RESEARCH COMMUNICATION

Control of androgen biosynthesis in the human through the interaction of Arg³⁴⁷ and Arg³⁵⁸ of CYP17 with cytochrome *b*₅

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The lyase activity of human CYP17 (17 α -hydroxylase-17,20-lyase also *P*-450c17 or *P*-450_{17 α}) is greatly dependent on the presence of cytochrome *b*₅, and this effect has been ascribed an important regulatory role [Lee-Robichaud, Wright, Akhtar and Akhtar (1995) *Biochem. J.* **308**, 901–908]. This facet was further investigated by site-directed mutagenesis of selected basic residues of human CYP17. The purified mutant proteins were subjected to detailed kinetic analysis. It was found that the mutation of

Lys⁸³, Arg³⁴⁷ and Arg³⁵⁸ produced proteins that were deficient in their responsiveness to cytochrome *b*₅, and the effect was most pronounced for the two arginine mutants (Arg³⁴⁷ → His and Arg³⁵⁸ → Gln) which have been found in male patients suffering from genital ambiguity. These residues are invoked to mediate protein–protein interaction between cytochrome *b*₅ and CYP17, which ‘awakens’ the lyase activity of the enzyme required for androgen formation.

INTRODUCTION

The multifunctional enzyme 17 α -hydroxylase-17,20-lyase (CYP17) lies at the crossroads of androgen and corticoid biosynthesis. It is endowed with two activities [1], promoting hydroxylation and side-chain cleavage, as represented by eqns. (1) and (2) respectively

The hydroxylation reaction, pregnenolone (1) → 17 α -hydroxypregnenolone (2) (1 → 2, see Scheme 1 in [2]) is common to corticoid and androgen biosynthesis, whereas the side-chain cleavage activity, 17 α -hydroxypregnenolone (2) → dehydroepiandrosterone (3) (lyase reaction 2 → 3, see Scheme 1 of [2]) is required only for the formation of androgens.

Figure 1 Schematic diagram showing the effect of cytochrome *b*₅ on the orientation of the iron-dioxygen ligand in the CYP17–17 α -hydroxypregnenolone complex

The rearrangement prepares the iron-peroxide species for a nucleophilic attack at C-20.

There is now a general consensus that the hydroxylation reaction by multifunctional *P*-450s, a class to which CYP17 belongs, is catalysed by an iron-monooxygen species, Fe^V=O or its equivalent [3–5], and the cleavage shown in eqn. (2) through the participation of an iron-peroxide intermediate (Fe^{III}-OO[•]) [6–10]. Our recent studies using homogeneous preparations of several CYP17s have shown that with the human isoform the lyase reaction is dependent on the presence of the membrane-bound form of cytochrome *b*₅, *M*_r 15 224 [11,12]. The experiments have led to the proposal that the interaction of cytochrome *b*₅ with the CYP17–17 α -hydroxypregnenolone complex causes protein conformational changes which culminate in the optimal juxtaposition of the peroxide iron with respect to C-20 of the substrate (Figure 1). The ensuing nucleophilic attack at the carbonyl group then produces a tetrahedral intermediate that follows a side-chain cleavage path [11].

In order to probe this hypothesis we have now performed the mutagenesis of basic amino acids of CYP17 which may interact with redox partners. The basic residues targeted included those which, from the X-ray structure of *P*-450_{BM3} [13], have been found in or immediately adjacent to B, C, J' and K helices. Together these helices form part of a depression on the proximal face of the haem (the face containing the thiolate ligand) that may function as a docking site for the redox partners NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅ [13].

Our results show that the mutation of residues Arg³⁴⁷ and Arg³⁵⁸ of human CYP17 produces proteins which are not responsive to activation by cytochrome *b*₅ and hence display loss of cytochrome *b*₅-dependent lyase activity. The findings complement a recent study which found that male patients with Arg³⁴⁷ → His or Arg³⁵⁸ → Gln mutations had reduced androgen levels and suffered from genital ambiguity [14].

EXPERIMENTAL

PCR mutagenesis [2], expression and purification of CYP17 proteins [15], purification of porcine NADPH-cytochrome *P*-450 reductase [11,16] and porcine cytochrome *b*₅ [11,17] and *K*_s

Abbreviation used: CYP17, 17 α -hydroxylase-17,20-lyase.

