Cytochrome b₅ modulation of 17 α 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 C₁₇ position to generate 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone, while the second enzymic activity cleaves the C₁₇–C₂₀ bond of 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone to form dehydroepiandrosterone and androstenedione respectively. The modulation of these two activities occurs through cytochrome b₅. Association of cytochrome b₅ 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 hydro-

phobic membrane regions of cytochrome b₅ and CYP17 are also thought to play a crucial role in the association of these two haemoproteins. Although cytochrome b₅ 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 b₅ promotes the cleavage by aligning the iron–oxygen complex attack onto the C₂₀ rather than the C₁₇ atom of the steroid substrate molecule. Thus, further understanding of the mechanism of modulation by cytochrome b₅ of the hydroxylase and lyase activities should shed new insights on developing therapeutic targets in CYP17-linked biochemical processes such as adrenarache, polycystic ovary syndrome and prostate cancer.

Journal of Endocrinology (2005) **187**, 267–274

Introduction

In humans, the endoplasmic reticular cytochrome P450, 17 α 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 b₅.

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 C₁₇ position to generate 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone. The second enzymic activity follows in cleavage of the C₁₇–C₂₀ bond of either 17 α -hydroxypregnenolone or 17 α -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 17 α -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^v=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,

Figure 1 Distinct steroidogenic roles of CYP17 in the human adrenal cortex. With cholesterol as the precursor, cholesterol desmolase catalyses the formation of pregnenolone, which is further reduced by 3β-hydroxysteroid dehydrogenase to yield progesterone. Progesterone and pregnenolone form vital intermediates in the synthesis of mineralocorticoids in the zona glomerulosa, corticoids in the zona fasciculata and androgens in the zona reticularis. Formation of the latter two groups of hormones occurs at a pivotal step catalysed by CYP17. This activity involves cleavage of the C₁₇-C₂₀ bond of 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.

Fe^v=O, species have both been suggested as possible candidates (Akhtar *et al.* 1994, Lee-Robichaud *et al.* 1995). Kinetic studies of the aromatase and the 14α-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. A further round of oxidative conversion of the hydroxylated form by CYP17 to form DHEA occurs in the zona reticularis but is absent from the zona fasciculata. Moreover, the dual activities of CYP17 in the zona reticularis are temporally expressed during the lifespan of an individual in a phenomenon known as adrenarche (Auchus & Rainey 2004).

Adrenarche occurs in males and females before the onset of puberty, typically around 8 years of age, when the adrenal gland begins to increase production of androgens. From childhood to early adulthood, there is a rise in androgen levels which correlates with an increase in

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 adrenocorticotrophic 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 b_5 modulates CYP17 activity

Membrane-bound cytochrome b_5 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 ($N_{out}-C_{in}$) (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 b_5 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 b_5 and CYP17 in the endoplasmic reticulum of the gonads and the zona reticularis of the adrenal cortex initially implicated the involvement of cytochrome b_5 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 b_5 . The hydroxylase and lyase activities of isolated adrenal and testicular microsomes were suppressed in the presence of cytochrome b_5 antibodies (Ishii-Ohba *et al.* 1984, Kominami *et al.* 1992). Further evidence for cytochrome b_5 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 b_5 content and CPR activity (Sakai *et al.* 1994). In contrast, gonadal and adenomal tissues overproducing androgen showed prominent distributions of staining of cytochrome b_5 that correlated with that of CYP17 (Yanase *et al.* 1998, Mapes *et al.* 1999). The involvement of cytochrome b_5 in selective stimulation of

the cleavage activity of CYP17 was eventually substantiated in a number of reconstituted assays where the presence of cytochrome b_5 stimulated the lyase activity by up to 10-fold, with insignificant stimulation of the hydroxylase activity (Katagiri *et al.* 1995).

