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The cationic charges on Arg^{347}, Arg^{358} and Arg^{449} of human cytochrome P450c17 (CYP17) are essential for the enzyme's cytochrome b_5-dependent acyl-carbon cleavage activities☆

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Abstract

CYP17 (17α-hydroxylase-17,20-lyase; also P450c17 or P450$_{17\alpha}$) catalyses the17α-hydroxylation of progestogens and the subsequent acyl-carbon cleavage of the 17α-hydroxylated products (*lyase* activity) in the biosynthesis of androgens. The enzyme also catalyses another type of acyl-carbon cleavage (*direct cleavage* activity) in which the 17α-hydroxylation reaction is by-passed. Human CYP17 is heavily dependent on the presence of the membrane form of cytochrome b_5 for both its *lyase* and *direct cleavage* activities. In the present study it was found that substitution of human CYP17 amino acids, Arg^{347}, Arg^{358} and Arg^{449}, with non-cationic residues, yielded variants that were impaired in the two acyl-carbon bond cleavage activities, quantitatively to the same extent and these were reduced to between 3 and 4% of the wild-type protein. When the arginines were replaced by lysines, the sensitivity to cytochrome b_5 was restored and the acyl-carbon cleavage activities were recovered. All of the human mutant CYP17 proteins displayed wild-type hydroxylase activity, in the absence of cytochrome b_5. The results suggest that the bifurcated cationic charges at Arg^{347}, Arg^{358} and Arg^{449} make important contributions to the formation of catalytically competent CYP17·cytochrome b_5 complex. The results support our original proposal that the main role of cytochrome b_5 is to promote protein conformational changes which allow the iron-peroxo anion to form a tetrahedral adduct that fragments to produce the acyl-carbon cleavage products.

Keywords: Cytochrome P450; P450c17; P450$_{17\alpha}$; 17α-Hydroxylase-17,20-lyase; Cytochrome b_5

1. Introduction

Three cytochrome P450 enzymes, involved in the biosynthesis of steroid hormones and sterols, form a distinctive group that catalyse not only the conventional P450 hydroxylation reaction, Eq. (1) [1–4], but also an acyl-carbon cleavage as shown in Eq. (2)

$$R - H + O_2 + NADPH + H^+ \rightarrow R - OH + H_2O + NADP^+ \quad (1)$$

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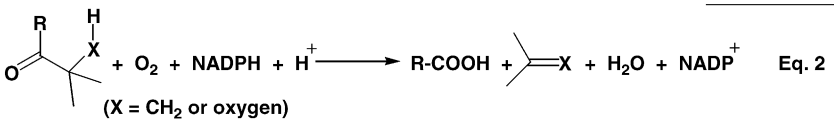
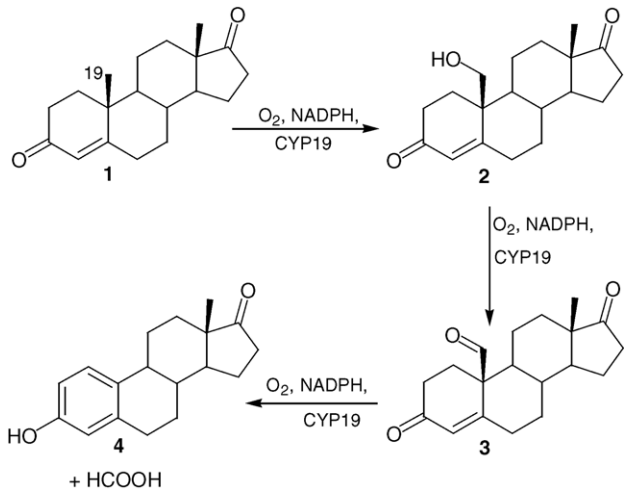


Eq. 2

The dual functionality was originally highlighted by our studies on aromatase (CYP19), which is responsible for the construction, from the androgen nucleus, of the aromatic ring A of the female hormones, oestrone and oestradiol (Scheme 1) [5–9]. The oxidative target for aromatase is the C-19 of androgens (**1**), which is hydroxylated to produce the alcohol (**2**), the latter is then converted into the 19-aldehyde (**3**), presumably via the intermediary of a gem diol. The discovery, that the formyl group of the 19-aldehyde (**3**) is eliminated as formic acid and that an atom of oxygen from molecular oxygen had been introduced into its carboxyl group (**3** → **4**), proved crucial for highlighting that aromatase is endowed with the property of catalysing two different generic reactions (Eqs. (1) + (2) at the same active site [6,9,10].

The duality was rationalised in terms of the model of Scheme 2 [10]. The main assumption underpinning the model is that, in the catalytic cycle of P-450s, the Fe^{III}–O–O^- species (**6**) is generated for conversion into the iron-oxo-derivative (**8**) that promotes hydroxylation by a free radical mechanism [11,12]. Although the general consensus is that P450-catalysed hydroxylations are achieved by an iron-oxo intermediate, **8**, there is evidence that some hydroxylations, where the C–H bond is weak, can be mediated by the hydroperoxo-iron species (**7**) that catalyses the insertion of an OH^+ equivalent [13]. However, when the target carbon atom of the substrate contains an electrophilic functionality, the iron-peroxide anion, expected to be a strong nucleophile, is trapped into producing an adduct (**11**) which decomposes by one of several closely related



