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## Evidence that cytochrome $b_5$ acts as a redox donor in CYP17A1 mediated androgen synthesis

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### Abstract

Cytochrome P450 17A1 (CYP17A1) is an important drug target for castration resistant prostate cancer. It is a bi-functional enzyme, catalyzing production of glucocorticoid precursors by hydroxylation of pregnene- nucleus, and androgen biosynthesis by a second C-C lyase step, at the expense of glucocorticoid production. Cytochrome  $b_5$  (cyt  $b_5$ ) is known to be a key regulator of the androgen synthesis reaction *in vivo*, by a mechanism that is not well understood. Two hypotheses have been proposed for the mechanism by which cyt  $b_5$  increases androgen biosynthesis. Cyt  $b_5$  could act as an allosteric effector, binding to CYP17A1 and either changing its selective substrate affinity or altering the conformation of the P450 to increase the catalytic rate or decrease unproductive uncoupling channels. Alternatively, cyt  $b_5$  could act as a redox donor for supply of the second electron in the P450 cycle, reducing the oxyferrous complex to form the reactive peroxo-intermediate. To understand the mechanism of lyase enhancement by cyt  $b_5$ , we generated a redox-inactive form of cyt  $b_5$ , in which the heme is replaced with a Manganese-protoporphyrin IX (Mn- $b_5$ ), and investigated enhancement of androgen producing lyase reaction by CYP17A1. Given the critical significance of a stable membrane anchor for all of the proteins involved and the need for controlled stoichiometric ratios, we employed the Nanodisc system for this study. The redox inactive form was observed to have no effect on the lyase reaction, while reactions with the normal heme-iron containing cyt  $b_5$  were enhanced ~ 5 fold as compared to reactions in the absence of cyt  $b_5$ . We also performed resonance Raman measurements on ferric CYP17A1 bound to Mn- $b_5$ . Upon addition of Mn- $b_5$  to Nanodisc reconstituted CYP17A1, we observed clear evidence for the formation of a  $b_5$ -CYP17A1 complex, as noted by changes in the porphyrin modes and alteration in the proximal Fe-S vibrational frequency. Thus, although Mn- $b_5$  binds to CYP17A1, it is unable to enhance the lyase reaction, strongly suggesting that cyt  $b_5$  has a redox effector role in enhancement of the CYP17A1 mediated lyase reaction necessary for androgen synthesis.

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## Keywords

CYP17A1; Cytochrome  $b_5$ ; Redox donor; Androgen synthesis; Nanodiscs

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## 1. Introduction

The role of cytochrome  $b_5$  in P450 mediated metabolism has been the subject of intense debate for decades. Although its essential role in the fatty acid biosynthetic pathways is well appreciated [1], early work with hepatic drug metabolizing enzymes often yielded conflicting results. For instance, Sato and coworkers suggested that the binding of cytochrome  $b_5$  (cyt  $b_5$ ) to cytochrome P450 elicited a structural change in the enzyme which activated product turnover, either by increasing the inherent catalytic rate or by inhibiting non-productive auto-oxidative shunt processes (Fig. 1) [2]. Alternatively, as cyt  $b_5$  contains a bis-imidazole coordinated heme, direct transfer of electrons from cyt  $b_5$  to P450 has been proposed [2,3]. In this latter role, the relatively high redox potential of cyt  $b_5$  (~ 0 mV *versus* Normal Hydrogen Electrode [4]) suggests that electron transfer to ferric P450 (redox potential ~ -300 mV vs. NHE) is unfavorable. Hence it was suggested that the redox function of cyt  $b_5$  involved electron transfer to the ferrous dioxygen intermediate which has a redox potential near 0 mV [5] thus providing the "second electron" in the normal monooxygenase stoichiometry. In an attempt to differentiate between these two roles, Coon and co-workers reconstituted apo-cyt  $b_5$  with manganese protoporphyrin IX [6]. They observed no reduced Mn- $b_5$  in the presence of cytochrome P450 reductase (CPR) and NADPH, whereas iron cyt  $b_5$  was rapidly reduced. Hence Mn- $b_5$  is incapable of any electron transfer to the P450. From their experiments on various P450 reactions they concluded that cyt  $b_5$  effects depend on the specific P450 in question, the substrate being examined, and molar ratio of CPR to P450. This suggested that their observations could not be explained solely by a simple electron transfer role and some effects may also be caused by possible conformational changes caused by cyt  $b_5$  binding. While the role of cyt  $b_5$  in drug metabolism continues to be explored, a more interesting and potentially critical function of cyt  $b_5$  is in human steroid biosynthesis.

