Cytochrome b$_5$ modulation of 17α hydroxylase and 17–20 lyase (CYP17) activities in steroidogenesis

M K Akhtar, S L Kelly and M A Kaderbhai

Institute of Biological Sciences, Cledwyn Building, University of Wales, Aberystwyth, Ceredigion SY23 3DD, UK

(Requests for offprints should be addressed to M A Kaderbhai; Email: mak@aber.ac.uk)

Abstract

CYP17 is a steroidogenic enzyme located in the zona fasciculata and zona reticularis of the adrenal cortex and gonad tissues and which has dual functions – hydroxylation and as a lyase. The first activity gives hydroxylation of pregnenolone and progesterone at the C$_{17}$ position to generate 17α-hydroxyprogrenenolone and 17α-hydroxyprogesterone, while the second enzymic activity cleaves the C$_{17}$–C$_{20}$ bond of 17α-hydroxyprogrenenolone and 17α-hydroxyprogesterone to form dehydroepiandrosterone and androstenedione respectively. The modulation of these two activities occurs through cytochrome b$_5$. Association of cytochrome b$_5$ and CYP17 is thought to be based primarily on electrostatic interactions in which the negatively charged residues pair up with positively charged residues on the proximal surface of the CYP17 molecule. Non-specific interactions of the hydrophobic membrane regions of cytochrome b$_5$ and CYP17 are also thought to play a crucial role in the association of these two haemoproteins. Although cytochrome b$_5$ is known to stimulate CYP activity by contributing the second electron in the catalytic cycle, in the case of CYP17, the mechanism of cleavage stimulation proceeds via an allosteric mode. It is hypothesised that cytochrome b$_5$ promotes the cleavage by aligning the iron–oxygen complex attack onto the C$_{20}$ rather than the C$_{17}$ atom of the steroid substrate molecule. Thus, further understanding of the mechanism of modulation by cytochrome b$_5$ of the hydroxylase and lyase activities should shed new insights on developing therapeutic targets in CYP17-linked biochemical processes such as adrenarche, polycystic ovary syndrome and prostate cancer.

Introduction

In humans, the endoplasmic reticular cytochrome P450, 17α hydroxylase, 17–20 lyase (CYP17), plays a key role in the biosynthesis of steroid hormones (Lieberman & Warne 2001). This 56 kDa steroidogenic CYP is located in the zona fasciculata and zona reticularis of the adrenal cortex (and the gonad tissues), where it catalyses the pivotal step in the formation of glucocorticoids in the former and androgens in the latter (Fig. 1); zona glomerulosa lacks CYP17 and consequently produces mineralocorticoids. CYP17 dysfunction has been associated with a number of diseases including polycystic ovary syndrome (Qin & Rosenfield 1998, Miller 2002, Strauss 2003), Cushing’s syndrome (Ogo et al. 1991), congenital adrenal hyperplasia (Maitra & Shirwalkar 2003) and prostate cancer (Lunn et al. 1999, Madigan et al. 2003). The key towards alleviation of these endocrine-related human disorders lies in deciphering the mode of functioning of this important enzyme. The purpose of this review is to highlight recent advances in our understanding of the modulation of CYP17 activity by cytochrome b$_5$.

Nakajin et al. (1981a, 1981b) first isolated CYP17 from neonatal pig testis and reported it to have two distinct enzymic activities (Fig. 1). The first activity is to catalyse hydroxylation of pregnenolone and progesterone at the C$_{17}$ position to generate 17α-hydroxyprogrenenolone and 17α-hydroxyprogesterone. The second enzymic activity follows in cleavage of the C$_{17}$–C$_{20}$ bond of either 17α-hydroxyprogrenenolone or 17α-hydroxyprogesterone to form dehydroepiandrosterone (DHEA) and androstenedione respectively. In comparison with the enzyme from rat and trout, the human form of CYP17 has a significantly lower affinity for 17α-hydroxyprogesterone, and hence the metabolic route of testosterone formation in humans favours pregnenolone as the starting precursor rather than progesterone (Brock & Waterman 1999).

