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Substrate-Specific Allosteric Effects on the Enhancement of CYP17A1 Lyase Efficiency by Cytochrome b_5

Yilin Liu,
Department of Chemistry, Marquette University, Milwaukee, Wisconsin 53233, United States

Ilia G. Denisov,
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, United States

Stephen G. Sligar,
Department of Biochemistry and Department of Chemistry, University of Illinois, Urbana, Illinois 61801, United States

James R. Kincaid
Department of Chemistry, Marquette University, Milwaukee, Wisconsin 53233, United States

Abstract

CYP17A1 is an essential human steroidogenic enzyme, which catalyzes two sequential reactions leading to the formation of androstenedione from progesterone and dehydroepiandrosterone from pregnenolone. The second reaction is the C17-C20 bond scission, which is strongly dependent on the presence of cytochrome b_5 and displays a heretofore unexplained more pronounced acceleration when 17OH-progesteone (17OH-PROG) is a substrate. The origin of the stimulating effect of cytochrome b_5 on C-C bond scission catalyzed by CYP17A1 is still debated as mostly due to either the acceleration of the electron transfer to the P450 oxy complex or allosteric effects of cytochrome b_5 favoring active site conformations that promote lyase activity. Using resonance Raman spectroscopy, we compared the effect of Mn-substituted cytochrome b_5 (Mn-Cytb_5) on the oxy complex of CYP17A1 with both proteins co-incorporated in lipid nanodiscs. For CYP17A1 with 17OH-PROG, a characteristic shift of the Fe-O mode is observed in the presence of Mn-b_5, indicating reorientation of a hydrogen bond between the 17OH group of the substrate from the terminal to the proximal oxygenatom of the Fe-O-O moiety, a configuration favorable for the lyase catalysis. For 17OHpregnenolone, no such shift is observed, the favorable H-bonding orientation being present even without Mn-Cytb_5. These new data provide a precise allosteric interpretation for the more pronounced acceleration seen for the 17OH-PROG substrate.

The role of cytochrome b_5 (Cytb_5) in cytochrome P450 catalysis has been a subject of spirited debate for more than 40 years.[1] This small ~15 kDa eukaryotic heme protein is a

Corresponding Authors: James R. Kincaid james.kincaid@mu.edu; **Stephen G. Sligar** s-sligar@illinois.edu.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c00581.

All experimental details including sample preparation and resonance Raman measurements (PDF)

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mitochondrial and microsomal membrane protein that is well-known for its role in electron transfer in several systems, such as fatty acid elongation.[2] Its role in several cytochrome P450 systems is less clear. Addition of Cytb_5 to reconstituted microsomal P450 drug metabolizing assays by using cytochrome P450 reductase (CPR) can result in activation or inhibition as well as in alterations of substrate specificity.[3–6] More recently, some of these varied results were successfully explained by the competition of two redox transfer partners for the same, or overlapping, binding sites on the proximal surface face of hepatic P450 CYP2B4 and CYP17A1.[7,8] Other works indicated an improvement of redox coupling and an acceleration of the protonation events that are necessary for formation of the active "compound I" intermediate.[9] These, as well as other investigations, have provided a large amount of information about the multiple aspects of cytochrome b_5 effects, but there is still a lack of mechanistic understanding in many cases, particularly for the human CYP17A1 involved in steroid biosynthesis.

