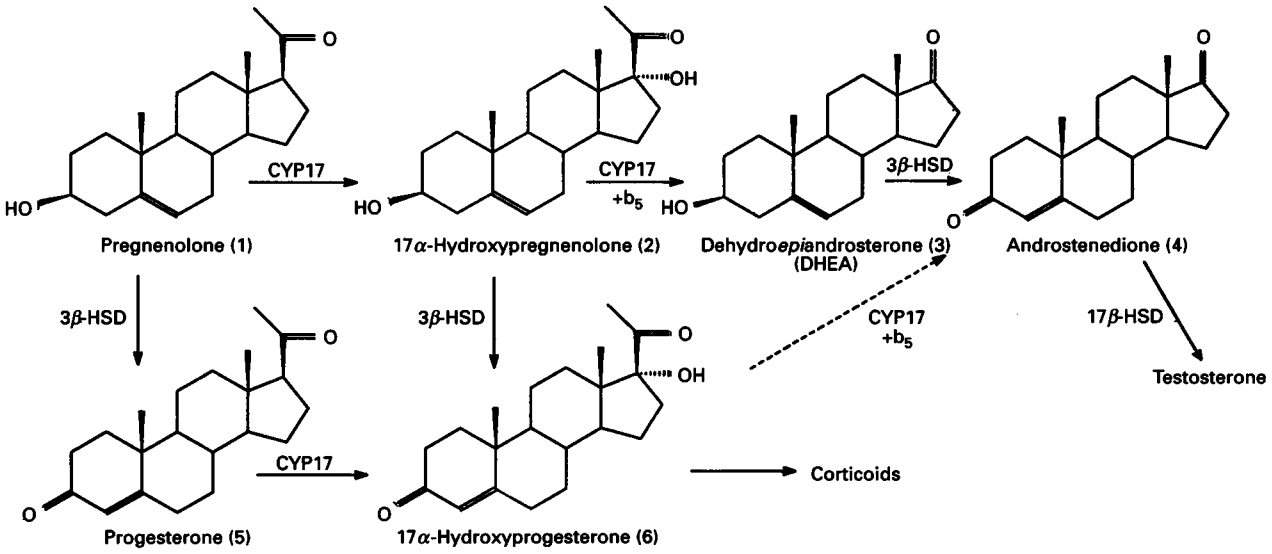
Modulation of the activity of human 17α-hydroxylase-17,20-lyase (CYP17) by cytochrome b_5: endocrinological and mechanistic implications

Peter LEE-ROBICHAUD, J. Neville WRIGHT, Monika E. AKHTAR and Muhammad AKHTAR*
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K.

Using NADPH-cytochrome *P*-450 reductase as electron donor the homogeneous pig 17α-hydroxylase-17,20-lyase (CYP17) was shown to catalyse the conversion of Δ^5, as well as Δ^4, steroids (pregnenolone and progesterone respectively) predominantly into the corresponding 17α-hydroxylated products. The latter were then cleaved by the lyase (desmolase) activity of the enzyme into androgens. Cytochrome b_5 stimulated both these activities, but its most noticeable effect was on the formation of Δ^{16}-steroids, which compulsorily required the presence of cytochrome b_5. These results on the pig enzyme confirm the original findings [Nakajin, Takahashi, Shinoda and Hall (1985) Biochem. Biophys. Res. Commun. **132**, 708–713]. The human CYP17 expressed in *Escherichia coli* [Imai, Globerman, Gertner, Kagawa and Waterman (1993) J. Biol. Chem. **268**, 19681–19689] was also purified to homogeneity and was found to catalyse the hydroxylation of pregnenolone and progesterone without requiring cytochrome b_5. Like the pig CYP17, the human CYP17 also catalysed the cytochrome b_5-dependent direct cleavage of pregnenolone into the $\Delta^{5,16}$-steroid, but unlike it the human enzyme did not cleave progesterone at all. 17α-Hydroxypregnenolone was, however, cleaved into the corresponding androgen but only in the presence of cytochrome b_5. 17α-Hydroxyprogesterone was a poor substrate for the human CYP17; although it was converted into androstenedione in the presence of cytochrome b_5 its K_m was 5 times higher and $V_{max.}$ 2.6 times lower than those for the hydroxylation of progesterone. The endocrinological and mechanistic implications of these results are discussed.

INTRODUCTION

The multifunctional enzyme 17α-hydroxylase-17,20-lyase (CYP17) lies at the crossroads of androgen and corticoid biosynthesis. It catalyses not only an hydroxylation reaction, which is common to both pathways, but also the cleavage of a carbon–carbon bond required only for the formation of androgens [1]. If, under normal physiological conditions, the adrenal gland is not to contribute to virilization there must exist either a stringent control so that 17α-hydroxysteroids (**2** and **6**; Scheme 1), formed from the hydroxylase activity of the enzyme, are prevented from undergoing carbon–carbon-bond cleavage to



Scheme 1 The involvement of human CYP17 and the role of cytochrome b_5 in corticoid and androgen biosynthesis

The full arrows indicate the preferred and the broken arrow the less favourable reactions. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

Abbreviations used: CYP17, 17α-hydroxylase-17,20-lyase; DHEA, dehydro*epi*androsterone.

* To whom correspondence should be addressed.

produce androgens, or so that these androgens are produced but in an inactive form. [The androgenic activity is associated with testosterone and to some extent with androstenedione but all C_{19} steroids, including the androgenically inactive dehydro*epi*androsterone (DHEA) and DHEA-sulphate, are known as androgens.] The precise mechanism underpinning the regulation is not known, although the role of NADPH-cytochrome *P*-450 reductase levels, the lipid environment and competition for substrate by other hydroxylases have been invoked [2–4]. Other possibilities for the absence of excessive production of the active androgen, testosterone, by the adrenal cortex may include the relatively low levels of 17β-hydroxysteroid dehydrogenase activity in this tissue and also the possible protection offered by the conversion of DHEA (**3**; Scheme 1) to its sulphoconjugate (see the Discussion and ref. [5]).