The synthesis of androgens, estrogens and corticosteroids involves multiple P450 catalyzed reactions. Cytochrome P450 17A1 (CYP17A1) is a key player in these reactions, being responsible for catalyzing 17 $\alpha$ -hydroxylation of pregnenolone and progesterone followed by C17-C20 bond scission in a separate lyase reaction to form dehydroepiandrosterone (DHEA) and androstenedione (AD) respectively (Fig. 2). The hydroxylation reactions occur throughout a person's lifetime, and when a person approaches adrenarche, the hydroxylated products can be shunted towards the subsequent step of formation of androgenic precursors by CYP17A1 in addition to the production of glucocorticoids [7]. The presence of membrane bound form of cyt  $b_5$  has been known to selectively and significantly enhance the rate of the lyase reaction [8]. Mutations of CYP17A1 surface residues: R347, R358 and K89 which are known to interact with cyt  $b_5$ , have been shown to cause impairment of lyase activity [9]. In a recent report, significant lyase impairment was shown in mice testicular Leydig cells lacking cyt  $b_5$  [10]. Additionally, male patients with mutations causing a lack of cyt  $b_5$  present with pseudo hermaphroditism, while high cyt  $b_5$  expression in adrenocortical

adenomas in Cushing's syndrome patients has been associated with increased androgen synthesis [11]. Clearly, *cyt b<sub>5</sub>* has a physiological significance in maintaining normal levels of androgen synthesis. The nature of this role has been long debated [8,12–14], with Auchus and coworkers studying lyase activity in recombinant yeast using apo *b<sub>5</sub>* which stimulated the lyase reaction, leading these workers to conclude that there is no redox role [15]. A similar report from Akhtar and coworkers mentions unpublished results demonstrating lyase enhancement in a reconstituted system utilizing Mn-*b<sub>5</sub>* [9]. On the contrary, Estabrook and others demonstrated in an *in vitro* reconstituted system in the presence of lipids that a zinc substituted derivative of *b<sub>5</sub>* did not stimulate the lyase activity of CYP17A1, and that the previous reports of rate enhancement by apo *b<sub>5</sub>* are the result of transfer of the heme group from the P450 to apo *b<sub>5</sub>* forming the holoenzyme [16]. Another property of *cyt b<sub>5</sub>* that complicates all previous reports is the need for an intact membrane to influence the P450 activity [17]. Given the key importance of *cyt b<sub>5</sub>* in the regulation of androgen synthesis, it is imperative that this disparity about the role of *cyt b<sub>5</sub>* be solved in a reproducible system representative of the membrane environment of these proteins. To this end, we employ the Nanodisc system to reconstitute CYP17A1, CPR and *cyt b<sub>5</sub>* in controlled stoichiometric ratios. We reconstituted *cyt b<sub>5</sub>* with manganese protoporphyrin IX and investigated the rate of lyase reaction by CYP17A1 in Nanodiscs, with iron containing *cyt b<sub>5</sub>*, or with Mn-protoporphyrin IX substituted form of *cyt b<sub>5</sub>* (Mn-*b<sub>5</sub>*) and compared each case *versus* the rate in the absence of *cyt b<sub>5</sub>*. In humans, androgens are primarily derived from the 17 $\alpha$ -hydroxypregnenolone substrate, therefore we decided to investigate this reaction to determine whether *cyt b<sub>5</sub>* is a redox donor in CYP17A1 mediated lyase reaction for androgen synthesis. Additionally, we probed the ferric resting state of CYP17A1 in the presence of native substrates to indicate formation of the *b<sub>5</sub>*-CYP17A1 complex.

## 2. Materials and methods

### 2.1 Expression and purification of recombinant proteins, Nanodisc assembly

The expression and purification of CYP17A1 was performed as described [18]. The expression and purification of membrane scaffold protein and *cyt b<sub>5</sub>* were performed as described [19,20]. Nanodiscs with CYP17A1 were assembled as documented [21]. Incorporation of CPR and *cyt b<sub>5</sub>* into purified CYP17A1 Nanodiscs was performed by direct addition of CPR and/or *cyt b<sub>5</sub>* in 2 to 4-fold molar excess, as documented [22–24].