Interactions of Cytb_5 with CYP17A1 attracts special attention because of a vitally important role in hormonal regulation. Human CYP17A1 is located at a branch point leading to androgen biosynthesis.[10–13] This is a two-step reaction, first involving a typical P450 hydroxylation of either progesterone (PROG) or pregnenolone (PREG) at the C-17 position to produce 17α-hydroxyprogesterone (17OH-PROG) or 17α-hydroxypregnenolone (17OH-PREG). In a second round of catalysis, these hydroxylated products undergo a C_{17}-C_{20} bond cleavage (lyase) reaction to form androstenedione (AD) or dehydroepiandrosterone (DHEA),[14] also critical precursors for glucocorticoid biosynthesis.[15] While the mechanism of this lyase reaction has been vigorously debated with arguments being made that it proceeds through a conventional compound I pathway,[16] a proposed peroxo-mediated mechanism of the lyase activity, supported by recent studies on cryo-trapped intermediates[17–21] and further reinforced by the present results, is shown in Scheme 1. The presence of substrate promotes the transfer of an electron from cytochrome P450 reductase (CPR), with the resulting ferrous CYP17A1 protein then binding oxygen to form the oxygenated (oxy) complex. The second electron transfer required can occur through either CPR or cytochrome b_5. In the case of the carbon-carbon lyase reaction, delivery of this second electron generates a nucleophilic ferric-peroxo intermediate that attacks the susceptible carbonyl group of the lyase substrate, forming a new intermediate, whose spectroscopic properties are consistent with the peroxo-hemiketal structure depicted in the center of Scheme 1,[18,20,21] which precedes an electrocyclic carbon-carbon (C-C) bond cleavage process to form the DHEA product.[14,22,23]

Significantly, it has been well documented that Cytb_5 (Scheme 1) plays a crucial physiological role by enhancing CYP17A1-mediated 17,20-lyase turnover by up to ~10 fold,[24–26] although the origin of this stimulation remains controversial.[8,27–31] One possibility is that Cytb_5 acts as a complementary electron donor, working in concert with CPR to more efficiently complete the lyase cycle[24] with the first electron transfer requiring CPR and the second electron being donated by either CPR or Cytb_5.[7,32,33] Clearly, the presence of a reduced Cytb_5 donor positioned near the newly formed oxygenated intermediate could offer a significant advantage.

In previous work we demonstrated that a redox-inactive form of Cytb_5, where the heme iron was replaced with manganese (Mn-Cytb_5),[34] does not enhance the rate of lyase reaction catalyzed by CYP17A1 when incorporated into nanodiscs.[24] In contrast, the normal iron Cytb_5 increases the reaction rate by ~5-fold, thus identifying an important role of Cytb_5 in electron transfer during the catalytic cycle.[24] Subsequently, we performed more detailed kinetic studies to directly compare the rates of the second electron transfer to CYP17A1 from CPR and Cytb_5.[35] In addition, the autoxidation rates were measured for the oxy-ferrous 17OH-PREG bound-CYP17A1 in the absence or presence of Cytb_5, of cytochrome P450 reductase (CPR), or both simultaneously when all are self-assembled into nanodiscs. It was observed that ferrous Cytb_5 reduces oxy-ferrous CYP17A1 ~10 times faster than CPR, clearly demonstrating a direct electron transfer from Cytb_5 to the oxy complex of CYP17A1. However, these results do not exclude the possibility of a significant allosteric interaction between Cytb_5 and CYP17A1 when in a protein-protein complex. Evidence for such an allosteric role was recently observed in our resonance Raman (rR) data acquired for 17OH-PREG bound samples. A 3 cm^{-1} upshift of the Fe-S stretching mode, which carries functional implications, was noted when the redox-inactive Mn-Cytb_5 was bound to ND:CYP17.[24] Furthermore, several studies of Cytb_5 impact on the lyase reactivity have also pointed to possible allosteric influences,[7,28,31] including reports that the level of enhancement is greater for 17-OH-PROG than for 17-OH-PREG.[26,36]

