Interaction of human CYP17 (P-450$_{17\alpha}$, 17α-hydroxylase-17,20-lyase) with cytochrome b_5: importance of the orientation of the hydrophobic domain of cytochrome b_5

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Human CYP17 (P-450$_{17\alpha}$, 17α-hydroxylase-17,20-lyase)-catalysed side-chain cleavage of 17α-hydroxyprogestogens into androgens is greatly dependent on the presence of cytochrome b_5. The native form of cytochrome b_5 is composed of a globular core, residues 1–98, followed by a membrane insertable C-terminal tail, residues 99–133. In the present study the abilities of five different forms of cytochrome b_5 to support the side-chain cleavage activity of CYP17 were compared. The five derivatives were: the native pig cytochrome b_5 (native pig), its genetically engineered rat counterpart (core–tail), the soluble core form of the latter (core), the core with the secretory signal sequence of alkaline phosphatase appended to its N-terminal (signal–core) and the latter containing the C-terminal tail of the native rat protein (signal–core–tail). When examined by Edman degradation and MS, the engineered proteins were shown to have the expected N-terminal amino acid sequences and molecular masses. The native pig was found to be acetylated at the N-terminal. The native pig and core–tail enzymes were equally efficient at enhancing the side-chain cleavage activity of human CYP17 and the signal–core–tail was 55% as efficient. The core and signal–core constructs were completely inactive in the aforementioned reaction. All the five derivatives were reduced to varying degrees by NADPH:cytochrome P-450 (NADPH-P450) reductase and the relative efficiencies of this reduction were reminiscent of the behaviour of these derivatives in supporting the side-chain cleavage reaction. In the side-chain cleavage assay, however, NADPH-P450 reductase was used in large excess so that the reduction of cytochrome b_5 derivatives was not rate-limiting. The results highlight that productive interaction between cytochrome b_5 and CYP17 is governed not only by the presence of a membrane insertable hydrophobic region on the cytochrome b_5 but also by its defined spatial orientation at the C-terminal.

INTRODUCTION

CYP17 (P-450$_{17\alpha}$, 17α-hydroxylase-17,20-lyase) is a multifunctional cytochrome P-450 (P450) that catalyses not only the conventional hydroxylation reaction but also a variety of carbon–carbon bond cleavages [1,1a,2]. These cleavages have been generalized as acyl-carbon bond fissions and conform to the stoichiometry of the reaction shown in Scheme 1 [3–5].

In the testis the essential function of CYP17 is to catalyse the conversion of progestogens into androgens using its two activities (**1** → **2** and **2** → **3**; Scheme 2) [1,1a], with its obligatory redox partner, NADPH-cytochrome P-450 (NADPH-P450) reductase, providing two single electrons from NADPH in each step. In the adrenal cortex, however, the major function of CYP17 is to catalyse the conversion of progestogens into 17α-hydroxylated steroids that are then utilized in glucocorticoid biosynthesis. This means that CYP17's side-chain cleavage activity (**2** → **3**; Scheme 2) needs to be attenuated in the adrenal in order to prevent uncontrolled formation of androgens by this tissue. We [6] and others [7] have shown that for human CYP17 to display significant side-chain cleavage activity (**2** → **3**; Scheme 2) cytochrome b_5 is required. Cytochrome b_5 functions as an electron transfer protein and readily accepts electrons from its native reductase, NADH-cytochrome b_5 reductase, and also NADPH-P450 reductase [8]. Its participation in electron transfer to P450s was first shown by Hilderbrandt and Estabrook [9] and later confirmed by others [10–13]. This haemoprotein has also been found to enhance the activities of the porcine and bovine CYP17 [14–16] as well as subtly altering the substrate specificity or modifying the catalytic efficiencies of other P-450s [10,17–22]. The observations on the human enzyme have raised the possibility that levels of cytochrome b_5 in the testis and adrenal cortex may play a central role in dictating the fate of 17α-hydroxyprogestogens (structure of type **2**; Scheme 2).

The significant stimulatory effect of the native cytochrome b_5 on the side-chain cleavage reaction (**2** → **3**; Scheme 2) catalysed by human CYP17 and the ease with which this process can be monitored using a recently developed radiochemical assay [6], have now paved the way to delineate the likely nature of interactions between the two haemoproteins. Using novel forms of cytochrome b_5, the work here has focused on highlighting the importance of the orientation of the hydrophobic tail of cytochrome b_5 in mediating its interaction with the human CYP17.