### 2.2 Reconstitution of *cyt b<sub>5</sub>* with manganese protoporphyrin IX

*Cyt b<sub>5</sub>* was reconstituted with Mn-protoporphyrin IX according to [6], with the following changes: After performing buffer exchange with G-25 column to separate unbound Mn-protoporphyrin IX, the eluate was run through a DEAE-cellulose column, equilibrated with 25 mM Tris acetate (pH 8.0), 1 mM EDTA and 10 mM sodium cholate. A linear salt gradient was formed using the equilibration buffer supplemented with 1M NaCl. Mn-*b<sub>5</sub>* fractions which were characterized on the basis of their observed R<sub>z</sub> ratios, were pooled and rigorously dialyzed against 100 mM potassium phosphate buffer (pH 7.4) and flash frozen in liquid nitrogen before being stored at –80 °C until use.

### 2.3 Spectroscopic characterizations of Nanodisc incorporated CYP17A1 and of Mn-*b*<sub>5</sub>

Nanodisc incorporated CYP17A1 was characterized by UV-visible spectroscopy in a Cary 300 UV-visible spectrophotometer. The amount of P450 was determined by change in spin shift upon pregnenolone binding using  $\epsilon_{390}-\epsilon_{417} = 100 \text{ mM}^{-1}\text{cm}^{-1}$  [25]. Mn-*b*<sub>5</sub> was quantified using UV-visible spectroscopy using  $\epsilon_{368}=50\pm 2 \text{ mM}^{-1}\text{cm}^{-1}$  and  $\epsilon_{468}=38\pm 1.5 \text{ mM}^{-1}\text{cm}^{-1}$  [6]. Purity and identity of all proteins was confirmed by denaturing electrophoresis and MALDI-mass spectrometry.

### 2.5 Determination of NADPH oxidation rates

NADPH oxidation rates and product formation rates were determined with 365 pmoles of CYP17A1 in Nanodiscs reconstituted with 4-fold CPR, with/without the addition of 4-fold cyt *b*<sub>5</sub>. The solution was brought to 1 mL by the addition of 100 mM potassium phosphate buffer (pH 7.4) containing 50 mM NaCl and 50  $\mu\text{M}$  17 $\alpha$ -hydroxypregnenolone (Sigma) in a stirred quartz cuvette. The solution was equilibrated at 37°C by incubation for 5 min and reaction was initiated by addition of NADPH solution. The NADPH consumption was monitored by change in absorbance at 340 nm for 15 min and the linearity was verified. At 15 min the reaction was quenched by adding 50  $\mu\text{L}$  8.9 N sulfuric acid. Each sample was flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until product analysis. These reactions were performed in a Cary 300 UV-visible spectrophotometer fitted with magnetic stirrer and Peltier temperature controller.

### 2.6 Determination of product formation rates

Frozen samples were thawed and extraction of analytes was performed as previously documented [23]. The product analysis was performed by gas chromatography with a DB-17 phenol substituted siloxane column and a flame ionization detector on a Hewlett-Packard 6890 gas chromatograph, and the chromatograms were processed with Grams/32 AI software.

### 2.7 Preparation of Raman samples

CYP17A1 incorporated in Nanodiscs in 100 mM potassium phosphate buffer (pH 7.4) was concentrated to 200  $\mu\text{M}$  and 2-fold excess of Mn-*b*<sub>5</sub> was added from a 400  $\mu\text{M}$  solution in the same buffer. The resultant solution incubated at 37°C for 15 minutes. Substrate was added to each sample from methanolic stocks such that the final concentration was 420  $\mu\text{M}$  [24]. Finally, ultrapure glycerol was added to a concentration of 15% (v/v); i.e., the final concentration of CYP17A1 was calculated to be 85  $\mu\text{M}$ . The samples were flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analyzed.