In this paper we show that human cytochrome CYP17 differs from the corresponding enzymes of other mammals and exhibits the carbon–carbon-bond cleavage activity preferentially with the Δ^5-3β-hydroxysteroid (**2**) when the reaction has an absolute requirement for cytochrome b_5. The low level of cytochrome b_5 in human adrenal cortex should therefore be one of the important factors in limiting the formation of hormonally active androgens by this tissue [6]. The work also provides information on the steric movements within the Michaelis complex allowed by cytochrome b_5 and allows speculation on how the ensuing interaction may control the nature of chemical reactions at the haem iron of the CYP17.

EXPERIMENTAL

Materials

[1,2,6,7-^{3}H]Progesterone, [1,2-^{3}H]17α-hydroxyprogesterone and [7-^{3}H]pregnenolone were obtained from DuPont (U.K.) Ltd. Optiphase Hisafe 3 was purchased from Wallac (U.K.). All other chemicals were purchased from Sigma Chemical Co. (U.K.).

Expression and purification of human CYP17

The expression of a modified form of human CYP17 in *E. coli*, using the plasmid pCW17mod, and the purification of the resulting enzyme to a specific content of 7.8 nmol of cytochrome *P*-450/mg of protein, referred to as P-450H17mod(His)$_4$ in the original paper, was performed as described therein [7]. The activities of the purified enzyme for various conversions, expressed as nmol of product formed/min per nmol of cytochrome *P*-450, are shown by the $V_{max.}$ values in Table 2.

Purification of pig CYP17, pig NADPH-cytochrome *P*-450 reductase and pig cytochrome b_5

Pig 17α-hydroxylase-17,20-lyase (pig CYP17) was purified from neonatal pig testes [1] to a specific content of 7.0 nmol of cytochrome *P*-450/mg of protein with activities summarized in Table 2. Pig NADPH-cytochrome *P*-450 reductase was purified from pig liver [8] and assayed in a final volume of 1 ml, at 35 °C, in potassium phosphate buffer (375 mM) containing cytochrome *c* (40 μM). The assay was started by the addition of NADPH (100 μM). The activity is calculated using an increase in absorbance at 550 nm to measure reduced cytochrome *c*, absorption coefficient 21 $mM^{-1} \cdot cm^{-1}$. A specific activity of 31.43 units/mg of protein (1 unit defined as 1 μmol of cytochrome *c* reduced per min) was recorded. Pig cytochrome b_5 was purified to a specific content of 43.4 nmol of haem/mg of protein, as described in the literature [9]. (We thank Professor J. Coon and Dr. A. D. N. Vaz for their helpful advice on the purification of cytochrome b_5.)

Chemical synthesis of radiolabelled substrates

17α-Hydroxy[21-^{3}H]pregnenolone and [16α-^{3}H]pregnenolone were prepared using the general methods developed for the preparation of deuteriated compounds [11] and [17α-^{3}H]pregnenolone from 17α-bromopregnenolone following debromination with Zn in acetic acid [12].

Enzyme assays

Two different principles of radiochemical assay were employed; the first was based on the release of [^{3}H]acetic acid or [^{3}H]water into the medium from appropriately tritiated substrates, and the second on the analysis of radiolabelled steroidal products following separation by TLC. In preliminary experiments the saturating concentrations of NADPH-cytochrome *P*-450 reductase and cytochrome b_5 required for the CYP17-catalysed reactions, as well as K_m and $V_{max.}$ values, were determined using the rapid and convenient tritium release assay. For this purpose the release of ^{3}H from [16α-^{3}H]pregnenolone, [17α,21-^{3}H]pregnenolone and [21-^{3}H]17α-hydroxypregnenolone was used to obtain kinetic parameters for the conversions: (i) pregnenolone $\rightarrow \Delta^{16}$-steroid (**12**; see Scheme 3); (ii) pregnenolone $\rightarrow$ 17α-hydroxypregnenolone + DHEA; and (iii) 17α-hydroxypregnenolone $\rightarrow$ DHEA respectively. Since this method may underestimate the $V_{max.}$ values for reactions (i) and (ii), due to an operation of the isotope effect, unambiguous data for these two reactions were obtained using the conventional method which involves the analysis of products following TLC separation. However, the ^{3}H-release method gave useful information on the period for which the reactions were linear and about the appropriate concentrations of substrates and enzymes to be employed. This information was exploited in the design of subsequent experiments.

Kinetic data on the cleavage of 17α-hydroxypregnenolone using the ^{3}H-release assay

DHEA production (**2** $\rightarrow$ **3**, Scheme 1) was monitored by measuring the release of tritiated acetate from [21-^{3}H]17α-hydroxypregnenolone (specific radioactivity 4.4×10^4 d.p.m./nmol) into the aqueous medium. Aliquots (190 μl) were removed from the incubation (for components of the incubation see below) at 0, 2, 4, 6 and 10 min and the reactions terminated by vortexing with 4 vol. of methylenechloride and 1 vol. of phosphate buffer. The organic layer containing the radiolabelled precursor was removed and the aqueous layer, containing the released tritium in the form of [^{3}H]CH_3COO^-, was vortexed with activated charcoal (this adsorbs any remaining unextracted steroid) and left for 4 h. After pelleting the charcoal by centrifuging in a benchtop Beckmann microfuge, 200 μl samples of the supernatants were counted in Optiphase (4 ml) using a liquid-scintillation counter. The specific radioactivity of 17α-hydroxypregnenolone was used to convert the measured radioactivity into nmol of acyl side-chain cleaved and released as tritiated acetate; 1 nmol of DHEA and 1 nmol of acetate are produced from 1 nmol of 17α-hydroxypregnenolone. Kinetic constants were derived from linear regression analysis of the Lineweaver–Burk plots.