To further address these two distinct roles for Cytb_5 in CYP17A1 carbon-carbon bond scission, in the present work, rR spectra were acquired for the 17-OH PREG and 17-OH PROG bound oxy complexes of CYP17A1 in nanodiscs in the absence and presence of oxidized Mn-Cytb_5. It is noted that the role of nanodisc technology is especially important in these studies of enzyme/reductase interactions inasmuch as the experimental protocols (Supporting Information) employed here not only eliminate undesired CYP17 aggregation but also ensure the formation of functional dyads, ND:CYP17/Cytb_5 in a membrane bilayer. In the case of 17-OH PREG (Figure 1), the $\upsilon(^{16}O\text{-}^{16}O)$ and $\upsilon(Fe\text{-}^{16}O)$ modes are observed at 1135 and 526 cm^{-1}, with the corresponding $\upsilon(^{18}O\text{-}^{18}O)$ and $\upsilon(Fe\text{-}^{18}O)$ modes occurring at 1070 and 497 cm^{-1}. These values are not changed from those observed without Mn-Cytb_5. It is noted that the $\upsilon(Fe\text{-}O)$ mode is 9 cm^{-1} lower than the value (535 cm^{-1}) observed for the sample containing the non-hydrogen-bonding PREG,[17] confirming the presence of a hydrogen-bond interaction of the C_{17}-OH group with the proximal oxygen atom (O_P) of the $Fe\text{-}O_P\text{-}O_T$ fragment.[17,18]

As argued previously,[17–20,22,37,38] such an arrangement, which has been shown to persist in the corresponding peroxo intermediate,[18] possesses a more nucleophilic terminal oxygen (O_t) of the peroxo fragment compared to that with an H-bonding interaction with the *terminal* oxygen, thereby facilitating peroxo attack on the C_{20} carbon. Clearly, the present observation that the CYP17A1:Cytb_5 complex with 17OH-PREG maintains an inherent nucleophilicity of the peroxo fragment as seen without the Cytb_5 redox partner suggests that the observed enhancement of lyase efficiency by Cytb_5 for this substrate is mainly ascribable to the faster second electron transfer from this redox partner, as suggested by earlier studies.[24]

In contrast with the above data, the binding of Mn-Cytb_5 to the oxygenated complex with 17OH-PROG bound CYP17A1 brings about obvious conformational changes, as evidenced in the rR difference spectra shown in Figure 2, where the $\upsilon(^{16}O\text{-}^{16}O)$ and $\upsilon(^{18}O\text{-}^{18}O)$ stretching modes occur at 1131 and 1066 cm^{-1}, consistent with H-bonded Fe-O-O fragments.[−17] Significantly, the rR spectrum of the sample including Mn-Cytb_5 reveals an active site rearrangement yielding a dominant fraction of the oxy complex possessing a $\upsilon(Fe\text{-}^{16}O)$ mode at 530 cm^{-1} compared to 542 cm^{-1}, a spectral shift consistent with transfer of the H-bonding interaction from the terminal to the proximal oxygen of the Fe-O-O fragment;[17] this structure favors the carbon-carbon bond scission reaction,[15–20] noting that similar conformers were described in the X-ray structures of CYP17A1 with OH-PREG and OH-PROG bound as substrates.[39] The positions of these two substrates are clearly different in the crystal structures, with OH-PROG bound further from heme iron and thus not in a favorable geometry for peroxo attack on the C20 carbonyl as the first step in C17-C20 bond scission, unlike the productive conformer observed for OH-PREG. These spectral changes observed for 17OH-PROG in the presence of Mn-Cytb_5 are quite consistent with a shift of the conformational equilibrium toward formation of an H-bond between the 17OH group of the substrate and the proximal oxygen atom of the Fe-O-O complex, i.e., the conformer well oriented to form the peroxo-hemiketal intermediate.[22] Finally, it may initially appear surprising that binding of Cytb_5 on the proximal side of CYP17 could differentially affect orientations of these two lyase substrates bound in the distal substrate binding pocket. However, recent NMR studies reported by Scott and coworkers reveal that binding of Cytb_5 causes conformational changes in a region containing several isoleusine residues (I205, I206, and I238),[27] all of which are in the vicinity of an asparagine (N202) that has been shown to play an important role in controlling the position of the H-bonding hydroxyl group of OH-PROG and OH-PREG substrates with respect to the Fe-O-O fragments of the oxy and peroxo intermediates.[39]