EXPERIMENTAL

Materials

17α-Hydroxy[21-^{3}H]pregnenolone (4.4×10^4 d.p.m./nmol) was prepared as described previously [23]. All other chemicals were purchased from Sigma Chemical Co. (U.K.). The plasmid, pCW17mod [24], containing the human CYP17 gene, was generously given by Professor M. R. Waterman, Vanderbilt University, Nashville, TN, U.S.A.

Abbreviations used: P450, cytochrome P-450; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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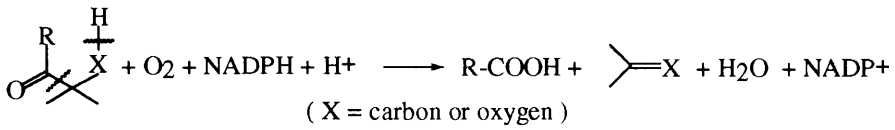
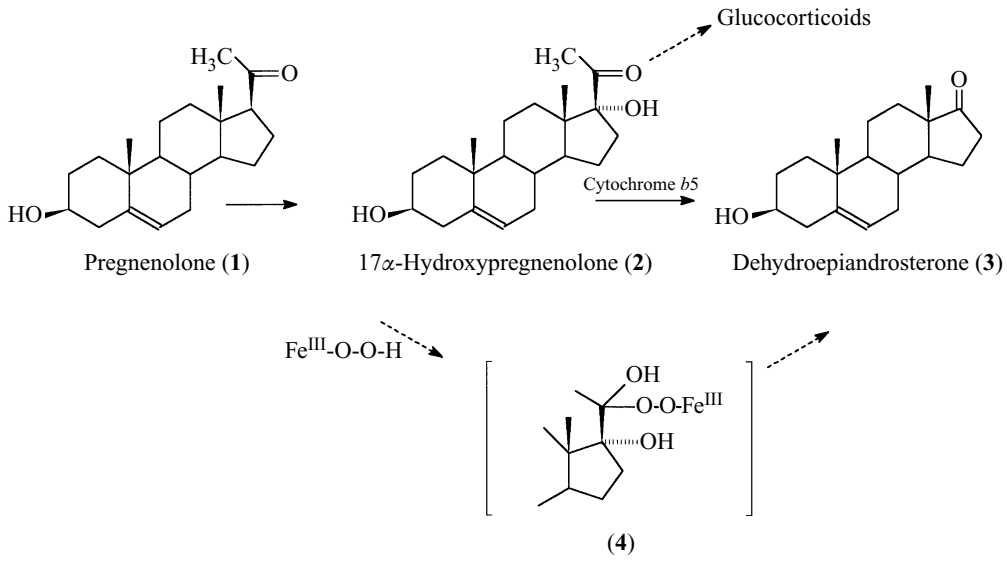


Scheme 1



Scheme 2 The hydroxylation (1 → 2) and side-chain cleavage (2 → 3) reactions catalysed by human CYP17

The 17α-hydroxysteroid of type **2**, after its conversion to the Δ^4-3-keto system by 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase, also produces androgens. In the adrenal gland, however, this steroid is predominantly directed to the biosynthesis of glucocorticoids. The Fe^{III}–OOH intermediate of CYP17 is invoked in the formation of the adduct **4**, which then fragments to furnish the androgen, **3**, and the acyl side-chain as acetic acid.

Expression and purification of proteins

NADPH-P450 reductase and cytochrome b_5 were purified from porcine liver, as described in the literature [25,26], to a specific activity of 31 units/mg of protein and specific content of 43.4 nmol of haemoprotein/mg of protein respectively. Human CYP17 was expressed in *Escherichia coli* and purified to a specific content of 7.8 nmol of haemoprotein/mg of protein using a modification [27] of a method described in the literature [24]. This specific content compares favourably with other reported values for the human 10.7 [24] and the pig 8.0 [1,1a] enzymes. These values are lower than the theoretical value of 18 nmol of P450/mg of protein because of variable loss of non-covalently bound haem during the extreme environmental changes that occur during purification. The recombinant rat cytochrome b_5 of the native type (core–tail) (Figure 1) and its core, signal–core, and signal–core–tail derivatives were expressed in *E. coli* and purified to near homogeneity as reported elsewhere [28–31].

