Lysine mutagenesis identifies cationic charges of human CYP17 that interact with cytochrome b_5 to promote male sex-hormone biosynthesis

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Human CYP17 (17α-hydroxylase-17,20-lyase; also cytochrome P450c17 or cytochrome $P450_{17\alpha}$) catalyses a hydroxylation reaction and another reaction involving the cleavage of a C–C bond (the lyase activity) that is required only for androgen production. Single amino acid mutations in human CYP17, $Arg^{347} \rightarrow$ His and $Arg^{358} \rightarrow$ Gln, have been reported to result in the loss of the lyase activity and to cause sexual phenotypic changes in 46XY male patients. By using site-directed mutagenesis we show here that another mutation in human CYP17, $Arg^{449} \rightarrow$ Ala, for which human variants have yet not been described, also leads to selective lyase deficiency. Furthermore, all the three types of mutants display a loss of responsiveness to cytochrome b_5, an interaction that is essential for lyase activity, and hence male sex-hormone biosynthesis. That the defect could be essentially reversed by lysine mutagenesis has led to the conclusion that the cationic charges on all three residues (at the positions of Arg^{347}, Arg^{358}, Arg^{449}) are vital for the functional interaction of CYP17 with cytochrome b_5 and that the loss of any one of these cationic charges is catastrophic.

Key words: CYP17, cytochrome b_5, mutagenesis, P450c17.

INTRODUCTION

The rapid and precise regulation of biochemical processes by hormones needs to be under stringent and fail-safe control. Similarly, the biosynthesis of hormones would be expected, to occur by specifically targeted mechanisms, as is generally true. One important exception to this model is the manner in which the carbon skeleton of progestogens (**1**, Scheme 1) is directed either towards corticoid or male sex-hormone biosynthesis, by processes that include the involvement, in a dual role, of CYP17 (17α-hydroxylase-17,20-lyase; also cytochrome P450c17 or cytochrome $P450_{17\alpha}$) [1]. The two types of activity (**1** $\rightarrow$ **2** and **2** $\rightarrow$ **3**, Scheme 1) associated with CYP17 have been hypothesized to depend on the use of two distinct iron–oxygen species; a high-valency oxo-derivative $[(^{+\cdot})Fe^{IV}=O \leftrightarrow Fe^{IV}–O^{\cdot} \leftrightarrow Fe^{V}=O]$ for the hydroxylase activity (**1** $\rightarrow$ **2**, Scheme 1) and an iron-peroxide anion, $Fe^{III}–O–O^{-}$, participating in the cleavage process (the lyase activity; **2** $\rightarrow$ **3**, Scheme 1) [2]. Since its inception in the early 1980s [3] the hypothesis (reviewed in [4,5]) has received increasing support from a wide range of studies [2,6–10].

During the course of our work on the elucidation of the chemical mechanism that links these apparently unrelated reactions, it was discovered that with the human isoform of CYP17 the lyase reaction was heavily dependent on the presence of the membrane-bound form of cytochrome b_5 [11], as has been reported simultaneously by others [12]. To explore the nature of the interactions between CYP17 and cytochrome b_5, several mutant forms of CYP17 had been engineered and subjected to detailed kinetic and mechanistic analysis. It was found that mutations of certain arginine residues of human CYP17, in particular Arg^{347} and Arg^{358} found in male patients suffering from genital ambiguity [13], markedly affected the lyase reaction [13,14] with comparatively little effect on the hydroxylation process [14]. We now report that mutation of another arginine residue of human CYP17, namely Arg^{449}, also results in a protein having selective impaired lyase activity. More significantly, we show that the molecular basis underlying this selective lyase deficiency displayed by all the three mutant CYP17 proteins is the same and involves the loss of essential cationic charges that are required for the interaction of CYP17 with cytochrome b_5.

EXPERIMENTAL

Site-directed mutagenesis, protein expression and purification

A plasmid, pCWH17mod [15], in which the cDNA of human *CYP17* was modified at its 5′ end to allow expression in *Escherichia coli* and also at the 3′ end to facilitate the purification of the enzyme, was a gift from Professor M. R. Waterman (Vanderbilt University, Nashville, TN, U.S.A.) and used to obtain human CYP17 to a specific P450 content of 10 nmol/mg of protein [8]. The CYP17 mutants were constructed by PCR-site-directed mutagenesis, the various proteins being expressed and purified as described elsewhere [16]. The mutant proteins were between 80% and 95% pure as judged by PAGE and contained between 6.5 and 9.7 nmol of P450/mg of protein. These values are lower than the theoretical P450 content of 18 and have been attributed [11] to a variable loss of the non-covalently bound haem that occurs during purification and to the fact that the preparations were not 100% homogeneous.