### 2.8 Resonance Raman spectroscopy

To determine the effect of cyt *b*<sub>5</sub> on the heme stretching modes in ferric CYP17A1, we employed the Mn reconstituted form of cyt *b*<sub>5</sub>. This was done to prevent interference from the heme of cyt *b*<sub>5</sub> as was done previously by Mak *et al.* in rR studies on CYP2B4 [26,27]. We acquired resonance Raman spectra after adding 2-fold and 4-fold excess of Mn-*b*<sub>5</sub>. The high frequency spectra were acquired using the 406.7 nm excitation line from a Krypton ion laser (Coherent Innova Sabre Ion Laser) and changes in the  $\nu(\text{Fe-S})$  stretching mode were

investigated using the 356.4 nm excitation line from the same laser, a wavelength known to selectively enhance this mode [28]. The RR spectra of all samples were measured using a Spex 1269 spectrometer equipped with a Spec-10 LN liquid nitrogen-cooled detector (Princeton Instruments, NJ). The laser power was adjusted to ~10 mW at the sample. All samples were measured in a spinning NMR tube to avoid local heating and protein degradation. The spectra were collected using a 180° backscattering geometry, with the laser beam being focused as a line image on the sample tube using a cylindrical lens. Spectra were calibrated with data acquired for fenchone and processed with Grams/32 AI software (Galactic Industries, Salem, NH).

### 3. Results

#### 3.1 Reconstitution and characterization of Mn-*b*<sub>5</sub>

Mn-*b*<sub>5</sub> was successfully reconstituted from a purified preparation of apo-cyt *b*<sub>5</sub>. The complete reconstitution was confirmed by UV-visible spectroscopy. SDS-PAGE analysis and MALDI-MS using a sinapinic acid matrix confirmed the presence of highly purified and full length protein construct of Mn-*b*<sub>5</sub> as well as cyt *b*<sub>5</sub> (data not shown).

#### 3.2 NADPH oxidation and catalytic turnover of 17 $\alpha$ -hydroxypregnenolone in the presence of cyt *b*<sub>5</sub> or Mn-*b*<sub>5</sub>

The rates of NADPH oxidation and product formation in the presence of cyt *b*<sub>5</sub> and Mn-*b*<sub>5</sub> were compared to the rates in the absence of any added cyt *b*<sub>5</sub>. The presence of cyt *b*<sub>5</sub> increases the product formation rate ~5 fold, while the addition of Mn-*b*<sub>5</sub> does not cause any change (Fig. 3A). The rates of NADPH oxidation were close in value in all three cases, 54 $\pm$ 1.3 min<sup>-1</sup> for cyt *b*<sub>5</sub>, 51 $\pm$ 1.0 min<sup>-1</sup> for Mn-*b*<sub>5</sub> and 46 $\pm$ 1.9 min<sup>-1</sup> for reactions in the absence of any *b*<sub>5</sub>. The efficiency of coupling was calculated as the ratio of product formation rate to the rate of NADPH consumption. This was seen to be maximal in the reactions with cyt *b*<sub>5</sub>, while reactions with no *b*<sub>5</sub> and with Mn-*b*<sub>5</sub> had similar coupling efficiency (Fig. 3B).

#### 3.3 Resonance Raman spectroscopy on Mn-*b*<sub>5</sub> binding to ferric CYP17A1

As was reported in an earlier work [29], binding of pregnenolone to substrate-free CYP17A1 causes a spin state conversion from almost pure low spin (LS) to largely high spin (HS) form, while binding of 17 $\alpha$ -hydroxypregnenolone generates a lower HS fraction. The persistence of more LS component being attributed to the tendency of the 17 $\alpha$ -hydroxy fragment of the substrate to directly interact with the heme iron or promote retention of distal pocket water molecules. In the present work, additions of Mn-*b*<sub>5</sub> to substrate-bound forms of CYP17A1, creating 2-fold excesses relative to the enzyme, cause relatively small increases of the LS component in these samples. This is reflected in the resonance Raman (rR) spectra illustrated in Fig. 4 for the samples bearing a 2-fold excess of Mn-*b*<sub>5</sub>. As can be seen in trace B, pregnenolone bound CYP17A1 interacting with Mn-*b*<sub>5</sub> shows a spectrum similar to that for the sample without Mn-*b*<sub>5</sub> (trace A); they both exhibit a strong isolated  $\nu_3$  mode at 1488 cm<sup>-1</sup> and a small signal for LS population near 1500 cm<sup>-1</sup>. Employing a procedure developed in our laboratory by Mak *et al.* [30], it is possible to estimate the spin state population using the intensity ratio of  $I_{HS}/I_{LS}$  equal to 1.24. Using this value, the HS

