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Human cytochrome CYP17A1. The structural basis for compromised lyase activity with 17-hydroxyprogesterone

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Abstract

The multifunctional enzyme, cytochrome P450 (CYP17A1), plays a crucial role in the production of androgens, catalyzing two key reactions on pregnenolone (PREG) and progesterone (PROG), the first being a 17-hydroxylation to generate 17-OH PREG and 17-OH PROG, with roughly equal efficiencies. The second is a C-C bond scission or “lyase” reaction in which the C17-C20 bond is cleaved, leading to the eventual production of powerful androgens, whose involvement in the proliferation of prostate cancer has generated intense interest in developing inhibitors of CYP17A1. For humans, the significance of the C-C bond cleavage of 17-OH PROG is lessened, because it is about fifty times less efficient than for 17-OH PREG in terms of kcat/Km. Recognizing the need to clarify relevant reaction mechanisms involved with such transformations, we first report studies of solvent isotope effects, results of which are consistent with a Compound I mediated PROG hydroxylase activity, yet exclude this intermediate as a participant in the formation of androstenedione (AD) via the lyase reaction. This finding is also supported by a combination of cryoreduction and resonance Raman spectroscopy that traps and structurally characterizes the key hemi-ketal reaction intermediates. Adding to a previous study of PREG and 17-OH PREG metabolism, the current work provides definitive evidence for a more facile protonation of the initially formed ferric peroxo- intermediate for 17-OH PROG-bound CYP17A1, compared to the complex with 17-OH PREG. Importantly, Raman characterization also reveals an H-bonding interaction with the terminal oxygen of the peroxo fragment, rather than with the proximal oxygen, as is present for 17-OH PREG. These factors would favor a diminished lyase activity of the sample with 17-OH PROG relative to the complex with 17-OH PREG, thereby providing a convincing structural explanation for the dramatic differences in activity for these lyase substrates in humans.

Graphical abstract

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Keywords

Cytochrome P450; CYP17A1; resonance Raman; cryoreduction; lyase; progesterone

INTRODUCTION

Located primarily in the tissues of the adrenal glands and sex organs, the steroidogenic cytochrome P450 (CYP17A1) plays a crucial role in the production of androgens. This multifunctional enzyme catalyzes two key reactions on pregnenolone (PREG) and progesterone (PROG) (depicted in Figure 1), the first being a 17-hydroxylation to generate 17-OH PREG and 17-OH PROG at roughly equivalent rates.^{1–3} In a subsequent physiologically crucial C-C bond scission, or “C-C lyase” reaction, the C17-C20 bond is cleaved to produce dehydroepiandrosterone (DHEA) from 17-OH PREG and androstenedione (AD) from 17-OH PROG. This lyase activity represents the crucial step in the eventual production of the powerful androgens, testosterone and dihydrotestosterone, the involvement of which in the proliferation of nearly 80% of prostate cancers^{4,5} has, not surprisingly, generated intense interest in developing inhibitors of CYP17A1.^{6,7}

While the hydroxylase activity of CYP17A1 is believed to be the result of a typical P450 “Groves rebound”⁸ mechanism, the identity of the reactive intermediate responsible for C-C bond scission has been the subject of much debate over the past several decades.^{9–15} While some have recently proposed involvement of a ferryl oxene in androgen formation,¹⁵ operating by hydrogen abstraction from the 17-alcohol,¹⁵ a significant body of evidence has emerged favoring involvement of a nucleophilic peroxoanion in the formation of DHEA. Support for a nucleophilic peroxoanion includes studies of active site mutants,³ observation of an inverse kinetic solvent isotope effect,^{15–17} trapping and rR interrogation of a peroxo-hemiketal intermediate,¹³ as well as a QM investigation which shows on-path hemiketal transition state and the inability of Compound I to elicit the hydrogen abstraction step required for the reaction to proceed through this intermediate.¹⁸

As shown in Figure 2, a key feature distinguishing Compound I mediated lyase chemistry from one utilizing the peroxoanion is the involvement of at least two protons in generating the former species. Kinetic solvent isotope effects (KSIE) observed in steady state product forming rates therefore have the potential to exclude one of these pathways. P450 enzymes operating through a Groves rebound mechanism typically exhibit a KSIE k_H/k_D of 1.2 –

3.^{19–23} An unusual inverse isotope effect in the formation of DHEA from 17-OH PREG strongly suggested a peroxoanion intermediate operating in the formation of this androgen product.¹²