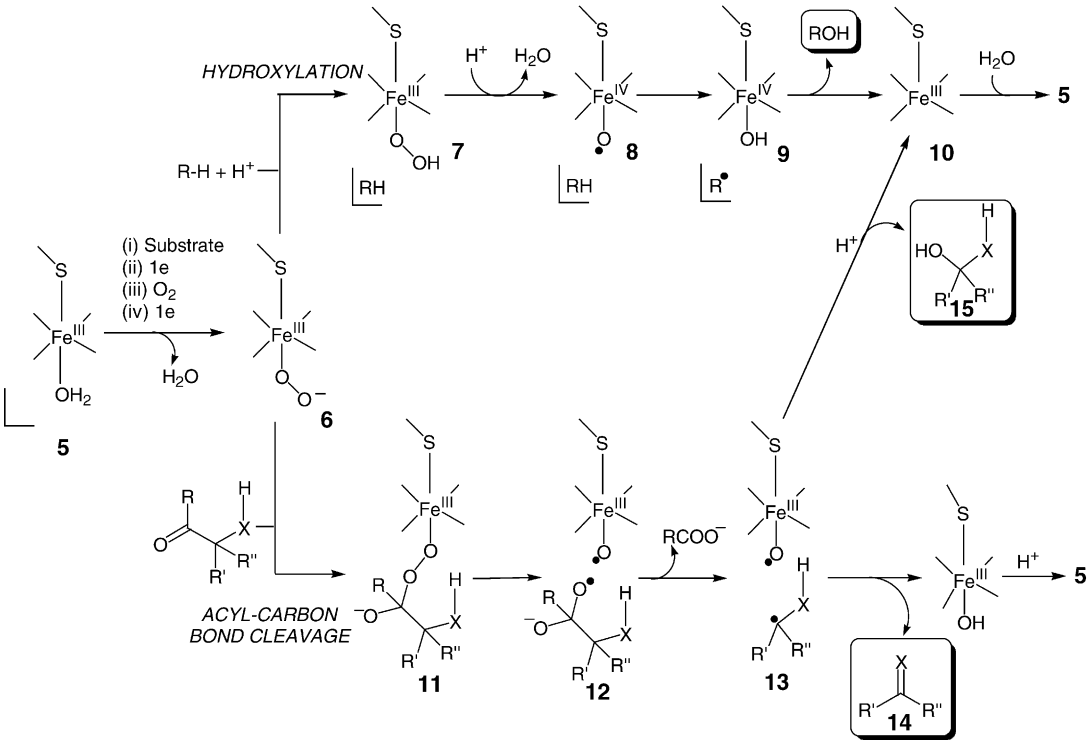
Scheme 1. CYP19 (aromatase)-catalysed transformations. CYP19 catalyses C-19 demethylation and aromatization of ring A of the androgen nucleus, via three sequential oxidative reactions.

pathways, to furnish the fragmentation products (**11**–**15**, Scheme 2).

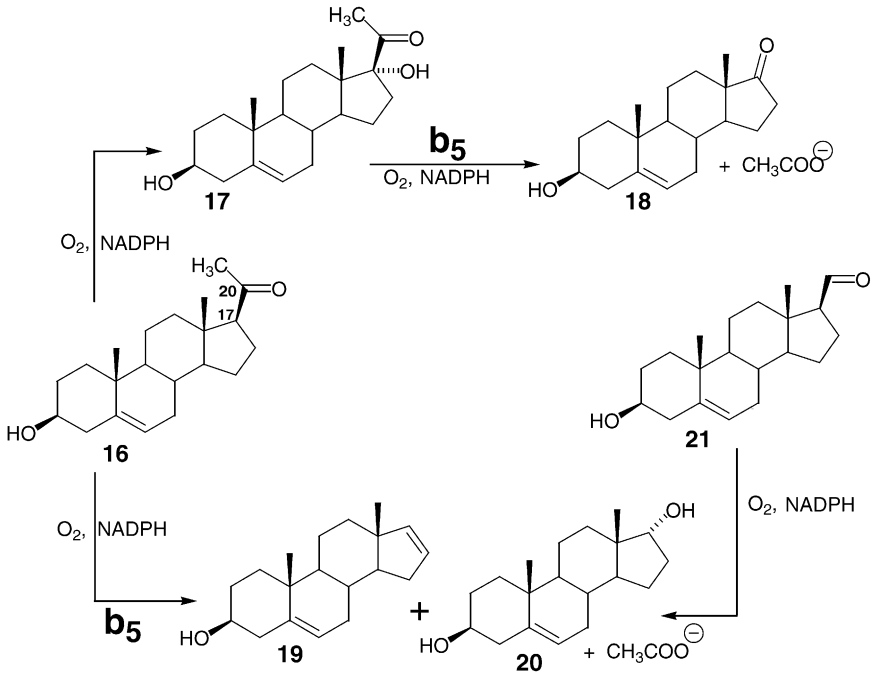
The same three-step sequence, found for aromatase (Scheme 1), was shown to be involved in the removal of the 14α-methyl group of lanosterol during the biosynthesis of animal, yeast and plant sterols [14]. This transformation is catalysed by 14α-demethylase (CYP51), the third step of which involves the removal of C-30 as formic acid and the formation of an 8,14-ene intermediate [15]. The latter, through a multi-step reaction, is then transformed into the unique sterol of each organism.

In broad terms the enzyme, 17α-hydroxylase-17,20-*lyase* (CYP17), that catalyses the formation of dehydro*epi*androsterone (**18**) from pregnenolone (**16**) (Scheme 3) also conforms to the aforementioned duality. CYP17 catalyses a hydroxylation reaction and then a side chain cleavage of the hydroxylated intermediate, the *lyase* reaction [16], which in essence is reducible to the acyl-carbon fission of Eq. (2) [17]. In the cases of aromatase and 14α-demethylase the hydroxylation and the acyl-carbon cleavage reactions are targeted to the same carbon atom. As implied above, the regulation of acyl-carbon cleavage is governed entirely by the reactivity of Fe^{III}–O–O^- species, formed during the catalytic cycle of P-450s, which is trapped by the electrophilic carbonyl group. However, the two types of reactions catalysed by CYP17 are targeted, not to the same but to adjacent carbon atoms. The iron-oxygen ligand for hydroxylation must be projected toward C-17, while according to our model, the Fe^{III}–O–O^- must attack at the neighbouring C-20 for the *lyase* reaction.

During the course of our studies on the elucidation of the chemical mechanism of these dual-function P450s, it was discovered that with the human isoform of CYP17 the androgen-producing *lyase* reaction (**17** → **18**, Scheme 3), was heavily dependent on the presence of the membrane-bound form of cytochrome b_5, as was reported simultaneously by others [18,19]. To explain the nature of interactions between CYP17 and cytochrome b_5, several mutant forms of CYP17 had been engineered and subjected to detailed kinetic and mechanistic studies [20]. It was found that the mutation of certain arginine residues of human CYP17, in particular Arg^{347}, Arg^{358} and Arg^{449}, dramatically impaired the cytochrome b_5-dependent *lyase* reaction, with comparatively little effect on the cytochrome b_5-*in*dependent hydroxylation process (**16** → **17**, Scheme 3) [21]. The mutated forms of two of these arginines, Arg^{347} and Arg^{358}, have been discovered in male patients who suffered from genital ambiguity and had reduced production of androgens [22]. According to our kinetic studies these diseased-state mutants were primarily defective in responding to the stimulatory



Scheme 2. The iron-peroxide species: both precursor and catalytic intermediate for P450-catalysed hydroxylation and acyl-carbon cleavage reactions, respectively. The Fe^{III}–O–O^- species (**6**) is generated for conversion into the oxo-derivative (**8**) that promotes hydroxylation by a free radical mechanism. However, when the target carbon atom of the substrate is an electrophilic carbonyl then **6** becomes the catalytic iron-oxygen intermediate and promotes the cleavage of the substrate acyl-carbon bond. The hydroxylated and acyl-carbon bond cleavage products are highlighted; x = oxygen or carbon.



