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Ruchia Duggal
University of Illinois at Urbana-Champaign

Yilin Liu
Marquette University

Michael C. Gregory
University of Illinois at Urbana-Champaign

Ilia G. Denisov
University of Illinois at Urbana-Champaign

James R. Kincaid
Marquette University, james.kincaid@marquette.edu

See next page for additional authors

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Authors
Ruchia Duggal, Yilin Liu, Michael C. Gregory, Ilia G. Denisov, James R. Kincaid, and Stephen G. Sligar

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Evidence That Cytochrome B_5 Acts as A Redox Donor in CYP17A1 Mediated Androgen Synthesis

Ruchia Duggal
Department of Biochemistry,
University of Illinois Urbana-Champaign,
Urbana, IL
Yilin Liu
Department of Chemistry, Marquette University,
Milwaukee, WI
Michael C. Gregory
Department of Biochemistry,
University of Illinois Urbana-Champaign,
Urbana, IL
Ilia G. Denisov
Department of Biochemistry,
University of Illinois Urbana-Champaign,
Urbana, IL
James R. Kincaid
Department of Chemistry, Marquette University,
Milwaukee, WI

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Stephen G. Sligar

Department of Biochemistry, Department of Chemistry,
University of Illinois Urbana-Champaign,
Urbana, IL

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.

Keywords: CYP17A1, Cytochrome b_5, Redox donor, Androgen synthesis, Nanodiscs

Abbreviations

- Cyt b_5, cytochrome b_5;
- Mn-b_5, manganese protoporphyrin IX substituted cyt b_5;
- DHEA, dehydroepiandrosterone;
- PREG, pregnenolone;
- OH-PREG, 17α-hydroxypregenolone

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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 Ref.[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.

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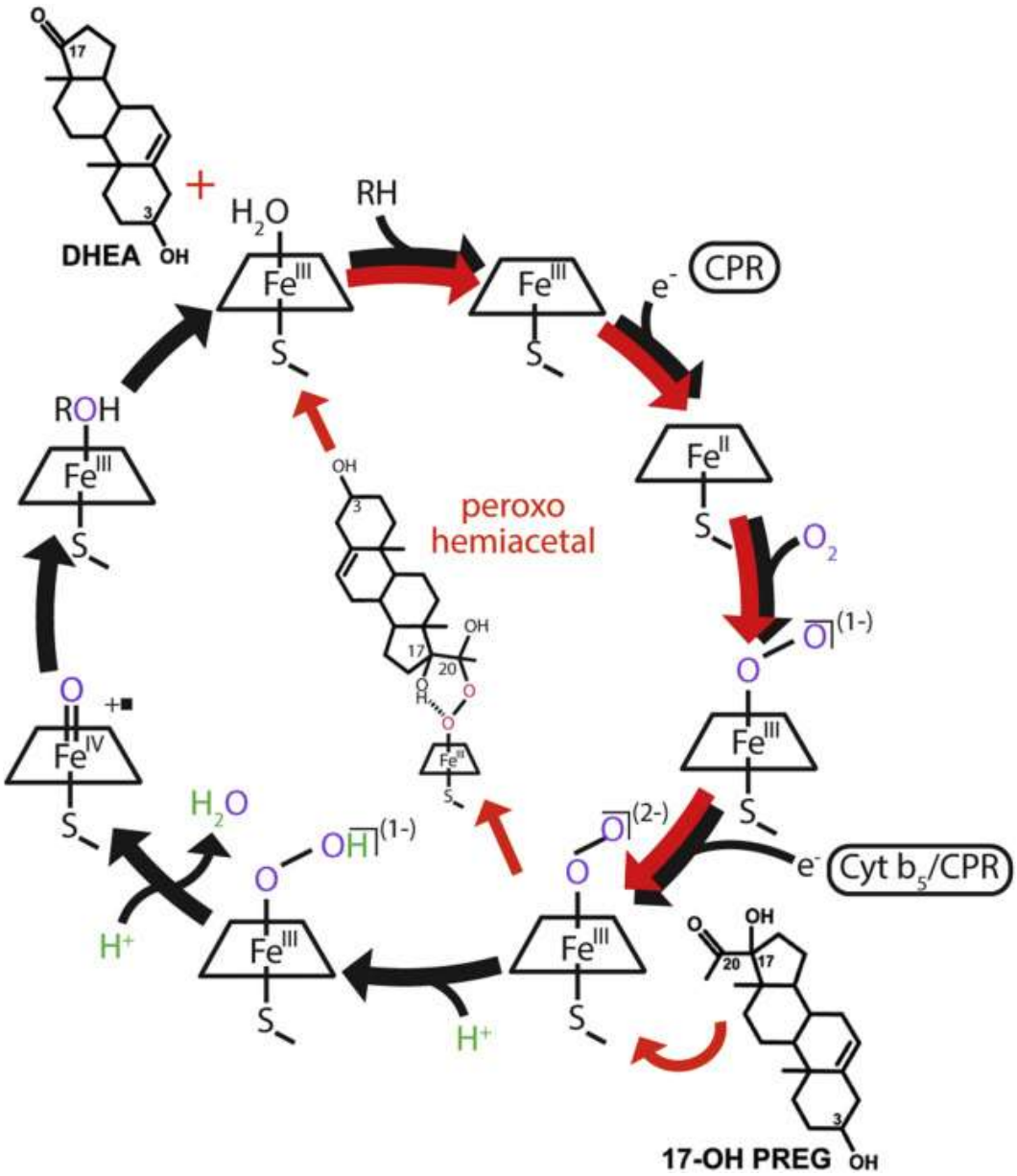