Both the hydroxylation and cleavage activities are catalysed sequentially at the common active site of CYP17 and proceed through transfer of two electrons from NADPH via its redox partner, cytochrome P450 reductase (CPR). The reaction mechanism for each activity is thought to involve formation of distinct iron–oxygen complexes. For the hydroxylation mechanism, the oxo-intermediate, Fe$^>$O, is considered to be the active catalytic oxygen-bound CYP complex (Atkinson & Ingold 1993), while for the acyl-carbon bond cleavage, participation of the iron-peroxo, Fe$^{III}$OOH, and iron-oxo,
species have both been suggested as possible candidates (Akhtar et al. 1994, Lee-Robichaud et al. 1995). Kinetic studies of the aromatase and the 14\(\beta\)-afili9825-demethylase reactions, which also involve similar cleavage of the acyl-carbon bonds, strongly favour the involvement of the latter complex (Akhtar et al. 1993, Shyadehi et al. 1996).

Role of CYP17 in human adrenal cortex physiology

Morphologically, the adrenal glands consist of an inner medulla and an outer cortex, the latter histologically subdivided into three zones of cells (Feige et al. 1998) known as the zona glomerulosa, zona fasciculata and zona reticularis (Fig. 1). Immunologically, CYP17 is localised in the latter two zones, where it hydroxylates both pregnenolone and progesterone to form DHEA and androsterone respectively. Although CYP17 is located in both the zona fasciculata and zona reticularis, where it is capable of undertaking hydroxylation of progesterone and pregnenolone, the cleavage activity of CYP17 is solely restricted to the zona reticularis. From immunohistochemical and reconstitution studies, it is now a currently accepted view that cytochrome \(b_5\) is somehow able to modulate the lyase activity of CYP17.
CYP17 cleavage activity. This activity gradually reaches peak levels between the ages of 25 and 35 and then steadily declines in later years of life. Although there is a rhythmic feedback control in the metabolic activity of the suprarenal glands via hypothalamic secretion of adrenocorticotropic hormone, there is no overall significant change in hormonal levels of cortisol in the lifespan of an individual. This implies that while CYP17 hydroxylase activity remains unaltered the lyase activity is somehow differentially regulated. The question then arises: how are the two distinct activities of CYP17 regulated in adrenarche?

Cytochrome b₅ modulates CYP17 activity

Membrane-bound cytochrome b₅ is a highly conserved electron-transfer protein found in the endoplasmic reticulum and the mitochondrion. It consists of two domains: a cytoplasmic, globular, haem-binding core domain (∼100 residues) and a carboxy-terminal membrane anchor (∼35 residues), and belongs to a class of widespread, integral proteins with a monotopic membrane topology (Nₒ–Cᵢ) (Kaderbhai et al. 2003). The endoplasmic reticular isoform is a multifunctional protein participating in a variety of electron-transfer reactions including desaturation of fatty acids (Oshino et al. 1971) and cholesterol biosynthesis (Fukushima et al. 1981).

It has also been known for some time that the endoplasmic reticular cytochrome b₅ augments the activities of numerous CYPs (Lamb et al. 2001, Yamazaki et al. 2002, Yamaori et al. 2003). A strong positive correlation between DHEA production and the significant co-localisation of cytochrome b₅ and CYP17 in the endoplasmic reticulum of the gonads and the zona reticularis of the adrenal cortex initially implicated the involvement of cytochrome b₅ as a potential modulator of the cleavage activity of CYP17 (Lu et al. 1974, 1975). Selective stimulation of CYP17 cleavage activity was first demonstrated in 1982 by Katagiri et al. (1982) who found that the extent of side-chain cleavage was dependent on the concentration of cytochrome b₅. The hydroxylase and lyase activities of isolated adrenal and testicular microsomes were suppressed in the presence of cytochrome b₅ antibodies (Ishii-Ohba et al. 1984, Kominami et al. 1992). Further evidence for cytochrome b₅ involvement arrived from histological studies of adenomal tissues retrieved from Cushing’s syndrome patients who had impaired production of androgens. These studies revealed that although the hydroxylase activity was similar to that of healthy adrenal glands, the lyase activity was significantly diminished along with reduced cytochrome b₅ content and CPR activity (Sakai et al. 1994). In contrast, gonadal and adrenal tissues overproducing androgen showed prominent distributions of staining of cytochrome b₅ that correlated with that of CYP17 (Yanase et al. 1998, Mapes et al. 1999). The involvement of cytochrome b₅ in selective stimulation of the cleavage activity of CYP17 was eventually substantiated in a number of reconstituted assays where the presence of cytochrome b₅ stimulated the lyase activity by up to 10-fold, with insignificant stimulation of the hydroxylase activity (Katagiri et al. 1995).