Scheme 3. Human CYP17-catalysed transformations. Each transformation requires a molar equivalent of molecular oxygen and NADPH, as well as the obligatory redox protein partner of CYP17, NADPH-cytochrome P450 reductase. **b_5** indicates an obligatory requirement for cytochrome b_5.

effect of cytochrome b_5. The finding bolstered our original proposal that the interaction of cytochrome b_5 with the CYP17-substrate complex causes protein conformational changes which culminate in directing the iron-oxygen ligand of the P450 away from C-17 and towards C-20 (Scheme 4) [18]. This facilitates a nucleophilic attack of the peroxide anion on the carbonyl carbon producing a tetrahedral adduct that follows the side chain cleavage path.

CYP17 catalyses another type of acyl-carbon cleavage (hereafter referred to as *direct cleavage* activity) in which the 17α-hydroxylation reaction is by-passed and leads to the formation of a 5,16-diene steroid, **19**, regarded as a male pheromone [23] and 17α-hydroxyandrogen (**20**), presumed to be the precursor of *epi* testosterone [24]. Extending the earlier observations of Hall and co-workers [25] we have shown that the formation of **19** as well as **20** is entirely dependent on the presence of cytochrome b_5 and our mechanistic studies established that these steroids are formed from a common intermediate in the cleavage pathway [18,26]. We argued that the main role of cytochrome b_5 is to regulate the orientation of the iron-oxygen ligand, away from C-17, towards C-20. If this is the case then the mutations affecting the *lyase* reaction, leading to androgen formation, may also profoundly influence the alternative *direct cleavage* reaction. This is because, according to our proposal, in both cases the product determining factor is the attack of the Fe^{III}–O–O^- species on the C-20 carbonyl group. The present paper examines this feature by studying the two types of cleavage reactions (**17** → **18** and **16** → **19** and **20**, Scheme 3) by various mutant forms of human CYP17.

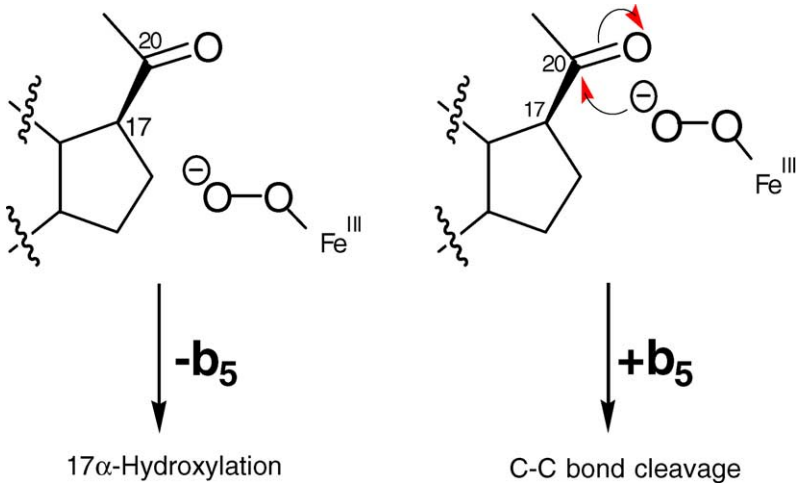
2. Materials and methods

2.1. Chemicals

[21-^{3}H]17α-Hydroxypregnenolone, and [20-^{3}H]3β-hydroxyandrost-5-ene-17β-carbaldehyde were prepared as described previously [26]; [17α-^{3}H]pregnenolone was prepared from 17α-bromopregnenolone [27], following debromination with Zn in CH_3COO^3H and [7-^{3}H]pregnenolone was purchased from DuPont (UK) Ltd. DNA primers and sequencing were synthesised and performed by Oswel DNA Services, University of Southampton (UK). $VENT_R$ DNA polymerase and restriction endonucleases were obtained from New England BioLabs (UK). All other chemicals were obtained from Sigma Chemical Co. (UK). The plasmid pCWH17mod, containing the modified human CYP17 cDNA, was a generous gift from Prof. Waterman, Vanderbilt University, Nashville, TN.

2.2. Polymerase chain reaction (PCR) site-directed mutagenesis

Human CYP17 mutant proteins were constructed, using PCR-site-directed mutagenesis, as described previously [28]. Briefly, 1051 bp *Xba*I-*Sac*I and 1236 bp *Xba*I-*Hind*III fragments encoding human CYP17 amino acids 102–452 and 102–547 respectively, were cut out of pCWH17mod and cloned into pBluescript SK(−) (Stratagene). Using these constructs as DNA templates, mutagenesis was achieved in two stages, involving three PCR reactions. Firstly, two separate PCR amplifications were performed, one using an outside flanking M13/reverse primer **A**, 5′-AACAGCTATGACCATG-3′, with a primer of the series **C** (see legend to Table 1) and the other a flanking M13(−20) primer **B**, 5′-GTAAAACGACGGCCAGT-3′, together with a primer of series **D** (Table 1). Secondly, the amplified products from these two PCR reactions were combined in equal molar ratios and served as template DNA for the final PCR reaction, which was performed using only the two flanking primers, **A** and **B**. All three PCR amplifications were performed in a final volume of 40 μl, containing the primers (300 ng each), template DNA (80 ng), dNTP (440 μM each), $MgSO_4$ (4.5 mM), KCl (10 mM), $(NH_4)_2SO_4$ (10 mM) Tri-



Scheme 4. Proposed effect of cytochrome b_5 on substrate re-positioning with respect to the active site haem-iron-peroxide anion species.