This discovery led us to interrogate the various iron-oxygen intermediates in the P450 catalytic cycle in the presence of PREG and 17-OH PREG substrates.¹³ In these previous studies with PREG (hydroxylation substrate), resonance Raman (rR) monitoring of the cryoradiolytically reduced dioxygen adduct of the CYP17A1 complex documented the initial formation of the peroxo intermediate, which upon annealing to higher temperatures, converted to the rR spectroscopically documented hydroperoxo intermediate, which eventually (at higher temperatures), disappeared, presumably generating the reactive Compound I which initiates hydrogen abstraction and radical rebound⁸ to form 17-OH PREG and regenerate the resting state CYP17A1. On the other hand, rR monitoring of the reaction cycle that evolved when 17-OH PREG (lyase substrate) was bound to CYP17A1, provided evidence for an initially formed peroxo intermediate, but no evidence for the hydroperoxo species upon annealing to higher temperatures; i.e., the cycle did not proceed along the periphery of the reaction cycle (grey arrows in Figure 2). Instead, the initially formed peroxo intermediate converted to a new intermediate (red arrows, Figure 2), depicted in the center of the cycle, a peroxo-hemiketal that had long been predicted,⁹ but never spectroscopically documented.

Because most of the flux through the CYP17A1 system is known to proceed through the PREG to DHEA pathway in humans, the majority of mechanistic interrogations have been performed with only the PREG and 17-OH PREG substrates. While it is known that a roughly 50-fold difference in k_{cat}/K_m distinguishes the C-C lyase activities of these two pathways, this is not true in all species in which CYP17A1 enzymes are operating.²⁴ Additionally, differences in coupling of pyridine nucleotide consumption to product forming rates³ exist in these two pathways suggesting the possibility of subtle mechanistic distinctions between the two C-C lyase reactions. One key finding made in the earlier works was that the 17-OH PREG substrate provided an H-bond donating 17-OH group that was oriented toward the proximal (O_p) atom of the $Fe-O_p-O_t$ oxy-complex fragment, whereas the 17-OH group of 17-OH PROG donates an H-bond to the terminal (O_t) atom of this fragment.²⁵ Based on elegant studies by Scott and others,^{26,27} this redirection of the active site 17-OH group of these otherwise structurally quite similar substrates is presumably controlled by the differential interactions of the $3\beta OH$ (PREG) and $C=O$ (PROG) groups with the N202 residue of the protein, an interaction that was confirmed in a recent study of the CYP17A1 Asn202Ser mutant that was found to reverse the PREG/PROG specificity.²⁸

Importantly, a subsequent work documented the fact that the H-bonding interaction between the 17-OH fragment of 17-OH PREG to the proximal oxygen atom (O_p) persisted when the oxy-complex was reduced by cryoradiolysis to the peroxo-anion.¹³ Therein we hypothesized that formation of a proximal H-bond may be an essential determinant in driving carbon-carbon bond scission: not only would the substrate be poised in an orientation favorable for transition state formation, but the distal oxygen atom would be free to attack the substrate C-20 carbonyl. Conversely, H-bonding to the distal oxygen could be expected to decrease the nucleophilicity of the peroxo fragment as well as perhaps participate in a distal pocket

proton delivery network that forms the catalytically incompetent hydroperoxo species with second proton transfer breaking the O-O bond and forming Compound I.

In this report we apply a variety of biochemical and spectroscopic analyses to the processes forming 17-OH PROG and AD. First, we demonstrate the presence of a normal isotope effect in the formation of 17-OH PROG, but an inverse kinetic solvent isotope effect for the formation of AD, as was observed in the PREG to 17-OH PREG pathway. We then document our detailed rR study of the reaction cycle for CYP17A1 in the presence of the PROG and 17-OH PROG substrates. The results confirm the presence of a peroxo-anion intermediate, yet reveal a persistent H-bonding interaction with the terminal oxygen of the peroxo fragment, which would be expected to diminish lyase activity relative to the complex with 17-OH PREG,^{18,29–31} thereby providing a convincing structural explanation for the significant differences in observed activity for these two lyase substrates.

EXPERIMENTAL SECTION

Expression and Purification of CYP17A1 and Incorporation in to Nanodiscs

A gene for full length human CYP17A1 was synthesized (DNA 2.0) including a C-terminal penta-histidine tag and modifications to the first twenty-four 5' bases as described by Imai et al.³² and ligated into the pCWori⁺ vector. DH5 α cells were co-transformed with the resultant plasmid as well as pGro7 containing the GroEL/ES chaperone system. Expression was carried out using the method devised by Waterman³³ and purification performed as documented previously.²⁵ The resultant detergent solubilized CYP17A1 was inserted into Nanodiscs with a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine membrane using a method described previously in detail.³⁴

Determination of Steady State Kinetic Solvent Isotope Effects

Reactions for steady state turnover were conducted as described previously.³ Briefly, 200 pmol CYP17 Nanodiscs and four-fold molar excesses of full length cytochrome b5 and full length CPR were contained in 1 mL of 0.1 M potassium phosphate at pH 7.4 at 37°C. D₂O experiments were performed under identical conditions except that the reconstitution system was contained in 1 mL of 0.1 M potassium phosphate buffered to pD 7.4 in 100% D₂O. Each reaction was initiated by addition of NADPH to a final concentration of 0.7 mM and were stopped after 15 minutes by addition of 50 μ L 8.9 N H₂SO₄. Products were extracted in dichloromethane, dried under a gentle stream of N₂ and reconstituted in methanol prior to analysis by reversed phase HPLC using a C₁₈ column (ACE). Separation was achieved using a linear gradient of 90:5:5 to 10:45:45 H₂O:methanol:acetonitrile and products quantitated at 240 nm.