Allosteric role of cytochrome b₅ in CYP17 catalysis

Two general mechanisms have been proposed to explain the enhanced action of cytochrome b₅ in CYP catalysis. Hildebrandt and Estabrook (Estabrook 1999) first suggested that during the reductive stages of CYP, the second electron for the completion of the catalytic cycle can be derived from cytochrome b₅, an alternative redox partner to the conventional CPR. The faster rate of the second electron transfer from cytochrome b₅ is thought to reduce the likelihood of the spontaneous decay of oxyhaemoprotein complex during the P450 oxidoreduction cycle, an event referred to as uncoupling. A second model suggested that cytochrome b₅ could serve as an allosteric modulator that could promote optimal interaction between CPR and CYP to enhance electron flow within the system, or promote facile breakdown of the CYP-substrate intermediate in the catalytic cycle (Schenkman & Jansson 2003).

These likely catalytic models in the augmentation of CYP17 activity were further investigated by Auchus and Miller (Auchus et al. 1998) in a reconstituted assay system employing variable molar ratios of cytochrome b₅ to CYP while maintaining CPR at a fixed concentration. Maximum stimulation of lyase activity was observed when the cytochrome b₅:CYP17 ratio ranged from 10:1 to 30:1. However, the stimulated lyase activity was both rapidly and substantially decreased in the presence of excess cytochrome b₅, suggesting that electron scavenging by cytochrome b₅ from CPR at the highest ratios may have reduced the potential of the first electron derived from CPR to reduce CYP17. This suggestion was substantiated using an alternative CPR electron acceptor, cytochrome c, which at an equimolar amount to cytochrome b₅, gave a similar pattern of inhibition of the lyase activity. The potential redox role of cytochrome b₅ was further investigated using a recombinant cytochrome b₅ that was devoid of haem. Surprisingly, the apo-cytochrome b₅ isoform expressed a similar stimulatory profile of CYP17 lyase activity to that of the holo-cytochrome b₅. Exceptionally, the cleavage activity was not inhibited when the apo-cytochrome b₅:CYP17 ratio exceeded the optimum expressed by the holo-cytochrome b₅ (Auchus et al. 1998). Similar observations were reported for a Mn²⁺-substituted cytochrome b₅ (Lee-Robichaud et al. 1998), implying that in these series of studies the modified cytochrome b₅ did not play a role in electron donation but rather functioned allosterically in modulating CYP17.
activity. Similar allosteric roles of cytochrome b5 have also been reported in the catalysis of a number of CYP isoforms including CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP3A4 and CYP3A5 (Yamazaki et al. 2002).

The membrane-anchoring tail of cytochrome b5 is crucial for its catalytically productive interaction with CYP17

NMR studies suggest that while the membrane-anchoring regions of the apo- and holo-forms of cytochrome b5 are structurally indistinguishable, the globular haem-binding domain of the apo-form is significantly more structurally disordered relative to the holo-form (Falzone et al. 1996). Lee-Robichaud et al. (1997) investigated the importance of the membrane-anchoring domain in the modulation of CYP17 lyase activity. These workers utilised the modified or engineered forms of cytochrome b5: the native rat cytochrome b5 (core-tail, residues 1–134), the globular, soluble domain (core, residues 1–99), the native cytochrome b5 appended to an alkaline phosphatase signal sequence at the N-terminus (signal-core-tail, residues 21–134) and the globular core appended to the alkaline phosphatase signal sequence (signal-core, residues 21–99) (Fig. 2). Apart from the core form, engineered cytochrome b5 was constructed such that each form when anchored in the membrane would impose a distinct orientation of the globular form for its interaction with CYP17. While the signal-core-tail variant showed 55% of the activity relative to the native cytochrome b5, the signal-core and core derivatives were inactive in promoting CYP lyase activity. The authors concluded that the decreased catalytic efficiency imposed by the tail-modified cytochrome b5 forms was attributed to their altered interaction with CYP17.