Fig. 1. Cytochrome P450 reaction cycle. Black arrows represent the path followed for CYP17A1 mediated hydroxylation chemistry, while red arrows represent the CYP17A1 mediated carbon-carbon scission reaction, the lyase chemistry. The first reduction is indicated as being carried out by CPR, while the second electron can be donated by either CPR or cytochrome b_5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 17α-hydroxylation of pregnenolone and progesterone

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followed by C17C20 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_5 has a physiological significance in maintaining normal levels of androgen synthesis. The nature of this role has been long debated,[8,12,13,14] with Auchus and coworkers studying lyase activity in recombinant yeast using apo b_5 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_5.[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_5 did not stimulate the lyase activity of CYP17A1, and that the previous reports of rate enhancement by apo b_5 are the result of transfer of the heme group from the P450 to apo b_5 forming the holoenzyme.[16] Another property of cyt b_5 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_5 in the regulation of androgen synthesis, it is imperative that this disparity about the role of cyt b_5 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_5 in controlled stoichiometric ratios. We reconstituted cyt b_5 with manganese protoporphyrin IX and investigated the rate of lyase reaction by CYP17A1 in Nanodiscs, with iron containing cyt b_5, or with Mn-protoporphyrin IX substituted form of cyt b_5 (Mn-b_5) and compared

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each case *versus* the rate in the absence of cyt b_5. In humans, androgens are primarily derived from the 17α-hydroxypregnenolone substrate, therefore we decided to investigate this reaction to determine whether cyt b_5 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_5-CYP17A1 complex.