Preparation of Samples for rR Spectroscopy

Resonance Raman samples contained 320 μ M CYP17A1 Nanodiscs in 100 mM potassium phosphate, pH 7.4; 250 mM sodium chloride; 30% (v/v) distilled glycerol; 6.24 μ M methyl viologen; and either 450 μ M PROG or 400 μ M 17OH-PROG. Deuterated samples were prepared by thorough exchange²⁵ into identical buffer adjusted to pD 7.4 (electrode calibrated by method of Glasoe and Long)³⁵ prepared with 100% D₂O and distilled

glycerol-D₃. Ferric samples were then contained in a 5 mm O.D. NMR tubes (WG-5 ECONOMY, Wilmad) and de-aerated under argon for 5 minutes followed by reduction under anaerobic conditions with a 1.5 fold molar excess of sodium dithionite. Each sample was then transferred to a dry ice-ethanol bath held at -15°C where it was cooled for 1 minute. Oxy-ferrous complexes were formed by addition of $^{16}\text{O}_2$ or $^{18}\text{O}_2$ gas for 10 seconds, followed by rapid freezing in liquid N₂. Frozen samples containing oxy-ferrous CYP17A1 were subsequently radiolytically reduced to the peroxo- state by a 3.5 MRad dose of gamma-rays in a Gammacell 200 Excel ^{60}Co source while immersed in liquid nitrogen as described previously.³⁶

Resonance Raman Measurements

Samples of irradiated oxy ND:CYP17A1 were excited using 441.6 nm line provided by a He-Cd laser (IK Series He-Cd laser, Kimmon Koha CO., LTD.) while the annealed to 190 K samples were measured with 406.7 nm excitation line from a Kr⁺ laser (Coherent Innova Sabre Ion Laser). The rR spectra of all samples were measured using a Spex 1269 spectrometer equipped with Spec-10 LN-cooled detector (Princeton Instruments). The slit width was set at 150 μm and the 1200 g/mm grating was used; with this grating, the resultant spectral dispersion is 0.46 $\text{cm}^{-1}/\text{pixel}$. The laser power was kept at ~ 1 mW or less to minimize photodissociation and the samples contained in NMR tubes were spanned to avoid laser-induced heating and protein degradation. The 180° backscattering geometry was used for all measurements and the laser beam was focused onto the sample using a cylindrical lens.³⁷ The NMR tubes were positioned into a double-walled quartz low temperature cell filled with liquid nitrogen. All measurements were done at 77 K and total collection time was around 4.0–4.5 hrs for the irradiated samples and approximately 6–7 hrs for the annealed samples. Spectra were calibrated with fenchone (Sigma-Aldrich, WI) and processed with Grams/32 AI software (Galactic Industries, Salem, NH).

Preparation of Optical Samples and Collection of Optical Absorption Spectra

Methods of preparation and collection of optical samples containing P450 in the peroxo-state have been described in detail previously.^{36,38} Briefly, CYP17A1:Nanodiscs in 100 mM potassium phosphate (pH 7.4, 15% (v/v) glycerol, and 400 μM PROG or 17-OH PROG) were anaerobically reduced with a 1.5 fold molar excess of sodium dithionite with the aid of methyl viologen at a 1:40 ratio of redox mediator to P450. Oxy-ferrous CYP17A1 was formed by rapid injection of this solution into 100 mM potassium phosphate, pH 7.4 buffer containing 67.5% (v/v) glycerol contained in a methacrylate cuvette (Fisher Scientific, Cat. No. 759075D) and chilled at 243 K methanol – dry ice bath. After 25 seconds of vigorous mixing, the sample was rapidly cooled to 210 K, and then to 77 K at a rate of ~ 4 K/min. The final concentration of CYP17A1:ND and glycerol was ~ 30 μM and 60% (v/v), respectively. Samples were irradiated as described above and then photobleached for 5–10 minutes under a 100W tungsten-halogen lamp behind a 450 nm long-pass filter while immersed in liquid nitrogen. Spectra were collected in a home-built optical cryostat³⁹ aligned within the beam path of a Cary 300 spectrophotometer as the temperature was increased linearly at a rate of ~ 1 K/min.