In the light of these findings, a topology-based membrane-interactive model of the haemoprotein with CYP17 was proposed (Fig. 2). Maximum stimulation of the lyase activity by the native rat cytochrome b5 was postulated to be due to optimal interaction and/or binding with CYP17 whereby the C-terminal tail laterally orients the globular domain to a favourable ‘equatorial’ positioning with the cognate binding site in CYP17 (Fig. 2A). The signal-core-variant, on the other hand, was potentially able to adopt three topological states, only one of which could provide productive interactions with CYP17 (Fig. 2C and D) whereas the core and the signal-core variants were inappropriately orientated with respect to CYP17 to allow any activity (Fig. 2B). The inability of the soluble haem core to stimulate the lyase activity is in agreement with other reconstituted CYP–CPR–cytochrome b5 studies, indicating that the membrane-anchoring region provides not only correct membrane-integrated topology of cytochrome b5 for maximal interaction with CYP17 but also facilitates protein–protein association through charge-based pairings (Lamb et al. 2001, Clarke et al. 2004). Moreover, Mulrooney et al. (2004) have recently provided evidence that the hydrophobic interactions of the membrane-anchoring regions occur via non-specific interactions. Importance of the membrane-anchoring domain for CYP catalysis is somewhat analogous to the requirement of the CPR N-terminal membrane-anchoring domain for enhanced activities of numerous microsomal CYPs and suggests crucial roles of the membrane-anchoring segments in promoting topologically relevant allosteric modulations (Black & Coon 1982).

Nature of interaction between cytochrome b5 and CYP17

Generally, clinically diagnosed mutations of human CYP17 exhibit an almost equal impairment of both the hydroxylase and cleavage activity (Yanase 1995). However, male patients with isolated lyase deficiency were identified with homozygous mutations for Arg347→His and Arg358→Gln, thus providing a focal point in the understanding of the lyase selectivity of CYP17 (Geller et al. 1997). Expression of these mutants in COS-1 cells, in the presence of cytochrome b5, showed approximately 65% of hydroxylase and 5% lyase activity (for pregnenolone metabolism) relative to cells transfected with...
the wild-type enzyme. In a separate study, another group of workers showed that substitution of arginine at these positions for lysine did not affect the hydroxylase or lyase activity suggesting that these surface positive charges in CYP17 are vital for lyase stimulation (Lee-Robichaud et al. 2004). Furthermore, since the substrate affinities at the active sites of the CYP17 mutants Arg$^{347}$→His and Arg$^{358}$→Gln were found to be unaffected raised crucial questions as to how the mutations were able to selectively impair lyase activity of CYP17 (Geller et al. 1999).

Redox partners such as CPR and cytochrome b$_5$, which contain clusters of surface negative charges, are generally believed to form ionic interactions with the positive surface residues of CYPs. This has been experimentally demonstrated for numerous CYP isoforms (Yamazaki et al. 2002), but particularly so in a study by Bridges et al. (1998), who elucidated the redox partner binding sites of CYP2B4. Of the 24 mutant constructs used in their study, only mutations of those residues located on the proximal surface of CYP2B4 promoted decreased binding to cytochrome b$_5$ as well as CPR. Therefore, it was concluded that the redox partner binding region for P450s was located on the proximal surface which partially overlapped the interactive zones for cytochrome b$_5$ and CPR. In light of these findings, the cationic residues Arg$^{347}$ and Arg$^{358}$ of CYP17 are thought to be positioned similarly on the proximal surface of the molecule where they electrostatically dock with CPR and/or cytochrome b$_5$ (Auchus & Miller 1999). Sequence alignment of human CYP17 with various P450s, including CYP2B4, reported to be stimulated by apo- and the holo-forms of cytochrome b$_5$, identifies eight conserved positively-charged residues Lys$^{26}$, Lys$^{83}$, Lys$^{253}$, Lys$^{327}$, Arg$^{362}$, Arg$^{388}$, Arg$^{440}$ and Arg$^{347}$. Of these only Arg$^{347}$, Arg$^{350}$ and Arg$^{449}$ have been so far demonstrated to selectively ablate lyase activity. Thus, the role of the remaining homologous conserved residues in interactive modulation with cytochrome b$_5$ remains to be established.