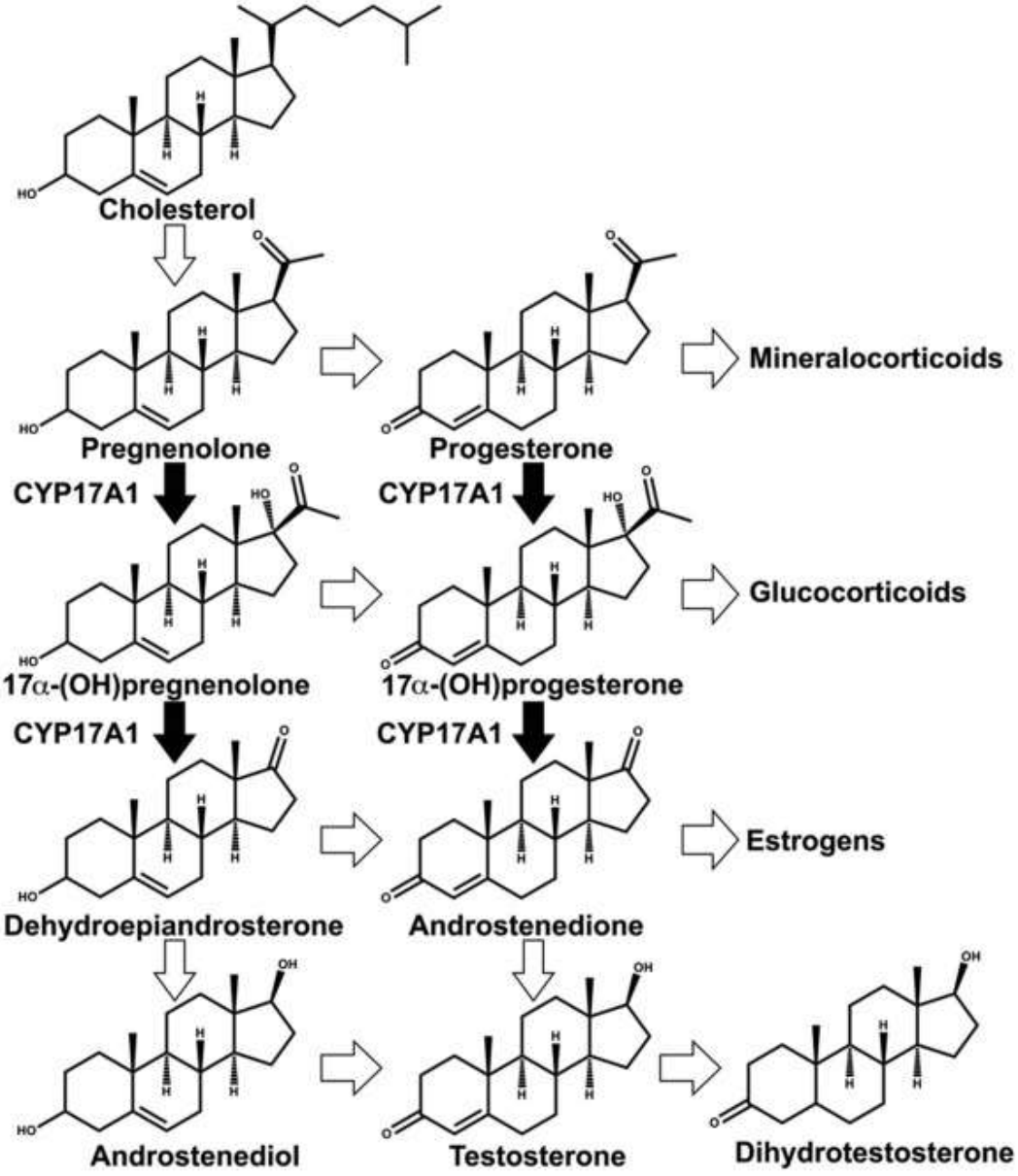

Fig. 2. CYP17A1 catalyzed reactions in steroidogenesis.

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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_5 were performed as described.[19,20] Nanodiscs with CYP17A1 were assembled as documented.[21] Incorporation of CPR and *cyt* b_5 into purified CYP17A1 Nanodiscs was performed by direct addition of CPR and/or *cyt* b_5 in 2–4-fold molar excess, as documented.[22,23,24]

2.2. Reconstitution of cyt b_5 with manganese protoporphyrin IX

Cyt b_5 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 1 M NaCl. Mn-b_5 fractions which were characterized on the basis of their observed Rz 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_5

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 $\varepsilon_{390}-\varepsilon_{417}$ = 100 mM^{-1} cm^{-1}.[25] Mn-b_5 was quantified using UV–visible spectroscopy using ε_{368} = 50 ± 2 mM^{-1} cm^{-1} and ε_{468} = 38 ± 1.5 mM^{-1} cm^{-1}.[6] Purity and identity of all proteins was confirmed by denaturing electrophoresis and MALDI-mass spectrometry.