RESULTS AND DISCUSSION

A. Measurement of Kinetic Solvent Isotope Effects in Steady State turnover

A key question regarding the carbon-carbon lyase activity of CYP17A1 is the nature of the reactive intermediate responsible for androgen formation. Though recent evidence involving the 17-OH PREG substrate suggests involvement of a nucleophilic attack of the C-20 carbonyl of the substrate by the peroxo-anion, a detailed investigation of this C-C lyase activity when 17-OH PROG is a substrate has not been conducted. In order to distinguish between these two possible pathways we have, in a previous report, documented an inverse kinetic solvent isotope effect for the case of 17-OH PREG.¹⁶ While the peroxoanion is formed immediately after reduction of the oxy-ferrous complex, formation of Compound I relies on two subsequent protonations of the iron bound dioxygen, generating first the hydroperoxo intermediate and, following an additional proton transfer and O-O bond scission, the ferryl oxene Compound I. The necessity of at least two protons opens the possibility of excluding one of these pathways by determining the KSIE of the steady state product forming rates in the presence of protiated versus deuterated solvent.

Such was the case in a previous report in which k_H/k_D of the steady state product forming rates of 17-OH PREG and DHEA were observed.¹⁶ Consistent with other cytochrome P450s operating through a Groves rebound mechanism which typically exhibit a partially masked KSIE of 1.2–3, hydroxylation of PREG displayed a k_H/k_D of 1.2. The C-C lyase activity responsible for DHEA formation however, displayed an unusual inverse KSIE of 0.39 as the product forming rates more than doubled in the presence of D_2O .¹⁶ This result was rationalized by recognizing that protonation of the peroxoanion is slower in the presence of D_2O , increasing the steady state concentration of the peroxo state. Though the microscopic catalytic rate of formation of the peroxo-hemiketal remains the same, the apparent rate increases as a result of the increased concentration of the peroxoanion species.

In order to determine if a similar process is operating in the conversion of 17-OH PROG to AD, we repeated these experiments with the new set of substrates. In the case of the PROG to 17-OH PROG conversion, in H_2O PROG was hydroxylated to 17-OH PROG at a rate of $4.74 \pm 0.06 \text{ min}^{-1}$. The same reaction carried out in deuterated solvent proceeded at $3.29 \pm 0.09 \text{ min}^{-1}$, giving a KSIE of 1.44 entirely consistent with other P450 enzymes utilizing Compound I chemistry. In the case of carbon-carbon bond scission forming AD, an inverse isotope effect was again observed, although smaller in magnitude to that measured during formation of DHEA. In H_2O 17-OH PROG was converted to AD at a rate of $0.258 \pm 0.008 \text{ min}^{-1}$ vs $0.319 \pm 0.006 \text{ min}^{-1}$ in D_2O , manifesting as a KSIE k_H/k_D of 0.81. These results are consistent with the behavior previously observed in the PREG to DHEA pathway and are consistent with Compound I mediated PROG hydroxylase activity, yet exclude this intermediate as a participant in the formation of AD. For this alternate pathway of androgen formation, these data similarly suggest inhibition of protonation of the peroxoanion leading to an increase in the apparent rate of product formation.

B. Detection and temporal evolution of enzymatic intermediates

It is first important to detect any intermediates encountered in the C-C bond cleavage process, monitoring their decay throughout the reaction cycle. To accomplish this, solutions of the oxy-ferrous CYP17A1:ND saturated with PROG or 17-OH PROG have been reduced by cryoradiolysis to obtain peroxo-ferric intermediate stabilized at 77 K.³⁶ Progress along the reaction pathway is monitored by optical absorption spectroscopy with gradual annealing from 77K (liquid nitrogen) to ~190 K in order to detect intermediates that arise.