population of pregnenolone bound CYP17A1 in the presence of a 2-fold excess of Mn-*b*<sub>5</sub> is calculated to be 80%, close to the value observed in the absence of Mn-*b*<sub>5</sub>. Similarly, it is noted that the HS component changes from ~57% to ~50% when a 2-fold excess of Mn-*b*<sub>5</sub> was added into 17 $\alpha$ -hydroxypregnenolone bound CYP17A1 (Fig. 4 right panel, trace D). These remained nearly the same even in the presence of 4-fold Mn-*b*<sub>5</sub>, call of CYP17A1 binding sites for cyt *b*<sub>5</sub> have been occupied (data not shown). In addition to the relatively minor effects on spin state populations, as can be seen in Fig. 4, it is important to emphasize that the frequencies observed for both the low energy and high energy internal modes of the heme prosthetic group do not shift relative to those observed for the corresponding samples without Mn-*b*<sub>5</sub>, implying that the interaction with Mn-*b*<sub>5</sub> does not induce significant structural changes of the heme macrocycle or its peripheral substituents in the substrate-bound ferric enzyme. Though minimal effects were observed for spin state populations and heme structure, it is noted that association of CYP17A1 with Mn-*b*<sub>5</sub> increases the strength of Fe-S linkage, demonstrating the formation of a CYP17A1:Mn-*b*<sub>5</sub> complex. This is evident from viewing the inserts for the low-frequency spectra in Fig. 4, where the  $\nu(\text{Fe-S})$  stretching modes occurring in samples with the excess Mn-*b*<sub>5</sub> appear at 350 cm<sup>-1</sup>, while those previously reported for the same samples of CYP17A1 in Nanodiscs without Mn-*b*<sub>5</sub> appear at 347 cm<sup>-1</sup> [29].

#### 4. Discussion

CYP17A1 is a critical enzyme in steroidogenesis, lying at the branch point of glucocorticoids synthesis and androgen synthesis. It performs hydroxylation on the pregnene- nucleus to form hydroxylated products which can either be diverted to glucocorticoid synthesis, or undergo a C-C bond lyase reaction by CYP17A1 to produce androgens. Cytochrome *b*<sub>5</sub> is known to be the chief regulator of CYP17A1 reactivities *in vivo*, yet the nature of interactions of CYP17A1 with cyt *b*<sub>5</sub> is not clearly understood. Cyt *b*<sub>5</sub> is known to enhance the lyase reactions from 5 to 10 fold. Whether cyt *b*<sub>5</sub> acts as an allosteric modulator or if it functions as a second electron donor has long been debated [15,31], and conflicting reports from various groups are complicated by the fact that these studies were carried out in uncontrolled conditions where aggregation states and stoichiometries of interactions were not known [15,32,33]. Scott and coworkers have used solution NMR with soluble forms of the proteins to study the conformational changes occurring in CYP17A1 bound to lyase substrates upon cyt *b*<sub>5</sub> binding and associated them with an allosteric role for cyt *b*<sub>5</sub> [12,34], although not directly addressing a possible redox role. The application of Nanodisc technology to this system is therefore an effective solution, enabling us to study reactions in controlled stoichiometries in a native-like membrane environment.

We reconstituted cyt *b*<sub>5</sub> with Mn-protoporphyrin IX, which is known to be redox inactive under these conditions [6], and tested the rate of lyase reaction in 17 $\alpha$ -hydroxypregnenolone in the presence and absence of cyt *b*<sub>5</sub>, and also in the presence of Mn-*b*<sub>5</sub>. Reactions with Mn-*b*<sub>5</sub> showed no enhancement of the rate of DHEA formation, which suggests very strongly that cyt *b*<sub>5</sub> has a definite redox donor role in the CYP17A1 lyase chemistry. Importantly, presence of cyt *b*<sub>5</sub> increases the coupling efficiency about 5-fold. Given the relatively high redox potential of cyt *b*<sub>5</sub> (~0 mV NHE) [4], its redox role is limited to



