Human cytochrome P450 17A1 Conformational Selection

MODULATION BY LIGAND AND CYTOCHROME b5

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**Background:** Crystallography provides a static structure of cytochrome P450 17A1 (CYP17A1). The distribution of CYP17A1 conformational states was influenced by temperature, binding of these two substrates, and binding of the soluble domain of cytochrome b5 (b5). Notably, titration of b5 to CYP17A1-pregnenolone induced a set of conformational states closely resembling those of CYP17A1-17α-hydroxypregnenolone without b5, providing structural evidence consistent with the reported ability of b5 to selectively enhance 17,20-lyase activity. Solution NMR thus revealed a set of conformations likely to modulate human steroidogenesis by CYP17A1, demonstrating that this approach has the potential to make similar contributions to understanding the functions of other membrane P450 enzymes involved in drug metabolism and disease states.

**Results:** Solution NMR reveals an ensemble of CYP17A1 conformational substates.

**Conclusion:** Ligand, cytochrome b5, or temperature alters the conformational CYP17A1 substates present.

**Significance:** Changes in conformations probably modulate human steroidogenesis by CYP17A1.

Crystalllographic studies of different membrane cytochrome P450 enzymes have provided examples of distinct structural conformations, suggesting protein flexibility. It has been speculated that conformational selection is an integral component of substrate recognition and access, but direct evidence of such substrate interconversion has thus far remained elusive. In the current study, solution NMR revealed multiple and exchanging backbone conformations for certain structural features of the human steroidogenic cytochrome P450 17A1 (CYP17A1). This bifunctional enzyme is responsible for pregnenolone C17 hydroxylation, followed by a 17,20-lyase reaction to produce dehydroepiandrosterone, the key intermediate in human synthesis of androgen and estrogen sex steroids. The distribution of CYP17A1 conformational states was influenced by temperature, binding of these two substrates, and binding of the soluble domain of cytochrome b5 (b5). Notably, titration of b5 to CYP17A1-pregnenolone induced a set of conformational states closely resembling those of CYP17A1-17α-hydroxypregnenolone without b5, providing structural evidence consistent with the reported ability of b5 to selectively enhance 17,20-lyase activity. Solution NMR thus revealed a set of conformations likely to modulate human steroidogenesis by CYP17A1, demonstrating that this approach has the potential to make similar contributions to understanding the functions of other membrane P450 enzymes involved in drug metabolism and disease states.

Human cytochrome P450 (P450) enzymes are membrane proteins that play key roles in the phase I metabolism of xenobiotic compounds and in the synthesis and interconversions of endogenous compounds, such as vitamins and steroids (1). These enzymes have been referred to as nature’s blowtorch (2) for their ability to oxidize chemically inert hydrocarbons with regio- and stereospecificity. Modern perspectives on enzyme catalysis recognize a series of conformational substates may exist for an enzyme interacting with a substrate as this system moves between E + S and E + P ground states. Studies of many enzymes reveal a role for conformational selection, wherein the presence of different conformational substates enables ligand selection and also reveal that the ligand and/or interacting partners influence the equilibrium between substates (3). In the cytochrome P450 field, a series of well defined chemical intermediates are known for the buried heme iron and its ligands, but relatively little is known about protein conformations that modulate the ability to perform chemistry at the iron, particularly for the membrane P450 enzymes found in humans and other mammals. In part because many individual human P450 enzymes oxidize multiple substrates with remarkably diverse small molecule scaffolds, the conformational ensemble for P450 enzymes should be expected to be particularly broad.

For mammalian P450 enzymes, which are all membrane proteins, an atomic level view of the protein structure was first available for an engineered rabbit P450, CYP2C5, as determined by x-ray crystallography (4). Building on this key advance, at least one structure is now available for most major human xenobiotic-metabolizing P450 enzymes and for some of those involved in key endogenous pathways (5). However, this seeming wealth of structural information is at a severe deficit compared with that needed to understand substrate binding and catalysis by these enzymes. Although x-ray structures are recognized to represent a snapshot of protein conformation, in the absence of other structural information, such static depictions can often be unconsciously overemphasized as the only functionally relevant conformation. Most membrane P450 structural snapshots represent liganded states because these are frequently more stable. However, much less information is available about enzyme conformation when ligands are absent (6). A number of the these ligands coordinate directly to the heme iron in an inhibitory state, which again increases stability of the complex for crystallography but does not fully reflect the situation for substrate binding or catalysis. Some x-ray structures show substrates with placement of the functionally observed oxidation site near the heme iron, but in other instances, substrates are observed in very different orientations.
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inconsistent with known metabolism products. Sometimes one or more channels are observed from the buried active site to the surface. In the absence of other information, much emphasis is placed on these channels with respect to potential substrate entry or product exit. A review of the available structures reveals that much of the variation among these structures occurs at or near what is thought to be the membrane-binding face (6). This exposed, generally hydrophobic surface is also the portion of the protein that almost invariably packs together for adjacent protein molecules in the crystal lattice, potentially limiting or influencing the observed structure of this region. For all of these reasons, one suspects that these static structures are only a small part of the protein conformational landscape that facilitates P450 catalysis. This idea is consistent with the well-established effects of amino acids far from the active site modulating catalysis. For example non-active site residues in CYP2B4 (7), CYP2A6 (8), CYP1A2 (9, 10), and CYP17A1 (reviewed in Ref. 11) are known to alter ligand binding, metabolism, and inhibition, some potentially by playing roles in conformational sampling and the transitions between substates necessary for ligand binding and catalysis.