The results in Figure 3 (top) for the sample with PROG, a typical hydroxylase substrate, are quite similar to those obtained in a previous study with PREG,¹³ another hydroxylase substrate, where it is observed that the strong absorbance band (~439 nm) associated with the peroxo-/hydroperoxo- species, decreases as the temperature increases from 160 K to 192 K, giving rise to a new feature, maximizing near 413 nm. It is noted that both the peroxo- and hydroperoxo- intermediates absorb at 435 – 440 nm, making it impossible to determine the extent of proton delivery using this detection method. This temperature dependent behavior is readily explained by reference to Figure 2. Following the *grey* arrows, upon annealing, the peroxo and hydroperoxo species are eventually converted to Compound I, which catalyzes hydrogen abstraction and radical rebound at C₁₇-H bond, producing the 17-OH fragment and regenerating the resting state CYP17A1, with its telltale So-ret peak appearing at 413 – 417 nm. On the other hand, temperature dependent spectral data for the sample containing 17-OH PROG, a lyase substrate, are presented in Figure 3 (bottom) as a set of difference traces, where a strong absorption band appears at 437 nm. Annealing the cryoreduced sample from 160 K up to about 192 K causes almost complete loss of this band corresponding to the peroxo/hydroperoxo intermediate(s) and appearance of a weaker absorption band near 407 nm. Now following the *red* arrows in Figure 2, some fraction of the trapped peroxo intermediate can attack the susceptible C₂₀ carbonyl to generate a new species shown in the center, whose characteristic absorption band appears near 407 nm, a value similar to that seen for the reaction with 17-OH PREG.¹³ Inasmuch as the absolute absorbance at 407 nm is quite weaker than that of the peroxo/hydroperoxo envelope, it is concluded that either the peroxo form yielded only partial conversion to a species absorbing near 407 nm or fully converted to a new species that possesses an inherently weaker molar absorptivity. In any case, decay of the peroxo intermediate gives rise to a new species absorbing near 407 nm. The structure depicted in the center of Figure 2 is that which is expected to arise from attack of the Fe-O-O peroxo fragment on the C₂₀ carbonyl, with the caveat that the 3 β -OH group of the central structure is actually a carbonyl in the case of 17-OH PROG. As is seen in the next section, resonance Raman spectroscopy provides definitive spectral evidence in support of this interpretation.

C. Structural definition of observed intermediates

As has been amply demonstrated in many earlier studies, rR spectroscopy can provide definitive structural characterization of the Fe-O-O fragments of these trapped cytochrome P450 enzymatic intermediates.^{13,25,40–43} While the raw spectra obtained for these rR studies of cryoreduced P450 enzymes are cluttered with strong heme modes that overwhelm the weaker internal modes of the Fe-O-O fragment, and are often plagued by the presence of fluorescence, generation of ¹⁶O-¹⁸O difference traces very effectively cancels out these

interferences yielding clear features corresponding to the $\nu(\text{O-O})$ and $\nu(\text{Fe-O})$ stretching modes, both of which provide revealing information regarding the nature of the intermediate and its functionally relevant interactions with the immediate active site environment.

1. Initial intermediates trapped at 77K—Focusing on the rR spectroscopic data obtained for the samples of interest here, the $^{16}\text{O}_2$ - $^{18}\text{O}_2$ difference traces of irradiated oxyCYP17 samples with PROG and 17-OH PROG are shown in Figure 4. The top two traces are for PROG bound samples, in H_2O (A) and D_2O (B) buffers, and the two bottom traces are for 17-OH PROG samples, also in H_2O (C) and D_2O (D) buffers. The PROG bound samples show a clear positive band at 772 cm^{-1} which shifts by 37 cm^{-1} upon $^{18}\text{O}_2$ substitution and exhibits a 4 cm^{-1} downshift in D_2O buffer, behavior which prompts its assignment to the $\nu(\text{O-O})$ mode of a hydroperoxo-intermediate. Indeed, the 772 cm^{-1} frequency of this band is very close to that which was observed previously for the $\nu(\text{O-O})$ mode of a hydroperoxo intermediate in a sample containing PREG (775 cm^{-1}).¹³ The corresponding $\nu(\text{Fe-O})$ mode is seen at 575 cm^{-1} and exhibits 26 cm^{-1} and 3 cm^{-1} shifts to lower frequency for samples prepared with $^{18}\text{O}_2$ and D_2O buffer, again behavior which is consistent with assignment to the hydroperoxo- intermediate. While it is true that the observed intensities of these bands are only slightly above the noise level, the extracted isotope frequency shifts are quite consistent with those expected, reinforcing their assignments. In further support of this assignment, it is also noted that the assigned $\nu(\text{Fe-O})$ mode is around 40 cm^{-1} higher than the $\nu(\text{Fe-O})$ mode of the oxy form (536 cm^{-1}) seen in our earlier work,²⁵ an upshift comparable to that also seen in earlier works for samples with PREG (535 cm^{-1} for the oxy form and 572 cm^{-1} for the hydroperoxo species).^{13,25}

Significantly, there is no evidence for the presence of an unprotonated peroxo intermediate in the PROG bound sample, a species which would be expected to exhibit an oxygen isotope sensitive band near 800 cm^{-1} , with no significant shift in the sample prepared in D_2O buffer. The lack of such a feature is in direct contrast to the situation that was seen previously for the PREG bound sample, where a $\nu(\text{O-O})$ mode associated with the peroxo species was clearly observed at 802 cm^{-1} .¹³ The most reasonable explanation for the failure to observe the peroxo intermediate for this substrate is that, when PROG is the bound substrate, a different hydrogen bonding pattern is formed that generates a more facile proton delivery network compared with that of the PREG-bound enzyme. Importantly, as will be seen below, a similarly more substantial proton transfer is indicated for 17-OH PROG compared to 17-OH PREG, noting that this difference could partially account for the lower lyase efficiency of CYP17A1 with 17-OH PROG compared to 17-OH PREG (*vide infra*); i.e., the consequence of increased proton delivery to the peroxo-intermediate is decreased lyase activity.

