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Active site proton delivery and the lyase activity of human CYP17A1

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ABSTRACT

Cytochrome P450 CYP17A1 catalyzes a series of reactions that lie at the intersection of corticoid and androgen biosynthesis and thus occupies an essential role in steroid hormone metabolism. This multifunctional enzyme catalyzes the 17α-hydroxylation of Δ4- and Δ5-steroids progesterone and pregnenolone to form the corresponding 17α-hydroxy products through its hydroxylase activity, and a subsequent 17,20-carbon–carbon scission of pregnene-side chain produce the androgens androstenedione (AD) and dehydroepiandrosterone (DHEA). While the former hydroxylation reaction is believed to proceed through a conventional "Compound I" rebound mechanism, it has been suggested that the latter carbon cleavage is initiated by an iron-peroxy intermediate. We report on the role of Thr306 in CYP17 catalysis. Thr306 is a member of the conserved acid/alcohol pair thought to be essential for the efficient delivery of protons required for hydroperoxoanion heterolysis and formation of Compound I in the cytochromes P450. Wild type and T306A CYP17A1 self-assembled in Nanodiscs were used to quantitate turnover and coupling efficiencies of CYP17's physiological Δ4- and Δ5-substrates. We observed that T306A co-incorporated in Nanodiscs with its redox partner cytochrome P450 oxidoreductase, coupled NADPH only by 0.9% and 0.7% compared to the wild type (97% and 22%) during the conversion of pregnenolone and progesterone, respectively, to the corresponding 17-OH products. Despite increased oxidation of pyridine nucleotide, hydroxylase activity was drastically diminished in the T306A mutant, suggesting a high degree of uncoupling in which reducing equivalents and protons are funneled into non-productive pathways. This is similar to previous work with other P450 catalyzed hydroxylation. However, catalysis of carbon–carbon bond scission by the T306A mutant was largely unimpeded by disruption of the CYP17A1 acid-alcohol pair. The unique response of CYP17A1 lyase activity to mutation of Thr306 is consistent with a reactive intermediate formed independently of proton delivery in the active site, and supports involvement of a nucleophilic peroxo-anion rather than the traditional Compound I in catalysis.

1. Introduction

The human microsomal cytochrome P450, CYP17A1, is a key enzyme in steroid hormone biosynthesis, which is capable of both hydroxylase and carbon–carbon lyase activity in a series of chemical transfomations of pregnenolone that give rise to corticoid precursors and androgens. In human adrenal steroidogenesis, the hydroxylation process predominates in the *adrenal zona fasciculata*, where Δ5 (pregnenolone, PREG) and Δ4 (progesterone, PROG) steroid are converted into the 17α-hydroxy products OH-PREG and OH-PROG, respectively, by insertion of an oxygen into a C–H bond. In the *adrenal zona reticularis*, each of these hydroxylated products are converted to the estrogen and testosterone precursors dehydroepiandrosterone (DHEA) and androstenedione (AD) via 17,20-carbon–carbon bond scission in which the 21-carbon 17α-hydroxysteroids are cleaved to 19-carbon, 17-ketosteroids, and acetic acid [1]. This compartmentalization of CYP17A1 behavior in the adrenal gland is thought to be controlled by co-localization with its putative allosteric effector cytochrome b_5 (cyt-b_5) in the *adrenal zona reticularis* [2–4]. Interaction between CYP17A1 and cyt-b_5 is known to substantially stimulate C–C lyase activity of this enzyme such that its presence can be considered essential for lyase chemistry. Furthermore, the absence of CYP17A1 in the *adrenal zona glomerulosa* helps to direct steroidogenesis in this region toward mineralocorticoid aldosterone production. As a result, varying expression levels of CYP17A1 and cyt-b_5 throughout the adrenal gland controls an important branch point in human steroidogenesis between glucocorticoid and sex hormone biosynthesis. It has been shown that both PREG and PROG are good substrates for human CYP17A1 for the 17α-hydroxylase reaction, however, 17-OH-PREG is preferred over 17-OH-PROG for the 17,20-lyase reaction [5–7].