Spectroscopy and other biophysical methods have been powerful approaches to probe P450 catalysis, leading to the current understanding of the catalytic cycle (12, 13). However, the present compendium of mammalian P450 x-ray structures represents only the first substrate-binding step in a multistep catalytic cycle. Subsequent P450 interactions with NADPH-cytochrome P450 reductase, required for electron delivery, and with cytochrome b5 still await structural definition, although progress is being made for soluble, bacterial P450 enzymes (14, 15). Due to the paucity of experimental data, computational techniques have sought to extrapolate protein dynamics and/or the docking of other substrates from current structures to explore further aspects of enzyme function (16–18).

Solution NMR spectroscopy is particularly sensitive to the experimental detection of conformational substates of proteins. This powerful tool has been used to reveal multiple conformational substates in liganded (19–22) and substrate-free (23) water-soluble, bacterial P450 enzymes that are relatively restrictive for a single substrate. Unfortunately, technical challenges have delayed the application of NMR to the large field of medically relevant human membrane P450 enzymes. Herein protein NMR reveals previously undiscovered conformational substates for the human steroidogenic cytochrome P450 CYP17A1.

CYP17A1 plays several key roles in human steroidogenesis. It hydroxylates cholesterol-derived pregnenolone and progesterone to generate 17α-hydroxy precursors for glucocorticoid and androgen production. Additionally, it accomplishes carbon-carbon bond cleavage to perform subsequent conversion of 17α-hydroxypregnenolone to dehydroepiandrosterone, the first androgen leading to production of male and female sex hormones. CYP17A1 is a clinically important drug target. Its inhibition by the active drug abiraterone for metastatic castration-resistant prostate cancer (24) recently provided an exciting new option for treating the second leading cause of cancer death in United States men (25). This enzyme is intriguing from a biochemical perspective because it binds and turns over a set of substrates and performs both hydroxylation and lyase reactions via distinct catalytic intermediates, with only the lyase reaction altered by the presence of cytochrome b5. The addition of b5 disproportionately enhances the efficiency of the 17,20-lyase reaction (as much as 10-fold), with only a 2-fold increase in the 17α-hydroxylase activity (26–29). This impact of b5 on the lyase reaction is a relationship that is physiologically important in human prepubertal development and tissue-specific steroid production.

Although the few existing x-ray structures of CYP17A1 are very similar to each other (11), examination by protein NMR reveals new data that directly indicate conformational heterogeneity. Key portions of the CYP17A1 structure adopt multiple conformations, which are modulated by temperature, ligand, and cytochrome b5. The conformations of CYP17A1 differ when binding the inhibitor abiraterone, a hydroxylase substrate, or a 17,20-lyase substrate. Finally, cytochrome b5 alters heterogeneous NMR signals, thus shifting the CYP17A1 conformational ensemble. The detection and interconversions of multiple conformational substates for CYP17A1 provide an orthogonal view of the secret life of this key steroidogenic enzyme, potentially revealing a broader target space for the design of improved therapeutic inhibitors. Additionally, the realization of solution NMR as a viable tool to probe ensembles of membrane CYP17A1 substates provides the potential to examine numerous human P450 enzymes with key roles in human drug metabolism and other endogenous processes.

EXPERIMENTAL PROCEDURES

Expression of CYP17A1 with Abiraterone—Escherichia coli JM109 cells containing the pCW17A1Δ19H1 plasmid (11) were used to express human CYP17A1 modified by deletion of the N-terminal membrane-spanning tail (residues 2–19), substitution of the native sequence with 20RRCP23 with 20AKKT23, and incorporation of four C-terminal histidines to facilitate purification. A 10-ml lysogeny broth culture inoculated with a single colony was grown for ~8 h, and 200 μl was used to inoculate a 200-ml lysogeny broth overnight culture (37 °C, 220 rpm). Inoculate from this culture (25 ml) was used to initiate growth of each liter of minimal medium. Ampicillin (100 μg/ml) was included in all cultures.

For the expression of uniformly 15N-labeled samples, cells were grown in minimal medium (30 °C at 37 °C with shaking at 220 rpm until reaching an optical density of 0.6–0.8, as measured at 600 nm. Cultures were then induced by adding isopropyl 1-thio-β-D-galactopyranoside (1 mM) and supplemented with the heme precursor γ-aminolevulinic acid (0.55 mM). For expression of CYP17A1 in complex with abiraterone, abiraterone was dissolved in DMSO and added to medium at induction to a final concentration of 50 μM. Abiraterone was generated as described previously (11) and was a generous gift from the laboratory of Dr. Jeffrey Aubé. Overexpression of CYP17A1 in complex with abiraterone was achieved by shaking at 180 rpm and 27 °C for 48–64 h.