Enzyme assays

NADPH:cytochrome P450 reductase (specific activity 42 units/mg of protein; 1 unit is defined as 1 μmol of cytochrome *c* reduced/min at 35 °C in 375 mM potassium phosphate buffer, pH 7.7) and cytochrome b_5 (specific content 43.4 nmol of haem/mg of protein) were purified and enzyme assays were performed as described previously [11]. In brief, two different principles of radiochemical assay were employed: the first was based on monitoring the release of [^{3}H]acetate from [21-^{3}H]17α-hydroxypregnenolone (specific radioactivity 5.6×10^4 d.p.m./nmol) into the aqueous medium and was used to measure the CYP17 lyase activity (**2** $\rightarrow$ **3**, Scheme 1); the second was based on the analysis of radiolabelled steroidal product, [7-^{3}H]17α-hydroxypregnenolone, from [7-^{3}H]pregnenolone (specific radioactivity 4.4×10^5 d.p.m./nmol), by TLC analysis. In the standard protocol,

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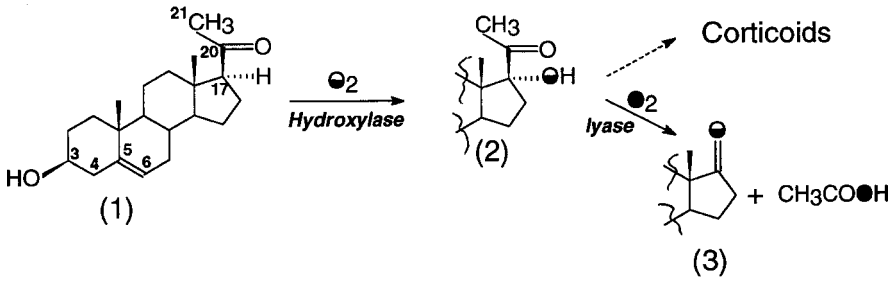

Scheme 1 The two reactions catalysed by CYP17

Series **a** steroids (**1a**, **2a**) are as above; in series **b** steroids (**1b**, **2b**), C-3 contains a carbonyl group and there is a double bond between C-4 and C-5. The characteristics of the two reactions are emphasized by the status of oxygen atoms [24]: the C-20 carbonyl oxygen is shown unfilled; the oxygen molecules depicted as half-filled and filled are used in reactions **1** → **2** and **2** → **3** respectively. Structures **3a** and **3b** are the androgens dehydroepiandrosterone and androstenedione respectively.

radiolabelled substrate (30 nmol, in 1–2 μl of dimethylformamide), dilauroyl L-α-phosphatidylcholine (80 μg), NADPH:cytochrome P450 reductase (3.5 units, 1.1 nmol), CYP17 (0.1 nmol), sodium phosphate buffer (50 mM, pH 7.2, containing 0.1 mM EDTA) and, when present, cytochrome b_5 (0.5 nmol) were mixed and reconstituted in 200 μl. The reconstituted mixture was preincubated at 37 °C for 1 min and the reaction was started by the addition of 800 μl of the preincubated buffer containing 3 mg of NADPH. The assays were terminated, worked up and analysed as described previously [11].

Measurement of K_s (dissociation constant) values

17α-Hydroxypregnenolone-binding spectra were recorded and K_s values were calculated as described previously [16].

Calculations of effect of cytochrome b_5

The effect of cytochrome b_5 on the two activities of the mutant human CYP17 proteins, shown in Table 1 as percentages of that displayed by the wild-type, were calculated as follows: for the hydroxylase activity, (fold stimulation $-1) \times 100/1.42$; for the lyase activity, (fold stimulation $-1) \times 100/9.92$. The factors of 1.42 and 9.92 are the fold stimulations of the wild-type activity by cytochrome b_5 minus one, calculated as $(3.36/1.39)-1 = 1.42$ and $(2.73/0.25)-1 = 9.92$.

This parameter allows the magnitude of cytochrome b_5-dependent stimulation of CYP17 activity, above that of the basal level, to be expressed as a percentage of that displayed by the wild-type enzyme and thus permits easy comparisons to be made between CYP17 variants.

