Hydrogen Bonding Control

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Differential Hydrogen Bonding in Human CYP17 Dictates Hydroxylation versus Lyase Chemistry**

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CYP17A1 is a member of the cytochrome P450 superfamily and occupies a central role in the biosynthesis of steroid hormones.[1,2] This enzyme catalyzes both the hydroxylation of its primary substrates pregnenolone and progesterone as well as the subsequent 17,20 carbon–carbon lyase chemistry which is the first committed step in the biosynthesis of androgens.[1–3] There is significant debate as to the chemical mechanisms of this lyase activity, with human CYP17 exhibiting a significant preference for 17-hydroxy pregnenolone over 17-hydroxy progesterone. In vivo, the predominant pathway forming androgens proceeds through the conversion of hydroxypregnenolone into dehydroepiandrosterone.[4–6] Herein we report newly acquired resonance Raman (rR) spectra of monomeric CYP17A1 self-assembled in nanodiscs, which reveal a distinct difference in hydrogen bonding to the ferrous dioxygen intermediate. With 17-hydroxyprogesterone, the oxygen vibrational modes indicate hydrogen bonding to the distal oxygen atom of the Fe-O-O fragment, whereas with 17-hydroxypregnenolone hydrogen bonding is to the proximal oxygen atom. To the extent that such interactions persist in the subsequent peroxo intermediate, the latter interaction is expected to inhibit O–O bond cleavage relative to the former, thus permitting nucleophilic attack of the peroxo intermediate on the 20-carbonyl. This observation of differential hydrogen-bonding interactions, alone, satisfactorily explains the dramatically lower activity of the Δ-4 progesterone substrate relative to that of the Δ-5 pregnenolone compound in androgen biosynthesis. These results constitute a definitive experimental confirmation of the role of substrate structure in directly controlling the metabolic processing of these dual function cytochrome P450s and support the role of the peroxoanion in maintaining high lyase activity.

The cytochromes P450 (CYPs) are heme-containing monooxygenases which participate in a wide range of physiologically important processes in both eukaryotic and prokaryotic organisms.[7] In vertebrates, one essential function of CYPs is the transformation of cholesterol into steroid hormones through the involvement of highly stereo- and regiospecific reactions ultimately yielding the corticoids, androgens, and estrogens.[1–3] These steroidogenic P450s act on a select number of compounds, some catalyzing only hydroxylation chemistry, with others also active in carbon–carbon bond cleavage. The chemical intermediates responsible for this variable reactivity have been the subject of much debate, thus yielding questions as to how these enzymes control multistate oxidative transformations.[8,9]

The membrane-bound CYP17 lies at the heart of steroid metabolism where it carries out standard monooxygenase chemistry, converting into 17α-OH pregnenolone (OH-PREG) and 17α-OH progesterone (OH-PROG), respectively, and apparently utilizing a "Compound I" to initiate hydrogen abstraction and radical recombination in the classic "Groves rebound" mechanism.[7–10] However, these hydroxylated products can also serve as substrates in a second oxidative cycle to cut the 17–20 carbon–carbon bond to form dehydroepiandrosterone and androstenedione (Figure 1).[1,2] This reaction represents a critical branch point in human steroidogenesis at which the hydroxylated products of CYP17 are either shunted towards production of corticoids or subjected to a second round of catalysis which constitutes the first committed step of androgen formation.

Clearly, elucidating the factors that control CYP17 product formation is crucial for understanding healthy and diseased human physiology. Particularly intense scrutiny of the C–C lyase activity of this enzyme has led to suggestions that nucleophilic attack on the C20 carbonyl by the early peroxo ferric intermediate is utilized in the formation of androgens.[11–13] These suggestions led us to seek structural features of the active site which could control a branch point between O–O bond scission/Compound I formation versus a heme oxygen intermediate reactive in C–C lyase chemistry. Since androgen formation is 50-fold greater with OH-PREG than for OH-PROG, and this substrate pair was used for our investigations.[4,6,14,15]