**Mechanism of stimulation of CYP17 cleavage activity**

Allosteric effect or allosterity is a phenomenon in which an allosteric effector (usually other than the substrate) interacting at one site of a protein induces conformational change in the protein molecule such that activity at the distant (active) site is altered. The effect of a ligand binding at a distant site from the active site on a protein/enzyme typically alters the kinetic and sometimes other biochemical properties of the protein. In the case of CYPs, this phenomenon can be brought about by a variety of factors such as lipid binding, pH, salt concentration etc., but is commonly observed in CYPs upon binding to specific substrates (Hlavica & Lewis 2001). Indeed, the redox partners such as cytochrome b$_5$ and CPR can also affect allostery by binding at the redox partner binding site of the CYP molecule and inducing changes in the CYP conformation. Clearly, the subtle structural alterations of the active site of CYP3A4 isofrom during attack of phenyl-diazene, through the availability of nitrogen atoms of the haem cofactor, was evident with the modulation of the activity in the presence of both cytochrome b$_5$ and CPR (Yamaguchi et al. 2004).

In the case of CYP17, allosteric modulation by cytochrome b$_5$ promotes the cleavage activity (Fig. 3). Precisely how this is achieved remains enigmatic. Nevertheless, there appears to be no significant conformational change brought about at the active site of CYP17 as evidenced by the unchanged $K_{m}$ and substrate-binding.

![Figure 3](Image 352x414 to 465x453)
affinities of the active site for the lyase activity. This does not, however, negate the possibility of subtle structural changes to the active site/interior of CYP17 upon cytochrome \( b_5 \) docking, whereby the substrate molecule is somehow placed in a position favouring \( C_{17}-C_{20} \) cleavage while maintaining its affinity for the substrate as initially suggested by Lee-Robichaud et al. (1998). It is this view that we favour for the explanation of the stimulation of CYP17 cleavage in the presence of cytochrome \( b_5 \). Molecular stimulation studies of CYP17 show that substrate binding occurs with the steroid molecule positioned in a proximity parallel to the haem ring (Auchus & Miller 1999). In the CYP17–CPR complex, closer proximity of the \( C_{17} \) atom to the attacking iron–oxygen intermediate would favour attack on this carbon atom, although hydroxylation at the \( C_{16} \) position, which has been reported in some cases, is also possible (Swart et al. 1993). An atom positioned too distant for oxygenation attack, as in the case of entiomeric forms of progesterone, regardless of a high-spin state, would serve to inhibit CYP17 activity (Auchus et al. 2003). This mode differs for the CYP17–CPR–cytochrome \( b_5 \) complex, where the conformation of CYP17 is altered by cytochrome \( b_5 \) so as to bring the iron–oxygen intermediate, through repositioning of either the haem moiety or substrate molecule, into alignment with the \( C_{20} \) position of the hydroxylated precursor. A subsequent nucleophilic attack is directed on the carbonyl group of the substrate to yield the cleaved steroid substrate and acetic acid (Lee–Robichaud et al. 1998). Thus, cytochrome \( b_5 \) could be essentially regarded as a switch which promotes certain conformational changes of CYP17 and turns lyase activity on and off. This notion of substrate realignment with the attacking iron–oxygen complex by cytochrome \( b_5 \) is supported by the recently discovered third 16–ene synthase activity of CYP17 (Soucy et al. 2003), which bypasses the hydroxylation step and directly cleaves pregnenolone to yield androstadienol (Fig. 1). This reaction proceeds at a rate 10-fold faster in the presence of cytochrome \( b_5 \) and occurs in Leydig cells, where it is considered to be an important precursor in the biosynthesis of androstenol. Although the significance of androstenol in humans is not yet fully understood, it has been speculated to modulate orphan receptors and/or act as pheromones, which are typically present in human sweat to attract the opposite sex.