RESULTS

Central to the understanding of the results presented here is a knowledge of some of the properties of the human isoform of CYP17. The enzyme uses the 3β-hydroxy-Δ^5 as well as the 3-keto-Δ^4 steroids, pregnenolone (**1a**) and progesterone (**1b**) respectively, as substrates. The kinetic parameters for the hydroxylation reaction are almost identical for the substrates in the two series [11]. However, the lyase reaction, in terms of K_{cat}/K_m, is 6-fold more favourable for 17α-hydroxypregnenolone (**2a**) than for 17α-hydroxyprogesterone (**2b**) [11]. In the present study, kinetic analysis was performed with substrates in the pregnenolone series because under the optimal conditions for each reaction these were catalysed at similar rates. This ensured that the two processes required similar rates of electron transfer for the reduction of O_2. The results shown in Table 1 confirm [14] that the hydroxylation reaction, which occurred at a good rate in the absence of cytochrome b_5, was stimulated 2.4-fold in its presence. In contrast, the relatively low basal lyase reaction was stimulated by approx. 11-fold in the presence of cytochrome b_5 to a level reaching almost that of the hydroxylation process, as reported previously [12,14,16].

Four of the entries in Table 1, from [14], highlighted that the $Arg^{347} \rightarrow Ala$, $Arg^{347} \rightarrow His$, $Arg^{358} \rightarrow Ala$ and $Arg^{358} \rightarrow Gln$ mutants (Table 1, entries 2, 3, 5 and 6 respectively), in the absence of cytochrome b_5, had similar hydroxylase activities to that of the wild-type CYP17. This shows that under the assay conditions, in which 0.1 μM P450 and 1.1 μM NADPH:cytochrome P450 reductase were present, the electron transfer process was not impaired in the mutants. The mutants, however, did not display the cytochrome b_5-dependent enhancement of the hydroxylase activity. The most marked aspects of these mutants was the low lyase activity, which was resistant to stimulation by cytochrome b_5. The effect of cytochrome b_5 on the two disease-state mutants, $Arg^{347} \rightarrow His$ and $Arg^{358} \rightarrow Gln$, was subsequently studied in yeast microsomes, where it was also found that these mutants had low lyase activities in the presence of cytochrome b_5 but that after increasing the molar excess of cytochrome b_5 to 10-fold and then 30-fold a partial restoration of activity was observed [17]. In our standard protocol the optimized concentrations of the three proteins were 0.1 μM CYP17, 1.1 μM NADPH:cytochrome P450 reductase and 0.5 μM cytochrome b_5 (giving molar ratios of 1:11:5). When the amount of cytochrome b_5 was progressively increased to a final concentration of 2.0 μM (to yield a molar ratio of CYP17 to reductase to cytochrome b_5 of 1:11:20), no significant increase in the lyase activity of the wild-type or disease-state mutants was observed (Figure 1). The titration experiments show that with the relatively high concentrations used in the standard protocol, a 1:5 molar ratio of P450 to cytochrome b_5 was sufficient to ensure that CYP17 was predominantly present in a complexed state. The discrepancy between our results and those recently reported and discussed above [17] cannot be explained at this stage because the authors reported only the molar ratios of the proteins, without specifying their concentrations.

We now show that the mutagenesis to alanine of another arginine residue, $Arg^{449} \rightarrow Ala$, also yielded a protein that expressed a similar level of hydroxylase activity to that of the wild-type enzyme but was not significantly stimulated by the presence of cytochrome b_5. The most significant feature of the resistance to cytochrome b_5 was a negligible lyase activity (Table 1, entry 8).

Table 1 Hydroxylase (1 → 2, Scheme 1) and lyase (2 → 3, Scheme 1) activities of wild-type and mutant CYP17

The enzyme assays were performed in triplicate; results are expressed as means ± S.E.M. The b_5 effect is calculated as described in the Experimental section. The results in entries 2, 3, 5 and 6 have been published previously [14].