For expression of 15N-selectively labeled CYP17A1, cells were grown as described above with the substitution of unlabeled NH4Cl. After reaching log phase, cells were collected via centrifugation at 6800 × g for 10 min and resuspended in prewarmed minimal medium containing either isoleucine or phe-
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nylalanine labeled with $^{15}$N and unlabeled versions of the other 19 amino acids (as described previously (31)). Following equilibration for 1 h with shaking at 220 rpm at 37 °C, cells were induced as described above.

Expression of CYP17A1 with Substrate—CYP17A1 bound to either substrate required co-expression with GroEL/GroES chaperones. Briefly, DH5α cells were transformed with both the CYP17A1 expression construct and the pGro7 plasmid (Takara Bio Inc.), selected for by the inclusion of ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml), respectively. Chaperone expression was induced by the addition of L-arabinose (25 mM) concurrent with CYP17A1 induction. Additionally, either pregnenolone (Acros Organics) or 17α-hydroxyxypregnenolone (Sigma-Aldrich) was added to expression medium at inoculation to a final concentration of 100 μM. Expression conditions were otherwise as described above.

CYP17A1 Purification—Typically cells from 2–6 liters of culture were harvested by centrifugation at 6800 × g for 10 min and resuspended in 30 ml of cold lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 20% glycerol, pH 8.0) and augmented with a small amount of solid phenylmethylsulfonyl fluoride. Resuspended cells were either stored at −80 °C until purification or subjected to flash freezing in liquid N2 and immediately thawed at room temperature for purification. Thawed cells were augmented again with a small amount of solid phenylmethylsulfonyl fluoride and sonicated on ice for 30-s bursts with 60-s of intermittent cooling for a total of six cycles. Lysed cells were centrifuged at 6800 × g for 15 min and subjected to detergent extraction with stirring for 1 h at 4 °C using 2% Emulgen-913 (Desert Biologicals). For extraction with substrate-bound CYP17A1, additional substrate was dissolved in DMSO and added to the extraction mixture to a final concentration of 200 μM. Subsequent purification of CYP17A1 was carried out by metal affinity and ion exchange chromatography as described (32). For generation of substrate-bound samples, 25 μM substrate was added to all purification buffers. Protein purity was evaluated by SDS-PAGE and UV-visible spectroscopy ($A_{420}/A_{280} > 1$ for abiraterone-bound sample and $A_{395}/A_{280} > 1$ for substrate-bound samples).

Mutagenesis—Mutation of individual amino acids to determine NMR assignments in CYP17A1 was carried out using the QuickChange Lightning site-directed mutagenesis kit (Stratagene). In order to preserve the structural integrity and stability of the protein, all substitutions were designed using similar side chains (I292V, I296V, F300L, I238V, I205V, and I206L). All mutants were expressed and purified in the presence of abiraterone as described above.

Expression and Purification of Cytochrome $b_5$—The soluble domain of microsomal cytochrome $b_5$ (residues 1–107) expressed was identical to the one used previously (32), in which the membrane-spanning C-terminal domain was replaced with a hexahistidine tag. Expression of unlabeled $b_5$ was carried out in Terrific Broth medium and was otherwise identical to the previous protocol (32). Final purity was evaluated by SDS-PAGE and UV-visible spectroscopy ($A_{412}/A_{280} > 4$).

NMR Spectroscopy and Titration of $^{15}$N/CYP17A1 with Unlabeled $b_5$—Purified $^{15}$N/CYP17A1 was transferred into a buffer consisting of 50 mM potassium phosphate, pH 6.5, 50 mM NaCl, 10% D$_2$O, and either 10 μM abiraterone or 50 μM substrate. Final CYP17A1 concentration was 0.2–0.5 mM. CYP17A1 saturation with ligands was verified by UV-visible spectroscopy. All $^1$H-$^{15}$N HSQC and $^1$H-$^{15}$N TROSY data sets were collected on a Bruker Avance 800-MHz spectrometer fitted with a TCI cryoprobe at 35 or 40 °C. Data were processed using NMRPipe (33) and analyzed with NMRViewJ (34) and CcpNmr (35). Increasing amounts of $b_5$ were added to samples containing $^{15}$N/CYP17A1 saturated with either the inhibitor abiraterone or one of the substrates. Due to significant line broadening when $b_5$ amounts approached concentrations equimolar with $^{15}$N/CYP17A1, all $b_5$ concentrations were kept between 0.2 and 0.5 molar eq. As an additional countermeasure to line broadening due to formation of the CYP17A1-$b_5$ complex, stepwise increments of $b_5$ were accompanied by increases in the number of scans acquired for each spectrum.