Kinetic data using the analysis of tritiated steroids by TLC

[1,2,6,7-^{3}H]Progesterone, [1,2-^{3}H]17α-hydroxyprogesterone and [7-^{3}H]pregnenolone (specific radioactivity 4.4×10^5 d.p.m./nmol) were used as substrates when measuring directly the formation of products with the retention of radiolabel in the steroid nucleus. The individual incubations (see below) were terminated after

Table 1 The effect of cytochrome b_5 on the profile of products from various precursors: pig (a) and human (b) CYP17

A final volume of 1 ml contained the steroid radiolabelled in the nuclear position(s) (specific activity 4.4×10^5 d.p.m./nmol) (15 nmol), CYP17 (100 pmol), NADPH-cytochrome *P*-450 reductase (1 unit, 400 pmol), L-α-phosphatidylcholine dilauroyl (80 μg), and when present, cytochrome b_5 (500 pmol). Following an incubation of 30 min the steroids were extracted and separated by TLC and quantified by the measurement of radioactivity as described in the Experimental section. Since during the enzymic conversion the functionalization status of rings A and B is not altered, the products in the Δ^4- and Δ^5-series are included in the same column. *In the hydroxylation of progesterone, 16α-hydroxyprogesterone is also produced and the percentage labelling of this is shown in parentheses.

Substrate	Cytochrome b_5	Percentage conversion into: 17α-hydroxy (2) or (6)	Androgen (3) or (4)	Δ^{16}-steroid of the type (12)
(a) Pig cytochrome *P*-450$_{17\alpha}$				
(1a) Pregnenolone	−	15	40	1
(1b) Pregnenolone	+	0	49	26
(2a) Progesterone	−	29 (4)*	31	1
(2b) Progesterone	+	9 (6)*	53	23
(3a)17α-Hydroxypregnenolone	−	−	27	0
(3b)17α-Hydroxypregnenolone	+	−	81	0
(4a)17α-Hydroxyprogesterone	−	−	48	0
(4b)17α-Hydroxyprogesterone	+	−	95	0
(b) Human cytochrome *P*-450$_{17\alpha}$				
(5a) Pregnenolone	−	18	0	0
(5b) Pregnenolone	+	26	25	13
(6a) Progesterone	−	35 (8)*	< 1	0
(6b) Progesterone	+	42 (11)*	< 2	0
(7a)17α-Hydroxypregnenolone	−	−	0	0
(7b)17α-Hydroxypregnenolone	+	−	35	0
(8a)17α-Hydroxyprogesterone	−	−	0	0
(8b)17α-Hydroxyprogesterone	+	−	8	0

10 min by vortexing with 4 ml of ethyl acetate/methanol (9:1, v/v). Steroids were extracted (with an efficiency of 85 ± 5%) in a further 10 ml of ethyl acetate/methanol (9:1, v/v), solvents were evaporated *in vacuo* and the steroids spotted on TLC plates (Merck kieselgel 60 F254), which were then run twice in chloroform/ethyl acetate (9:2, v/v) for the progesterone (Δ^4) series and twice in benzene/acetone (10:1, v/v) followed by twice in chloroform/ethyl acetate (9:2, v/v) for the pregnenolone (Δ^5) series. The TLC plates were scanned and separated steroids were counted using a Berthold TLC scanner/counter. The radiochemical data were used to obtain percentage conversions and these, together with the knowledge of the specific radioactivity of the original substrate, gave the amount of each product formed. The kinetic constants were derived from linear regression analysis of the Lineweaver–Burk plots.

Incubation details

Both assays were performed at 37 °C, in 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 mM EDTA in a final volume of 1 ml. In kinetic experiments radiolabelled substrate (0.083, 0.125, 0.25, 0.50 and 4.0 nmol, or 0.4, 1.0, 2.0, 8.0 and 25.0 nmol for 17α-hydroxyprogesterone only), in 1–2 μl of dimethylformamide, was mixed with CYP17 (30 pmol), NADPH-cytochrome *P*-450 reductase (1 unit), L-α-phosphatidylcholine dilauroyl (40 μg), and when present, cytochrome b_5 (150 pmol). The mixture was made up to 200 μl with the buffer and left to reconstitute for 3 h at 4 °C. It was found that a 5-fold molar excess of cytochrome b_5 over CYP17 and 1 unit of NADPH-cytochrome *P*-450 reductase were saturating and gave optimal activities. The reconstituted mixture was pre-incubated at 37 °C for 5 min and the reaction started by the addition of pre-incubated NADPH-generating buffer (800 μl) [sodium phosphate (50 mM), pH 7.2, EDTA (0.1 mM), $NADP^+$ (3 mM), glucose 6-phosphate (14 mM), glucose-6-phosphate dehydrogenase (4 units)].

The product profile in Table 1 was studied using 15 μM substrates and an incubation period of 30 min, since under these conditions the salient features of all 16 incubations were clearly revealed.

RESULTS

The human and pig CYP17s were purified to give single bands on SDS/polyacrylamide gel, with the cytochrome b_5 and NADPH-cytochrome *P*-450 reductase being purified to greater than 95% homogeneity (Figure 1). In control experiments the incubation of cytochrome *P*-450 without the reductase and vice versa, and also

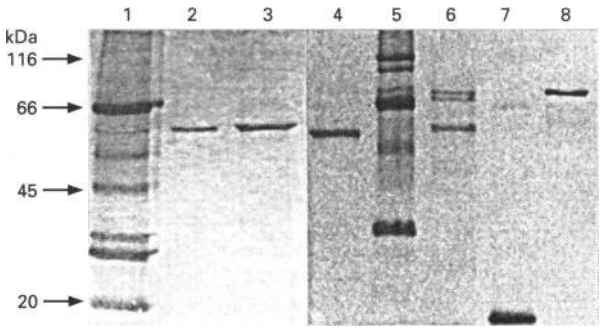


Figure 1 Coomassie Blue-stained SDS/10%-PAGE of purified proteins

Lanes 1 and 5, molecular-mass standards; lanes 2 and 3, human CYP17 (0.45 and 0.8 μg, 55 kDa); lane 4, pig CYP17 (1 μg, 54 kDa); lane 6, semi-purified human CYP17 from Ni^{2+}–agarose (1.7 μg); lane 7, cytochrome b_5 (2.0 μg, 16 kDa); lane 8, NADPH-cytochrome *P*-450 reductase (0.8 μg, 77 kDa).