			Hydroxylase		Lyase	
Entry	CYP17 variant	Cytochrome b_5	Activity (nmol/min per nmol of P450)	Cytochrome b_5 effect (%)	Activity (nmol/min per nmol of P450)	Cytochrome b_5 effect (%)
1	Wild-type	−	1.39 ± 0.10	–	0.25 ± 0.03	–
		+	3.36 ± 0.20	100	2.73 ± 0.07	100
2	Arg^{347} → Ala	−	1.70 ± 0.01	–	0.10 ± 0.02	–
		+	2.09 ± 0.01	16	0.11 ± 0.01	1
3	Arg^{347} → His	−	1.55 ± 0.07	–	0.11 ± 0.01	–
		+	1.62 ± 0.02	3	0.14 ± 0.01	3
4	Arg^{347} → Lys	−	1.57	–	0.16 ± 0.02	–
		+	3.50 ± 0.20	87	0.98 ± 0.04	52
5	Arg^{358} → Ala	−	1.24 ± 0.14	–	0.09 ± 0.02	–
		+	1.56 ± 0.03	18	0.11 ± 0.02	2
6	Arg^{358} → Gln	−	1.44 ± 0.06	–	0.07 ± 0.01	–
		+	1.56 ± 0.02	6	0.08 ± 0.01	1
7	Arg^{358} → Lys	−	1.64 ± 0.05	–	0.27 ± 0.02	–
		+	3.58 ± 0.13	84	2.70 ± 0.07	91
8	Arg^{449} → Ala	−	1.22 ± 0.02	–	0.12 ± 0.01	–
		+	1.45 ± 0.04	13	0.13 ± 0.01	1
9	Arg^{449} → Lys	−	1.60 ± 0.08	–	0.27 ± 0.01	–
		+	2.70 ± 0.10	49	0.78 ± 0.03	19

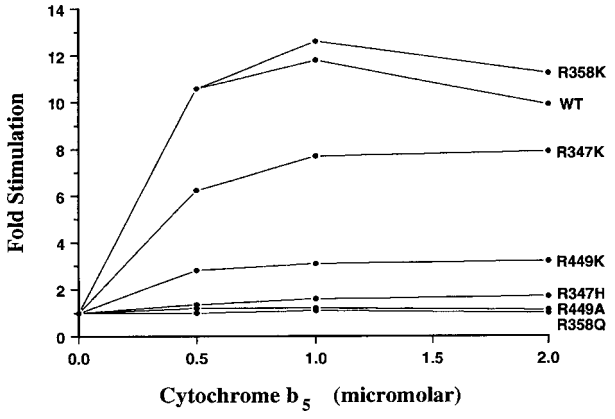

Figure 1 Effect of various concentrations of cytochrome b_5 on the lyase activity of 0.1 μM CYP17 proteins

Enzyme assays were performed in duplicate as described in the Experimental section. Mutants are identified by one-letter amino acid codes. Abbreviation: WT, wild type.

Table 2 Spectrally determined 17α-hydroxypregnenolone (2a)-binding constants for wild-type and mutant human CYP17

Results are means of two independently determined binding constants.

CYP17 variant	K_s (μM)
Wild-type	1.22
Arg^{358} → Ala	1.66
Arg^{358} → Gln	1.57
Arg^{358} → Lys	1.40
Arg^{347} → Ala	1.39
Arg^{347} → His	1.90
Arg^{347} → Lys	2.11
Arg^{449} → Ala	1.10
Arg^{449} → Lys	1.05

When the results on the alanine and disease-state mutants are analysed in terms of the effect of cytochrome b_5, the mutant enzymes displayed 3–18 % of the effect on the wild-type CYP17 for the hydroxylase activity and approx. 1–3 % for the lyase reaction. Thus the main defect in these proteins was the lack of responsiveness of their two activities to cytochrome b_5. Spectrally determined K_s values (Table 2) provided the assurance that the impairment of the catalytic activities was not due to altered substrate affinity. That the efficiency of electron transfer to CYP17 from NADPH:cytochrome P450 reductase had not been impaired by the mutations is shown by the fact that the level of hydroxylase activity displayed by the wild-type protein in the absence of cytochrome b_5 was essentially retained by all the CYP17 variants. Furthermore, we have previously demonstrated that a cleavage reaction with a pseudo-substrate, which uses the same chemistry as that involved in the removal of the side chain of pregnenolone (the lyase activity), is catalysed by mutant proteins at rates exceeding those found for the physiological reaction catalysed by the wild-type enzyme [14]. This indicates that the rate of electron transfer from the reductase to the CYP17 mutants is not rate-limiting for the cleavage reaction. Cumulatively, these results can be interpreted to suggest that the optimization of catalysis brought about by the interaction of cytochrome b_5 with CYP17 is greatly dependent on the presence of Arg^{347}, Arg^{358} and Arg^{449} that is lost by the aforementioned mutations.

Of all the different substitutions attempted in these positions (alanine, histidine, glutamine and lysine) those with lysine gave the most interesting results (Table 1, entries 4, 7 and 9). The Arg^{358} → Lys mutant showed the largest recovery; in the presence of cytochrome b_5 both the activities were stimulated to similar extents, as observed for the wild-type CYP17. With the Arg^{347} → Lys mutant, the cytochrome b_5-dependent component of the hydroxylase activity recovered to approx. 90 % and the lyase

activity to approx. 50 % of the wild-type values. The $Arg^{449} \rightarrow$ Lys protein also regained sensitivity to cytochrome b_5 but the effect on lyase activity was a more modest 19 %; the enzyme was nonetheless a fair catalyst for the lyase reaction (displaying 29 % of the maximal lyase activity achieved by the wild-type enzyme; the alanine mutant displayed barely 1 % of the cytochrome b_5 effect).

