5, as indicated by a weak Glu-48 and Glu-49 signal even with 1.5 molar equivalents of CPR. *C*, when 17α-hydroxypregnenolone is the CYP17A1 ligand, more CPR is required to liberate b_5 than in the substrate-free situation, as indicated by weak signals in the 1:1:1 condition, but the free b_5 signal fully reappears upon increasing CPR to the 1.5 molar equivalent (*1:1:1.5*).

DISCUSSION

As a whole, the body of literature describing the ability of b_5 to affect P450 rates of catalysis provides an intriguing, yet occasionally confounding picture. The b_5 effect, for example, can be P450- or substrate-specific (3). Furthermore, the orientation of b_5 binding may be different for different P450 isoforms (24). Therefore, to provide insight into b_5 function in general and interactions with CYP17A1 specifically, solution NMR spectroscopy was employed as a high-resolution technique to investigate the interaction between the soluble domains of cytochrome b_5 and CYP17A1. This particular b_5/P450 system is particularly important for a number of reasons. First, the presence of b_5 selectively enhances the CYP17A1-mediated 17,20-lyase activity but not its 17α-hydroxylase activity, although both occur with very similar substrates in the same active site. Elucidating b_5 function in this system may also provide insight into other monooxygenase systems, such as CYP2B4, in which the b_5 effect appears to be partially substrate-dependent (4). Secondly, the detection of clinical isolated lyase deficiency associated with some CYP17A1 mutations (39) and the selective enhancement of 17,20-lyase activity by b_5 suggest that the two catalytic activities of CYP17A1 can be controlled independently, which is of clinical utility. The current CYP17A1 inhibitor and prostate cancer drug abiraterone inhibits both hydroxylase and lyase activities (40–42), with the hydroxylase inhibition leading to undesirable side effects as a result of mineralocorticoid and glucocorticoid imbalances (43). As such, there is considerable interest in novel 17,20-lyase selective inhibitors. Characterizing the structural biology underlying the b_5-driven 17,20-lyase enhancement of CYP17A1 may provide substrate-specific information that could inform the design of such lyase-selective inhibitors.

Although isolated b_5 is a well studied system (32, 44), to our knowledge, the this work represents the first use of multidimensional solution NMR to study this important protein/protein interaction in a mammalian (membrane) monooxygenase system. Simple mixing of the soluble ^{15}N-b_5 and CYP17A1 proteins resulted in a combination of line broadening and small chemical shift perturbations (Fig. 1), suggesting that binding occurs on an intermediate exchange time scale. Modulation of resonances for individual residues supported an interaction mediated primarily by surface electrostatics of both binding partners. Specifically, the b_5 anionic residues Glu-48 and Glu-49 in the α2 helix and the cationic Arg-347, Arg-358, and Arg-449 residues on the proximal surface of CYP17A1 were important for b_5-CYP17A1 complex formation (Fig. 2). These findings are in keeping with previous functional and genetic analysis of mutations at these same sites in the full-length proteins (11, 20, 23), thus indicating that the soluble domains interact in a manner consistent with the full-length variants. Additionally, direct biophysical evidence was provided for mutually exclusive, reversible binding between b_5 and CPR to CYP17A1 (Fig. 3), for which the simplest explanation is overlapped or partially overlapped binding sites. This binding relationship has

been proposed for several P450 enzymes (4, 6, 24), including CYP17A1 (19), but clear biophysical evidence of a direct interaction has been limited.

Ligands in the buried CYP17A1 active site modulated CYP17A1 surface interaction with b_5. The complex formed from a 1:1 molar ratio of b_5 and CYP17A1 bound to the iron-coordinating CYP17A1 inhibitor abiraterone enhanced most b_5 peak intensities relative to that of substrate, likely because this low-spin CYP17A1:ligand complex has reduced paramagnetism. In contrast, the presence of substrates in the CYP17A1 active site caused enhanced relaxation of additional NMR resonances near the protein-protein interface. Although this effect could be attributable to differences in b_5 conformational exchange when bound to substrate bound CYP17A1, given the highly specific clustering of affected residues near the confirmed binding site, the most likely cause is paramagnetism emanating from the CYP17A1 active site. However, reducing the b_5/CYP17A1 ratio to 1:0.5 reduced this enhanced relaxation effect and allowed observation of key residues (Fig. 5). For example, the resonances corresponding to Asp-65 and His-68 were uniquely shifted when b_5 was bound to CYP17A1 with the substrate present. The location of these b_5 residues opposite the primary α2 CYP17A1 binding surface may suggest either a secondary protein-protein contact site or a change in positioning of the b_5 heme in the complex, both of which are apparently influenced by the presence of substrate in the CYP17A1 active site. Additionally, these experiments also indicate that b_5 binds to CYP17A1 differently, depending on which substrate is present. For example, residues His-44 (the heme-coordinating histidine facing CYP17A1) and the binding determinant Glu-49 are both affected in a substrate-dependent manner. For example, the amide signal for His-44 is substantially more broadened at the b_5/CYP17A1 1:0.5 titration point when the CYP17A1 active site contains 17α-hydroxypregnenolone compared with His-44 at the same 1:0.5 titration point when the active site contains pregnenolone.