reduction of the oxy-complex [5], giving rise to the nucleophilic peroxo-species, the reactive intermediate responsible for carbon-carbon bond scission [23,35]. An enhanced reduction rate of the oxy-complex is expected to increase the steady-state concentration of the peroxoanion, which in the absence of cyt *b*<sub>5</sub> is depleted through non-productive release of hydrogen peroxide. Our resonance Raman data with Mn-*b*<sub>5</sub> binding to ferric CYP17A1 indicate no major structural changes of the heme planar modes, whereas on the proximal side, Fe-S bond was observed to get strengthened when Mn-*b*<sub>5</sub> was bound. Clearly, Mn-*b*<sub>5</sub> binds to CYP17A1, but is incapable of enhancing the lyase activity, the latter being enhanced if the native iron containing cyt *b*<sub>5</sub> is bound. Taken together, these experiments provide strong evidence that cyt *b*<sub>5</sub> is serving as a redox partner in CYP17A1 catalytic cycle, most likely delivering a second electron to the oxy-complex and facilitating formation of the peroxo-ferric complex, which recent evidence shows to be the catalytically active intermediate in the lyase reaction [35]. This result is in agreement with conclusions on the role of cyt *b*<sub>5</sub> in CYP2B4 catalysis [14,36]. Although our results cannot disprove the presence of functionally important allosteric interactions between cyt *b*<sub>5</sub> and CYP17A1, the lack of any effect of Mn-*b*<sub>5</sub> on the rate of turnover of 17 $\alpha$ -hydroxypregnenolone, as compared with 5-fold acceleration of this reaction in the presence of cyt *b*<sub>5</sub>, suggests that these allosteric interactions do not play significant role in the human CYP17A1 mediated catalysis of lyase reaction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>Cyt <i>b</i><sub>5</sub></b>	cytochrome <i>b</i> <sub>5</sub>
<b>Mn-<i>b</i><sub>5</sub></b>	manganese protoporphyrin IX substituted cyt <i>b</i> <sub>5</sub>
<b>DHEA</b>	dehydroepiandrosterone
<b>PREG</b>	pregnenolone
<b>OH-PREG</b>	17 $\alpha$ -hydroxypregnenolone

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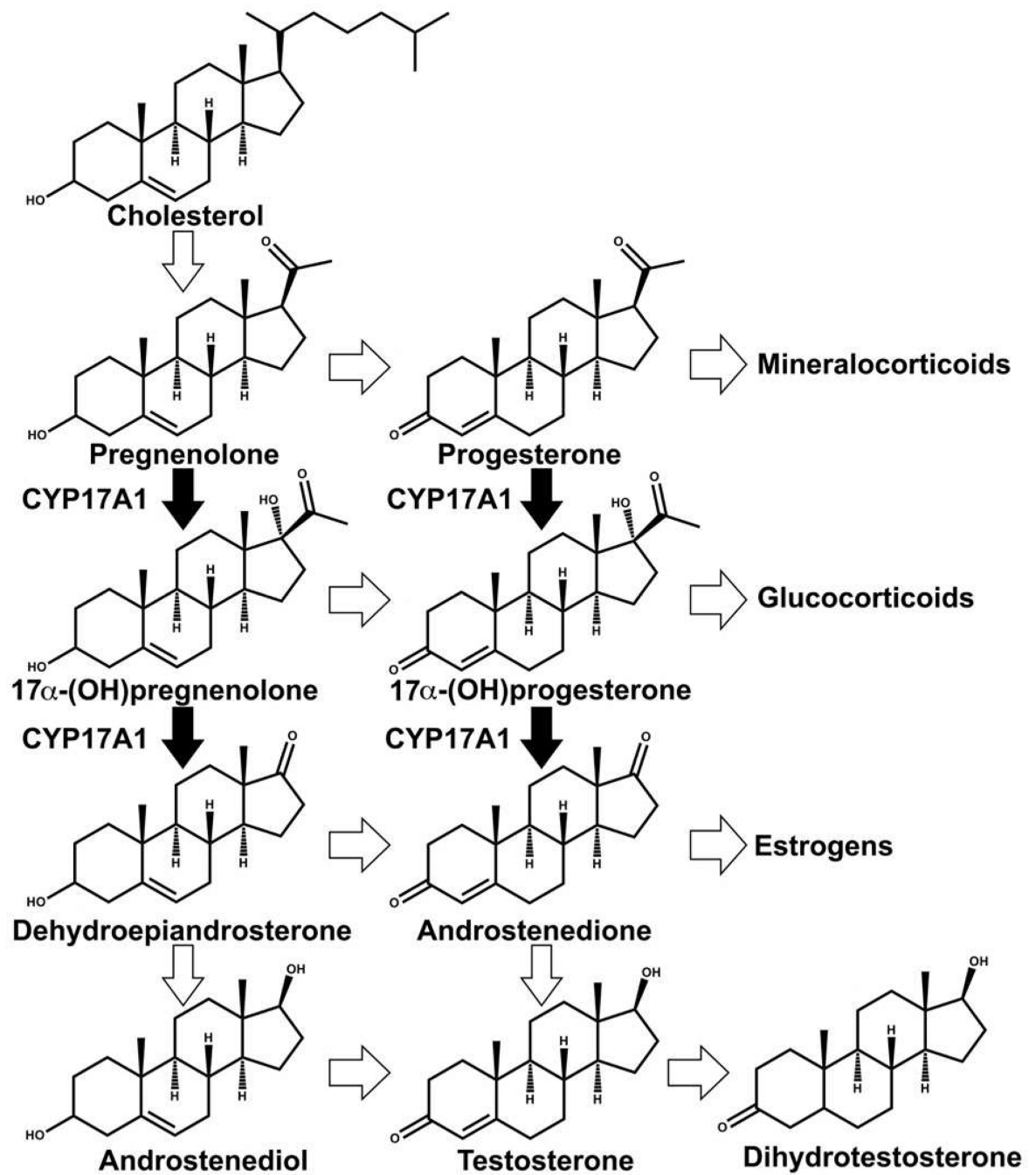