Allosteric role of cytochrome b_5 in CYP17 catalysis

Two general mechanisms have been proposed to explain the enhanced action of cytochrome b_5 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 b_5 , an alternative redox partner to the conventional CPR. The faster rate of the second electron transfer from cytochrome b_5 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 b_5 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 b_5 to CYP while maintaining CPR at a fixed concentration. Maximum stimulation of lyase activity was observed when the cytochrome b_5 :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 b_5 , suggesting that electron scavenging by cytochrome b_5 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 b_5 , gave a similar pattern of inhibition of the lyase activity. The potential redox role of cytochrome b_5 was further investigated using a recombinant cytochrome b_5 that was devoid of haem. Surprisingly, the apo-cytochrome b_5 isoform expressed a similar stimulatory profile of CYP17 lyase activity to that of the holo-cytochrome b_5 . Exceptionally, the cleavage activity was not inhibited when the apo-cytochrome b_5 :CYP17 ratio exceeded the optimum expressed by the holo-cytochrome b_5 (Auchus *et al.* 1998). Similar observations were reported for a Mn^{2+} -substituted cytochrome b_5 (Lee-Robichaud *et al.* 1998), implying that in these series of studies the modified cytochrome b_5 did not play a role in electron donation but rather functioned allosterically in modulating CYP17

activity. Similar allosteric roles of cytochrome *b*₅ 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 *b*₅ 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 *b*₅ 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 *b*₅: the native rat cytochrome *b*₅ (core-tail, residues 1–134), the globular, soluble domain (core, residues 1–99), the native cytochrome *b*₅ 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 *b*₅ 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 *b*₅, 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 *b*₅ 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 *b*₅ 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 *b*₅ studies, indicating that the membrane-anchoring region provides not only correct membrane-integrated topology of cytochrome *b*₅ for maximal interaction with CYP17 but also facilitates protein–protein association through charge-based pairings (Lamb *et al.* 2001, Clarke *et al.* 2004). Moreover,

Figure 2 Models showing the likely topologically relevant interaction of membrane-bound variants of cytochrome *b*₅ with CYP17 (A); wild-type cytochrome *b*₅ anchored to the membrane via its C-terminal domain shows optimal interaction with CYP17. (B) Cytochrome *b*₅ devoid of its C-terminal membrane anchor and N-terminally appended with the alkaline phosphatase signal sequence annuls catalytically positive interaction with CYP17. With two membrane-anchoring regions at opposing termini (C, D), cytochrome *b*₅ can adopt one of three membrane topological states. The two (C) states are identical to (A) and (B), while (D) forms a weak interaction with CYP17. For the sake of clarity, the authors omitted CPR in the proposed model. Adapted from Lee-Robichaud *et al.* (1997).

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 *b*₅ and CYP17

Generally, clinically diagnosed mutations of human CYP17 exhibit an almost equal impairment of both the hydroxylation 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 *b*₅, showed approximately 65% of hydroxylase and 5% lyase activity 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 Arg³⁴⁷→His and Arg³⁵⁸→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 Arg³⁴⁷ and Arg³⁵⁸ 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 Lys²⁶, Lys⁸³, Lys²⁵³, Lys³²⁷, Arg³⁶², Arg³⁸⁸, Arg⁴⁴⁰ and Arg³⁴⁷. Of these only Arg³⁴⁷, Arg³⁵⁰ and Arg⁴⁴⁹ 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 allostery 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

Figure 3 A model proposing cytochrome b_5 -mediated modulation of CYP17 oxidative activity. The redox proteins, CPR and cytochrome b_5 , pair up with CYP17 via electrostatic charges. Redox partner interaction with CYP17 may be aided by non-specific interaction between the membrane-anchoring regions. Two electrons are sequentially transferred from NADPH to the FAD and FMN domains of CPR. The formation of CPR–CYP17 complex suffices a molecular alignment for oxidative attack of CYP17 to hydroxylate at the C₁₇ position but not at C₂₀. In a second round of catalytic cycle, the hydroxylated substrate may undergo the lyase activity. For the generation of the lyase activity, it is envisaged that CYB (holo- or apo-forms of cytochrome b_5) interacts with the CYP17–CPR complex to induce a conformational change at the active site of CYP17 such that the oxidative attack by the iron–oxygen intermediate of CYP17 is now redirected to C₂₀, culminating in dissociation of the C₁₇–C₂₀ bond; it is suggested that the attacking iron–oxygen intermediate (*) may be Fe–O–O.