RESULTS

Generation of CYP17A1 NMR Spectra and Spectral Assignments—The major technical difficulties involved in the application of solution protein NMR to membrane cytochrome P450 enzymes have been 1) the ability to generate the required quantities of appropriately labeled protein in minimal medium and 2) the moderate stability and solubility of these enzymes in conditions suitable for the NMR experiment. Technical improvements in the expression and stabilization of these proteins employed originally for x-ray crystallography were applied herein, including truncation of the N-terminal helix, co-expression with chaperones, expression and/or purification with ligands, and efficient and high yield purification steps. The CYP17A1 construct used in these studies is identical to the one used to generate the only available crystal structures of CYP17A1, those in complex with the steroidal inhibitors TOK-001 and abiraterone (11) (Fig. 1A). Iterative optimization of CYP17A1 expression in minimal medium resulted in sufficient quantities of isotopically labeled, liganded CYP17A1 to initiate solution NMR experiments. Typically 50–100 nmol of highly purified CYP17A1 could be generated per liter of expression medium with $^{15}$N labeling either uniformly throughout the protein or for the Ile or Phe residues only. Additionally, CYP17A1 appears to have advantages in terms of stability and solubility in NMR-compatible solution conditions, without glycerol or detergent, especially in the presence of ligands, permitting the collection of NMR data, which typically occurred over 4–8 h. Comparison of the reduced CO difference spectra before and after at least 12 h at the highest temperatures used herein (40 °C for CYP17A1-abiraterone and 35 °C for CYP17A1-pregnenolone complex) yielded minimal changes in the spectra, with no obvious increase in absorbance at 420 nm that would indicate generation of the inactive P420 form.

A typical $^1$H-$^{15}$N TROSY spectrum of CYP17A1 in complex with the inhibitor abiraterone (Fig. 1B) shows the large degree of spectral overlap expected for a protein with 494 amino acids (55.6 kDa). Two approaches were applied to simplify this crowded spectrum and to link observed resonances to individual amino acids. First, CYP17A1 was generated with $^{15}$N labeling for only a select type of amino acid. Analysis revealed that the distribution of Ile and Phe residues throughout the protein...
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CYP17A1. The wild type and single mutant spectra were very similar, with essentially only a single missing resonance in the spectrum of the mutated protein, which could thus be ascribed to the specific mutated residue. The set of residues assigned in this way (Fig. 1, black side chains in A, labeled resonances in B) includes representatives from structural elements predicted to be variable in different protein conformations and included residues from the F helix (Ile-205 and Ile-206), the G helix (Ile-238), and three sequential turns of the I helix (Ile-292, Ile-296, and Phe-300). Changes in both these assigned residues and other unassigned residues were subsequently monitored under various conditions to obtain an initial overview of human CYP17A1 conformational states.

Conformational Heterogeneity—Initial evaluation of CYP17A1 structure by NMR was accomplished in the presence of the inhibitor and prostate cancer drug abiraterone. This drug consists of a steroid scaffold with a pyridine ring substituent at C17 (Fig. 1). The pyridine nitrogen lone pair forms a ligand/metal coordination complex with the heme iron, resulting in a hexacoordinate, low spin state (11). Abiraterone binds with a nanomolar affinity (11) and significantly stabilizes CYP17A1, permitting NMR data collection at room temperature and at higher temperatures. Temperature could thus be used to perturb the CYP17A1-abiraterone complex. A series of $^1$H-$^{15}$N TROSY spectra were collected at 35 and 40 °C using either $^{15}$N uniform or $^{15}$N Ile labeling for the different residues. In general, the increased temperature did not induce a dramatic change in the protein NMR spectrum, thus suggesting that the global fold of CYP17A1 was not affected. However, a small number of signals displayed temperature-induced changes in peak line width and shape. For example, at 35 °C, a number of the amide resonances for which assignments have been made appeared as broad or irregularly shaped peaks (Fig. 2, left), consistent with a variety of backbone conformations. However, several of these resonances resolved into more distinct backbone resonances when the spectrum was measured at 40 °C (Fig. 2, right). Residues Ile-292, Ile-296, and Phe-300 are found in three sequential turns of the N-terminal I helix, and all are present as two more distinct peaks at the higher temperature. Such changes are indicative of a change in environment for the backbone of these residues, most directly interpreted as two major conformational states for these residues. Interestingly, the distance between these split resonances increased incrementally from the center of the I helix (Phe-300) toward the peripheral N-terminal end (Ile-292), suggesting that the two environments for these residues increasingly diverge along the I helix. This change may extend its effect to Ile-206 of the F helix, where the higher temperature clarifies the appearance of up to three apparent conformers, whereas less change is observed for Ile-205. However, the second shell residue Ile-238 of the adjacent G helix appears as a single resonance at either temperature, suggesting that the conformational distributions observed for the I helix are localized and not global.