17α-Hydroxylase-17,20-lyase assay

Side-chain cleavage activity of 17α-hydroxylase-17,20-lyase was monitored by measuring the release of tritiated acetate from 17α-hydroxy[21-^{3}H]pregnenolone into the aqueous medium. This assay proved quicker and less involved than the conventional assay which involves the analysis of products following TLC separation. The selectivity and efficiency of the release of acetate was indirectly determined to be $> 90\%$ by admixing 17α-hydroxy[21-^{3}H]pregnenolone with a trace amount of [7-^{3}H]pregnenolone (approx. 1 μCi) and quantifying the products following TLC separation [6]. The assay was performed at 37 °C in a 1 ml final volume as previously described [6], but containing the following optimal quantities of the various components: 17α-hydroxy[21-^{3}H]pregnenolone (25 pmol, 4.4×10^4 d.p.m./nmol), CYP17 (50 pmol), NADPH-P450 reductase (400 pmol), didodecanoyl L-α-phosphatidylcholine (60 μg) and a given amount of cytochrome b_5 or its derivative.

Enzymic reduction of cytochrome b_5

The activity of NADPH-P450 reductase-catalysed electron transfer from NADPH to cytochrome b_5 was monitored as follows. A 1 ml volume containing potassium phosphate buffer (370 mM, pH 7.7), sodium cholate (0.15%, w/v), cytochrome b_5 (200 pmol) and either 0.8 pmol or 400 pmol of NADPH-P450 reductase, was equally divided into a reference and sample cuvette and the assay initiated by the addition of 200 μM NADPH to the sample cuvette. The reduced minus oxidized absorption difference spectrum was recorded after 10 s and every 30 s thereafter, and the rate of cytochrome b_5 reduction was determined using an absorption coefficient of 185 $mM^{-1} \cdot cm^{-1}$