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 isoform 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

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 C₁₇–C₂₀ 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 C₁₇ atom to the attacking iron–oxygen intermediate would favour attack on this carbon atom, although hydroxylation at the C₁₆ 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 enantiomeric 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 C₂₀ 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 C₁₇–C₂₀ 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 Glu⁴² 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 b_5 is able to promote a subtle conformational change of CYP17.

It is important to note that the modulation by cytochrome b_5 is strictly dependent on the formation of a CPR–CYP17 complex, since cytochrome b_5 , 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 b_5 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 b_5 will undoubtedly provide more meaningful data in deciphering the mode(s) of interactions involving CYP17.

Elucidating the catalytic switching of CYP17 by cytochrome b_5 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.

Acknowledgements

This work was supported in part by a BBSRC grant. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Akhtar M, Njar VC & Wright JN 1993 Mechanistic studies on aromatase and related C–C bond cleaving P-450 enzymes. *Journal of Steroid Biochemistry and Molecular Biology* **44** 375–387.
- Akhtar M, Corina D, Miller S, Shyadehi AZ & Wright JN 1994 Mechanism of the acyl-carbon cleavage and related reactions catalyzed by multifunctional P-450s: studies on cytochrome P-450(17) α . *Biochemistry* **33** 4410–4418.
- Atkinson JK & Ingold KU 1993 Cytochrome P450 hydroxylation of hydrocarbons: variation in the rate of oxygen rebound using cyclopropyl radical clocks including two new ultrafast probes. *Biochemistry* **32** 9209–9214.
- Auchus RJ & Miller WL 1999 Molecular modeling of human P450c17 (17 α -hydroxylase/17,20-lyase): insights into reaction mechanisms and effects of mutations. *Molecular Endocrinology* **13** 1169–1182.
- Auchus RJ & Rainey WE 2004 Adrenarche – physiology, biochemistry and human disease. *Clinical Endocrinology* **60** 288–296.
- Auchus RJ, Lee TC & Miller WL 1998 Cytochrome b_5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *Journal of Biological Chemistry* **273** 3158–3165.
- Auchus RJ, Kumar AS, Boswell CA, Gupta MK, Bruce K, Rath NP & Covey DF 2003 The enantiomer of progesterone (ent-progesterone) is a competitive inhibitor of human cytochromes P450c17 and P450c21. *Archives of Biochemistry and Biophysics* **409** 134–144.
- Biason-Lauber A, Zachmann M & Schoenle EJ 2000a Effect of leptin on CYP17 enzymatic activities in human adrenal cells: new insight in the onset of adrenarche. *Endocrinology* **141** 1446–1454.
- Biason-Lauber A, Kempken B, Werder E, Forest MG, Einaudi S, Ranke MB, Matsuo N, Brunelli V, Schonle EJ & Zachmann M 2000b 17 α -hydroxylase/17,20-lyase deficiency as a model to study enzymatic activity regulation: role of phosphorylation. *Journal of Clinical Endocrinology and Metabolism* **85** 1226–1231.
- Black SD & Coon MJ 1982 Structural features of liver microsomal NADPH-cytochrome P-450 reductase. Hydrophobic domain, hydrophilic domain, and connecting region. *Journal of Biological Chemistry* **257** 5929–5938.
- Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G & Waskell L 1998 Identification of the binding site on cytochrome P450 2B4 for cytochrome b_5 and cytochrome P450 reductase. *Journal of Biological Chemistry* **273** 17036–17049.
- Brock BJ & Waterman MR 1999 Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species. *Biochemistry* **38** 1598–1606.
- Chudaev MV, Gilpe AA & Usanov SA 2001 Site-directed mutagenesis of cytochrome b_5 for studies of its interaction with cytochrome P450. *Biochemistry (Moscow)* **66** 667–681.
- Clarke TA, Im SC, Bidwai A & Waskell L 2004 The role of the length and sequence of the linker domain of cytochrome b_5 in stimulating cytochrome P450 2B4 catalysis. *Journal of Biological Chemistry* **279** 36809–36818.
- Estabrook RW 1999 Discovering the functions of cytochrome P450 in drug metabolism: the role of Alfred Hildebrandt. *Drug Metabolism Reviews* **31** 317–331.
- Falzone CJ, Mayer MR, Whiteman EL, Moore CD & Lecomte JT 1996 Design challenges for hemoproteins: the solution structure of apocytochrome b_5 . *Biochemistry* **35** 6519–6526.
- Feige JJ, Vilgrain I, Brand C, Bailly S & Souchelitsky S 1998 Fine tuning of adrenocortical functions by locally produced growth factors. *Journal of Endocrinology* **158** 7–19.
- Fukushima H, Grinstead GF & Gaylor JL 1981 Total enzymic synthesis of cholesterol from lanosterol. Cytochrome b_5 -dependence of 4-methyl sterol oxidase. *Journal of Biological Chemistry* **256** 4822–4826.
- Geller DH, Auchus RJ, Mendonca BB & Miller WL 1997 The genetic and functional basis of isolated 17,20-lyase deficiency. *Nature Genetics* **17** 201–205.
- Geller DH, Auchus RJ & Miller WL 1999 P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b_5 . *Molecular Endocrinology* **13** 167–175.
- Hlavica P & Lewis DF 2001 Allosteric phenomena in cytochrome P450-catalyzed monooxygenations. *European Journal of Biochemistry* **268** 4817–4832.
- Ishii-Ohba H, Matsumura R, Inano H & Tamaoki B 1984 Contribution of cytochrome b_5 to androgen synthesis in rat testicular microsomes. *Journal of Biochemistry* **95** 335–343.
- Kaderbhai MA, Morgan R & Kaderbhai NN 2003 The membrane-interactive tail of cytochrome b_5 can function as a stop-transfer sequence in concert with a signal sequence to give inversion of protein topology in the endoplasmic reticulum. *Archives of Biochemistry and Biophysics* **412** 259–266.
- Katagiri M, Suhara K, Shiroo M & Fujimura Y 1982 Role of cytochrome b_5 in the cytochrome P-450-mediated C21-steroid 17,20-lyase reaction. *Biochemical and Biophysical Research Communications* **108** 379–384.
- Katagiri M, Kagawa N & Waterman MR 1995 The role of cytochrome b_5 in the biosynthesis of androgens by human P450c17. *Archives of Biochemistry and Biophysics* **317** 343–347.
- Kominami S, Ogawa N, Morimune R, De-Ying H & Takemori S 1992 The role of cytochrome b_5 in adrenal microsomal steroidogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **42** 57–64.
- Lamb DC, Kaderbhai NN, Venkateswarlu K, Kelly DE, Kelly SL & Kaderbhai MA 2001 Human sterol 14 α -demethylase activity is enhanced by the membrane-bound state of cytochrome b_5 . *Archives of Biochemistry and Biophysics* **395** 78–84.