The observation that specific resonances are better resolved with a small increase in temperature reflects a change in local dynamics invoked by overcoming an energy barrier to conformational exchange. Although faster exchange often results in peak averaging, for the I helix, these new states are distinct and
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FIGURE 2. Temperature-dependent conformational changes in residues in the F, G, and I helices of the CYP17A1-abiraterone complex. Comparison of uniform and selectively labeled 1-H-15N TROSY spectra collected at 35 and 40 °C suggests that residues in F and I helices undergo a change in conformational dynamics, as indicated by differences in NMR signal line shape for individual residues. Higher temperature results in additional conformations for the active site residues Ile-205 and Ile-206 as well as a clear second conformation for the evenly spaced I helix residues Ile-292, Ile-296, and Phe-300. This effect does not extend to the G helix.

Concomitantly, appearance of new peaks is observed in the 1-H NMR spectra for CYP17A1 in the absence of abiraterone. First, line broadening does not occur uniformly across the spectrum. Instead, at a CYP17A1/b₅ ratio of 1:0.3, a subpopulation of CYP17A1 resonances are significantly broadened, e.g. Phe-300 and Ile-205 (F300 and I205) in Fig. 3, whereas other resonances are broadened to a much smaller extent. This differential line broadening would be consistent with a b₅-induced effect that disproportionately changes the chemical exchange rate for particular regions of the protein. The assigned residues (Fig. 4) are some distance away from the b₅ binding site, yet they show various responses to the addition of b₅. For example, Phe-300 and Ile-205, which line the active site, were severely broadened beyond 0.3 molar eq of b₅ (Fig. 3). Although substantial line broadening would not have been unexpected near the b₅ binding interface, where chemical exchange should be most affected, these residues are remote from the b₅ binding surface on the proximal face of CYP17A1 (Fig. 1A), consistent with b₅ producing a long range allosteric effect on the CYP17A1 active site residues.

Other peaks, however, demonstrate the opposite effect. Instead of broadening, some resonances are narrowed or sharpened upon the addition of b₅ (Fig. 3, dashed arrows and inset). Closer inspection of these peaks reveals that what initially appears to be a chemical shift perturbation is actually progressive narrowing of the signal toward one side of the broader CYP17A1 peak observed in the absence of b₅. Such peak intensification is consistent with a reduction of motion or reinforcement of a single conformation for these portions of the CYP17A1 backbone upon b₅ binding. Although these resonances have not been assigned, such a response would be consistent with rigidification of residues that compose the CYP17A1 portion of the CYP17A1-b₅ interface.

Temperature-dependent conformational changes in residues in the F, G, and I helices of the CYP17A1-abiraterone complex.
heme and forms a “wall” of the active site. I helix residues Ile-292, Ile-296, and Phe-300 are located on the side of the I helix facing away from the active site. Here 0.3 molar eq of $b_5$ (Fig. 4, red spectra) causes Phe-300 nearest to the active site to lose heterogeneity and present as a single peak, whereas Ile-292 and Ile-296, although perturbed, maintain two primary conformations. However, the continued addition of $b_5$ to 0.4 molar eq results in further complex changes, with Phe-300 appearing to reacquire part of its original signal, whereas Ile-292 and Ile-296 continue to exchange between existing and potentially new resonances. This dynamic effect upon $b_5$ binding is consistent with evidence supporting the labile nature of the N-terminal end of the I helix. Notably, this degree of change was not induced by alterations in temperature (Fig. 2) and is probably a specific outcome of the $b_5$ interaction. In general, these results suggest that the multiple conformations observed for CYP17A1-abiraterone are affected by $b_5$ binding in a way such that particular subpopulations are selected or are introduced, whereas other subpopulations remain unaffected or are reduced.

**Substrate Influences CYP17A1 Conformational States**—In a previous study, CYP17A1 interactions with $b_5$ and cytochrome P450 reductase were altered by the specific substrate in the CYP17A1 active site (32), implying that substrates might modulate CYP17A1 conformational states. To probe this directly in solution, HSQC and TROSY NMR spectra were collected for $[^{15}N]$CYP17A1 bound to either the hydroxylase substrate pregnenolone or the lyase substrate 17α-hydroxypregnenolone. Due to reduced stability of CYP17A1 when bound to substrate (versus abiraterone), all spectra were collected at 35 °C instead of 40 °C. Some of the active site resonances (Phe-300, Ile-205, Ile-206) were no longer detectable in the spectra. Whereas the heme iron is in the six-coordinate, low spin state in the CYP17A1-abiraterone complex, in the CYP17A1-substrate complexes, the iron is five-coordinate or high spin. Apparent

**FIGURE 3.** NMR spectra of $[^{15}N]$CYP17A1 abiraterone titrated with cytochrome $b_5$. Portions of the spectra (40 °C) are overlaid, demonstrating effects upon the stepwise addition of unlabeled $b_5$. For different residues, binding of $b_5$ is observed to cause differential line broadening (e.g. Phe-300 and Ile-205) and conformational selection (dashed arrows; see inset). Spectra were collected using incremental increases in scans in order to compensate for signal loss due to line broadening.