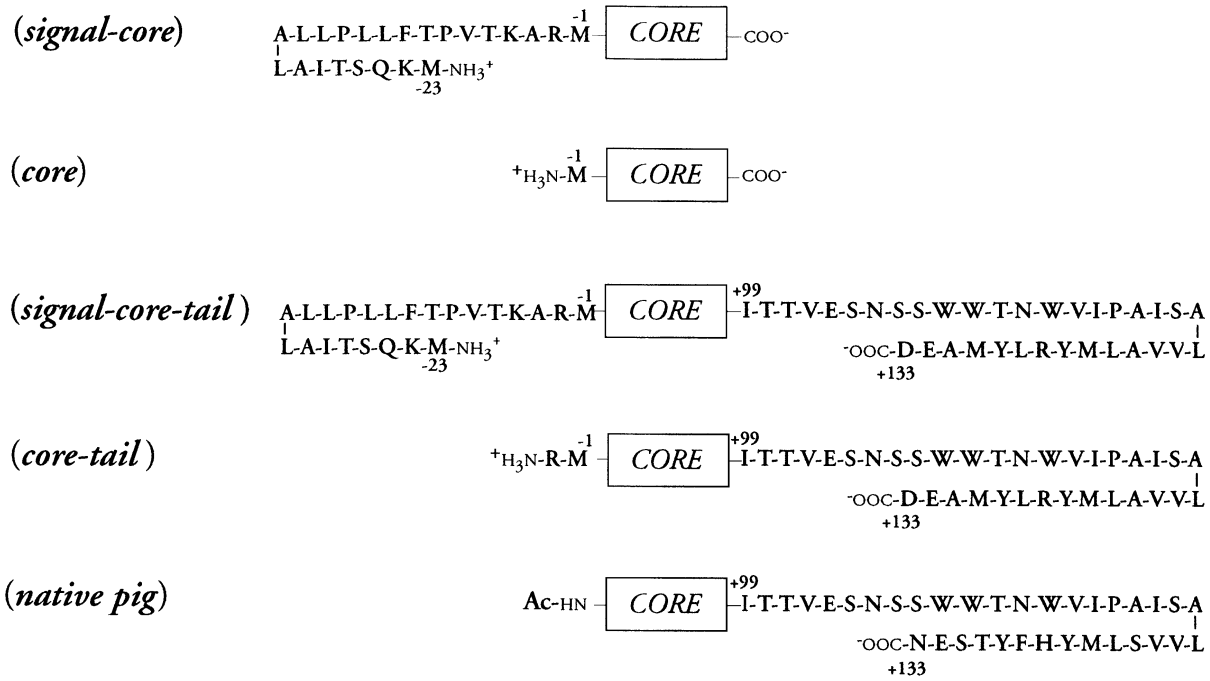


Figure 1 Protein sequences of the various cytochrome b_5 derivatives

The native pig is the sequence of the pig cytochrome b_5 [32]. Core–tail represents the full-length sequence of recombinant rat cytochrome b_5 with two additional amino acids, Arg^{-2} and Met^{-1}, at the N-terminal. The Met^{-1} residue encoded by the eukaryote gene is post-translationally removed in eukaryotes but is unprocessed in *E. coli*. The Arg^{-2} residue acts as a linker between the rat cytochrome b_5 sequence and the secretory signal sequence of alkaline phosphatase. The 'core' represents amino acid residues 1–98 of the native isoform [32], comprising the redox active haem-domain of the cytochrome. Residues +99 to +133 and −3 to −23 represent the sequence of the C-terminal hydrophobic tail of the native isoform and the secretory signal sequence of alkaline phosphatase respectively.

(424_{max} and 409_{min}). The inclusion of sodium cholate in the assay buffer was required to prevent aggregation of the hydrophobic proteins and maintain them in solution.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS

Preparations of cytochrome b_5 (1 nmol) in 100 μl of potassium phosphate buffer (100 mM, pH 7.4) and sodium cholate (0.1 % w/v) were extensively (18 h) dialysed against aqueous sodium cholate (0.1 % w/v); failure to deplete potassium phosphate in the samples impeded the flight trajectories of the haemoprotein molecules during the subsequent MS. A 5 μl sample of the dialysed cytochrome b_5 was admixed with an equal volume of acetonitrile/water/trifluoroacetic acid (300:700:1, by vol.) that had been saturated with sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid). This mixture (2 μl) was spotted on to a sample plate, air-dried and analysed using a Perceptive Biosystems MALDI-TOF mass spectrometer. The spectra were calibrated with myoglobin. Theoretical protein masses were calculated using the average chemical mass of each amino acid.

Electrospray MS

Mass spectra were recorded in the positive ion mode using a VG Quattro II instrument (Fisons Instruments, Manchester, U.K.). The probe was operated with a capillary voltage of 3.04 kV. Water, pumped at 10 μl/min, was used as the solvent into which 100 pmol of pig cytochrome b_5 dissolved in 10 μl of 1 % (v/v) formic acid was applied. Scans were acquired in a continuous mode with a 1 s scan duration and an interscan delay of 0.11 s.

Protein sequencing

Protein sequencing was performed on an Applied Biosystems 477A pulse liquid sequencer with on-line phenylthiohydantoin detection using an Applied Biosystems 120A analyser.

RESULTS

Characterization of cytochrome b_5 and its derivatives

The native porcine and rat cytochrome b_5 comprise 133 amino acids and are organized in two domains. The residues 1 to 92 are folded to create a compact globular domain, whereas the C-terminal sequence, comprising residues 93 onwards, forms a hydrophobic tail [32] which protrudes through the membrane bilayer [33]. The globular domain, which can be obtained by the proteolytic digestion of the membrane-embedded protein [34–36], was one of the first proteins to be examined by X-ray diffraction [37]; the three-dimensional structure of the complete molecule awaits determination.

In this work, the native cytochrome b_5 was purified from pig liver [26] and the rat enzyme (core–tail, Figure 1), together with its various derivatives, core, signal–core and signal–core–tail (Figure 1), were expressed in *E. coli* and purified to near homogeneity as previously described [28–31]. All four rat cytochrome b_5 derivatives were shown to have their predicted amino acid sequence for the first five N-terminal residues, and MALDI-TOF MS of the proteins gave [M (the molecular ion) + H]$^+$ ions

Table 1 Some physical and enzymic characteristics of native and recombinant cytochrome b_5 derivatives

Enzyme activities were measured as described in the Experimental section. A unit of reduction by NADPH-P450 reductase is defined as 1 pmol of cytochrome b_5 reduced by 1 pmol of reductase in 1 min. Values marked with an asterisk (*) were obtained using 1:250 molar ratio of NADPH-P450 reductase (0.8 pmol)/cytochrome b_5 (200 pmol) and are expressed as the mean of duplicate measurements ± half the difference between the duplicate values.