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2.4. 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_5. 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 μM 17α-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 μL 8.9 N sulfuric acid. Each sample was flash frozen in liquid nitrogen and stored at −80 °C until product analysis. These reactions were performed in a Cary 300 UV–visible spectrophotometer fitted with magnetic stirrer and Peltier temperature controller.

2.5. 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.

2.6. Preparation of Raman samples

CYP17A1 incorporated in Nanodiscs in 100 mM potassium phosphate buffer (pH 7.4) was concentrated to 200 μM and 2-fold excess of Mn-b_5 was added from a 400 μM solution in the same buffer. The resultant solution incubated at 37 °C for 15 min. Substrate was added to each sample from methanolic stocks such that the final concentration was 420 μM.[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 μM. The samples were flash frozen in liquid nitrogen and stored at −80 °C until analyzed.

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2.7. Resonance Raman spectroscopy

To determine the effect of cyt b_5 on the heme stretching modes in ferric CYP17A1, we employed the Mn reconstituted form of cyt b_5. This was done to prevent interference from the heme of cyt b_5 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_5. 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 v(FeS) 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_5

Mn-b_5 was successfully reconstituted from a purified preparation of apo-cyt b_5. 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_5 as well as cyt b_5 (data not shown).

3.2. NADPH oxidation and catalytic turnover of 17α-hydroxypregnenolone in the presence of cyt b_5 or Mn-b_5

The rates of NADPH oxidation and product formation in the presence of cyt b_5 and Mn-b_5 were compared to the rates in the absence of any added cyt b_5. The presence of cyt b_5 increases the product formation rate ~5 fold, while the addition of Mn-b_5 does not

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cause any change (Fig. 3A). The rates of NADPH oxidation were close in value in all three cases, 54 ± 1.3 min^{-1} for cyt b_5, 51 ± 1.0 min^{-1} for Mn-b_5 and 46 ± 1.9 min^{-1} for reactions in the absence of any b_5. 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_5, while reactions with no b_5 and with Mn-b_5 had similar coupling efficiency (Fig. 3B).