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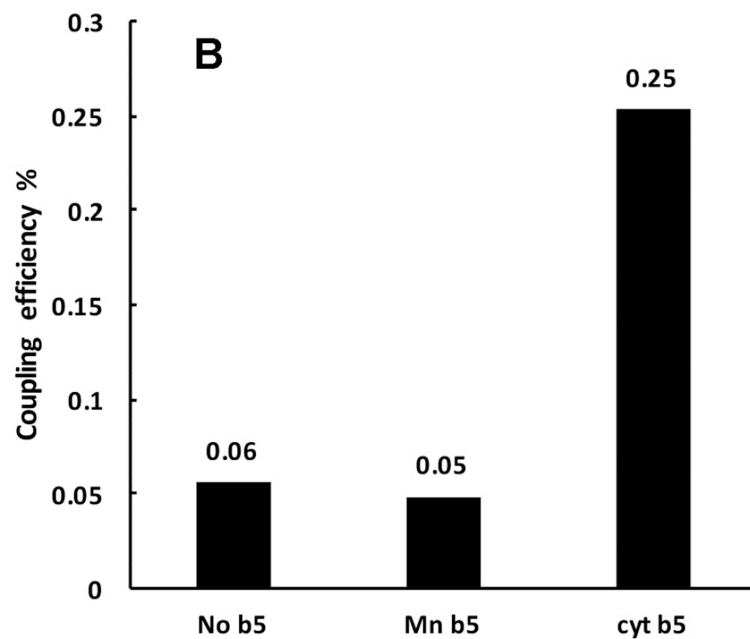
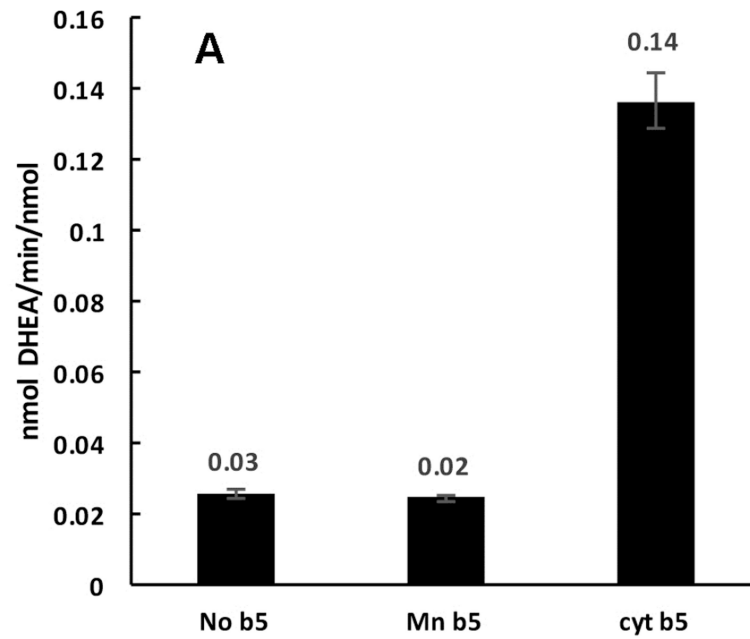
### Highlights

- Cyt  $b_5$  role in human CYP17A1 mediated androgen synthesis was probed in Nanodiscs.
- Native cyt  $b_5$  enhances androgen synthesis by CYP17A1.
- Redox inactive Mn cyt  $b_5$  does not enhance androgen synthesis by CYP17A1.
- Interactions with Cyt  $b_5$  perturb Fe-S and heme Raman modes of CYP17A1.
- Cyt  $b_5$  acts as a redox donor for CYP17A1 mediated androgen synthesis.