Property	Cytochrome b_5 derivative ...	Value				
		Signal–core	Core	Signal–core–tail	Core-tail	Native pig
Theoretical or predicted $[M+H]^+$ (Da)		13737	11342	17751	15513	15223
MALDI-TOF-MS-obtained $[M+H]^+$ (Da)		13741	11342	17748	15519	15224
Deviation from the predicted mass (Da)		4	0	3	6	1
Rate of reduction by NADPH-P450 reductase (pmol/min per pmol)*		5.2 ± 0.47	6.3 ± 0.09	15.0 ± 0.35	21.3 ± 0.26	25.3 ± 0.13
Relative rate (%)		22	25	59	84	100
Stimulation of CYP17 lyase activity (%)		0	0	55	100	100

Table 2 Effect of various cytochrome b_5 derivatives on human CYP17 lyase activity

Assays were performed as described in the Experimental section using 200 pmol of cytochrome b_5, except for the core–tail + signal–core incubation, which contained both core–tail (200 pmol) and signal–core (1 nmol). CYP17 lyase activity is expressed as the mean of duplicate measurements ± half the difference between the duplicate values. A unit of CYP17 lyase activity is defined as 1 nmol of 17α-hydroxypregnenolone cleaved by 1 nmol of P450 in 1 min.

Cytochrome b_5	CYP17 lyase activity (nmol/min per nmol of P450)
None	0.14 ± 0.04
Native pig	1.60 ± 0.06
Core–tail	1.63 ± 0.07
Signal–core–tail	0.89 ± 0.05
Signal–core	0.10 ± 0.03
Core	0.10 ± 0.02
Signal–core + core–tail (5:1)	1.45 ± 0.06

that were in excellent agreement with their theoretical masses (Table 1).

The analysis of the native pig cytochrome b_5 by MALDI-TOF MS gave the $[M+H]^+$ ion at m/z 15224, which is comparable with the value of $[M+H]^+$ 15223 that is derived from the predicted mass of the N-terminally acetylated protein (native pig, Figure 1). In a previous study [38] cytochromes b_5 from a number of species were shown to be N-terminally acetylated, although the modification was reported to be absent from the pig enzyme. To confirm the MALDI-TOF-derived mass, the pig cytochrome b_5 was subjected to electrospray MS analysis. This gave a series of multiply charged ions, which, after deconvolution, gave a mass of 15222 Da, indicative of the presence of an acetyl group. This, together with our inability to detect the release of a phenylthiohydantoin-amino acid during Edman sequencing, suggests that the native pig cytochrome b_5 must be capped at the N-terminal by an acetyl group.

Side-chain cleavage activity of CYP17 is determined by the orientation of the membrane-anchoring domain in cytochrome b_5

The purified forms of the four genetically engineered derivatives of rat cytochrome b_5 and the native pig cytochrome b_5 were spectrally identical and were used to study the side-chain cleavage reaction catalysed by human CYP17. The data presented in Table 2 confirm the previous findings that the cleavage reaction is clearly dependent on cytochrome b_5. At a 4:1 molar ratio of native pig cytochrome b_5 to CYP17, the reaction was enhanced about 10-fold. The engineered core–tail form of the rat cytochrome b_5 showed a similar level of stimulatory activity to its wild-type porcine counterpart. Such a stimulatory effect was unprovoked by the signal–core or the core cytochrome b_5 forms. However, the signal–core–tail haemoprotein, containing two potential membrane spanning hydrophobic segments, showed 55% of the activity of the native cytochrome b_5. The catalytic interaction between CYP17 and the various cytochromes b_5 forms were further investigated by titration experiments. Figure 2 shows that with both the recombinant rat core–tail and signal–core–tail cytochrome b_5 forms, the side-chain cleavage activity increased with progressive elevation in the cytochrome b_5/CYP17 molar ratio, displaying hyperbolic profiles typical of Michaelis–Menten kinetics. The core species was ineffective at all of the ratios studied here. The relative affinities of CYP17 for core–tail and signal–core–tail species were obtained by calculating the cytochrome b_5/CYP17 molar ratios that were required to achieve 50% of maximal stimulation. These ratios, derived from the double reciprocal plots, were 0.7 and 1.4 for the recombinant rat core–tail and signal–core–tail cytochrome b_5 species respectively. In addition to having a 2-fold greater affinity, the wild-type core–tail rat cytochrome b_5 also promoted a 1.4-fold greater stimulation of the CYP17 activity compared with its signal–core–tail counterpart.