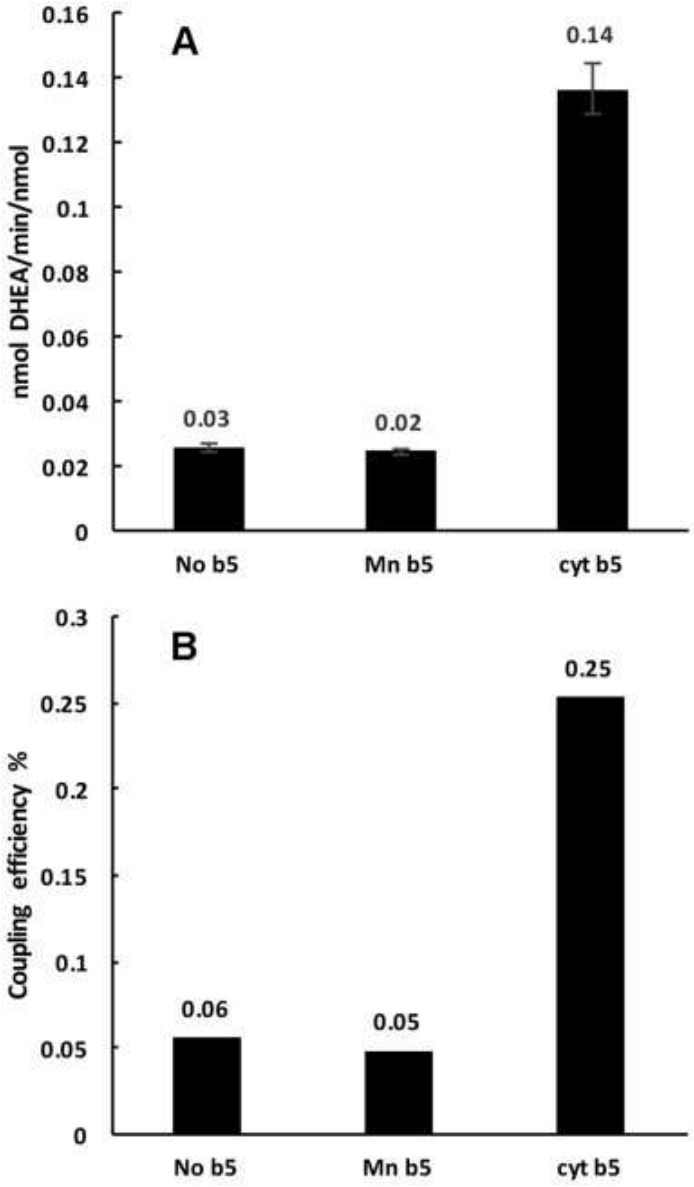

Fig. 3. Comparison of CYP17A1 catalyzed lyase reactions with 17α-hydroxy-pregnenolone as substrate, with no cytochrome b_5 present, or with Mn-b_5, or native cytochrome b_5. (A) DHEA product formation rates (B) Coupling efficiencies (calculated as the percent ratio of amount of product formed to the amount of NADPH consumed).

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3.3. Resonance Raman spectroscopy on Mn-b_5 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 17α-hydroxypregnenolone generates a lower HS fraction. The persistence of more LS component being attributed to the tendency of the 17α-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_5 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_5. As can be seen in trace B, pregnenolone bound CYP17A1 interacting with Mn-b_5 shows a spectrum similar to that for the sample without Mn-b_5 (trace A); they both exhibit a strong isolated ν_3 mode at 1488 cm^{-1} and a small signal for LS population near 1500 cm^{-1}. 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_5 is calculated to be 80%, close to the value observed in the absence of Mn-b_5. Similarly, it is noted that the HS component changes from ~57% to ~50% when a 2-fold excess of Mn-b_5 was added into 17α-hydroxypregnenolone bound CYP17A1 (Fig. 4 right panel, trace D). These remained nearly the same even in the presence of 4-fold Mn-b_5, indicating that all of CYP17A1 binding sites for cyt b_5 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_5, implying that the interaction with Mn-b_5 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_5 increases the strength of Fe—S linkage,

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demonstrating the formation of a CYP17A1:Mn-b_5 complex. This is evident from viewing the inserts for the low-frequency spectra in Fig. 4, where the v(Fe—S) stretching modes occurring in samples with the excess Mn-b_5 appear at 350 cm^{-1}, while those previously reported for the same samples of CYP17A1 in Nanodiscs without Mn-b_5 appear at 347 cm^{-1}.[29]