**Additional factors that modulate CYP17 cleavage activity**

The cleavage activity of CYP17 has also been reported to be considerably influenced by its post-translational modification (Biaison-Lauber et al. 2000b). In COS-1 cells, cAMP-mediated phosphorylation of CYP17 was found to stimulate its lyase activity, whereas dephosphorylation with phosphatase totally abolished this activity (Zhang et al. 1995). The retention of the hydroxylase activity during (de)phosphorylation negated the likelihood of proteolysis, active site modification or phosphate removal from NADPH as the cause of the loss of lyase activity following dephosphorylation. In view of the electrostatic association of CYP17 and its redox partners as mentioned previously, it was proposed that the modified negatively charged phosphorylated residues served to enhance electrostatic attraction between the redox partners so as to engage a stronger interaction of CPR with CYP17. Recently, the enhancement of 17,20 lyase by serine phosphorylation and cytochrome \( b_5 \) has been shown to occur independently of each other (Pandey & Miller 2005).

Selective promotion of the \( C_{17}-C_{20} \) cleavage activity also has been demonstrated with adipocyte-derived fat-regulating peptide hormone, leptin (Biaison-Lauber et al. 2000a). This hormone is thought to promote the intracellular cAMP–dependent kinase-mediated phosphorylation of CYP17 via the extracellular leptin receptor OB-R, which in turn triggers the serine and threonine phosphorylation in the signal transduction pathway.

Interestingly, a CYP17 from *Xenopus laevis* displayed unusually high lyase activity in the absence of either frog- or human-derived cytochrome \( b_5 \) (Yang & Hammes 2005). Moreover, the rate of cleavage activity of *Xenopus laevis* CYP17 in the absence of cytochrome \( b_5 \) was found to be similar to human CYP17 assayed in the presence of human cytochrome \( b_5 \). These findings suggest that modulation of the CYP17 cleavage activity cannot simply be explained by its sole interaction with cytochrome \( b_5 \), and other factors, possibly those of the signal-transduction pathways remain to be elucidated.

**Future prospects**

There have been relatively few studies on cytochrome \( b_5 \) mutagenesis regarding its influence on CYP catalysis (Chudaev et al. 2001, Clarke et al. 2004) and as yet there have been no reports on the effect of cytochrome \( b_5 \) mutagenesis on the steroidal activity of CYP17. Earlier work by Usanov’s group (Usanov & Chashchin 1991) showed that substitution of the residue Glu\(^{42}\) with Lys on cytochrome \( b_5 \) resulted in reduction of the cholesterol side-chain cleavage activity of CYP11 by almost 40%, suggesting that the anionic residue of cytochrome \( b_5 \) was important for electrostatic interaction with CYP11. The identity of such residues on the surface of cytochrome \( b_5 \) may similarly be vital for CYP17 cleavage. More recently, residues 16–41 in human cytochrome \( b_5 \) were found to be necessary in the specific regulation of the lyase activity of human CYP17, possibly serving as an interacting domain with the enzyme (Yang & Hammes 2005). Indeed, further detailed identification of the key residues for CYP17 and cytochrome \( b_5 \) interaction will give insight into the nature of interaction between cytochrome \( b_5 \) and CYP17 and...
shed light on how cytochrome b₅ is able to promote a subtle conformational change of CYP17.

It is important to note that the modulation by cytochrome b₅ is strictly dependent on the formation of a CPR–CYP17 complex, since cytochrome b₅, on its own, is unable to exert any effect on the CYP17 lyase activity. Thus, CPR is likely to have considerable influence on the overall interaction between cytochrome b₅ and CYP17. For instance, in CYP2D6 it was concluded that CPR allowed closer positioning of the phenyl ring of neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, to the haem iron of CYP (Modi et al. 1997). Further studies with kinetic/binding studies incorporating wild-type and mutant forms of CYP17, CPR and cytochrome b₅ will undoubtedly provide more meaningful data in deciphering the mode(s) of interactions involving CYP17.

Elucidating the catalytic switching of CYP17 by cytochrome b₅ will open the way to its exploitation as a therapeutic target in the development of more effective treatments of a number of CYP17-associated hormonal disorders such as polycystic ovary syndrome, congenital adrenal hyperplasia and prostate cancer.

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