The C–C lyase reaction catalyzed by CYP17A1 is notable not only for the profound rate enhancement exerted by association

Abbreviations: PREG, pregnenolone; OH-PREG, 17α-hydroxy-pregnenolone; PROG, progesterone; OH-PROG, 17α-hydroxy-progesterone; DHEA, dehydroepiandrosterone; AD, androstenedione; cyt-b_5, cytochrome b_5; Cpd I, Compound I.

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with cyt-b_5, but also in the chemistry performed. While the hydroxylase activity of CYP17A1 is expected to proceed through the "Compound I" initiated hydrogen abstraction observed in other members of the P450 superfamily, significant debate exists regarding the nature of the intermediate responsible for catalysis of carbon–carbon bond scission. The nucleophilic peroxoanion attacking the C-20 carbonyl of OH-PREG and OH-PROG with subsequent decomposition to form the androgen product was suggested [8–10]. On the other hand, the traditional mechanism catalyzed by Compound I (Cpd I) has also been proposed [11].

Decades of interrogation of the cytochromes P450, including the mechanistic exemplar for this family of enzymes, P450cam, has yielded a wealth of information regarding the key catalytic intermediates and active site environment necessary for the generation of the Cpd I high-valent porphyrin cation radical utilized in hydrogen abstraction and substrate hydroxylation, thought to operate in all P450s (Fig. 1) [12–14]. Of a particular relevance to this study is the structural motif of the I-helix and its conserved residue Threonine (Thr), located in the distal side of the heme-plane [15]. Thr252 in P450cam plays an important role in proton transfer essential for O–O bond scission required for Cpd I formation [16–18], and dioxygen activation during catalytic turnover [19,20]. It has been shown that mutation of this Thr residue to Ala results in a dramatic inhibition of substrate hydroxylation catalyzed by several P450s such as CYP102A1 (P450BM3) [21], CYP2E1 [22,23], CYP2D6 [24] and CYP1A2 [25]. The critical role of this threonine in successful formation of Cpd I and productive catalysis in cytochromes P450 is now well established [18,26,27].

In this work, we evaluate the mechanistic differences in the two steps reaction comprised with hydroxylation and lyase activity performed by CYP17A1 using the analogous mutation, T306A, within steroid metabolizing P450. Previously, Akthar's group studied the T306A mutant of CYP17A1, using Δ5-steroids (PREG and 17-OH-PREG) as substrates [9]. Other reports of CYP17A1 activity used various subsets of Δ4- and Δ-5-steroids as substrates and detergent solubilized CYP17A1 reconstituted with CPR and cyt-b_5 at different stoichiometries [8,9,28–30], which makes a direct comparison of the hydroxylase and lyase activity for specific Δ4- or Δ5-steroids difficult. To overcome these shortfalls, we incorporated CYP17A1 and its variant T306A in Nanodiscs and reconstituted it with redox partners in a well controlled stoichiometry [31,32]. We also employed the consistent substrate sets, the Δ5 (PREG and 17-OH-PREG) and Δ4 (PROG and 17-OH-PROG) steroids to study the hydroxylase and lyase activity using the same preparation of cytochrome P450 reductase (CPR) and cyt-b_5 throughout the *in vitro* reconstitution experiments. Importantly, we also documented the preference of substrate and coupling efficiency during the hydroxylation and lyase reaction by the wild type CYP17A1 and its mutant T306A.