Table 1
DNA primers used to generate the various CYP17 mutant proteins

Amino acid change	Primer sequence (series **D**)
$Arg^{347} \rightarrow Ala$	5′-CCAACTATCAGTGAC**gc**TAACCGTCTCCTC-3′
$Arg^{347} \rightarrow His$	5′-CCAACTATCAGTGACC**ac**AACCGTCTCCTC-3′
$Arg^{347} \rightarrow Lys$	5′-CCAACTATCAGTGAC**aag**AACCGTCTCCTC-3′
$Arg^{358} \rightarrow Ala$	5′-GCCACCATC**gc**AGAGGTGCTTCGCCTCAGG-3′
$Arg^{358} \rightarrow Gln$	5′-GCCACCATCC**ag**GAGGTGCTTCGCCTCAGG-3′
$Arg^{358} \rightarrow Lys$	5′-GCCACCATC**aa**AGAGGTGCTTCGCCTCAGG-3′
$Arg^{449} \rightarrow Ala$	5′-CTGGCC**gc**CCAGGAGCTCTTCCTCATCATG-3′
$Arg^{449} \rightarrow Lys$	5′-GGTGAGATCCTGGCC**aag**CAGGAGCTCTTCCTC-3′

Each primer shown represents the sense mutant primer and of the series **D**, series **C** comprises the antisense primers that have a complementary sequence to those displayed in the table. The mutagenic base changes are highlighted by bold lowercase face.

ton X-100 (0.1%, v/v), $VENT_R$ DNA polymerase (1 unit) and Tris–HCl (20 mM, pH 8.8 at 25 °C). For the first two PCR reactions the temperature was cycled between 94 °C (60 s), 55 °C (40 s) and 72 °C (45 s) for 30 cycles, whereas for the final PCR stage the temperature was cycled between 94 °C (60 s), 48 °C (70 s), 65 °C (60 s) for 5 cycles and 94 °C (60 s), 50 °C (45 s) and 72 °C (45 s) for a further 30 cycles. The final PCR product was subcloned back into pCWH17mod and following the selection of the mutant clones the entire subcloned fragments (*Xba*I-*Sac*I and *Xba*I-*Hind*III) were sequenced to confirm that only the desired mutation had been introduced.

2.3. Expression and purification of proteins

The native and mutant human CYP17 were expressed in *Escherichia coli* and purified as described previously [26]. The mutant proteins were between 80 and 95% homogeneous, as judged by Coomassie-stained SDS-PAGE and contained between 6.5 and 9.7 nmol P450/mg of protein. The wild-type protein was purified to 95% homogeneity and had a specific content of 10.5 nmol P450/mg. These values are lower than the theoretical P-450 content of 18 and have been attributed to the variable loss of the non-covalently bound haem that occurs during purification and because the preparations were not 100% homogeneous. NADPH-cytochrome P450 reductase was purified from porcine liver microsomes according to the method of Strobel and Dignam [29] to a specific activity of 42 units/mg; 1 unit is defined as 1 μmol of cytochrome *c* reduced per minute at 35 °C in 375 mM potassium phosphate buffer, pH 7.7. Cytochrome b_5 (43.4 nmol haem/mg of protein) was previously purified from detergent-solubilised porcine liver microsomes [18].

2.4. Enzyme assays

The different types of activities of the CYP17 variants were determined using two different radiochemical assays. The first involved monitoring the release into the aqueous medium of $[^3H]H_2O$, $[^3H]CH_3COOH$ and $[^3H]HCOOH$ from [17α-^{3}H]pregnenolone (**16**, specific activity 6.3×10^3 d.p.m./nmol), [21-^{3}H]17α-hydroxypregnenolone (**17**, specific activity 4.4×10^4 d.p.m./nmol) and [20-^{3}H]3β-hydroxandrost-5-ene-17β-carbaldehyde (**21**, specific activity 1.0×10^5 d.p.m./nmol), respectively, being used to measure the hydroxylase, *lyase* and aldehyde deformylation activities. The second type of radiochemical assay, which was used to determine the direct cleavage activity (**16** → **19** + **20**) for the CYP17 variants, involved the analysis of radio-labelled steroidal product, [7-^{3}H]3β-hydroxyandrosta-5,16-diene (**19**) and [7-^{3}H]17α-hydroxyandrogen (**20**), after their separation and purification, using silica thin layer chromatography, from the substrate, [7-^{3}H]pregnenolone (**16**, specific activity 4.4×10^5 d.p.m./nmol). Both types of assays were performed as described previously [18] but using CYP17:NADPH-cytochrome P450 reductase:cytochrome b_5 protein ratios of 1:11:5.

2.5. Protein analysis

Protein concentration was determined by the Lowry method after trichloroacetic acid precipitation of the proteins in the presence of sodium deoxycholate [30]. The specific content of cytochrome b_5 was determined by measuring the difference spectrum (reduced minus oxidised) using an extinction coefficient of 185 $mM^{-1}\,cm^{-1}$ ($Abs_{424\,minus\,409\,nm}$) [31]. The concentration of P450 was determined from the reduced carbon monoxide-binding minus the reduced difference spectrum, using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ ($Abs_{450\,minus\,490\,nm}$) [32].

3. Results

3.1. The catalytic properties of the arginine mutants of human CYP17

Attention is first drawn to the catalytic features of the wild-type human CYP17. The native enzyme, in the presence of NADPH, NADPH-cytochrome P450 reductase and O_2, displays about 50% of its maximal hydroxylase activity, which is modestly stimulated approximately two-fold in the presence cytochrome b_5 (Fig. 1A, WT). The *lyase* activity, producing **18**, is low without cytochrome b_5 and is enhanced by over 10-fold in its presence (Fig. 1B, WT). The *direct cleavage* activity (**16** → **19** + **20**) has absolute requirement for cytochrome b_5 (Fig. 1C, WT). Unlike the two preceding side chain cleav-