DISCUSSION

Previous studies on human patients [13] as well as laboratory manipulations of the human CYP17 gene [14] have identified Arg^{347} and Arg^{358} as important residues that are involved in the expression of the lyase activity of the enzyme. Here a third arginine residue, Arg^{449}, has been shown to perform a similar role. The key feature of the results in Table 1 is that the two disease-state mutants (entries 3 and 6, Table 1) and their counterparts substituted with alanine, as well as the new $Arg^{449} \rightarrow$ Ala mutant, showed similar hydroxylase activities to that of the wild-type protein. However, unlike the latter protein, the mutants did not show an increase in the hydroxylase activity in the presence of cytochrome b_5. Most of the lyase activity of the wild-type human CYP17 is dependent on cytochrome b_5; the main defect in these mutants is their lack of responsiveness to the stimulatory effect of cytochrome b_5. It is therefore the cytochrome b_5-dependent component of the two activities that is impaired in these mutants.

The main discovery in this study, that all three Arg $\rightarrow$ Lys mutants regained sensitivity to cytochrome b_5, strongly suggests that the modulation of the active-site chemistry of human CYP17 by cytochrome b_5 is predominantly dependent on ionic interactions between the two proteins, in which the cationic arginine residues at positions 347, 358 and 449 of CYP17 are involved. In the X-ray crystal structure of $P450_{bm\text{-}3}$ [18], the residues corresponding to the three arginine residues of human CYP17 are suitably located at the proximal face of the haem, for interaction with negatively charged proteins such as cytochrome b_5. Regarding the mechanism, we have previously proposed that CYP17's productive interaction with cytochrome b_5 positions the sensitive C-20 atom of the substrate (C-20 carbon of structure **2**, Scheme 1) for trapping by the iron-peroxide anion, so that it is directed to the cleavage path (**2** $\rightarrow$ **3**, Scheme 1) and hence sex-hormone biosynthesis (see Scheme 4 in [11]). However, information on the precise iron–oxygen chemistry underpinning the modulatory effect of cytochrome b_5 is not important to the main theme of the present paper.

It should be noted that the mutation of residues, however benign the substitution, can alter the structure of proteins in a microscopic sense, thus affecting their catalytic behaviour in an unpredictable manner. It is this uncertainty, inherent in site-directed mutagenesis experiments, that makes it problematic to estimate separately the contribution made to the overall effect by (1) the microscopic structural change in the protein and (2) the substitution of an amino acid pre-ordained to perform a specific role. In view of this difficulty we make no attempt to conjecture which of the three cationic sites Arg^{347}, Arg^{358} or Arg^{449} makes the largest contribution to the interaction of human CYP17 with cytochrome b_5. However, from the lower recovery of the cytochrome b_5 effect, shown by the $Arg^{449} \rightarrow$ Lys mutant, it seems that Arg^{449} of CYP17 might be important not only for its cationic character but also for engaging in a bidentate interaction with a complementary site on cytochrome b_5, a role that cannot be fulfilled by a lysine residue. More importantly, all three cationic residues are essential for the correct docking of the protein to cytochrome b_5: the loss of any one of them is catastrophic.

The original hypothesis that cytochrome b_5 modulates the lyase activity of human CYP17 [11,12] has received indirect support from studies on patients with Cushing's syndrome [19,20] and from other recent enzymological studies [14,21,22]. The results described above, when taken in conjunction with the low level of male sex-hormone synthesis found in human patients with $Arg^{347} \rightarrow$ His or $Arg^{358} \rightarrow$ Gln mutations in CYP17 [13], raise the serious possibility that the defect in these disease-state proteins might also be due to the impairment of their interaction with cytochrome b_5; the phenomenon described here is therefore physiologically significant.

In conclusion, in living systems a wide spectrum of mechanisms exist for the regulation of biochemical pathways as well as the activities of enzymes. In some cases the biological goal is realized by the interaction of a protein with a specially designed ligand; in others, such as that considered here or that recently reported for a plant hydroxylase system [23], an otherwise abundant molecular entity (cytochrome b_5) is recruited by a protein and exploited equally effectively.

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