Decreased stimulation of CYP17 side-chain cleavage activity by the modified cytochrome b_5 derivatives is due to their altered interaction with CYP17

Were the distinct stimulatory patterns of the various cytochrome b_5 derivatives in promoting the side-chain cleavage activity of CYP17 due to an altered mode of interaction with CYP17 or indirectly through altered electron coupling with NADPH-P450 reductase? This was answered by measuring the reduction of cytochrome b_5 by P450 reductase in the absence of CYP17. Using a NADPH-P450 reductase/cytochrome b_5 molar ratio of 2:1, as used in the CYP17 side-chain cleavage activity assay, all of the cytochrome b_5 derivatives were fully reduced within 20 s. However, for a more critical comparison, reduction of the cytochrome b_5 derivatives was conducted by decreasing the amount of P450 reductase by 500-fold. Under these conditions (P-450 reductase/cytochrome b_5 molar ratio of 1:250) the native pig and the rat core–tail cytochrome b_5 forms were reduced at the highest rates, whereas the signal–core–tail, core and signal–core species dis-

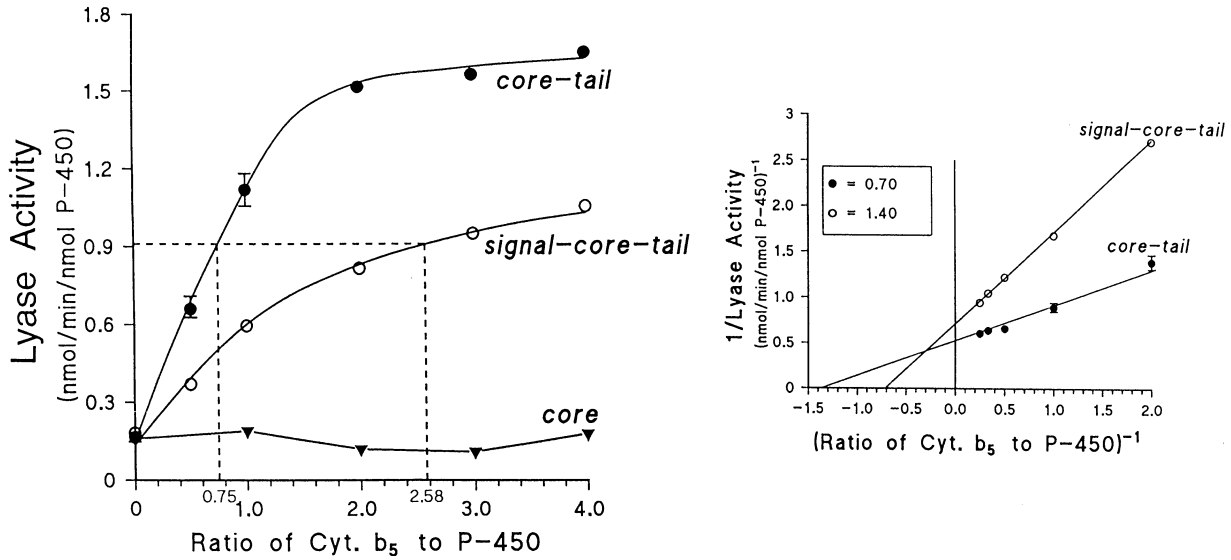


Figure 2 Effect of increasing amounts of recombinant rat cytochrome b_5 (core–tail), its double-tailed (signal–core–tail) and soluble (core) derivatives on human CYP17 lyase activity

Assays were performed as described in the Experimental section but using varying amounts of cytochrome b_5 derivative (0, 25, 50, 100, 150 and 200 pmol). The right-hand graph represents the double reciprocal plots of the left-hand graph and displays the cytochrome b_5/CYP17 ratio required to achieve 50% of the maximal stimulation of CYP17 lyase activity. The broken lines in the left-hand graph indicate the ratio of cytochrome b_5/CYP17 required to achieve 50% of the maximal stimulation achieved by the recombinant rat cytochrome b_5.