In summary, earlier functional studies showed the principal role of Cytb_5 on P450 catalysis by CYP17A1 is as an electron donor.[24,40,41] The rR studies reported herein provide structural evidence for an additional allosteric role when 17OH progesterone (OH-PROG) is the substrate for carbon-carbon bond scission. This additional allosteric enhancement is not expected, and not seen, when 17OH-PREG is at the active site. It is satisfying to note that these new structural revelations obtained through resonance Raman spectroscopy are entirely consistent with the results derived from detailed functional studies of the enzyme with both lyase substrates. Early studies of CYP17A1 in conventional media showed that the coupling efficiency for the OH-PREG substrate is enhanced by a factor of ~8 in the presence of Cytb_5, whereas the enhancement for OH-PROG was larger, ~20.[26] Also, detailed studies of the nanodisc CYP17A1/Cytb_5 complex reveal that the presence of Cytb_5 accelerates the lyase activity by ~5-fold for 17-OH PREG but by ~7-fold for 17-OH-PROG.[36] Thus, the present work confirms that, in addition to its redox partner role in CYP17A1 function, Cytb_5 binding also repositions 17OH-PROG in an optimal orientation to form a peroxo-hemiketal catalytic intermediate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

17OH-PROG	17α-hydroxyprogesterone
17OH-PREG	17α-hydroxypregnenolone
AD	androstenedione
DHEA	dehydroepiandrosterone
CPR	cytochrome P450 reductase
Cytb_5	cytochrome b_5
Mn-Cytb_5	manganese protoporphyrin IX substituted cytochrome b_5

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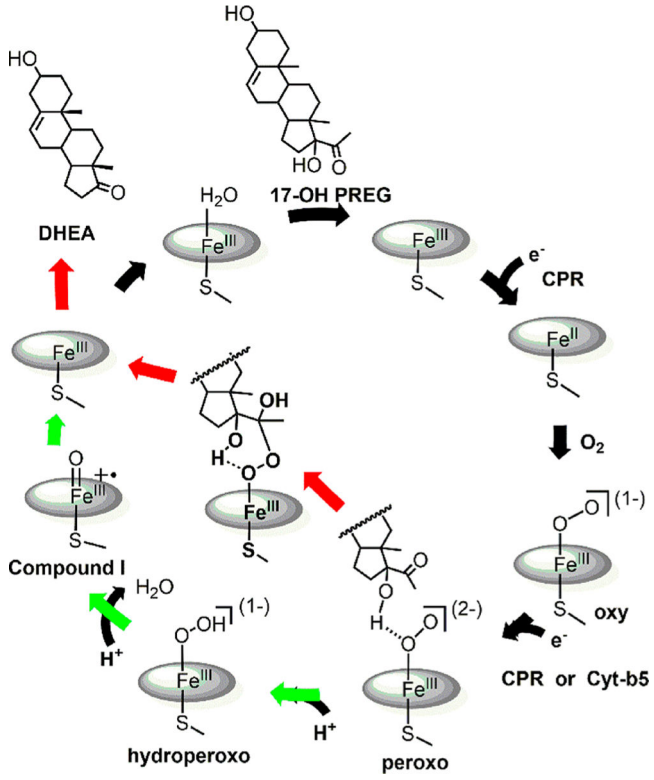

Scheme 1.

CYP17A1 Catalytic Cycle Showing a Proposed Lyase Pathway (Black and Red Arrows) for 17-OH PREG through a Peroxoanion Intermediate[18,20,21] versus the Possibility of C-C Scission Mediated by Compound I (Black and Green Arrows)[16] [a] [a]An analogous scheme applies to 17OH-PROG, with the caveat that the 17-OH hydrogen bond is directed toward the *terminal* oxygen of the Fe-O-O fragment of the peroxo intermediate.