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Table 1 Wild-type and mutant CYP17 hydroxylase (1 → 2), lyase (2 → 3) and carbaldehyde deformylation (4 → 5 + 6) activities (see Scheme 1 of [2] for structures 1–6)

Experiments were performed in triplicate with the data expressed as means $\pm$ S.E.M. The assays were performed and the percentage b_5 effect was calculated as described in the Experimental section. ND*, not determined (see text).

CYP17 variant	Activity (nmol/min per nmol P-450)					
	b_5	Hydroxylase	b_5 effect (%)	Lyase	b_5 effect (%)	Carbaldehyde (4) deformylation
Wild type	—	1.37 $\pm$ 0.10	—	0.27 $\pm$ 0.03	—	16.02 $\pm$ 0.36
	+	3.31 $\pm$ 0.20	100	2.95 $\pm$ 0.07	100	ND*
Lys ⁸³ → Ala	—	0.95 $\pm$ 0.01	—	0.05 $\pm$ 0.01	—	5.80 $\pm$ 0.44
	+	1.23 $\pm$ 0.05	21	0.27 $\pm$ 0.01	44	ND*
Lys ⁸⁸ → Ala	—	0.98 $\pm$ 0.03	—	0.11 $\pm$ 0.01	—	9.07 $\pm$ 0.09
	+	1.48 $\pm$ 0.04	36	0.74 $\pm$ 0.09	58	ND*
Lys ⁹¹ → Ala	—	1.37 $\pm$ 0.15	—	0.06 $\pm$ 0.01	—	10.74 $\pm$ 0.20
	+	2.13 $\pm$ 0.12	39	0.68 $\pm$ 0.06	104	11.30
Arg ¹²⁶ → Ala	—	1.26 $\pm$ 0.12	—	0.07 $\pm$ 0.02	—	9.07 $\pm$ 0.13
	+	2.25 $\pm$ 0.19	55	0.58 $\pm$ 0.06	73	ND*
Arg ³⁴⁷ → Ala	—	1.70 $\pm$ 0.01	—	0.10 $\pm$ 0.02	—	9.30
	+	2.09 $\pm$ 0.01	16	0.11 $\pm$ 0.01	1	ND*
Arg ³⁵⁸ → Ala	—	1.24 $\pm$ 0.14	—	0.09 $\pm$ 0.02	—	10.39
	+	1.56 $\pm$ 0.03	18	0.11 $\pm$ 0.02	2	ND*
Arg ³⁴⁷ → His	—	1.55 $\pm$ 0.07	—	0.11 $\pm$ 0.01	—	10.40 $\pm$ 1.0
	+	1.62 $\pm$ 0.02	3	0.14 $\pm$ 0.01	3	ND*
Arg ³⁵⁸ → Gln	—	1.44 $\pm$ 0.06	—	0.07 $\pm$ 0.01	—	7.50 $\pm$ 0.23
	+	1.56 $\pm$ 0.02	6	0.08 $\pm$ 0.01	1	ND*

measurements [2] were performed as described in the respective references. In the present study, however, greater attention was paid to ensure that the reductase preparation was completely free of contamination from cytochrome b_5 . This was necessary to observe the maximum stimulatory effect of cytochrome b_5 on the activities of CYP17. Enzymic assays were performed as described previously [2] but using a CYP17/reductase/cytochrome b_5 molar ratio of 1:13:5. The effect of cytochrome b_5 on the two activities of the mutant CYP17 proteins, shown in Table 1 as percentages of that seen in the wild type, were calculated as follows: (fold-stimulation – 1) $\times$ 100/1.42*, for the hydroxylase activity; (fold-stimulation – 1) $\times$ 100/9.93*, for the lyase activity. The asterisk (*) represents the fold-stimulation of wild-type activity by cytochrome b_5 minus one, calculated as (3.31/1.37) – 1 = 1.42 and (2.95/0.27) – 1 = 9.93

RESULTS AND DISCUSSION

A number of mutants for the redox-partner binding region of CYP17 were generated by PCR mutagenesis. Initially, the residues targeted for mutation were changed to alanine. However, the report published during the course of the work, that in two patients with sex hormone defects, the CYP17 contained Arg³⁴⁷ → His or Arg³⁵⁸ → Gln mutations [14], also prompted the preparation of proteins containing these modifications. Human CYP17 was expressed in *Escherichia coli* and purified to a specific content of 10 nmol P-450/mg of protein. This specific content compares favourably with other reported values of 10.7 for the human enzyme [18] and 8.0 for the pig enzyme [1]. Using the same method [15], the various mutant human CYP17 proteins were purified to between 80% and 95% homogeneity, and had specific contents ranging between 6.5 and 9.7 nmol P-450/mg of protein. These values are lower than the theoretical value of 18 because of variable loss of the non-covalently bound haem during the extreme environmental changes that occur during purification and because the preparations were not 100% homogeneous.