The cryoreduced 17-OH PROG sample (Figure 4, traces C and D) exhibits two sets of oxygen sensitive modes in the $700\text{--}800\text{ cm}^{-1}$ region, one seen at 790 cm^{-1} that shifts to 752 cm^{-1} upon $^{18}\text{O}_2$ exchange (38 cm^{-1}) and does not exhibit H/D sensitivity, while the second one, seen at 771 cm^{-1} , shifts 37 cm^{-1} for the sample containing $^{18}\text{O}_2$ and around 3 cm^{-1} in D_2O buffer. These modes are most reasonably assigned to a peroxo (790 cm^{-1}) and a hydroperoxo (771 cm^{-1}) species. Though the spectral quality in the low frequency region is relatively poor, candidate features for the corresponding $\nu(\text{Fe-O})$ modes are seen at 562 cm^{-1}

$^{-1}$ (peroxo-) and 576 cm^{-1} (hydroperoxo-); again, though of weak intensity, the relative frequency positions and extracted isotopic shifts are consistent with expectations. It is noted that these results are quite different than those observed previously for irradiated CYP17A1 with the other lyase substrate, 17-OH PREG,¹³ where only one band (showing no H/D sensitivity) was observed at 796 cm^{-1} , appropriately assigned to a peroxo intermediate, *with no modes associated with the hydroperoxo intermediate being detected*.

The essential point is that in the case of 17-OH PREG the initially formed peroxo intermediate accumulates in the absence of any hydroperoxo species, whereas in the case of 17-OH PROG, the formation of the peroxo intermediate is accompanied by formation of the hydroperoxo- species. While this behavior is obviously consistent with apparently more facile proton delivery compared to the sample bound with OH-PREG (as was seen above in the comparison of PROG and PREG), another plausible explanation is that the initially formed peroxo intermediate for the 17-OH PROG bound enzyme is less potent with respect to nucleophilic attack on the C₂₀ carbon than is that for the 17-OH PREG bound enzyme. From this perspective, for both lyase substrates, the initially formed peroxo- intermediate is poised to undergo nucleophilic attack on the carbonyl, but the tendency for this process is controlled by the inherent reactivity of the peroxo-fragment as well as by positioning of the substrate C₂₀ carbonyl atom. The fate of a persistent (lower reactivity) peroxo- fragment is to be transformed into the hydroperoxo-intermediate, an event that eliminates peroxo-hemiketal formation and depends on the efficiency of proton transfer. The key question arising then is what structural elements can manipulate the reactivity of the Fe-O-O peroxo fragment?

As was emphasized in several previous works by us and others,^{13,18,25} one of the key structural features that is effective in manipulating the reactivity of the peroxo intermediate is the relative orientation of nearby H-bond donors which interact with the reactive Fe-O-O fragment; i.e., in this case, comparing 17-OH PREG and 17-OH PROG. As was demonstrated in two of these earlier works,^{13,25} rR spectroscopy is especially effective in distinguishing H-bonding interactions to the proximal vs terminal oxygen atoms of the Fe-O_p-O_t fragment, where introduction of an H-bond to the proximal oxygen leads to a shift to lower frequency of the $\nu(\text{Fe-O})$ mode, while H-bonding to the distal oxygen atom causes an increase of the $\nu(\text{Fe-O})$ mode relative to a non-H-bonded Fe-O-O fragment, these spectral results being consistent with earlier experimental work and computational predictions.
25,29,44–53

In the present case, while no peroxo intermediate was trapped for the PROG sample, it is reasonable to expect that its $\nu(\text{Fe-O})$ mode would occur near 554 cm^{-1} , the frequency observed for the peroxo form of the PREG sample;¹³ i.e., it was previously shown that the $\nu(\text{Fe-O})$ modes of the PROG and PREG-bound dioxygen adducts of CYP17A1 exhibited virtually identical frequencies, owing to the fact that neither substrate introduces polar groups into the vicinity of the Fe-O-O fragment.²⁵ Then the spectral pattern of the peroxo intermediate of the 17-OH PROG sample, with its $\nu(\text{Fe-O})$ mode at 562 cm^{-1} , would imply an $\sim 8\text{ cm}^{-1}$ shift to higher frequency, a shift consistent with H-bonding of the 17-OH fragment to the *distal* oxygen of the Fe-O-O fragment.⁵³ It is further noted that the rR data for the 17-OH PREG bound samples provided convincing evidence that this substrate led to

H-bonding interactions with the proximal oxygen atom of the Fe-O_p-O_t fragment of both the dioxygen and peroxo- intermediates.^{13,25}