An important step towards the understanding of androgen formation was the recently reported crystal structure by DeVore and Scott of human CYP17A1 complexed with promising anticancer drugs.[16] These authors used molecular modeling to suggest that interactions of the 3β-alcohol or corresponding ketone fragments of PREG and PROG with active-site hydrogen-bonding residues place the substrate in correct orientation with respect to the heme prosthetic group, also making the point that the active site topology might be

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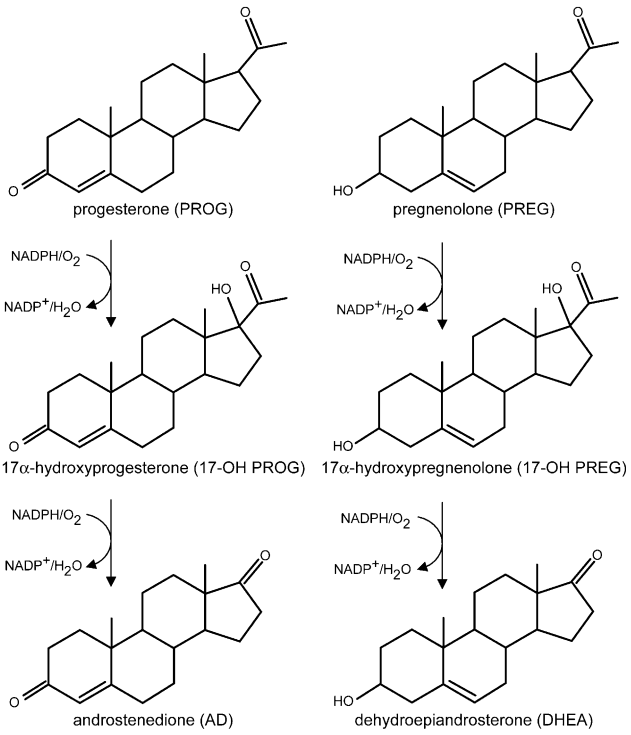


Figure 1. The proposed pathway for biosynthesis of androstenedione and dehydroepiandrosterone catalyzed by human CYP17A1. The full enzymatic cycle is shown in Figure S1.

altered by substrates and stressed the pressing need for further experimental work.[16]

The Nanodisc system allows functional incorporation of these membrane proteins into homogenous and monodisperse membrane environments to yield exceptionally well-behaved ligand binding properties, thus showing clean conversions of spin-state populations, and also enhances stability of their dioxygen adducts.[17–20] Combination of this system with the power of rR spectroscopy to interrogate active-site structure in heme proteins presents an especially effective approach to explore the complex mechanism of CYP17A1. Specifically addressed in this report is a particularly intriguing aspect of CYP17A1 structure, wherein the choice of substrate controls hydrogen bonding which defines the catalytic channel for product formation.[4–6,15] Herein rR spectroscopy convincingly demonstrates that the single difference at the 3β position of OH-PREG and OH-PROG leads to unequivocal changes in active-site hydrogen-bonding interactions with the key Fe-O-O fragment of enzymatic intermediates, thus leading to alterations in electronic structure which then control substrate processing.

Shown in Figure 2A are the rR spectra obtained for the $^{16}O_2$ adduct of PROG-bound CYP17. In addition to structure-sensitive heme modes, including ν_4, ν_3, and ν_2, the key $\nu(^{16}O$-$^{16}O)$ mode is observed at 1140 cm^{-1}, as confirmed by the uncluttered $^{16}O_2/^{18}O_2$ difference spectrum. The ν(Fe-O) stretching mode appears at 536 cm^{-1}, as documented in the $^{16}O_2/^{18}O_2$ difference trace (Figure 2A). These frequencies for the Fe-O-O fragment are quite similar to those observed when this fragment is weakly hydrogen bonded to P450