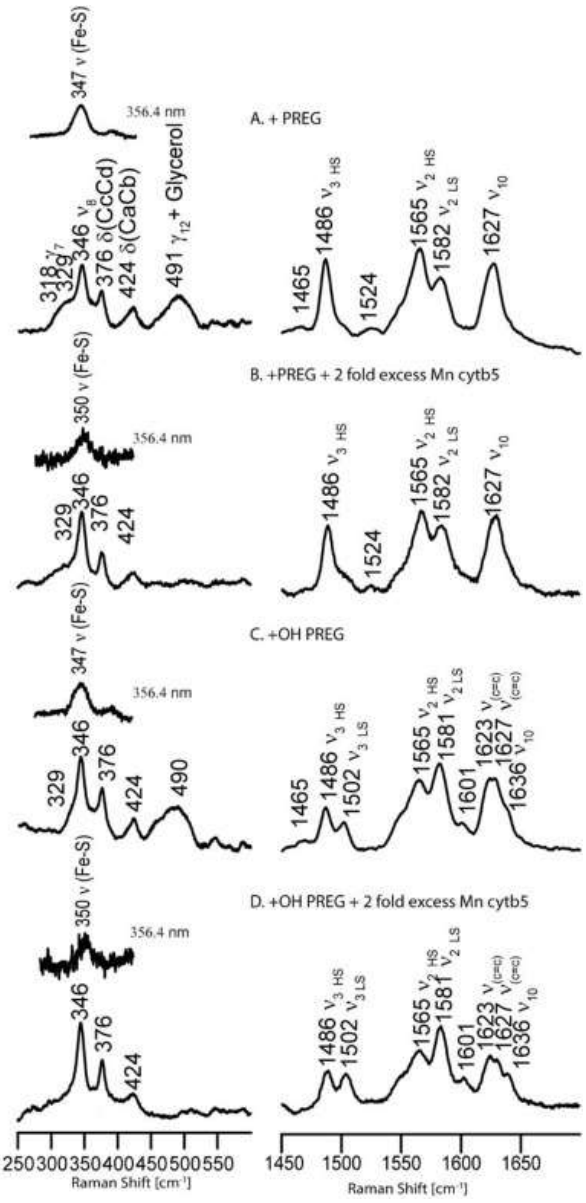

Fig. 4. Resonance Raman Spectra of CYP17A1 ± Mn-b_5. The right panel shows the high-frequency region of the acquired spectra, while the left panel shows the low frequency region; the spectra were acquired using the 406.7 nm excitation line from a Krypton ion laser, noting that the inset bands in the low frequency region were acquired with the 356.4 nm excitation line from the same laser, a wavelength that

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selectively enhances the v(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_5.

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_5 is known to be the chief regulator of CYP17A1 reactivities *in vivo*, yet the nature of interactions of CYP17A1 with cyt b_5 is not clearly understood. Cyt b_5 is known to enhance the lyase reactions from 5 to 10 fold. Whether cyt b_5 acts as an allosteric modulator or if it functions as a second electron donor has long been debated,[15,16] 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.[15,31,32] 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_5 binding and associated them with an allosteric role for cyt b_5,[12,33] 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_5 with Mn-protoporphyrin IX, which is known to be redox inactive under these conditions,[6] and tested the rate of lyase reaction in 17α-hydroxypregnenolone in the presence and absence of cyt b_5, and also in the presence of Mn-b_5. Reactions with Mn-b_5 showed no enhancement of the rate of DHEA formation, which suggests very strongly that cyt b_5 has a definite redox donor role in the CYP17A1 lyase chemistry. Importantly, presence of cyt b_5 increases the coupling efficiency about 5-fold. Given the relatively high redox potential of cyt b_5 (~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.[23,34] An enhanced reduction rate of the oxy-complex is

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expected to increase the steady-state concentration of the peroxoanion, which in the absence of cyt b_5 is depleted through non-productive release of hydrogen peroxide. Our resonance Raman data with Mn-b_5 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_5 was bound. Clearly, Mn-b_5 binds to CYP17A1, but is incapable of enhancing the lyase activity, the latter being enhanced if the native iron containing cyt b_5 is bound. Taken together, these experiments provide strong evidence that cyt b_5 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.[34] This result is in agreement with conclusions on the role of cyt b_5 in CYP2B4 catalysis.[14,35] Although our results cannot disprove the presence of functionally important allosteric interactions between cyt b_5 and CYP17A1, the lack of any effect of Mn-b_5 on the rate of turnover of 17α-hydroxypregnenolone, as compared with 5-fold acceleration of this reaction in the presence of cyt b_5, suggests that these allosteric interactions do not play significant role in the human CYP17A1 mediated catalysis of lyase reaction.

Acknowledgments

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Corresponding author. University of Illinois at Urbana-Champaign 505 S. Goodwin MC119, Urbana, IL 61801, United States.

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