Table 2 Kinetic parameters of purified human and pig CYP17

Activity assays were performed as described in the Experimental section and the data processed using Lineweaver–Burk plots. Activities are expressed as nmol of product formed/min per nmol of cytochrome *P*-450, K_m data are in μM and $k_{cat.}/K_m$ units are $M^{-1} \cdot s^{-1}$.

	Kinetic parameters					
	Without cytochrome b_5			With cytochrome b_5		
	K_m	$V_{max.}$	$k_{cat.}/K_m$	K_m	$V_{max.}$	$k_{cat.}/K_m$
Pig						
Δ^5-series						
Pregnenolone → 17α-hydroxypregnenolone	0.33	1.00	5.1×10^4	0.33	1.75	8.8×10^4
→ 5,16-diene	–	0.00	0.00	0.33	1.50	7.6×10^4
17α-Hydroxypregnenolone → DHEA	0.32	2.29	1.2×10^5	0.32	5.52	2.9×10^5
Δ^4-series						
Progesterone → 17α-hydroxyprogesterone	0.44	3.10	1.2×10^5	0.44	3.33	1.3×10^5
→ 4,16-diene	–	0.00	0.00	0.40	1.33	5.5×10^4
17α-Hydroxyprogesterone → androstenedione	0.70	3.64	8.7×10^4	1.40	10.00	1.2×10^5
Human						
Δ^5-series						
Pregnenolone → 17α-hydroxypregnenolone	0.65	2.20	5.6×10^4	0.65	3.90	1.0×10^5
→ 5,16-diene	–	0.00	0.00	0.65	0.39	1.0×10^4
17α-Hydroxypregnenolone → DHEA	–	0.00	0.00	1.18	2.45	3.5×10^4
Δ^4-series						
Progesterone → 17α-hydroxyprogesterone	1.00	3.33	5.6×10^4	1.00	4.66	7.8×10^4
→ 4,16-diene	–	0.00	0.00	–	0.00	0.00
17α-Hydroxyprogesterone → androstenedione	–	0.00	0.00	5.00	1.80	6.0×10^3

of cytochrome b_5 without the cytochrome *P*-450, confirmed that the purified enzymes were not cross-contaminated (data not included). The specific contents of the human and pig CYP17 (7.8 and 7.0 nmol of cytochrome *P*-450/mg of protein respectively) compare favourably with other reported values for the human 10.7 [7] and the pig 8.0 [1] enzymes. These values are lower than the theoretical value of 18 nmol of cytochrome *P*-450/mg because of variable loss of non-covalently bound haem during the extreme environmental changes that occur during purification procedures.

Pig CYP17

The results on the pig enzyme, in qualitative terms, are similar to those described previously [1,13,14]. These, however, are included to provide direct quantitative comparison of the properties of the two enzymes. Pig CYP17 converted pregnenolone and progesterone into the corresponding 17α-hydroxylated species that, as expected using the second activity of the enzyme, were cleaved to the androgens, dehydro*epi*androsterone (DHEA) and androstenedione [Table 1; entries (1) and (2)]. The latter assumption was confirmed by showing the direct cleavages of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone; furthermore these conversions were found to be stimulated by the presence of cytochrome b_5 [Table 1; entries (3) and (4)]. This stimulatory effect of cytochrome b_5 on cleavage was most noticeable with pregnenolone and progesterone as substrates when the 17α-hydroxylated steroids, produced by the hydroxylase activity of the enzyme, were almost completely converted into the corresponding androgens [Table 1; compare (1a) with (1b) and (2a) with (2b)]. The most significant observation was, however, regarding the formation of Δ^{16}-steroids (**12**; see Scheme 3) from pregnenolone and progesterone, which compulsorily depended on the presence of cytochrome b_5, as had been shown previously [13]. Our previous work has established that the Δ^{16}-steroids arise from the direct cleavage of the side chain of pregnenolone and progesterone [15,16]. The detailed kinetic parameters for each of these reactions are summarized in Table 2.

Human CYP17

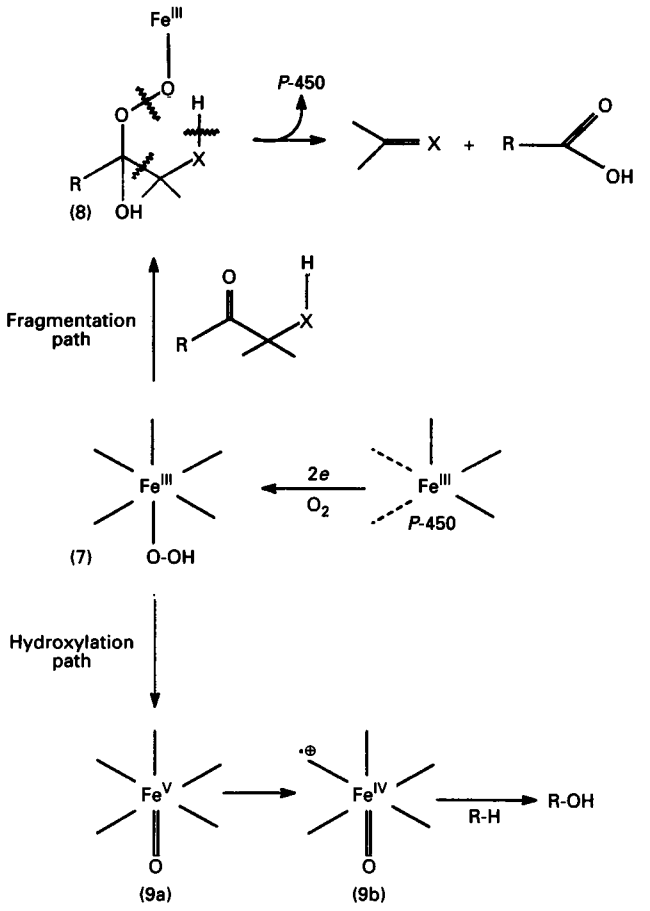
The source of the human CYP17 was a plasmid, pCWH17mod, [7], in which the 5′-end of the coding sequence was modified to allow the expression of the enzyme in *E. coli*. Furthermore, 12 nucleotides coding for four histidine residues were added to the 3′-terminal to facilitate the purification of the cloned enzyme using affinity chromatography on a Ni^{2+} matrix. To differentiate it from the wild-type sequence the enzyme has been referred to as P-450H17mod$(His)_4$[7] and its purity is shown in Figure 1.