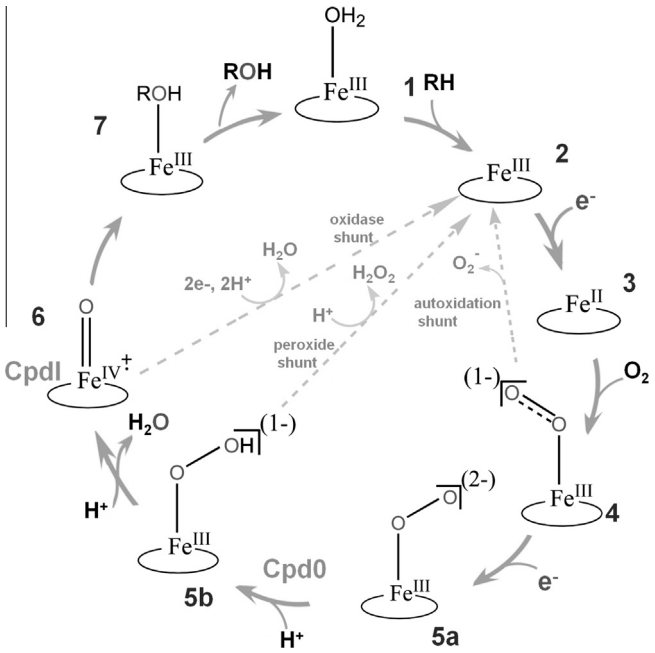

Fig. 1. A reaction cycle of cytochrome P450 catalysis. The seven steps indicating the binding of substrate (**1**), formation of a high spin ferric state (**2**), transfer of an electron from the redox partner to form the ferrous protein (**3**), generation of the ferrous oxygenated (oxy-ferrous) state (**4**), reduction to form the peroxo states after receiving second electron (**5a**), transfer of proton to the distal oxygen atom and production of the "hydroperoxo" state (**5b**), a second protonation and formation of a higher valent metal-oxo species "Compound I" (Cpd I) (**6**), and the hydroxylation and release of product (**7**) of P450 catalysis are illustrated. The three unproductive pathways are shown in grey. (For interpretation of color in this Figure, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Construction of T306A

In order to obtain the conserved I-helix variant of human CYP17A1 T306A, the pCWori_CYP17A1 construct [7] was mutated using primers 5′-ggt gcc ggt gtg gaa gcc acc acc agc gtc gtc-3′ (forward) and 5′-gac gac gct ggt ggt ggc ttc cac acc ggc acc-3′ (reverse). The sequence of the mutant was verified by automated DNA sequencing (ACGT, Inc.).

2.2. Expression and purification of recombinant proteins

The expression and purification of T306A was performed as described for wild type CYP17A1 [7,33]. The expression and purification of membrane scaffold protein (MSP), rat P450 reductase (CPR) and cyt-b_5 was performed as described [34–36].

2.3. Nanodisc assembly of T306A

The detailed protocol for the incorporation of CYP17A1 into Nanodiscs has been previously reported [7,33] and same procedure was employed for the T306A mutant.

2.4. Spectroscopic characterization

UV–visible spectra for CYP17A1 and T306A were recorded at room temperature using a Cary 300 UV–visible spectrophotometer. The P450 concentration was estimated by CO-difference spectra assuming $\Delta\varepsilon(450–490) = 91\ mM^{-1}\ cm^{-1}$ as described [37].

2.5. NADPH oxidation

Incorporation of CPR into preformed and purified CYP17A1 (or T306A) Nanodiscs was made by direct addition of oligomeric CPR at 1:4 CYP17A1 (or T306A)/CPR molar ratio, as described [38]. Briefly, 1 ml of CYP17A1 (or T306A) and CPR solution, in presence or absence of cytochrome b_5 (1:4:4 M ratio), in 100 mM potassium phosphate buffer, pH 7.4, containing 50 mM NaCl and 50 μM substrate (PROG, 17-OH-PROG, PREG or 17-OH-PREG) was brought 37 °C in a stirred quartz cuvette. The sample was incubated for 5 min and the reaction was initiated by addition of 100 μM of NADPH. The consumption of NADPH was monitored by recording the absorbance at 340 nm for 5 min. The reaction was stopped by adding 50 μl of 9 M sulfuric acid to bring the pH below 4.0. The sample was removed from the cuvette, flash frozen in liquid nitrogen, and stored at −80 °C until product analysis. The optical