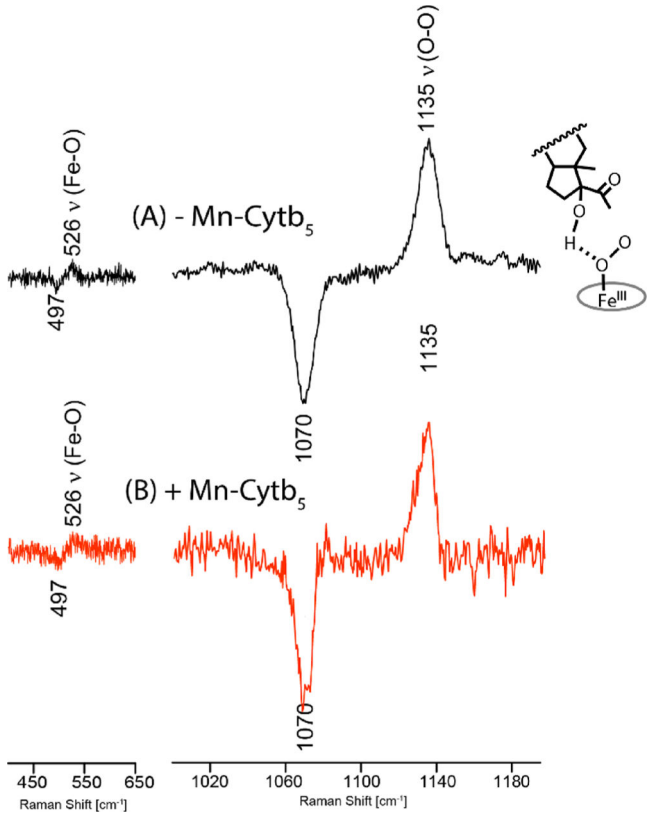

Figure 1.

^{16}O-^{18}O rR difference spectra of the 17OH-PREG bound CYP17A1 oxy complex without (A)[17] and with (B) Mn-Cytb_5; the spectrum in trace A was taken from raw data reported in ref 15. The [$^{16}O_2$-$^{18}O_2$] difference trace was generated by subtracting the two absolute spectral traces, where all (many) nonshifted features (i.e., those not associated with the Fe-O-O fragment) effectively cancel, with only the targeted υ(Fe-O) and υ(O-O) modes being observed.

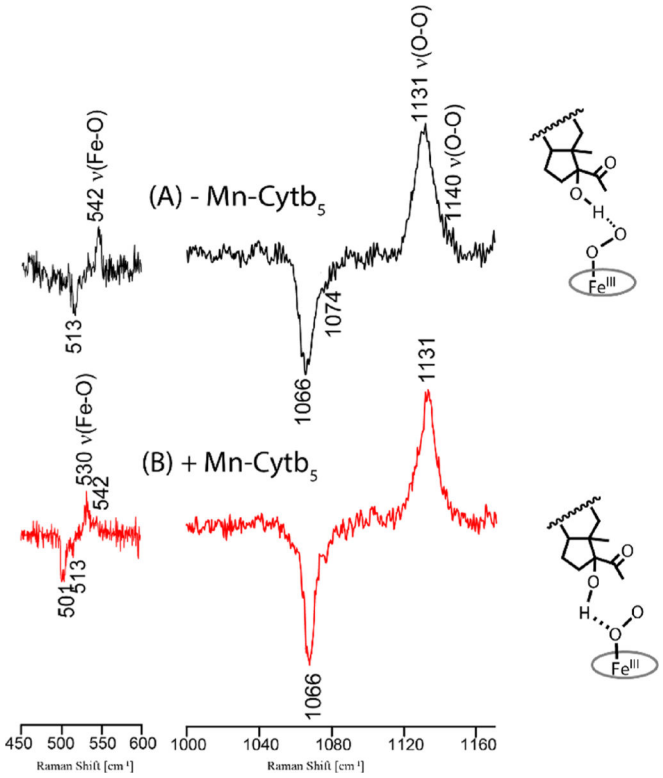

Figure 2.

^{16}O-^{18}O rR difference spectra of 17OH-PROG bound ND: CYP17A1 oxy complex without (A)[17] and with (B) Mn-Cytb_5; the spectrum in trace A was taken from raw data reported in ref 15. The [$^{16}O_2$-$^{18}O_2$] difference trace was generated as described in the caption to Figure 1.