**FIGURE 4.** In the CYP17A1-abiraterone complex, the conformational heterogeneity of residues in the F and I helices is affected by $b_5$ binding. The stepwise addition of $b_5$ is shown for key residues by spectra representing CYP17A1/$b_5$ molar ratios of 1:0 (black), 1:0.3 (red), and 1:0.4 (green). The peak contours for each titration point were lowered in order to detect signal weakened by the interaction.
loss of resonances near the heme iron is consistent with rapid relaxation of signal in the high spin state.

Overall comparison of the spectra for CYP17A1/h18528 pregnenolone and CYP17A1/h18528 17/19251-hydroxypregnenolone revealed very similar NMR spectra, as might be anticipated for two substrates that differ by a single hydroxyl at C17, which is expected to be oriented toward the heme iron. This suggests an overall conformation for CYP17A1 that is conserved. However, a subset of peaks displayed notable differences between the two substrates, including three that have been assigned: Ile-292 and Ile-296 on the I helix and Ile-238 on the G helix. For all three residues, the presence of 17α-hydroxypregnenolone (Fig. 5, blue spectra) results in a more heterogeneous signal compared with pregnenolone. This is most evident for Ile-238. Both substrates result in a common conformation, but 17α-hydroxypregnenolone also yields a second discrete resonance. A similar effect is observed for the Ile-296 resonance, where two distinct signals can be resolved with the 17α-hydroxypregnenolone complex, one of which is similar to the resonance observed for pregnenolone. Thus, although the overall protein conformation is similar when each of the two substrates are bound, a subset of resonances representing parts of the G helix and I helix support conformational differences in these regions of CYP17A1.

CYP17A1 Substrate Conformations Are Further Modulated by Cytochrome b5—To probe the allosteric mechanism proposed for significant increases in the CYP17A1 lyase reaction when b5 is present, the CYP17A1-substrate complexes examined above were then titrated with unlabeled b5. Overall, the effects of b5 on the CYP17A1-substrate-bound spectra resemble those observed for the b5 addition to the CYP17A1-abiraterone complex, in that a combination of peak narrowing and differential line broadening occurs across most of the spectrum. This suggests that the interaction between CYP17A1 and b5 is gen-

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**FIGURE 5.** The conformational dynamics of CYP17A1 I helix and G helix residues are modulated by both substrate and b5 binding. Conformations are distinct when pregnenolone (black) versus 17α-hydroxypregnenolone (blue) is the substrate in the active site. The addition of b5 to either substrate-bound sample further perturbed the conformations for these residues (top and bottom panels). Notably, serial titration of b5 to CYP17A1 saturated with the hydroxylase substrate pregnenolone (1:0.3 (red) and 1:0.5 (green)) induced a shift toward conformations more closely resembling those observed for CYP17A1 saturated with the lyase substrate 17α-hydroxypregnenolone state in the absence of b5. Vertical arrows emphasize differences between spectra.
erally similar with either abiraterone or either substrate in the active site. However, key resonances assigned for this study reveal intriguing differences in the I helix and G helix.

For example, the addition of \( b_5 \) (1:0.3) to the CYP17A1-pregnenolone complex results in an initial chemical shift perturbation for I helix resonance Ile-292 (Fig. 5, I292, black versus red), whereas additional \( b_5 \) (1:0.5) results in the appearance of a minor substrate in slow chemical exchange (Fig. 5, I292, red versus green). By comparison, the addition of 0.3 molar eq of \( b_5 \) to the CYP17A1-17\( \alpha \)-hydroxy pregnenolone resulted in a similar second resonance for this residue but without significant perturbation of the original signal (Fig. 5, I292, brown versus blue).

Residue Ile-238 in the G helix is also affected. The progressive addition of \( b_5 \) to CYP17A1-pregnenolone resulted in a dividing of the primary Ile-238 peak (Fig. 5, I238, black versus red), followed by migration of the new peak along the \( ^1H \) axis (Fig. 5, I238, red versus green). Notably, this final \( b_5 \)-containing spectrum with CYP17A1-pregnenolone resembled the CYP17A1-17\( \alpha \)-hydroxy pregnenolone conformation without \( b_5 \) (Fig. 5, I238, blue) more than it resembled the original CYP17A1-pregnenolone data (Fig. 5, I238, black). A similar effect was observed for Ile-296, in which the addition of \( b_5 \) to the pregnenolone-bound sample results in a dual resonance peak similar to that of CYP17A1-17\( \alpha \)-hydroxy pregnenolone bound without \( b_5 \) (Fig. 5, I296, black \( \rightarrow \) red \( \rightarrow \) green versus blue). This type of correlation between features of the CYP17A1-pregnenolone-\( b_5 \) spectra and features of the CYP17A1-17\( \alpha \)-hydroxy pregnenolone spectra without \( b_5 \) was observed for additional unassigned resonances and may provide evidence of a structural link between CYP17A1-\( b_5 \) binding and CYP17A1-substrate interactions that modulate catalysis.