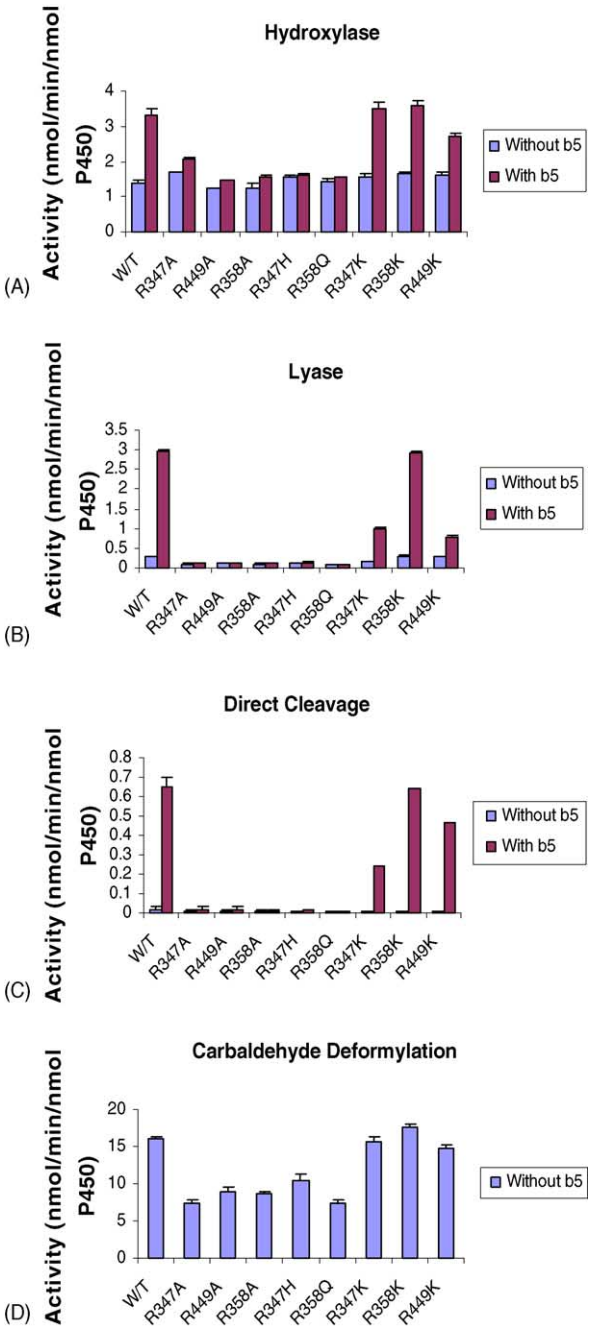


Fig. 1. Native and mutant human CYP17 hydroxylase (**16** → **17**), *lyase* (**17** → **18**), direct cleavage (**16** → **19** + **20**) and carbaldehyde deformylation (**21** → **19** + **20**) activities. Experiments were performed in triplicate as described in Section 2.4. The data is expressed as means ± S.E. bars.

ages, the deformylation of a substrate analogue (**21**), into **19** and **20** is not dependent on cytochrome b_5 [26]. Interestingly, in the absence of cytochrome b_5, the cleavage of **21** occurs at a level which is four-fold greater than that of any other reaction catalysed by human CYP17 (Fig. 1).

The mutation of Arg^{347}, Arg^{358} and Arg^{449} to alanine residues produced the most significant results. All of the three mutant proteins had the same basal hydroxylase activity, in the absence of cytochrome b_5, as displayed by the wild-type CYP17, but unlike the latter the mutants were resistant to stimulation by cytochrome b_5 (Fig. 1A). However, the main feature of the three mutants, was their very low intrinsic *lyase* activity, which was insensitive to stimulation by cytochrome b_5 (Fig. 1B). In the presence of cytochrome b_5 the ratio of hydroxylase to *lyase* activity is approximately 1 for the wild-type protein but ranges between 10 and 15 for the mutants. In other words, the mutants, by losing sensitivity to cytochrome b_5, had been converted from what in the wild-type was a bifunctional protein, into ones which were predominantly a hydroxylase. The same behaviour was shown by the constructs, Arg^{347} → His, Arg^{358} → Gln, corresponding to the mutations found in patients who had low androgen levels and suffered from genital ambiguity [22]. All the five mutants were also found to be totally impaired in the cytochrome b_5-dependent *direct cleavage* side of pregnenolone, which produces the two unusual steroids **19** and **20** (Fig. 1C). Furthermore, the sensitivity to cytochrome b_5 was restored for these mutants when cationic functionality was restored. Thus, the Arg^{347} → Lys and Arg^{358} → Lys mutants displayed 33–37 and 98–99% of the two wild-type acyl-carbon bond cleavages respectively, while in the Arg^{449} → Lys mutant exhibited a higher regain of activity for the cleavage of pregnenolone than for 17α-hydroxypregnenolone (72 and 26%, respectively). In contrast to the side chain cleavage of pregnenolone and 17α-hydroxypregnenolone, the third reaction of this class, the deformylation of the aldehyde analogue (**21**) lacking the 21-methyl group, was catalysed by all the five mutants at rates which were between 47 and 65% that of the wild-type enzyme (Fig. 1D).

3.2. Are the arginine mutants of CYP17 impaired in interaction with the NADPH-cytochrome P450 reductase?

The human CYP17-catalysed transformations studied above have varying requirements for cytochrome b_5 (Scheme 3) but depend on the obligatory participation of NADPH-cytochrome P450 reductase. We asked the question whether the impairment of the side chain cleavage reaction, found for the arginine mutants, was due to the alteration of the affinity of the CYP17 construct for the reductase or cytochrome b_5. Since the hydroxylation reaction is only partially stimulated by cytochrome b_5, this transformation can be used to study the affinity of CYP17 for the reductase, in the absence of cytochrome b_5. Under the routine assay conditions used to obtain the data displayed in Fig. 1 the incubation mixture contained 0.1 and 1.1 μM CYP17 and reductase respectively. The profiles of the hydroxylation rates versus the concentration ratios of the two proteins (Fig. 2, top) shows that, in the standard assay, the reductase was mostly saturating for the Arg^{347} → His, Arg^{358} → Gln and Arg^{449} → Ala mutants which were grossly crippled in the two side chain cleavage activities. The apparent affinity for the reductase is the same for both the wild-type CYP17