The enzymes were assayed, in the presence of NADPH, O₂ and NADPH-cytochrome P-450 reductase, for the hydroxylase (1 → 2, see Scheme 1 in [2]), lyase (2 → 3, see Scheme 1 in [2]) and a third activity involving the deformylation of 3 β -hydroxyandrost-5-ene-17 β -carbaldehyde (4) (4 → 5 + 6, see Scheme 1 in [2]), a specifically designed substrate analogue [15]. The deformylation of the carbaldehyde (4) occurs using the same basic chemistry that is involved in the side-chain cleavage of the physiological substrate (2), but this process is not dependent on cytochrome b_5 [15]. For the sake of economy, therefore, the kinetic experiments with the carbaldehyde (4) were performed only in the absence of cytochrome b_5 , after having confirmed that cytochrome b_5 had a negligible effect with the Lys⁹¹ → Ala mutant (5% stimulation, Table 1). Table 1 shows that for the wild-type human CYP17, the hydroxylation reaction was stimulated 2.4-fold in the presence of cytochrome b_5 , whereas the low basal lyase activity was increased 10.9-fold by cytochrome b_5 , which is similar to previous reports [11,19] and has also recently been confirmed [20]. The carbaldehyde (4) was deformylated at a rate that was 4.8-fold greater than any of the physiological reactions catalysed by CYP17; cytochrome b_5 had no significant effect on the process [15]. Mutation of the residues postulated to be involved in interaction with redox partners, in general, had a greater effect on the lyase reaction than on the hydroxylase or deformylation of the carbaldehyde. This feature is clearly seen with the Lys⁸³ → Ala mutant. However, the most dramatic alteration in the hydroxylase:lyase ratios were found for mutations involving Arg³⁴⁷ and Arg³⁵⁸, as has been previously observed in the COS-1 cell system for the human [14,21] and rat [22] CYP17 isoforms.

A closer examination of Table 1 shows that the Arg³⁴⁷ and Arg³⁵⁸ mutants had similar hydroxylase activities to the wild-type CYP17, but that these activities were only poorly stimulated by cytochrome b_5 . This feature is most convincingly noticed when these arginine residues are replaced by those found in genetic diseases (i.e. Arg³⁴⁷ → His and Arg³⁵⁸ → Gln). Using the quantitative data of Table 1 it may be predicted that the maximum hydroxylase activity for the Arg³⁴⁷ → His and Arg³⁵⁸ → Gln

Figure 2 17 α -Hydroxypregnenolone-binding spectra for the wild-type and the disease-state mutants Arg³⁴⁷ → His and Arg³⁵⁸ → Gln

The spectra were recorded and K_s values were calculated using 17 α -hydroxypregnenolone concentrations of 1, 2, 4, 7 and 10 μ M as previously described [2]. The K_s values were 1.22, 1.57 and 1.90 for wild-type, Arg³⁵⁸ → Gln and Arg³⁴⁷ → His CYP17 proteins respectively.

mutants *in vivo*, if cytochrome b_5 is available, should be 50 % of that of the wild-type enzyme, similar to that reported in the whole COS-1 cells [14].

For the lyase reaction, the Arg³⁴⁷ and Arg³⁵⁸ mutants displayed between 26 and 41 % of the basal wild-type activity, but the most significant aspects of these mutants was their lack of responsiveness to the stimulatory effect of cytochrome b_5 . Thus when comparison is made using the optimal activity achievable in the presence of cytochrome b_5 then the two mutants (Arg³⁴⁷ → His and Arg³⁵⁸ → Gln) were found to be 3 % and 5 % as active as the wild-type CYP17, as has been essentially reported for the COS-1 cell system [14].

That the low lyase activity, found for the Arg³⁴⁷ and Arg³⁵⁸ mutants, is not due to the impairment of the fundamental chemistry required for side-chain cleavage, is shown by the data on the cleavage of the carbaldehyde (**4**). The deformylation of (**4**) by these mutants occurred at 47–65 % of the rate of the wild-type enzyme and, more importantly, these rates were greater than those observed for any of the physiological reactions catalysed by the wild-type protein. This consideration forcefully emphasizes that the primary defect in these four mutants is not the efficiency of electron transfer from the reductase, since this process is common to all the three conversions studied in this work. Also, the mutant enzymes were not deficient in binding the lyase substrate 17 α -hydroxypregnenolone, as is shown by the spectral K_s values, which were similar for the wild-type and mutant proteins (see Figure 2).