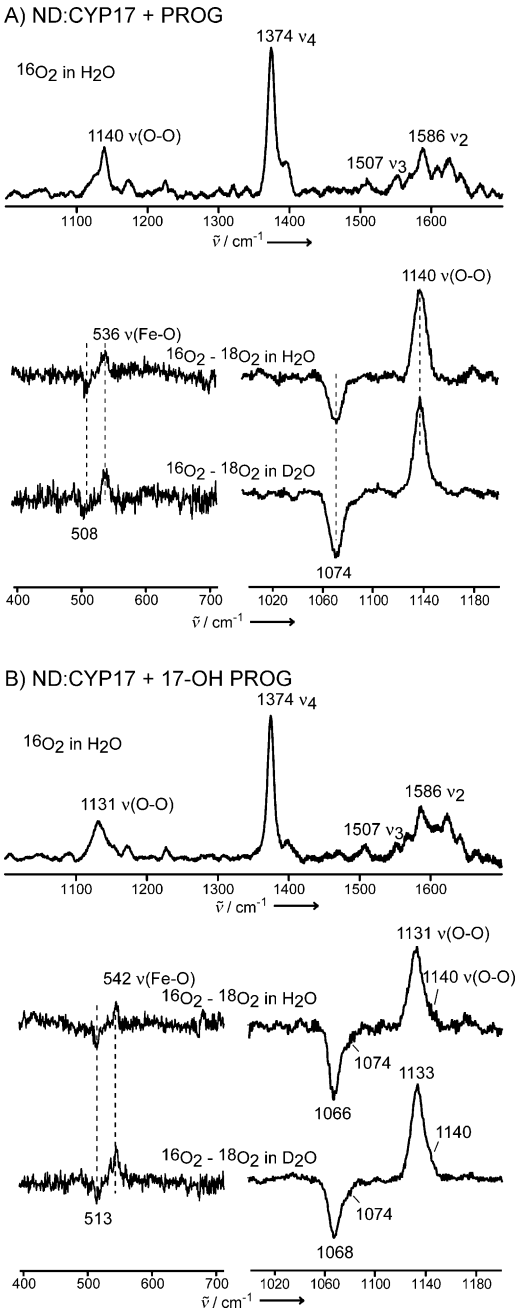


Figure 2. The rR spectra of PROG- and 17-OH-PROG-bound $^{16}O_2$ adducts of ND:CYP17 in H_2O buffer (panel A and B, respectively). The lower section of each panel shows $^{16}O_2$-$^{18}O_2$ difference plots in H_2O (upper) and D_2O (lower) buffers.

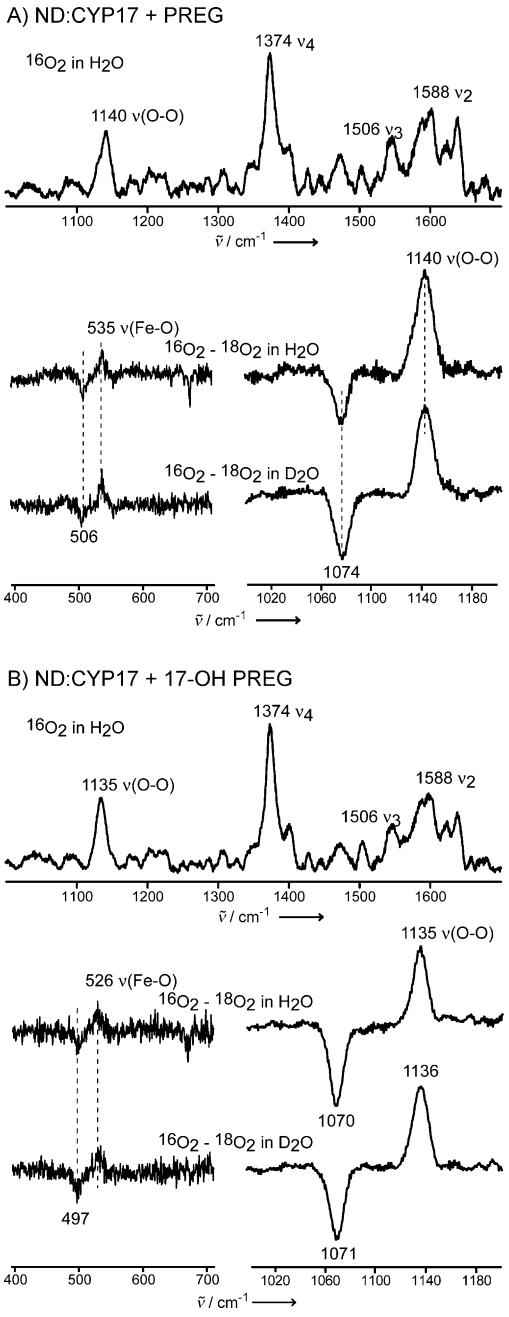
active-site residues.[21,22] Significantly, spectra acquired for this sample in solutions prepared in D_2O (left and right traces in lower section of Figure 2A) showed no difference for this non-hydrogen-bonding substrate.