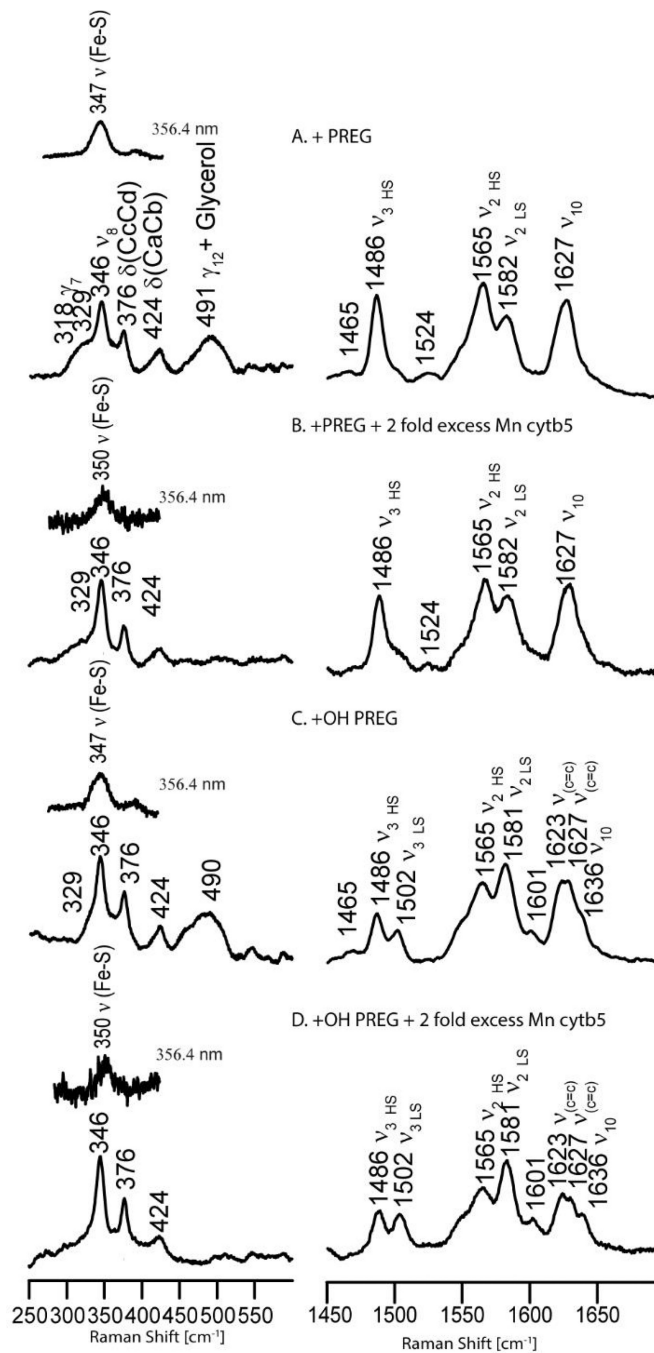


**Figure 2.**  
CYP17A1 catalyzed reactions in steroidogenesis.



**Figure 3.**

Comparison of CYP17A1 catalyzed lyase reactions with  $17\alpha$ -hydroxypregnenolone as substrate, with no cytochrome  $b_5$  present, or with Mn- $b_5$ , or native cytochrome  $b_5$ . (A) DHEA product formation rates (B) Coupling efficiencies (calculated as the percent ratio of amount of product formed to the amount of NADPH consumed).



**Figure 4.**

Resonance Raman Spectra of CYP17A1 +/- Mn-*b*<sub>5</sub>. The right panel shows the high-frequency region of the acquired spectra, while the left panel shows the low frequency region; the spectra were acquired using the 406.7 nm excitation line from a Krypton ion laser, noting that the inset bands in the low frequency region were acquired with the 356.4 nm excitation line from the same laser, a wavelength that selectively enhances the  $\nu(\text{Fe-S})$



stretching mode [28]. Spectra traces in B and D are the resulting traces after subtraction of spectra obtained for samples containing the equivalent concentration of Mn-*b*<sub>5</sub>.

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