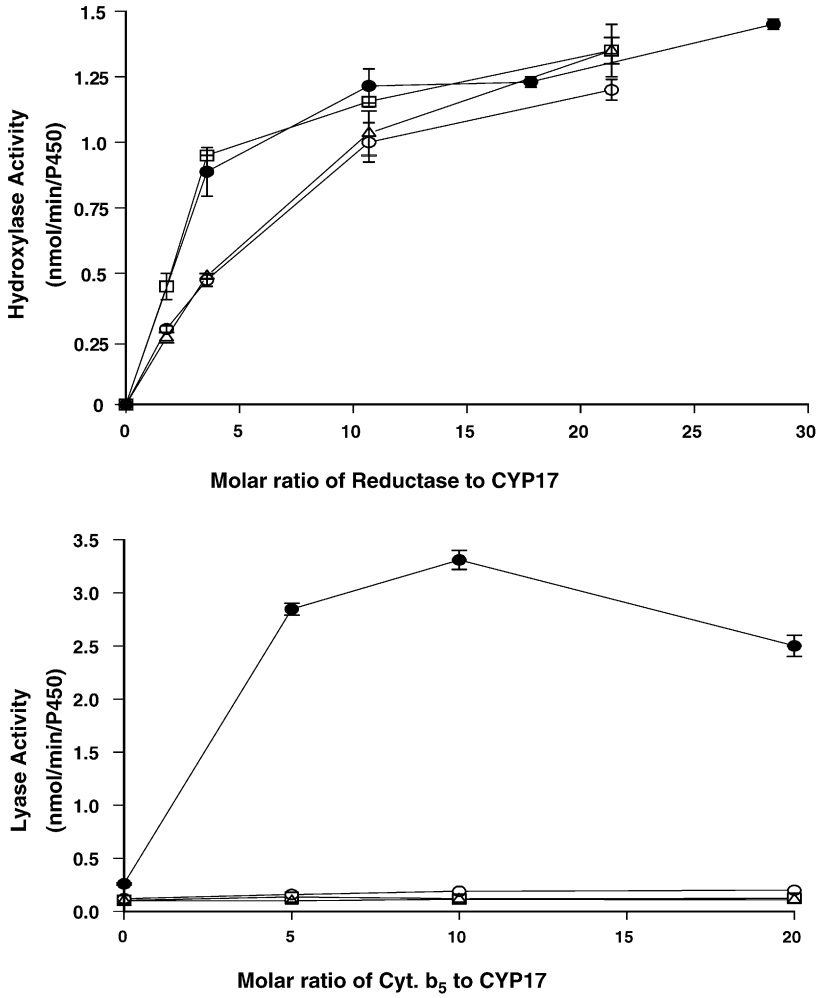


Fig. 2. The effect of increasing concentration of NADPH-cytochrome P450 reductase and cytochrome b_5 on the activities of native and mutant human CYP17. Top: shows the variation in hydroxylase activity of 0.1 μM CYP17 proteins with increasing molar ratio of reductase to CYP17. Bottom: shows the variation in *lyase* activity of 0.1 μM CYP17 proteins with increasing molar ratio of cytochrome b_5 to CYP17; the molar ratio of reductase to CYP17 was kept constant at 11. CYP17 proteins were: native (●), $Arg^{358} \rightarrow Gln$ (□), $Arg^{347} \rightarrow His$ (◯) and $Arg^{449} \rightarrow Ala$ (△). Each data point is the mean of three values ± S.E.

and the $Arg^{358} \rightarrow Gln$ mutant but is slightly reduced for the $Arg^{449} \rightarrow Ala$ and $Arg^{347} \rightarrow His$ variants.

Fig. 2, bottom, shows the effect of varying the concentration of cytochrome b_5 on the lyase activity of the wild-type CYP17 and its arginine mutants. In these experiments the concentration of CYP17 and its derivatives, as well as that of the reductase were fixed, while the molar ratios of cytochrome b_5 to CYP17 were varied from 0 to 20. The figure clearly shows that the mutants which were found to lack the lyase activity under the standard assay system, when the ratio of cytochrome b_5 to CYP17 was 5 (Fig. 1), were also totally inactive when the ratio was increased to 20. Attention should be drawn to the biphasic nature of the profile for the wild-type CYP17 in Fig. 2, bottom, when increasing concentration of cytochrome b_5 progressively stimulated the lyase activity but then began to inhibit at 20 μM concentrations. The simplest explanation of this inhibition is the possibility of the partitioning of the reductase between CYP17 and cytochrome b_5, through the formation of a reductase·cytochrome b_5 binary complex; it is known that the Fe^{III} form of cytochrome b_5 is reduced by the reductase, in the presence of NADPH [33]. Another possibility is that the reductase and cytochrome b_5 occupy the same or overlapping sites on CYP17 and the presence of higher concentrations of cytochrome b_5 impairs the formation of the CYP17·reductase complex, a mandatory requirement for the catalytic cycle. Irrespective of which of these mechanisms operate the overall consequence is the same; a decrease in the steady state concentration of the CYP17·reductase complex.

These considerations also point to the obvious conclusion that the initial stimulation of the cleavage activity for the wild-type CYP17 (Fig. 2, bottom) must be the net consequence of two factors: (i) the activation of CYP17, for cleavage, by cytochrome b_5 and (ii) the involvement of cytochrome b_5 in decreasing the availability of the reductase for electron transfer to the P450. The latter feature is more clearly demonstrated by studying the deformylation of the aldehyde analogue (**21**); a cleavage reaction which is

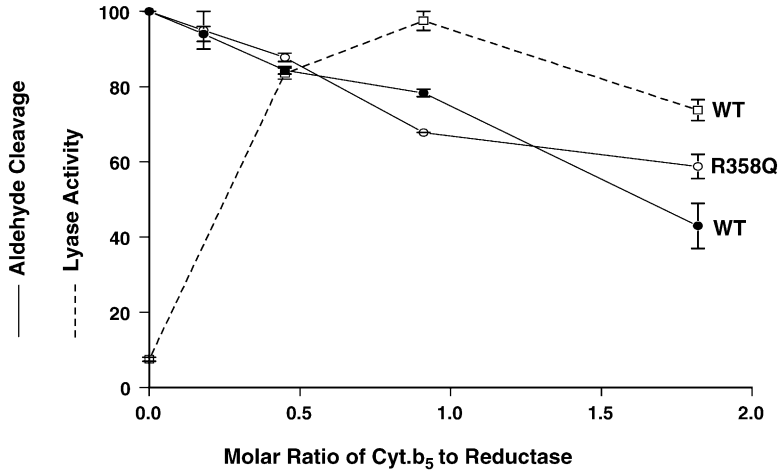


Fig. 3. The effect of increasing molar ratio of cytochrome b_5 to NADPH-cytochrome P450 reductase on aldehyde deformylation. Experiments were performed as described in Section 2.4 but using increasing quantities of cytochrome b_5 (0, 0.5, 1.0 and 2.0 μM) with fixed amounts of CYP17 (0.1 μM) and reductase (1.1 μM). Activities are expressed as a percentage of the maximum wild-type activity for each cleavage reaction. Each data point is the mean of three values ± S.E.

not dependent on cytochrome b_5 and is *not* significantly impaired by the arginine mutants (Fig. 1).