In addition to the mutants described in Table 1, we have prepared several other derivatives of CYP17 containing mutations in the putative active-site region of the enzyme [2]. These proteins possessed a range of lyase activities, but in all cases these were significantly stimulated by cytochrome b_5 (6.4–12-fold). Therefore, the loss of the stimulatory effect of cytochrome b_5 , observed to some extent with the Lys⁸³ → Ala mutant and more convincingly with the mutations involving Arg³⁴⁷ and Arg³⁵⁸, is highly significant.

The cumulative findings thus suggest that the main defect in the Arg³⁴⁷ and Arg³⁵⁸ mutant proteins is their unresponsiveness to cytochrome b_5 . In our view, it is the interaction of cytochrome b_5 with CYP17 that triggers the rearrangement seen in Figure 1, which is necessary for the side-chain cleavage of the physiological substrate that is impaired in the Arg³⁴⁷ and Arg³⁵⁸ mutants.

Protein–protein interactions normally occur through multiple contacts and are complex in nature. However, the present work

suggests that Arg³⁴⁷ and Arg³⁵⁸ of CYP17 are two of the important groups which may participate in the formation of a productive complex between CYP17 and the Fe^{II} form of cytochrome b_5 ; the latter will be the predominant species under the reducing conditions used in our assays. The subtle and unpredictable nature of protein–protein interactions is exemplified by two types of mutation introduced at Arg³⁴⁷ and Arg³⁵⁸. When the arginines were replaced by alanine, the resulting proteins retained some responsiveness to cytochrome b_5 in the hydroxylase reaction (16–18 % compared with wild-type; Table 1), but negligible responsiveness (1–2 %; Table 1) in the lyase reaction. However, Arg³⁴⁷ → His or Arg³⁵⁸ → Gln mutations found in patients suffering from genital ambiguity, yielded proteins which had lost nearly all responsiveness to cytochrome b_5 in both reactions (3–6 % for hydroxylase and 1–3 % for lyase; Table 1).

Whether the interaction between cytochrome b_5 and CYP17 promotes conformational changes only, or also facilitates electron transfer, specifically for the side-chain cleavage process, is not directly answered by the present work. However, the demonstration that the cleavage of the carbaldehyde, which uses the same chemistry as that involved in the removal of the side-chain of the physiological substrate [15], occurs at a high rate with the mutant proteins would indicate that electron transfer from the reductase to CYP17 is not rate-limiting for the cleavage reaction. The assistance of an additional redox partner is therefore not necessary. Cytochrome b_5 , though a potential electron carrier, may elicit its effect of regulating the activity of CYP17 by inducing specific and profound conformational changes in CYP17, as was originally suggested [11]. Indeed, recent work has shown that apocytochrome b_5 could replace the holo protein in altering the substrate kinetics of two *P*-450 proteins, *P*-450 3A4 and *P*-450 2C9 [23], and in stimulating the lyase activity of CYP17 [20]. In broad agreement with these observations we have found that the Mn-substituted cytochrome b_5 stimulated the lyase activity of human CYP17 2–3-fold (P. Lee-Robichaud, M. E. Akhtar and M. Akhtar, unpublished work).

Conclusion

There has been a long-standing debate regarding the paradoxical involvement of CYP17 in corticoid as well as androgen biosynthesis. In our earlier work we had hypothesized that the flux of the key intermediate, 17 α -hydroxyprogesterone [structure of

the type **2** (see Scheme 1 in [2]), towards either androgen or corticoid biosynthesis may be regulated by cytochrome b_5 [11]. Indirect support for this possibility is provided by the work of Sakai et al. [24,25], who found that of two groups of patients suffering from Cushing's Syndrome, one produced excessive corticoids only, whereas in the other, adrenal androgens were also produced in large amounts, which could be correlated with the expression of increased amounts of cytochrome b_5 in the adenomas of these patients. It should be noted that high levels of cytochrome b_5 in human testis have been shown [26]. The work presented here provides strong validation for the unusual function assigned to cytochrome b_5 . It also offers an explanation that the genital ambiguity seen in two genetically normal males (46XY) containing Arg³⁴⁷ → His or Arg³⁵⁸ → Gln mutations in CYP17, is due to the unresponsiveness of these proteins to the stimulatory effect of cytochrome b_5 .

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