Thus, the rR data for the 17-OH PROG bound enzyme indicate the presence of an H-bond interaction to the *terminal* oxygen atom of the Fe-O-O peroxo fragment, whereas previous work confirmed that the peroxo- intermediate of the 17-OH PREG-bound enzyme experiences an H-bonding interaction with the *proximal* oxygen. Furthermore, the results of earlier and recent computational studies,^{18,25,30,31} provide strong arguments that H-bonding to the proximal oxygen enhance nucleophilic reactivity, while terminal oxygen H-bonding interactions diminish such reactivity. Thus, data obtained in this work provide evidence for increased proton transfer efficiency to and diminished nucleophilicity of the Fe-O-O fragment of the OH PROG bound sample relative to the sample bound with OH PREG, both factors favoring increased lyase reactivity of CYP17A1 for OH PREG compared to OH PROG, in agreement with carefully documented studies of product formation.^{3,16,24,54,55}

2. Annealed 17-OH PROG samples—Inasmuch as the results of the optical studies, displayed in Figure 3, confirm that upon annealing to 190 K the sample with PROG simply shows a vanishing hydroperoxo intermediate (absorbing at 442 nm excitation) and evolution of a spectrum corresponding to the resting state enzyme (absorbing at 413 nm), there is no evidence for accumulation of a subsequent intermediate; i.e., as in the case of PREG, upon annealing the reaction efficiently proceeds through Compound I, generating product and resting state enzyme, the latter absorbing near 413 nm. Consequently, no rR studies of the annealed sample with PROG were undertaken. However, recalling that annealing of the irradiated 17-OH PREG sample, studied in the earlier work,¹³ produced a species absorbing near 406 nm, documented as a peroxo-hemiketal intermediate by rR spectroscopy, efforts were made here to carefully document changes in the rR spectroscopic data acquired upon annealing samples containing 17-OH PROG, noting that corresponding optical studies of samples containing this substrate, shown in Figure 3B above, also show evidence for a species absorbing near 406 nm. Figure 5, (trace A) shows the ¹⁶O₂–¹⁸O₂ difference spectra of samples prepared in H₂O buffer measured with 406 nm excitation, at 77K, before annealing; the data confirm that the irradiated sample contains some traces of residual oxy form, but no peroxo-hemiketal intermediate has yet been formed. On the other hand, upon annealing to 165K, the same sample (traces B and C) shows formation of a new species exhibiting a $\nu(^{16}\text{O}-^{16}\text{O})$ at 785 cm⁻¹, with a 40 cm⁻¹ shift upon ¹⁸O₂ substitution and no sensitivity to H/D exchange. This feature is most reasonably assigned to the peroxo-hemiketal intermediate formed with 17-OH PROG since it is quite comparable to that seen at 791 cm⁻¹ in the case of 17-OH PREG.¹³ It is also important to note that the same set of samples (annealed to 165 K), but measured with 442 nm excitation line (traces D and E for H₂O and D₂O buffers, respectively), shows that the peroxo species (790 cm⁻¹) has nearly disappeared, as compared to the 77 K samples (Figure 4, traces C and D), while the hydroperoxo species now dominates the rR spectrum. Though difficult to prove without an effective internal standard, it is reasonable to suggest that upon raising the temperature, the peroxo-intermediate may be depleted by either forming the peroxo-hemiketal intermediate or undergoing protonation to form more of the hydroperoxo-intermediate, as depicted in Figure 2.

As is shown in Figure 6, after further annealing of the irradiated samples to 190 K, the intensity of the peroxo hemiketal rR signal increases relative to that of the residual dioxy precursor, whose concentration is not expected to change upon the annealing process. It is also noted that rR interrogation of the samples annealed to 190 K, using the 442 nm excitation line that enhances the internal modes of the peroxo- and hydroperoxo-intermediates, reveals that these species have disappeared, the former generating the peroxo hemiketal and the latter degrading via release of free hydrogen peroxide or via Compound I formation and its unproductive degradation.

As a final note, while it may be tempting to suggest that the peroxo-like intermediate identified here as the peroxo hemiketal, arising from peroxo attack on the susceptible C₂₀ carbonyl, might instead be an alkyl peroxo intermediate that arises by Compound I attack on the -OH fragment of the 17-OH PROG (or 17-OH PREG), as has been proposed in a recent work by others,¹⁵ such arguments are flawed on multiple counts. First, this suggestion is inconsistent with recent computational studies,¹⁸ which indicate that this latter reaction pathway is highly unfavorable. Furthermore, this suggestion is convincingly ruled out by the rR data acquired here and already published in our earlier work with 17-OH PREG.¹³ Thus, the initial peroxo- intermediate generated with ¹⁸O₂ would react to form a peroxo hemiketal bearing a Fe-¹⁸O-¹⁸O-C₂₀ fragment, which is indeed observed in the rR data shown in Figures 5 and 6 (and previously in the case of 17-OH PREG).¹³ However, when using ¹⁸O₂, the suggested alternative reaction pathway would necessarily generate the proposed alternative intermediate bearing a Fe-¹⁸O-¹⁶O-C₁₇ fragment, which would give rise to a band near 765 cm⁻¹, shifted by ~20 cm⁻¹ from the $\nu(^{16}\text{O}-^{16}\text{O})$ seen at 785 cm⁻¹; tellingly, no bands are observed near this frequency neither here nor in our earlier work. ⁽⁸⁾