Using pregnenolone as the substrate the human enzyme, like its pig counterpart, showed the 17α-hydroxylase activity but differed from the pig CYP17 in not further cleaving the hydroxylated steroid to the corresponding androgen, DHEA [Table 1; compare entry (1a) with (5a)]. It is to be noted, however, that in the presence of cytochrome b_5, the 17,20-lyase activity of the human enzyme was awakened and the corresponding androgen, DHEA, was formed as well as androst-5,16-diene-3β-ol [Table 1; entry (5b)]. This feature was further emphasized by the experiment in which 17α-hydroxypregnenolone was used, when its cleavage to DHEA was found to be dependent on the presence of cytochrome b_5 [Table 1; entry (7)].

When progesterone was used as the substrate, 16α- and 17α-hydroxyprogesterone were produced, in the ratio of 1:4 [Table 1; entry (6)] as previously noted with the wild-type human CYP17 expressed in COS cells [17]. It is noteworthy that the 17α-hydroxyprogesterone, produced by the first activity of the human enzyme, underwent negligible cleavage to androstenedione even

in the presence of cytochrome b_5 [Table 1; entry (6)]. The most dramatic results, however, were obtained using 17α-hydroxyprogesterone as the substrate. In the absence of cytochrome b_5 the cleavage activity was not observed at all, while in its presence androstenedione could be clearly detected only when the substrate concentration was increased to 15 μM [Table 1; entry (8)]. In order to allow a global comparison of the accumulation of products in the experiments of Table 1, the same high substrate concentration (15 μM) was used in all cases. That 17α-hydroxyprogesterone is poorly cleaved by the human enzyme is further supported by the kinetic data in Table 2 which show that its K_m is 5-fold greater and $V_{max.}$ 2.6-fold lower than that for the hydroxylation of progesterone. Thus the $k_{cat.}/K_m$ value for the cleavage of 17α-hydroxyprogesterone is an order of magnitude smaller than that of the hydroxylation of progesterone. This may explain why no significant amount of androstenedione was formed in the incubation when progesterone was the substrate [Table 1; entry (6)].

It should be noted that the highest $k_{cat.}/K_m$ value (2.9×10^5 $M^{-1} \cdot s^{-1}$) recorded here for the cleavage of 17α-hydroxypregnenolone to DHEA, catalysed by the pig CYP17 is 2–3 orders of magnitude smaller than that found for enzymes which operate at the limit of diffusion control. However, the enzymes in the latter class, of which triose-phosphate isomerase is an example, catalyse relatively simple chemical transformations and often use a single substrate. In contrast cytochrome *P*-450-catalysed reactions occur through a large number of intermediates and involve Michaelis complexes consisting of several components. Given this complexity the $k_{cat.}/K_m$ values for these enzymes are not insignificant (Table 2) and are within striking distance of those found for some enzymes involved in intermediary metabolism, for example, fructose-1,6-diphosphate aldolase, with a $k_{cat.}/K_m$ of 5.5×10^5 $M^{-1} \cdot s^{-1}$.



Scheme 2 The Fe^{III}-OOH intermediate at the crossroads of hydroxylation and acyl-carbon bond cleavage

DISCUSSION

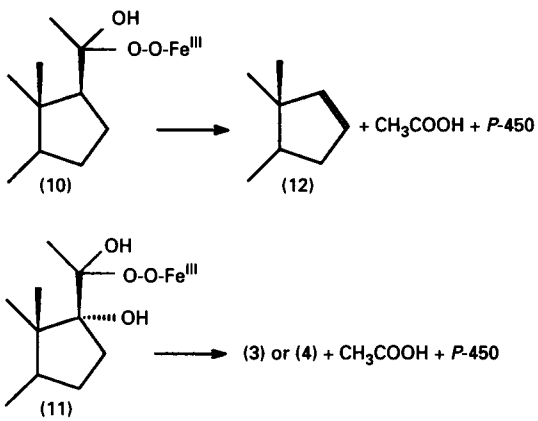
The detailed kinetic studies with purified rat CYP17 have not been performed; however, a great deal of information is available on the cloned enzyme expressed in COS cells [18]. Using the latter system it was found that Δ^4- and Δ^5-steroids are substrates for the two activities, hydroxylase and C–C-bond cleavage, of the enzyme, culminating in the production of the corresponding androgens. Our results on the pig CYP17 confirmed the earlier findings [3,13,14] that the enzyme also displays both the activities on the two classes of steroids (Δ^4 and Δ^5). Cytochrome b_5 stimulated these activities to different extents but its most noteworthy feature was to give rise to the formation of the Δ^{16}-steroids; the latter process had an absolute requirement for cytochrome b_5. It has been found that the Δ^{16}-steroid of the type (**12**; see Scheme 3) is formed directly from the cleavage of the side chain of pregnenolone, without involving the participation of any hydroxylated intermediate [15,16,19]. This fact, together with other mechanistic investigations, has led to the proposal that the formation of the Δ^{16}-steroid (**12**, see Scheme 3) from pregnenolone and of 17-ketosteroids from 17α-hydroxyprogestogens are examples of an acyl-carbon cleavage reaction represented by the fragmentation path in Scheme 2 [15,16].