Fig. 3 shows that, in the presence of fixed amounts of CYP17 and reductase, the deformylation reaction catalysed by both the wild-type CYP17 and also the mutant, $Arg^{358} \rightarrow Gln$, was progressively inhibited with increasing concentration of cytochrome b_5. That the $Arg^{358} \rightarrow Gln$ mutant is totally devoid of the two cytochrome b_5-dependent side chain cleavage activities, **17** → **18** and **16** → **19** + **20**, indicates that this mutant is unable to make a catalytically productive interaction with cytochrome b_5. Yet, in the deformylation assay, the mutant showed the same sensitivity, as the wild-type enzyme, to the increasing concentrations of cytochrome b_5. This indicates that the observed inhibition is likely to occur by a mechanism which does not involve a direct, productive interaction of cytochrome b_5 with CYP17, but rather by the propensity of cytochrome b_5 to complex with the reductase and so compete out electron transfer to CYP17.

4. Discussion

CYP17 is endowed with two catalytic activities, hydroxylation at C-17 and an acyl-carbon bond cleavage which must entail the attack of an activated oxygen species at the neighbouring C-20 of the C_{21} progestogen substrate molecules. There are physiological as well as chemical reasons for carefully modulating these activities. Chemically CYP17 is designed to catalyse the hydroxylation reaction and so must direct the relevant iron-oxygen ligand towards C-17. The change in the direction of attack, for side chain cleavage, would then depend on either the inherent flexibility in the active site region of the enzyme or the participation of an external agent. It is, however, the physiological need to ensure that both the reactions catalysed by CYP17 occur in the testes, ovaries and the adrenal zona reticularis, while the hydroxylation reaction is the only predominant event in the adrenal zona fasciculate [34], that necessitates that the modulation of these activities are achieved through the intervention of a regulatory molecule. We and others have proposed this regulatory molecule to be cytochrome b_5 [18,19]. Indirect support for this hypothesis comes from studies on patients with Cushing's syndrome, where it has been shown that those patients who produced large amounts of adrenal androgens also expressed increased amounts of cytochrome b_5 in their adenomas [35–37]. High levels of cytochrome b_5 in human testis have also been shown [38].

Cytochrome b_5 has a range of diverse effects on various P-450s but the molecular basis of these effects, which are often small, is not fully understood [39–47]. The protein is a substrate for NADH-cytochrome b_5 reductase [39–43,46] and plays a clear cut, electron transfer role for certain oxidases, for example in fatty acid desaturation [41,48]. The possibility has been considered that when cytochrome b_5 affects the behaviour of P-450s this may be due to an enhanced efficiency of electron transfer to a key iron-oxygen species involved in the catalytic cycle [46,49,50]. Evidence against a role for cytochrome b_5 participating directly in electron transfer is provided by work from Guengerich's laboratory; his group showed that redox inactive *apo*-cytochrome b_5 could still stimulate P4503A4 catalysed hydroxylation of testosterone [51]. However, a recent report suggests this observation is due to rapid haem transfer reactions within the *apo*-cytochrome b_5:P450:P450 reductase:lipid reconstitution assay mixture, leading to the formation of redox active *holo*-cytochrome b_5 [52]. Our results with the aldehyde analogue **21** provide a forceful argument against the direct involvement of cytochrome b_5 in an electron transfer process during the side chain cleavage catalysed by CYP17. The deformylation of the aldehyde analogue occurs by the same chemical mechanism as the side chain cleavage of the two physiological

substrates [26]. The former process occurs at over four-times the rate of any oxidase reaction catalysed by CYP17, yet this transformation is not dependent on the presence of cytochrome b_5. The oxidative cleavage of the C-17–C-20 bond, therefore, does not depend on the electron transfer properties of cytochrome b_5.

We have proposed that cytochrome b_5 promotes the lyase activity of CYP17 by facilitating the nucleophilic attack of the iron-peroxide anion (**8**) onto the C-20 carbonyl by initiating a conformational change in CYP17, that results in the juxtaposition of the iron-oxygen species and the C-20 carbonyl (Scheme 4). The resulting adduct, **11**, decomposes to furnish the side chain as acetic acid and the ketone functionality at C-17 (**17** → **18**, Scheme 3). The role for a *nucleophilic* iron-peroxide anion intermediate (**8**) in acyl-carbon cleavage was originally highlighted by our studies on aromatase (CYP19). It was found that the third reaction catalysed by aromatase, in the biosynthesis of oestrogens, is attended by the incorporation of an atom of oxygen from O_2 into the released formic acid [9,53]. This was rationalised by invoking the intermediary of an adduct, formed by the reaction of the 19-aldehyde with an iron-peroxy anion, Fe^{III}–O–O^- [10]. The latter is an acknowledged precursor of the hydroxylating agent, the oxo-derivative [54]. In subsequent studies on the acyl-carbon cleavage catalysed by CYP17 [17,26,55] and CYP51 [14,15], the analogous incorporation of an atom of oxygen into the expelled carboxylic acid was shown. A strong support for the involvement of a Fe^{III}–O–O^- species in the cleavage reaction (Eq. (2)) was provided by the finding that the genesis of 17α-hydroxyandrogen (**20**) from pregnenolone (**16**) involves the cleavage of only one bond of the latter, C-17–C-20, and leads to the incorporation of an atom of oxygen into each of the two products, steroid and acetic acid [17,26]. The process, then, can be regarded as a *dioxygenase* reaction and can only be rationalised by invoking the participation of a peroxy species such as Fe^{III}–O–O^-. Such a species has been detected by ESR following radiolytic reduction [56]. Further endorsement of the role of Fe^{III}–O–O^- has been provided by the studies of Coon and Vaz, who found that certain liver P-450s, normally involved in the hydroxylation of xenobiotics, could catalyse an acyl-carbon cleavage (Eq. (2)) when challenged with aldehydic substrates, under conditions when the peroxy species was the predominant oxidant [57–59].