Shown in Figure 2B (top trace), is the rR spectrum obtained for the $^{16}O_2$ adduct of CYP17 harboring OH-PROG at its active site. The $\nu(^{16}O$-$^{16}O)$ mode now appears approximately 9 cm^{-1} lower at 1131 cm^{-1}. Expansion of this spectral region, and comparison with the spectrum acquired with $^{18}O_2$, shows evidence for a minor conformer which is apparently

quite similar to the form observed when PROG is bound (Figure 2A). As is evidenced by a telltale 2 cm^{-1} shift to higher frequency in solutions prepared in D_2O,[21] the dominant form, occurring at 1131 cm^{-1}, is hydrogen bonded (Figure 2B, lower right). It is most reasonable to attribute the clearly observed hydrogen-bonding interaction to the newly introduced C_{17}-OH(D) functionality. Notably, this lowering of the ν(O-O) mode is correlated with a corresponding 6 cm^{-1} increase in the frequency of the ν(Fe-O) band relative to its value in the PROG-bound enzyme.

Corresponding spectral data for the PREG- and 17-OH-PREG-bound enzymes are shown in Figure 3. As for the PROG-bound enzyme, the ν(O-O) and ν(Fe-O) modes of the PREG-bound enzyme are observed near 1140 and 535 cm^{-1} with no evidence for hydrogen bonding. As shown in Figure 3B, for the OH-PREG sample, the ν(O-O) mode is shifted down by only 5 cm^{-1} compared with its value for the PREG-bound form and exhibits a barely detectable upshift in deuterated solvents. Now however, the ν(Fe-O) is observed at 526 cm^{-1}, thus exhibiting a 9 cm^{-1} shift to lower frequency compared to the value observed for the sample containing non-hydrogen-bonding PREG. The main point is that rather dramatic differences are observed when comparing the samples bound with OH-PROG and OH-PREG. Though introduction of the 17-OH group causes downshifts of the ν(O-O) modes for both OH-PROG and OH-PREG, the corresponding ν(Fe-O) modes shift in opposite directions, that is, the 17-OH-PROG yields a 6 cm^{-1} upshift while the 17-OH-PREG shows a 9 cm^{-1} downshift. It is emphasized that rR spectra acquired with a $\lambda = 356$ nm excitation for all four samples showed no evidence for changes in the trans Fe–S bond strength (data not shown), thus indicating that the effects on the Fe-O-O fragment arise mainly from distal side interactions.