CONCLUSIONS

This work employed solvent isotope effects, yielding results which are consistent with Compound I mediated PROG hydroxylase activity, yet exclude this intermediate as a participant in the formation of AD. In addition, cryoreduction and resonance Raman spectroscopy were used to trap and structurally characterize the key reaction intermediates involved in the lyase reaction sequence for 17-OH PROG. The current work provides definitive evidence for a different active site hydrogen bonding configuration that could reflect a more efficient proton delivery network for 17-OH PROG-bound CYP17A1, compared to the complex with 17-OH PREG and reveals an H-bonding interaction with the terminal oxygen of the peroxo fragment, rather than with the proximal oxygen, as is present for 17-OH PREG. These factors would favor a diminished lyase activity of the sample with 17-OH PROG relative to the complex with 17-OH PREG, thereby providing a convincing structural explanation for the significant differences in activity for these lyase substrates in humans.

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ABBREVIATIONS

CYP17A1	Cytochrome P450 CYP17A1
PROG	progesterone
17-OH PROG	17 α -hydroxyprogesterone
AD	Androstenedione
PREG	pregnenolone
17-OH PREG	17 α -hydroxypregnenolone
DHEA	Dehydroepiandrosterone
HS	High Spin
LS	Low Spin
rR	resonance Raman

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Figure 1.
Proposed pathway for biosynthesis of adrostenedione and DHEA catalyzed by human CYP17A1.

Figure 2. Cytochrome P450 enzymatic cycle and formation of a peroxohemiketal intermediate with 17-OH PREG as a substrate.¹³

Figure 3.

Thermal annealing of peroxo-ferric intermediates with gradual increase of temperature from 160 K to 192 K, monitored by optical absorption spectroscopy in CYP17A1 with different substrates PROG (A) and 17-OH PROG (B), the latter showing a gradual appearance (and possible subsequent disappearance) of peroxo-hemiketal from 160 K to 192 K, with the disappearance being completed at higher temperatures (not shown).

Figure 4.

The $^{16}\text{O}_2$ - $^{18}\text{O}_2$ difference traces of irradiated oxygenated ND:CYP17A1 containing PROG (A, B) and OHPROG (C, D) in H_2O buffer (A, C) and D_2O buffer (B, D). Spectra were measured at 77 K using 442 nm excitation line and the total collection time for each rR spectrum used to generate the difference trace was 4.0-4.5 hrs. The subtraction was performed in a way to obtain the cleanest difference trace. In some traces, though, there is some positive band at around 674 cm^{-1} . It arises most probably from higher intensity of the ν_7 mode in the $^{16}\text{O}_2$ samples, most probably caused by higher amounts of residual ferric form in these samples as compared to the $^{18}\text{O}_2$ samples.

Figure 5.

The $^{16}\text{O}_2$ - $^{18}\text{O}_2$ difference trace of irradiated oxygenated ND:CYP17A1 containing 17-OH PROG in H_2O immediately after irradiation measured with 406 nm excitation line (A), the same H_2O sample (B) and corresponding sample in D_2O buffer (C) after annealing at 165 K measured with 406 nm excitation line, the same 165 K annealed samples measured with 442 nm excitation line, in H_2O (D) and D_2O (E) buffers. Spectra were measured at 77 K. The difference patterns seen around 106-1140 cm^{-1} in the traces measured with 406 nm excitation line arise from the $\nu(\text{O-O})$ modes of the residual parent oxy adducts that are strongly enhanced with this line.

Figure 6.

The $^{16}\text{O}_2$ - $^{18}\text{O}_2$ difference trace of irradiated oxygenated ND:CYP17A1 containing 17-OH PROG in H_2O (A and C) and in D_2O (B and D) buffers after irradiation and annealing at 190 K measured with 406 nm excitation line (A, B) and with 442 nm (C, D) excitation line. Spectra were measured at 77 K. The difference patterns seen around 1106-1140 cm^{-1} in the traces measured with 406 nm excitation line arise from the $\nu(\text{O-O})$ modes of the residual parent oxy adducts that are strongly enhanced with the 406 nm line.