played 59%, 25% and 22% rates of reduction of the native pig cytochrome b_5 respectively (Table 1). These results indicate partial impairment of interaction between some of the modified forms of cytochrome b_5 and the NADPH-P450 reductase. However, this step is unlikely to be rate-limiting in the side-chain cleavage reaction, since under the conditions used in this assay (having 400 pmol of P450 reductase present) the rates of electron transfer from NADPH-P450 reductase to the various forms of cytochrome b_5 were in the range 2080–10120 pmol/min, compared with an optimal side-chain cleavage activity of 80 pmol/min. Furthermore, it was also shown that the signal–core derivative did not compete to depress the stimulatory activity of the native rat (core–tail) cytochrome b_5 (Table 2), even when the signal–core species was present at a 5-fold molar excess over the active core–tail protein. This result eliminates another related possibility that the lack of side-chain cleavage by CYP17 in the presence of core and signal–core derivatives was due to their preferential association with *P*-450 reductase, so that the latter was not available for interaction with CYP17. It should also be highlighted that, in the experiment just described, the signal–core derivative is in a 20-fold excess over CYP17. These data allow the conclusion to be drawn that the decreased stimulation of CYP17 lyase activity by the modified cytochrome b_5 derivatives is due to their altered interaction with CYP17 and not as a result of impaired electron coupling with P450 reductase.

DISCUSSION

The results described here show that the orientation of the hydrophobic segment of cytochrome b_5 plays a crucial role in its interaction with human CYP17, as measured by the side-chain cleavage activity of the P450. The observation that the core species was unable to stimulate the cleavage activity of CYP17 agrees with other studies in which proteolytically solubilized cytochrome b_5, corresponding to the core species, was found not to affect the activities of microsomal *P*-450s [18,20,21]. In contrast, a recent study [39] showed that the side-chain cleavage activity of bovine CYP17 was stimulated by an engineered derivative of rat cytochrome b_5 with a sequence similar to, but not identical with, that of the core species used in the present work. However, it should be noted that in their work [39] a recombinant protein in which bovine CYP17 was fused to rat NADPH-P450 reductase was used (a system deviating from the conventional reconstituted assays performed with the individually isolated components as studied here and by others [18,20,21]).

The stimulation of CYP17 lyase activity by the native forms of cytochrome b_5 but not by the core or signal–core derivatives clearly demonstrate that the C-terminal hydrophobic segment of cytochrome b_5 plays a crucial role in mediating the interaction of the catalytically active globular domain with the human CYP17. The N-terminally membrane-anchored signal–core cytochrome b_5, in which the globular head is spatially displaced (see Figure 3B), neither stimulated the side-chain cleavage activity of human CYP17 nor competed to depress the stimulatory activity of the native cytochrome b_5 (Table 2). These findings imply that a productive interaction between cytochrome b_5 and CYP17 is governed not only by the presence of a membrane-insertable hydrophobic segment but also by a defined spatial orientation of the exposed globular domain. This view is further supported by the kinetics of stimulation of CYP17 lyase activity by the signal–core–tail cytochrome b_5 which is membrane-insertable by either or both of the hydrophobic domains. In comparison with the native pig or core–tail cytochrome b_5 forms, the signal-core–tail form exhibited a 2-fold lower affinity for CYP17 and approximately 55% of the lyase stimulatory activity. These findings can be reconciled by invoking two modes of membrane assembly of the signal–core–tail in which either a proportion of the molecules are membrane-embedded via the native tail portion or dual anchorage via the N-terminal signal and the tail segments of the globular cytochrome b_5 domain distorts its optimal