The ability of CYP17 and related cytochrome *P*-450s to catalyse two different types of generic reactions, hydroxylation and the cleavage process (fragmentation path, Scheme 2), has been rationalized by the model of Scheme 2 [11,15,16,20–23].

The main assumption underpinning the model is that in the catalytic cycle of cytochrome *P*-450s, the Fe^{III}-OOH species is generated for conversion into the oxo-derivative (**9**; Scheme 2) that promotes hydroxylation. However, when a suitably juxtapositioned electrophilic centre is available, the iron peroxide is trapped, producing the adduct of the type (**8**; Scheme 2), that then decomposes to promote a cleavage reaction, for example that represented by the fragmentation path in Scheme 2.

The hypothesis has received wide-ranging support and seems to be applicable to a diverse range of cytochrome *P*-450s [24–28]. In this context we have demonstrated that pig CYP17, when challenged with a substrate analogue in which the C-20 position contains a highly electrophilic aldehyde group, leads to the alteration in the entire course of the enzymic reaction and culminates in the exclusive formation of Δ^{16}-steroids [29]. The observation provides chemical evidence in favour of the involvement of the Fe^{III}-OOH species in the formation of the Δ^{16}-steroid and hence by implication in that of androgens. The adducts involved in the two cleavage reactions, using the physiological substrates, are shown in Scheme 3.

In order to explain the product profile observed in this and previous [1,13] studies it may be speculated that when pregnenolone interacts with pig CYP17 the geometry within the enzyme–substrate complex is such that the Fe^{III}-OOH intermediate, formed during the catalytic cycle of the enzyme, is not accessible to the C-20 carbonyl group of the substrate and decomposes to the oxo-derivative that hydroxylates the 17-position (cf. the conversion **7** → **9**, Scheme 2). The situation is somewhat similar



Scheme 3 The structures of the peroxy adducts involved in androgen and 16,17-ene formation

The illustration shows that the conversions (**10**) → (**12**) and (**11**) → (**3** or **4**) occur by the same generic reaction, symbolized by the fragmentation path in Scheme 2.

with the corresponding Δ^4-steroid, progesterone, when hydroxylated products, 16α- and 17α-hydroxyprogesterone, are produced. In the next stage when the 17α-hydroxypregnenolone or 17α-hydroxyprogesterone become the substrates for the pig CYP17, the acyl-carbon bond cleavage of both these compounds occurs in the absence of cytochrome b_5. The presence of the 17α-hydroxyl group in the two steroids thus seems sufficient to position, in the enzyme–substrate complex, the C-20 of the substrate for attack by Fe^{III}-OOH. The binding of cytochrome b_5 seems to aid this process and stimulates the cleavage approximately 2.5-fold [Table 1; compare (3a) with (3b) and (4a) with (4b)]. Returning to the pregnenolone–CYP17 and progesterone–CYP17 complexes, here cytochrome b_5 is obligatorily required to steer the C-20 of the side chain towards the peroxide group, Fe^{III}-OOH, so that the formation of the peroxy adduct (**8**; Scheme 2) now favourably competes with the hydroxylation process; consequently cleaved (Δ^{16}-steroids) as well as hydroxylated products are formed; i.e. both the limbs of Scheme 2 are used.

Differing preferences for hydroxylation versus C–C-bond cleavage reactions with the two classes of progestogens (Δ^4 and Δ^5) and modulation of these activities by cytochrome b_5 for the pig and also bovine CYP17 [14,30] seem to have reached a state of almost perfection with the human isoform. The human enzyme, in the absence of cytochrome b_5, does not promote the cleavage of any of the four steroids (**1**, **2**, **5**, and **6**; Scheme 1), suggesting the possibility that, in the enzyme–substrate complexes (**13**, **15** and **17**; Scheme 4) the interactions between the protein and the substrate are sufficiently precise to ensure that the C-20 of the steroid is protected from nucleophilic attack by Fe^{III}-OOH.

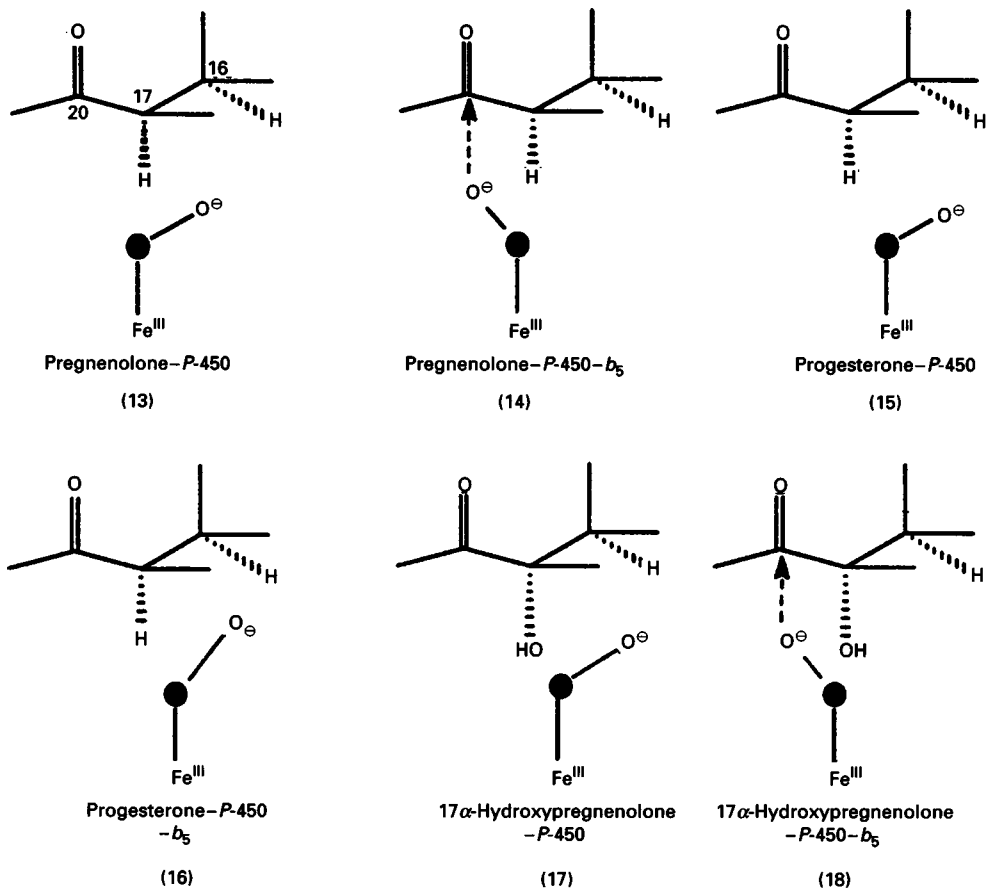
The situation is altered in the presence of cytochrome b_5 and now the interactions within the Michaelis complexes are such (**14** and **18**, Scheme 4) that the human enzyme is able to promote the cleavage of substrates in the Δ^5-series. However, the C-20 of the Δ^4-steroid, progesterone, is protected from attack by the peroxide and negligible cleavage is observed, while 17α-hydroxyprogesterone is poorly cleaved to androstenedione when the maximum rate of cleavage is less than 20% of its pig counterpart and the kinetic parameters (K_m and $V_{max.}$) for the cleavage are unfavourable (Table 2). These relatively poor kinetic parameters may explain the observation that when the substrate presented to the human enzyme is progesterone we see negligible cleavage of the intermediary 17α-hydroxyprogesterone, in the absence or presence of cytochrome b_5 [Table 1; entry (6)]. If such a scenario is extended to the human adrenal tissue and it is assumed that the natural flux through the pathway of Scheme 1 is such that progesterone is available to saturate the CYP17 for the hydroxylation reaction, then the cleavage of 17α-hydroxyprogesterone will be greatly impaired.