Furthermore, substrate-modulated modes of CYP17A1 binding to b_5 translate into a difference in the ability of rat CPR to disrupt the b_5-CYP17A1 complex (Fig. 6) so that more CPR is required to outcompete b_5 for binding to CYP17A1 when it contains substrate than with unliganded CYP17A1. This relationship goes a step further in that more CPR is necessary to disrupt the complex between b_5 and CYP17A1 when pregnenolone is in the CYP17A1 active site than when 17α-hydroxypregnenolone is present. The implication of these findings is that the presence of 17α-hydroxylase pregnenolone substrate results in stronger relative affinity for b_5 over CPR, whereas, by comparison, the presence of the 17,20-lyase substrate 17α-hydroxypregnenolone results in a decreased relative affinity for b_5 *versus* CPR. Because the data suggest that binding sites for b_5 and CPR on the proximal face of CYP17A1 may be at least partially overlapping, it is not possible to distinguish between CYP17A1 substrate-modulated increases in CYP17A1/b_5 association and decreases in CYP17A1/CPR association using this approach. Nonetheless, the rank ordering of these CPR competition experiments was unexpected. Given the ability of b_5 to enhance 17,20-lyase activity, the association of b_5 with CYP17A1:17α-hydroxypregnenolone might be expected to be the strongest.

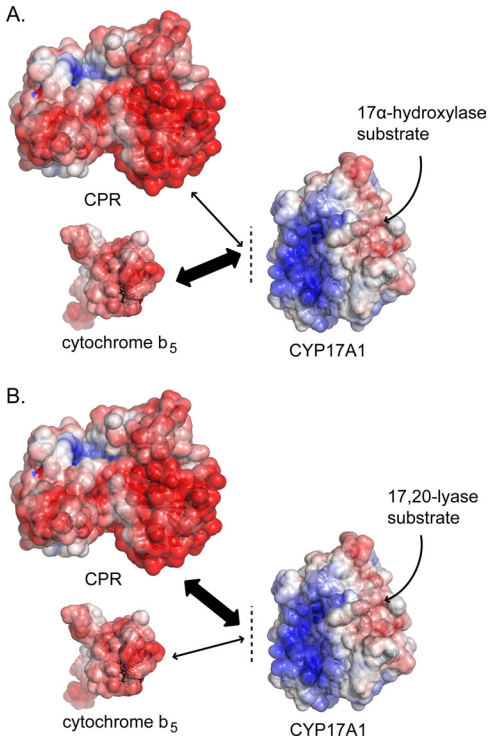

FIGURE 7. **Model describing the second-order effects of 17α-hydroxylase and 17,20-lyase substrates on modulation of the b_5-CYP17A1 and CPR-CYP17A1 complexes.** *A*, the current data support a scheme in which, when in the presence of pregnenolone, the *b5*-CYP17A1 electrostatically driven association is enhanced relative to the same interaction in the absence of substrate, thus resulting in decreased access to electron transfer via CPR. *B*, the scales of this balance tip in the presence of 17α-hydroxypregnenolone, in which a weaker *b5*:CYP17A1 association (relative to pregnenolone) results in enhanced electron transfer via CPR.

These findings indicate the opposite, with the hydroxylase substrate apparently resulting in a higher association between b_5 and CYP17A1. One explanation of these results can be envisaged in the model for b_5 modulation of CYP2B4, as put forth by Waskell and colleagues (3), in which b_5 might be able to both enhance rates of catalysis via an allosteric effect on the CYP17A1 active site while also inhibiting P450 activity by physically blocking electron donation via CPR. In systems where the stimulatory and inhibitory activities are approximately equal, there is no measurable b_5 enhancement. One can imagine, then, how modulation of the relative associations between the three monooxygenase components can, in effect, tip the scales of this balance. The data presented here suggest that such a relationship may exist in CYP17A1 (Fig. 7), in which the relatively weaker association between b_5 and CYP17A1 promoted by 17,20-lyase substrate binding results, in turn, in enhanced association between CPR and CYP17A1, thereby increasing the lyase reaction. The CYP17A1 lyase reaction is reportedly more sensitive than the hydroxylase reaction to naturally occurring CPR mutations so that the inability to generate androgens results in severe clinical steroidogenic defects, whereas effects on other CPR-mediated reactions are more moderate (45, 46). Additionally, *in vitro* assays with increased CPR preferentially increase the lyase reaction over the hydroxylase reaction (47).

Aside from the substrate-driven specific rank ordering of these relative affinities, this study clearly indicates the ability of

substrate binding to affect changes at the proximal surface of CYP17A1. This raises the distinct possibility of allostery occurring in the opposite direction, in which cytochrome b_5 binding may induce a conformational change that is communicated to the CYP17A1 active site. This b_5 allosteric effect has been postulated extensively (5, 8), posed as a potential means of reorienting lyase substrate to facilitate the acyl-carbon cleavage between C17 and C20 (48) to facilitate 17,20-lyase activity. Clearly, additional studies are required in which the CYP17A1 active site can be observed for specific changes that correlate to b_5 and substrate binding.

In summary, a key component to understanding and manipulating CYP17A1 function is to determine how b_5 interaction facilitates CYP17A1 lyase activity but not hydroxylation. This study employs high-resolution NMR to not only monitor the reversible interaction between cytochrome b_5 and CYP17A1 but to identify the specific residues involved in the interface, validate mutually exclusive binding of b_5 and CPR, and reveal differences in b_5-CYP17A1 complex formation, depending on the CYP17A1 substrate, thus providing biophysical evidence of allosteric communication between the CYP17A1 active site and its proximal superficial b_5 binding site.

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