The lack of dependence on cytochrome b_5 for the deformylation of the aldehyde (**21**) may be attributed to two factors: (i) the carbonyl group of the aldehyde is more reactive towards a nucleophilic attack than is that of the ketone in the physiological substrate and (ii) the 21-methyl of the physiological substrates makes important contribution to the binding interaction with CYP17 and its absence in the aldehyde analogue may allow a free rotation of the C-17–C-20 bond, thus permitting the trapping of the Fe^{III}–O–O^- species by its carbonyl group. Together, these two factors eliminate the need for the proposed conformational changes normally promoted by cytochrome b_5, when the aldehyde (**21**) is the substrate, as is

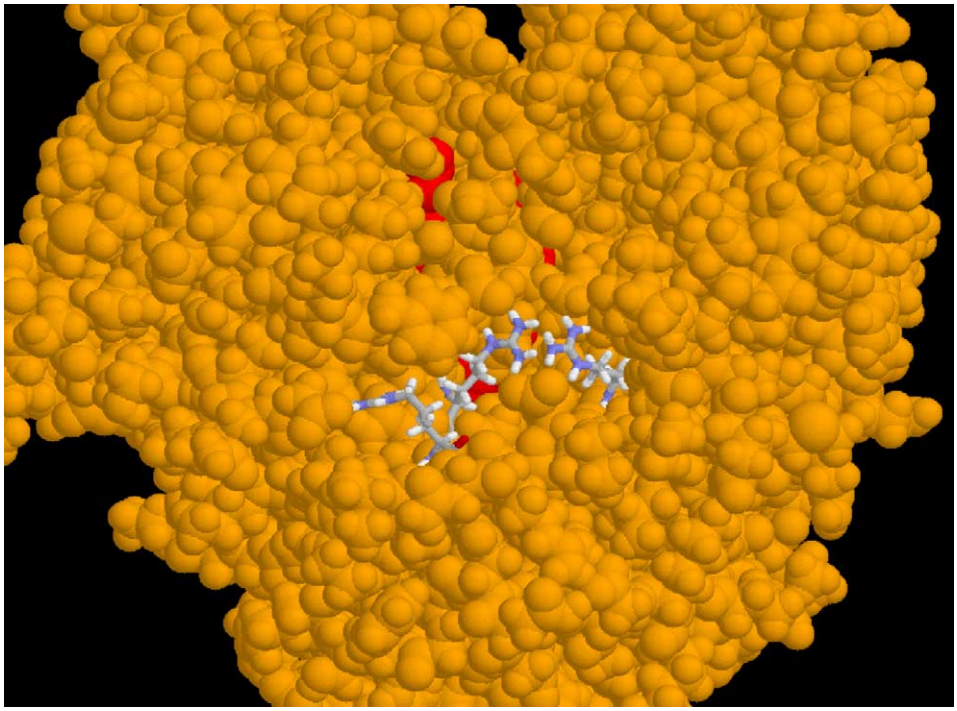


Fig. 4. Proximal face of human CYP17 (PDB code 2c17) [60] showing the cationic side chains of Arg^{347}, Arg^{358} and Arg^{449} exposed to the surface. The side chains are depicted in their stick form with the guanidinium nitrogen atoms coloured blue and clearly visible at the protein surface. The haem group is coloured red and can be partially seen below the arginine cluster.

shown by the fact that mutants of CYP17, unresponsive to cytochrome b_5, retained 47–65%, of the activity of the wild-type enzyme for the deformylation reaction (cf. B and C with D, Fig. 1).

Of course we cannot eliminate the possibility that cytochrome b_5 binding may promote conformational change in CYP17 that could be responsible for reducing the efficiency of proton delivery to the iron-oxygen species and so may result in an increase in the half-life of the iron-peroxide anion. This may increase the likelihood of this iron-peroxy species becoming trapped into nucleophilic attack onto the electrophilic C-20 substrate carbonyl; the first step in the oxidative cleavage of the C-17–C-20 bond [17]. Alternatively, the hydroperoxo-iron species could be the intermediate that undergoes nucleophilic attack onto the substrate should its protonation (**7** → **8**) be more affected than the protonation of its more basic precursor (**6** → **7**). This appears to be the case for a variant of cytochrome $P450_{cam}$ where the active site threonine residue, T252, that is thought to be involved in proton delivery during catalysis, has been substituted with the aprotic side chain of alanine. Protonation of the iron-peroxide anion to yield the hydroperoxo-iron derivative was not hindered in this T252A mutant but the second protonation of the distal oxygen, requisite for the formation of the iron-oxo species, appears to be impaired [56].

Using the computer generated three-dimensional model of human CYP17, that is based on the X-ray crystal structure of cytochrome $P450_{bm-3}$ and constructed by Auchus [60], the three cationic residues, Arg^{347}, Arg^{358} and Arg^{449}, that we have shown here to be essential for the cytochrome b_5-dependent activities of human CYP17, were found to be clustered at the surface of the proximal face of the protein, one in each of the *J′*-, *K*- and *L*-helices, with their bifurcated cationic guanidinium side groups projecting out from the protein surface (Fig. 4). Such a conformation would facilitate their ionic interaction with anionic carboxylate side groups on cytochrome b_5. Using linear amino acid-sequence alignments, Arg^{449} can be mapped to Arg^{443} and Arg^{364} in cytochromes P4502B4 and $P450_{cam}$, respectively; the latter two cationic residues have been implicated in forming electrostatic interaction with NADPH-cytochrome P450 reductase and cytochrome b_5, respectively [61,62]. Arg^{358} in human CYP17 can be aligned with Lys^{381} in cytochrome $P450_{scc}$, which is one of the cationic residues in this mitochondrial P450 found to interact with its redox partner, adrendoxin [63].

5. Conclusion

The results suggest that the bifurcated cationic charge at Arg^{347}, Arg^{358} and Arg^{449} makes important contribution to the formation of catalytically competent CYP17·cytochrome b_5 complex. The mutations of these residues have similar effects on all the cytochrome b_5-sensitive parameters, in particular the two cleavage reactions which heavily depend on cytochrome b_5 (**17** → **18** and **16** → **19** + **20**). Not only is the chemistry for cleavage the same for the two reactions, but also is the need to direct the iron-oxygen ligand of the enzyme to the same C-20. According to our earlier work the ligand is a peroxo-iron species which makes a nucleophilic attack [26], with CYP17 acting like a dioxygenase. We believe that the main effect of the binding of cytochrome b_5 to CYP17 is to regulate the geometry of this attack. Cumulatively, the engineering of mutants of CYP17, insensitive to the effect of cytochrome b_5, further support of our original hypothesis that the latter may be the regulatory substance through which side chain cleavage activity is physiologically modulated for androgen biosynthesis.

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