- Lee-Robichaud P, Shyadehi AZ, Wright JN, Akhtar ME & Akhtar M 1995 Mechanistic kinship between hydroxylation and desaturation reactions: acyl-carbon bond cleavage promoted by pig and human CYP17 (P-450(17) α ; 17 α -hydroxylase-17,20-lyase). *Biochemistry* **34** 14104–14113.
- Lee-Robichaud P, Kaderbhai MA, Kaderbhai N, Wright JN & Akhtar M 1997 Interaction of human CYP17 (P-450(17) α), 17 α -hydroxylase-17,20-lyase with cytochrome b₅: importance of the orientation of the hydrophobic domain of cytochrome b₅. *Biochemical Journal* **321** 857–863.
- Lee-Robichaud P, Akhtar ME & Akhtar M 1998 Control of androgen biosynthesis in the human through the interaction of Arg³⁴⁷ and Arg³⁵⁸ of CYP17 with cytochrome b₅. *Biochemical Journal* **332** 293–296.
- Lee-Robichaud P, Akhtar ME, Wright JN, Sheikh QI & Akhtar M 2004 The cationic charges on Arg347, Arg358 and Arg449 of human cytochrome P450c17 (CYP17) are essential for the enzyme's cytochrome b₅-dependent acyl-carbon cleavage activities. *Journal of Steroid Biochemistry and Molecular Biology* **92** 119–130.
- Lieberman S & Warne PA 2001 17-Hydroxylase: an evaluation of the present view of its catalytic role in steroidogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **78** 299–312.
- Lu AY, West SB, Vore M, Ryan D & Levin W 1974 Role of cytochrome b₅ in hydroxylation by a reconstituted cytochrome P-450-containing system. *Journal of Biological Chemistry* **249** 6701–6709.
- Lu AY, Levin W, West SB, Vore M, Ryan D, Kuntzman R & Conney AH 1975 Role of cytochrome b₅ in NADPH- and NADH-dependent hydroxylation by the reconstituted cytochrome P-450- or P-448-containing system. *Advances in Experimental Medicine and Biology* **58** 447–466.
- Lunn RM, Bell DA, Mohler JL & Taylor JA 1999 Prostate cancer risk and polymorphism in 17 hydroxylase (CYP17) and steroid reductase (SRD5A2). *Carcinogenesis* **20** 1727–1731.
- Madigan MP, Gao YT, Deng J, Pfeiffer RM, Chang BL, Zheng S, Meyers DA, Stanczyk FZ, Xu J & Hsing AW 2003 CYP17 polymorphisms in relation to risks of prostate cancer and benign prostatic hyperplasia: a population-based study in China. *International Journal of Cancer* **107** 271–275.
- Maitra A & Shirwalkar H 2003 Congenital adrenal hyperplasia: biochemical and molecular perspectives. *Indian Journal of Experimental Biology* **41** 701–709.
- Mapes S, Corbin CJ, Tarantal A & Conley A 1999 The primate adrenal zona reticularis is defined by expression of cytochrome b₅, 17 α -hydroxylase/17,20-lyase cytochrome P450 (P450c17) and NADPH-cytochrome P450 reductase (reductase) but not 3 β -hydroxysteroid dehydrogenase/ Δ 5–4 isomerase (3 β -HSD). *Journal of Clinical Endocrinology and Metabolism* **84** 3382–3385.
- Miller WL 2002 Androgen biosynthesis from cholesterol to DHEA. *Molecular and Cellular Endocrinology* **198** 7–14.
- Modi S, Gilham DE, Sutcliffe MJ, Lian LY, Primrose WU, Wolf CR & Roberts GC 1997 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine as a substrate of cytochrome P450 2D6: allosteric effects of NADPH-cytochrome P450 reductase. *Biochemistry* **36** 4461–4470.
- Mulrooney SB, Meinhardt DR & Waskell L 2004 The α -helical membrane spanning domain of cytochrome b₅ interacts with cytochrome P450 via nonspecific interactions. *Biochimica et Biophysica Acta* **1674** 319–326.
- Nakajin S, Hall PF & Onoda M 1981a Testicular microsomal cytochrome P-450 for C21 steroid side chain cleavage. Spectral and binding studies. *Journal of Biological Chemistry* **256** 6134–6139.
- Nakajin S, Shively JE, Yuan PM & Hall PF 1981b Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17 α -hydroxylase and 17,20-lyase) associated with one protein. *Biochemistry* **20** 4037–4042.