While reference data for the vibrational modes of dioxygen adducts of heme proteins are relatively scarce, certain fundamental patterns have emerged.[21–26] Studies of globin mutants indicate that hydrogen bonds to the inner (proximal) O of the Fe-O-O fragment cause a decrease in the ν(Fe-O) mode, with similar but smaller effects on the ν(O-O), that is, a positive correlation,[23] whereas hydrogen bonding to the outer (terminal) O often leads to significant lowering of the ν(O-O) fragment in P450s and related enzymes.[21,24–26] Importantly, recent DFT calculations on histidine-ligated oxy complexes predict that,[22] other factors being held constant, hydrogen-bond donation to the proximal oxygen atom (O^P) will weaken both bonds by withdrawing of electrons into the nonbonding sp^2 orbital on the O^P, thus causing both the ν(Fe-O) and the ν(O-O) modes to shift in concert to lower frequency. In contrast, hydrogen-bond donation to the terminal oxygen atom, O^T, results in a straightforward increase in back-bonding, that is, one expects a negative correlation, where the ν(O-O) decreases while the ν(Fe-O) increases. Therefore, collectively, these rR results provide convincing evidence that the two hydroxylated substrates interact at opposite ends of the Fe-O-O fragment as depicted in Figure 4, thus leading to crucial implications for CYP17A1 function. That is, it is anticipated that these interactions will dictate the preference for the hydroxylation and lyase pathways. Such hydrogen-bonding interactions with O^T are expected to promote O–O bond cleavage and Compound I formation, whereas the H···O^P interaction should prolong the lifetime of the ferric peroxo intermediate which is suggested to be the active fragment in the lyase reaction.[27,28] Indeed, such differences in O^T versus O^P hydrogen-bonding interactions have been invoked for the nitric oxide synthase reaction. In the first cycle the arginine substrate is suggested to provide a H···O^T interaction and facilitate Compound I formation, whereas in the second cycle the hydroxylated



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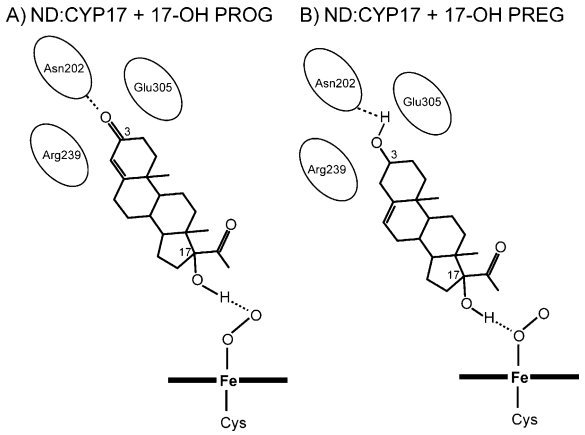

Figure 4. The human CYP17A1 protein–substrate interaction derived from these newly acquired rR data.

substrate (NOHA) is believed to provide a H···O^{P} interaction and proceed through a peroxo intermediate.[26,29,30]

In summary, rR spectra of Nanodisc-incorporated CYP17A1 clearly define the active-site hydrogen bonding, where the only change being compared is a difference between a 3β-OH group versus a corresponding ketone in the natural CYP17A1 substrates. Significantly, this single difference at a relatively remote site is sufficient to alter the hydrogen-bonding interactions with the critical Fe-O-O fragment in such a way as to dictate its predisposition towards one of two alternative reaction pathways. Though providing a structural explanation for the differential reactivity of CYP17A1 substrates is itself a significant contribution, the present results also convincingly illustrate the impressive power of rR spectroscopy to identify localized structural interactions which dictate reactivity patterns within the enzymatic cycles of cytochromes P450 and related systems.

Experimental Section

Full length human CYP17A1 was expressed from a synthetic gene in DH5α co-transformed with the GroEL/ES chaperone system (Takara Bio), purified to electrophoretic homogeneity and incorporated into Nanodiscs as previously described[31–33] and are summarized in details in the Supporting Information. Resonance Raman spectra of frozen samples were obtained using the $\lambda = 413.1$ nm excitation line from a Kr^{+} laser, which effectively enhances heme modes and internal modes of the Fe-O-O fragment (see the Supporting Information for details). The spectra were calibrated with fenchone (Sigma–Aldrich, WI) and processed with Grams/32 AI software (Galactic Industries, Salem, NH).

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