The results described here with the human P-450H17mod$(His)_4$, modified at the two termini, will need to be verified using the native enzyme, when it becomes available in amounts required for purification. In the meantime it should be emphasized that our findings, in general terms, interlock with those obtained with the wild-type human enzyme expressed in COS cells [17]. Although in the latter work the effect of cytochrome b_5 on the course of the enzyme reaction was not studied the profile of product formation was reminiscent of that found in this present work. Thus COS cells containing the human CYP17 gene converted pregnenolone into 17α-hydroxypregnenolone and DHEA, whereas progesterone was only converted into its hydroxylated product without the subsequent cleavage of the C17–C20 bond to produce its corresponding androgen, androstenedione. An interesting finding in the work, confirmed here, was that progesterone, in addition to being converted into 17α-hydroxyprogesterone, also produced some 16α-hydroxyprogesterone. This observation further emphasizes that the steroid, containing conjugated ketone functionality in ring A, binds to human CYP17 in such a fashion that the oxygen ligand of the cytochrome is projected, not between the C-20–C-17, but the C-17–C-16 bond (**15** and **16**; Scheme 4). Furthermore that this binding mode is relatively uninfluenced by the involvement of cytochrome b_5, as shown by the absence of $\Delta^{4,16}$-steroid formation.

Conclusions

In the light of the findings and argument presented above it is plausible that a low level of cytochrome b_5 in the human adrenal may be one of the factors in ensuring that this gland does not contribute to the unwanted production of active androgens (i.e. testosterone and androstenedione) under normal physiological conditions. Indirect information on the levels of cytochrome $b_5$and its role in hormone biosynthesis in the human adrenal gland may be deduced from recent work [6,31] that studied two groups of patients suffering from Cushing's Syndrome. One of the groups produced excessive corticoids only, while, 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. A corollary from the preceding line of argument is the requirement for the presence of adequate amounts of cytochrome b_5 in human testis for androgen production. High levels of cytochrome b_5 have indeed been reported in human testes [32].

The results described in this paper, together with existing information in the literature, allow the consideration of factors which may down-regulate the production of active androgens (androstenedione and testosterone) by the human adrenal cortex. Human CYP17 compulsorily requires the presence of cytochrome b_5 for its lyase activity. The level of cytochrome b_5 in a tissue will therefore be expected to profoundly influence androgen biosynthesis. Cytochrome b_5 levels in human endocrine tissues have not yet been quantified but the work of Sakai et al. and others [6,31] implies it to be low in the human adrenal cortex, which will have a moderating effect on the lyase activity of CYP17. In



Scheme 4 The effect of cytochrome b_5 on the orientation of the oxygen ligand in various complexes involving human CYP17

The stylized diagram shows that the peroxide anion is suitably juxtapositioned for a nucleophilic attack on the carbonyl carbon only in complexes (**14**) and (**18**) containing cytochrome b_5. The peroxide anion has been dragged away from the carbonyl group in other complexes which do not promote the C–C-bond cleavage reactions (**13**, **15**, **16** and **17**). This is an exaggeration used only for illustrative convenience. In reality the formation of the peroxy adduct (of type **8**, Scheme 2) may be prevented by a relatively minor rotation of the C-17–C-20 bond, so that the stringent geometry for a nucleophilic attack on the carbonyl group, required by Dunitz and colleagues [34], is not available. The structures for the 17α-hydroxyprogesterone–cytochrome *P*-450 and 17α-hydroxyprogesterone–cytochrome *P*-450–cytochrome b_5 complexes are not shown but should be similar to the 17α-hydroxypregnenolone complexes depicted in the diagram. The filled oxygen (●) in the structures denotes the oxygen atom that is destined to be the part of the oxo-derivative used in the hydroxylation reaction.

addition, we have shown in this study that the kinetic parameters for the cytochrome b_5-dependent cleavage are more favourable for the cleavage of 17α-hydroxypregnenolone than that of 17α-hydroxyprogesterone (the specificity, as determined by the $k_{cat.}/K_m$ values, is 5–6-fold higher for 17α-hydroxypregnenolone). The consequences of which, if this pattern is followed *in vivo*, should be the preferential cleavage of 17α-hydroxypregnenolone to produce DHEA, which using the sulphotransferase activity of the adrenal cortex is secreted as the corresponding sulpho-conjugate [5]. Furthermore, any androstenedione formed from the direct cleavage of 17α-hydroxyprogesterone is not converted into the most active androgen, testosterone [33], presumably because the 17β-hydroxysteroid dehydrogenase activity in the adrenal is low. These inter-related and carefully co-ordinated mechanisms may provide the safety net which enables the female of the human species to escape from the physiological ramifications promoted by the male hormone.