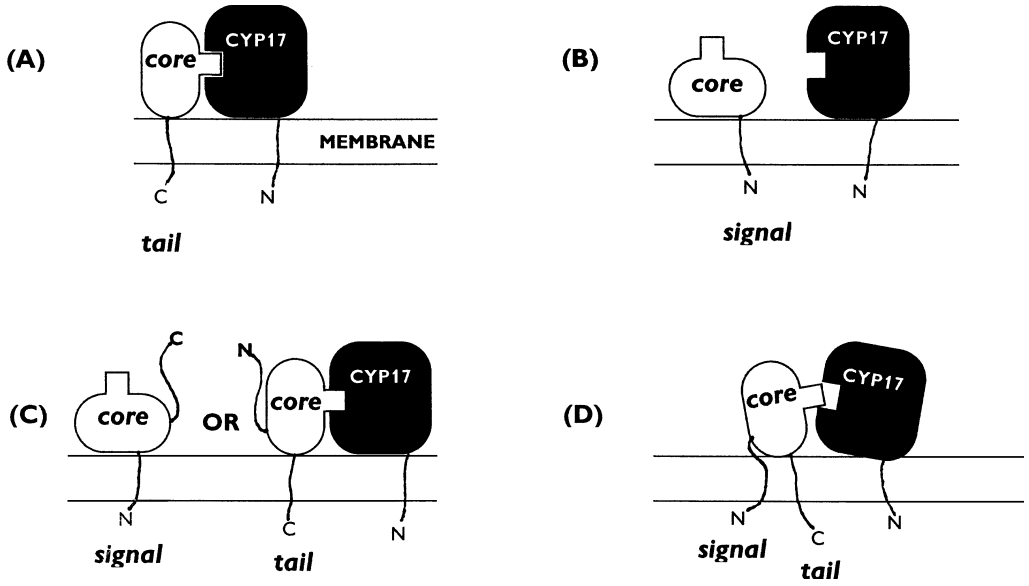


Figure 3 Proposed model showing the interaction of the various cytochrome b_5 derivatives with CYP17

(**A**) The core domain of the wild-type cytochrome b_5 is shown to be anchored to the membrane via its C-terminal hydrophobic tail for optimal interaction with CYP17. (**B**) The core domain of the signal–core derivative is anchored to the membrane via its N-terminal signal and is incorrectly orientated for productive interaction with CYP17. (**C**) Only half of the signal–core–tail molecules are anchored to the membrane via their C-terminal tails and have their core domain spatially orientated for optimal interaction with CYP17. (**D**) The core domain is anchored by both its N-terminal signal and C-terminal tail which restricts movement of the core with respect to the plane of the membrane and leads to a weaker interaction with CYP17.

interaction with CYP17 (see Figures 3C and 3D). Both models signify the importance of the membrane anchor being orientated at the C-terminus, in determining a topography of the exposed globular region for productive interaction with CYP17.

For quantitative comparison, the reduction of the cytochrome b_5 derivatives was studied using limiting quantities of NADPH-P450 reductase. The pattern of the rates thus obtained (Table 1) was reminiscent of the efficiencies of the cytochrome b_5 derivatives in promoting the side-chain cleavage reaction catalysed by CYP17. The important difference being that the core and the signal–core species, which were completely inactive in stimulating the CYP17 cleavage activity, still showed up to 25% of the activity as substrates for reductase. Cumulatively these results highlight the fact that the hydrophobic tail of cytochrome b_5 contributes to establishing interactions with CYP17 as well as the P450 reductase, though the structural requirements are less stringent for interaction of cytochrome b_5 with reductase than with CYP17, as has also been found for another system involving the oxidation of methoxyflurane by P450 LM2 (2B4) [40].

The importance of ionic interactions in binary complex formation between cytochrome b_5 and certain *P*-450s has been emphasized in several previous studies [41–45]. These observations can be reconciled with the present findings by assuming that the juxtapositioning of the C-terminal membrane 'anchor' of cytochrome b_5 and the transmembrane N-terminal domain of CYP17 could initiate co-operative associations between the subunits, eventually stabilized by complementary charge-pairing and patches of hydrophobic interactions.

Finally, it should be emphasized that the precise molecular mechanism by which cytochrome b_5 regulates the side-chain cleavage activity of human CYP17 remains unknown. This may occur through either a protein conformational change or a redox-driven process. Irrespective of which of these mechanisms operate, our previous work [6,27] indicates that cytochrome b_5 directs the nucleophilic oxygen of the Fe^{III}–OOH intermediate of CYP17 to be orientated towards the C-20 carbonyl group of the substrate to allow the formation of an enzyme–substrate adduct of the type **4** (Scheme 2), which then fragments to furnish the androgen and releasing the side chain as acetic acid.

In summary, over the past two decades it has been shown that the C-terminal hydrophobic domain of cytochrome b_5 is essential for cytochrome integration into microsomal membranes and for optimal interaction with its redox partners, NADH-cytochrome b_5 reductase, P450 reductase and various P450s [18,20,21,35,40]. The study presented here goes further and shows that productive interaction between cytochrome b_5 and P450s is governed not only by the presence of a membrane insertable hydrophobic region on the cytochrome b_5 but also by a defined spatial orientation of the exposed catalytic globular domain.

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