- Ogo A, Haji M, Ohashi M & Nawata H 1991 Markedly increased expression of cytochrome P-450 17 α -hydroxylase (P-450c17) mRNA in adrenocortical adenomas from patients with Cushing's syndrome. *Molecular and Cellular Endocrinology* **80** 83–89.
- Oshino N, Imai Y & Sato R 1971 A function of cytochrome b₅ in fatty acid desaturation by rat liver microsomes. *Journal of Biochemistry* **69** 155–167.
- Pandey AV & Miller WL 2005 Regulation of 17,20 lyase activity by cytochrome b₅ and by serine phosphorylation of P450c17. *Journal of Biological Chemistry* **280** 13265–13271.
- Qin KN & Rosenfield RL 1998 Role of cytochrome P450c17 in polycystic ovary syndrome. *Molecular and Cellular Endocrinology* **145** 111–121.
- Sakai Y, Yanase T, Hara T, Takayanagi R, Haji M & Nawata H 1994 *In vitro* evidence for the regulation of 17,20-lyase activity by cytochrome b₅ in adrenocortical adenomas from patients with Cushing's syndrome. *Clinical Endocrinology* **40** 205–209.
- Schenkman JB & Jansson I 2003 The many roles of cytochrome b₅. *Pharmacology and Therapeutics* **97** 139–152.
- Shyadehi AZ, Lamb DC, Kelly SL, Kelly DE, Schunck WH, Wright JN, Corina D & Akhtar M 1996 The mechanism of the acyl-carbon bond cleavage reaction catalyzed by recombinant sterol 14 α -demethylase of *Candida albicans* (other names are: lanosterol 14 α -demethylase, P-45014DM, and CYP51). *Journal of Biological Chemistry* **271** 12445–12450.
- Soucy P, Lacoste L & Luu-The V 2003 Assessment of porcine and human 16-ene-synthase, a third activity of P450c17, in the formation of an androstene precursor. Role of recombinant cytochrome b₅ and P450 reductase. *European Journal of Biochemistry* **270** 1349–1355.
- Strauss JF 3rd 2003 Some new thoughts on the pathophysiology and genetics of polycystic ovary syndrome. *Annals of the New York Academy of Sciences* **997** 42–48.
- Swart P, Swart AC, Waterman MR, Estabrook RW & Mason JI 1993 Progesterone 16 α -hydroxylase activity is catalyzed by human cytochrome P450 17 α -hydroxylase. *Journal of Clinical Endocrinology and Metabolism* **77** 98–102.
- Usanov SA & Chashchin VL 1991 Interaction of cytochrome P-450 scc with cytochrome b₅. *FEBS Letters* **278** 279–282.
- Yamaguchi Y, Khan KK, He YA, He YQ & Halpert JR 2004 Topological changes in the CYP3A4 active site probed with phenyldiazene: effect of interaction with NADPH-cytochrome P450 reductase and cytochrome b₅ and of site-directed mutagenesis. *Drug Metabolism and Disposition* **32** 155–161.
- Yamaori S, Yamazaki H, Suzuki A, Yamada A, Tani H, Kamidate T, Fujita K & Kamataki T 2003 Effects of cytochrome b(5) on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7. *Biochemical Pharmacology* **66** 2333–2340.
- Yamazaki H, Nakamura M, Komatsu T, Ohya K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M *et al.* 2002 Roles of NADPH-P450 reductase and apo- and holo-cytochrome b₅ on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein Expression and Purification* **24** 329–337.
- Yanase T 1995 17 α -Hydroxylase/17,20-lyase defects. *Journal of Steroid Biochemistry and Molecular Biology* **53** 153–157.
- Yanase T, Sasano H, Yubisui T, Sakai Y, Takayanagi R & Nawata H 1998 Immunohistochemical study of cytochrome b₅ in human adrenal gland and in adrenocortical adenomas from patients with Cushing's syndrome. *Endocrinology* **45** 89–95.
- Yang WH & Hammes SR 2005 *Xenopus laevis* CYP17 regulates androgen biosynthesis independent of the cofactor cytochrome b₅. *Journal of Biological Chemistry* **280** 10196–10201.
- Zhang LH, Rodriguez H, Ohno S & Miller WL 1995 Serine phosphorylation of human P450c17 increases 17,20-lyase activity: implications for adrenarche and the polycystic ovary syndrome. *PNAS* **92** 10619–10623.

Received in final form 11 August 2005
Accepted 19 August 2005