We thank Professor Michael R. Waterman for his generosity in providing us with the plasmid containing the modified human CYP17 gene. His group has independently studied the effect of cytochrome b_5 on the activity of this CYP17 and the results are being prepared for publication. The work was supported by a grant from the Science and Engineering Research Council to M.A. and a Wellcome Trust Studentship in Toxicology to P.L.-R.

REFERENCES

1 Nakajin, S. and Hall, P. F. (1981) J. Biol. Chem. **256**, 3871–3876
2 Yanagibashi, K. and Hall, P. F. (1986) J. Biol. Chem. **261**, 8429–8433
3 Suhara, K., Fujimura, Y., Shiroo, M. and Katagiri, M. (1984) J. Biol. Chem. **259**, 8729–8736
4 Cooke, G. M. (1992) J. Steroid Biochem. Mol. Biol. **41**, 99–107
5 Kime, D. E., Vinson, G. P., Patricia, W. M. and Kilpatrick, R. (1980) in General, Comparative and Clinical Endocrinology of the Adrenal Cortex (Chester Jones, I. and Henderson, I. W., eds.), pp. 183–264, Academic Press, London
6 Sakai, Y., Yanase, T., Hara, T., Takayanagi, R., Haji, M. and Nawata, H. (1994) Clin. Endocrinol. (Oxford) **40**, 205–209
7 Imai, T., Globerman, H., Gertner, J. M., Kagawa, N. and Waterman, M. R. (1993) J. Biol. Chem. **268**, 19681–19689
8 Strobel, H. W. and Dignam, J. D. (1978) Methods Enzymol. **52**, 89–96
9 Strittmatter, P., Fleming, P., Connors, M. and Corcoran, D. (1978) Methods Enzymol. **52**, 97–101
10 Reference deleted.

11 Akhtar, M., Corina, D. L., (the late) Miller, S. L., Shyadehi, A. Z. and Wright, J. N. (1994) J. Chem. Soc. Perkin Trans. 1, 263–267
12 Kremers, P., Denoel, J. and Lapieve, C. J. (1974) Steroids **23**, 603–613
13 Nakajin, S., Takahashi, M., Shinoda, M. and Hall, P. F. (1985) Biochem. Biophys. Res. Commun. **132**, 708–713
14 Meadus, W. J., Mason, J. I. and Squires, E. J. (1993) J. Steroid Biochem. Mol. Biol. **46**, 565–572
15 Corina, D. L., Miller, S. L., Wright, J. N. and Akhtar, M. (1991) J. Chem. Soc., Chem. Commun. 782–783
16 Akhtar, M., Corina, D., Miller, S., Shyadehi, A. Z. and Wright, J. N. (1994) Biochemistry **33**, 4410–4418
17 Swart, P., Swart, A. C., Waterman, M. R., Estabrook, R. W. and Mason, J. I. (1993) J. Clin. Endocrinol. Metab. **77**, 98–102
18 Koh, Y., Buczko, E. and Dufau, M. L. (1993) J. Biol. Chem. **268**, 18267–18271
19 Shimizu, K. and Nakada, F. (1976) Biochim. Biophys. Acta **450**, 441–449
20 Miller, S. L., Wright, J. N., Corina, D. L. and Akhtar, M. (1991) J. Chem. Soc., Chem. Commun. 157–158
21 Akhtar, M., Njar, V. C. and Wright, J. N. (1993) J. Steroid Biochem. Mol. Biol. **44**, 375–387
22 Stevenson, D. E., Wright, J. N. and Akhtar, M. (1988) J. Chem. Soc. Perkin Trans. 1, 2043–2052
23 Akhtar, M. and Wright, J. N. (1991) Nat. Prod. Rep. 527–551
24 Cole, P. A. and Robinson, C. H. (1988) J. Am. Chem. Soc. **110**, 1284–1285
25 Vaz, A. D. N., Roberts, E. S. and Coon, M. J. (1991) J. Am. Chem. Soc. **113**, 5886–5887
26 Roberts, E. S., Vaz, A. D. N. and Coon, M. J. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8963–8966
27 Swinney, D. C. and Mak, A. Y. (1994) Biochemistry **33**, 2185–2190
28 Marletta, M. A. (1994) J. Med. Chem. **37**, 1899–1907
29 Robichaud, P., Wright, J. N. and Akhtar, M. (1994) J. Chem. Soc., Chem. Commun. 1501–1503
30 Shet, M. S., Fisher, C. W., Arlotto, M. P., Shackleton, C. H. L., Holmans, P. L., Martin-Wixtrom, C. A., Saeki, Y. and Estabrook, R. W. (1994) Arch. Biochem. Biophys. **311**, 402–417
31 Sakai, Y., Yanase, T., Takayanagi, R., Nakao, R., Nishi, Y., Haji, M. and Nawata, H. (1993) J. Clin. Endocrinol. Metab. **76**, 1286–1290
32 Mason, J. I., Estabrook, R. W. and Purvis, J. L. (1973) Ann. N.Y. Acad. Sci. **212**, 406–419
33 Wieland, R. G., Decourcy, C., Levy, R. P., Zala, A. P. and Hirschmann, H. (1965) J. Clin. Invest. **44**, 159–168
34 Schweizer, W. B., Procter, G., Kaftory, M. and Dunitz, J. D. (1978) Helv. Chim. Acta **61**, 2783

Received 11 November 1994/3 February 1995; accepted 9 February 1995