**DISCUSSION**

Current structural knowledge of mammalian P450 enzymes derives primarily from x-ray crystallographic structures (6), which provide detailed but static snapshots of the intramolecular interactions across the protein and with the sequestered catalytic heme and often a ligand of interest in the adjacent cavity. This wealth of information nonetheless forms an incomplete background for interpretation of the rich diversity of dynamic substrate binding and reorientation, protein-protein interactions, and catalysis accomplished for these enzymes. In the consensus catalytic cycle, a P450 must bind substrate, be reduced by one electron from NADPH-cytochrome P450 reductase, bind oxygen, and finally accept a second electron from reductase before dual protonation of the distal oxygen can result in cleavage of the \( O_2 \) bond to form a molecule of water and the catalytic iron oxo intermediate. This iron oxo intermediate is then competent to abstract a hydrogen from the substrate, followed by a rebound reaction to form the metabolite.

In aggregate, structures of different membrane P450 enzymes suggest that flexible regions include the F-G helices with a fulcrum at their base; the B’ helix, which is often poorly packed against the rest of the protein; and the N terminus of the I helix, which has a noncanonical hydrogen bonding kink in the middle. Unfortunately, sets of structures for the same enzyme in various states often are not available, and generalizations are hampered by the extreme diversity of substrates, active site volumes, and topography (6). Additionally, the tendency of the hydrophobic F/G regions to pack together for adjacent molecules in the crystalline state may limit or prejudice the conformations that are observed. Other biophysical approaches (36–38) support conformational heterogeneity but must be interpreted within the context of current static structures or that of molecular dynamics simulations whose predictions about molecular motions are not easily verifiable. Although NMR studies for soluble bacterial systems (20, 39) and the smaller and more NMR-amenable \( b_5 \) protein (32, 40) have begun to demonstrate the potential for NMR to provide a high resolution perspective on P450 conformation(s) free of crystalization constraints, protein NMR is a new approach to investigating membrane P450 conformational heterogeneity.

In the present study, production of isotopically labeled human CYP17A1 allowed NMR analysis of this bifunctional steroidogenic enzyme whose activity controls the physiological balance of mineralocorticoids, glucocorticoids, and androgen and estrogen sex steroids. When the identical CYP17A1 protein examined herein is crystallized with inhibitors (steroidal or nonsteroidal) or any of its primary physiological substrates, the same overall protein conformation is observed, with only small variations observed in the protein backbone at the N terminus, the F/G region, and an adjacent region of the \( \beta \) system, portions of the protein involved in crystal packing contacts. In contrast, when observed through the technique of protein NMR, the uniformly and selectively \( ^{15}N \)-labeled spectra herein reveal that the backbone amides of residues in specific structural elements experience a change in environment upon modulations in temperature, substrate, and/or the presence or absence of cytochrome \( b_5 \). All of the residues assigned and monitored in the current studies are parts of \( \alpha \)-helices that are intact secondary structure units in structures of known P450 structures. This suggests that the changes in environment are most likely due to repositioning of the F (residues 205 and 206) and G (residue 238) helices and the N-terminal portion of the I helix (residues 292, 296, and 300) as a unit rather than disruption of these helices. These types of structural differences have been observed in x-ray structures of other membrane P450 enzymes (6), even if not for CYP17A1. Thus, the observed changes in specific NMR resonances most likely indicate that CYP17A1 exists in solution as a family of conformational states.

Although many features of the overall spectra of CYP17A1 bound to the different active site ligands support conservation of the global conformation, a number of residues have multiple resonance peaks and are different in ligand-specific ways. A subset of CYP17A1 residues was assigned, and they support ligand-specific adaptations of the N terminus of the I helix and of the F and G helices. For example, when the enzyme is bound to abiraterone, I helix residues Ile-292, Ile-296, and Ile-300 all demonstrate multiple backbone conformations (Fig. 2), but different patterns are observed when pregnenolone or 17\( \alpha \)-hydroxy pregnenolone are bound.

The presence of \( b_5 \) has been demonstrated to facilitate CYP17A1 lyase activity as much as 10-fold with minimal effects on the enzyme’s hydroxylase activity (26–29). The binding of reductase and \( b_5 \) to CYP17A1 are mutually exclusive (32), and
b₅ cannot deliver electrons to CYP17A1 (41). Therefore, the interaction of CYP17A1 with b₅ is assumed to be reversible, and b₅ has been suggested to function as an allosteric regulator of CYP17A1. Thus, CYP17A1 was first saturated with each individual ligand, and the resulting complex was used as a starting point for stepwise additions of the unlabeled soluble domain of b₅. Although the resonances in the resulting spectra were broadened overall as expected, this effect was more intense for a subset of residues, suggesting increased exchange between conformations on the microsecond to millisecond time scale of the NMR experiment. Other resonances narrowed in response to b₅ binding, suggesting the rigidification of certain regions of CYP17A1. Although these resonances remain unassigned at present, it is reasonable to expect that at least some might be CYP17A1 residues that directly form part of the protein/protein interface. For the set of residues that have been assigned, the significant conformational heterogeneity observed for the CYP17A1-abiiraterone and CYP17A1-17α-hydroxypregnenolone complexes even in the absence of b₅ was further exacerbated when b₅ was added, and in some cases, new states were observed that are unique to this substrate/b₅ combination. Multiple conformations were less apparent when CYP17A1 was saturated with the hydroxylase substrate pregnenolone, but the addition of b₅ did promote the appearance of new resonance states. There is substantial similarity between some of the resonance states observed for CYP17A1-pregnenolone/b₅ and CYP17A1-17α-hydroxypregnenolone even without b₅ (Fig. 5). This suggests that a set of conformational states is being observed and modulated by different combinations of substrate and b₅.

The limited set of residues assigned for this study allows only partial interpretation of the conformational diversity represented within the CYP17A1 NMR spectra but nonetheless substantiates participation of residues in the I, F, and G helices in substrate and b₅-mediated conformational transitions. The ability of b₅ to promote different conformational states in the presence of different substrates is consistent with the biological function of b₅, but the mechanisms for this have not been determined to date. The current study provides an entry point to begin to assess such molecular mechanisms.

One mechanism supported by the current findings is that b₅ binding might communicate with the active site via the NMR-detected changes in the I helix conformation. Like most P450 enzymes, the long protein-spanning I helix of CYP17A1 has a noncanonical hydrogen bonding arrangement just as it passes over the heme. In existing structures of some P450 enzymes (14, 23, 39), this results in an I helix that has been observed in either a straight conformation or with a kink arising at the midpoint, allowing movement of the N-terminal half of the I helix. In CYP17A1, the carbonyl of Ala-302 clearly hydrogen-bonds to the side chain hydroxyl of Thr-306 rather than the Thr-306 backbone. Similarly, the carbonyl of the subsequent Gly-303 residue appears to hydrogen-bond with the side chain hydroxyl of Thr-306 before the typical i → i + 4 hydrogen bonding pattern resumes. As a result, the CYP17A1 I helix has a slight kink, with the N-terminal end slightly displaced toward the active site in the available structures. The current NMR evidence supports at least two conformations for residues on the N terminus of the I helix and indicates that b₅ binding modulates these. Which conformation (straight or kinked) might be favored by b₅ binding cannot be determined by the present data.

A second, not necessarily independent, mechanism for b₅ effects is suggested by the observed influence on conformations for residues in the F and G helices. These helices and residues in the intervening loop are on the opposite side of CYP17A1 from the b₅ binding site and form the “roof” of the active site, the exterior surface of which is embedded in the membrane bilayer. This and variable observations of channels penetrating from the buried active site to the protein surface in this region (6) have supported the idea that hydrophobic substrates enter via this face of at least some P450 enzymes. Because b₅ alters conformations for F and G helix residues in a substrate-specific way, the entry, binding, and/or orientation of substrates in the active site could reasonably be altered. In the case of CYP17A1, these different conformations might also be related to observed decreases in the amount of reducing equivalents consumed without productive metabolite formation, a process called uncoupling. Although pregnenolone hydroxylation is highly coupled (97% (42)), the 17,20-lyase reaction for 17α-hydroxy-pregnenolone is not (4% (42)). At low concentrations of b₅ like those used in the described NMR experiments, single turnover experiments demonstrate that catalysis is stimulated due to decreased uncoupling (43, 44). Small, b₅-driven modulations in CYP17A1 conformation, leading to slight decreases in lyase uncoupling, would therefore be expected to substantially impact the production of androgens without large changes in the generation of hydroxylase metabolites.

In summary, the detection of changes in the CYP17A1 conformations of specific residues, as described here for abiraterone-, substrate-, and b₅-bound states, offers an advanced perspective on protein function. Although a structural basis is unambiguously provided, substantiating an allosteric role for b₅, it is clear that conformational exchange also occurs in the absence of b₅. Some of those same conformational states are substrate-specific and further modulated by b₅, but overlap. Interconversions between these states suggest that at least some of the energy required for b₅ allostery is provided by intrinsic conformational equilibrium between substrates. Reversible b₅ binding would be envisioned to shift the ensemble of CYP17A1 conformations to promote the lyase reaction without interfering with reductase-mediated electron delivery. Documentation of conformational equilibrium as an integral component of mammalian P450 interactions with substrates and protein partners provides an expanded context for the interpretation and analysis of cytochrome P450 function and is likely to facilitate comparisons between different P450 enzymes, recognition of the effects of disease-causing mutations, and the design of drugs both metabolized by and targeted to P450 enzymes.

Acknowledgments—Abiraterone was a gift synthesized by Charlie Fehl (supported by National Institutes of Health Grant T32 GM08545) in the laboratory of Dr. Jeff Aubé, University of Kansas Department of Medicinal Chemistry. NMR data were collected at the University of Kansas Biomolecular NMR Laboratory, supported in part by the Center of Biomedical Research Excellence in Protein Structure and Function (National Institutes of Health Grants RR01778 and GM103420).
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