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A single amino acid residue, Ala 105, confers 16 α -hydroxylase activity to human cytochrome P450 17 α -hydroxylase/17,20 lyase

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ABSTRACT

In adrenal steroidogenesis, CYP17 catalyses the 17 α -hydroxylation of pregnenolone and progesterone and the subsequent 17,20-lyase reaction, yielding adrenal androgens. The enzyme exhibits distinctly different selectivities towards these substrates in various species. CYP17 has also been shown to exhibit 16 α -hydroxylase activity towards progesterone in some species, with only human and chimp CYP17 catalysing the biosynthesis of substantial amounts of 16-OHprogesterone. The 16 α -hydroxylase activity was investigated by introducing an Ala105Leu substitution into human CYP17. The converse mutation, Leu105Ala was introduced into the baboon, goat and pig enzymes. Wt human CYP17 converted ~30% progesterone to 16-OHprogesterone while the Ala105Leu mutant converted negligible amounts to 16-OHprogesterone (~9%), comparable to wt CYP17 of the other three species when expressed in COS-1 cells. The ratio of 17-hydroxylated products to 16-OHprogesterone of human CYP17 was 2.7 and that of the mutant human construct 10.5. Similar ratios were observed for human and goat CYP17 with the corresponding Ala or Leu residues. Although the Leu105Ala mutation of both baboon and pig CYP17 exhibited the same trend regarding the ratios, the rate of progesterone conversion was reduced. Coexpression with cytochrome b₅ significantly decreased the ratio of 17-hydroxylated products to 16-OHprogesterone in the Leu105 constructs, while effects were negligible with Ala at this position. Homology models show that Ala105 faces towards the active pocket in the predicted B'-C domain of CYP17. The smaller residue allows more flexibility of movement in the active pocket than Leu, presenting both the C16 and C17 of progesterone to the iron-oxo complex.

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1. Introduction

Cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) plays a vital role in both adrenal and gonadal steroidogenesis in mammals. CYP17 catalyses two distinct mixed-function oxidase reactions, namely the 17 α -hydroxylation of the C21 steroids, pregnenolone (PREG) and progesterone (PROG), followed by the cleavage of the C17–20 bond to produce the C19 steroids, dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively. The 17,20-lyase activity of CYP17 is enhanced by allosteric interactions with cytochrome b₅ and possibly by post-translational phosphorylation [1]. In the adrenal, the dual activity of CYP17 places this enzyme at a key branch point in the synthesis of mineralocorticoids, glucocorticoids and adrenal androgens, while in the gonads CYP17 is essential in the production of the sex steroids required for mammalian reproduction.

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In addition to the 17 α -hydroxylation of PROG, the human and chimpanzee enzymes have been shown to convert significant amounts of PROG to 16-OHPROG [2,3]. The reaction is catalysed in a common active site via the same reaction mechanism, while the enzyme does not hydroxylate pregnenolone at the C16 position [2]. The ratio of 16-OHPROG:17-OHPROG production by human CYP17 has been reported to be 1:4 when expressed in COS-1 cells, while expression in *Pichia pastoris* yielded a ratio as high as 1:2 (16-OHPROG:17-OHPROG) [4].

Although the physiological importance of the mineralocorticoids, glucocorticoids and sex steroids are well established, the role of 16-OHPROG and the mechanism of its production remains unclear. 16 α -Hydroxyprogesterone was first reported in 1965 when significant amounts were detected when feminizing testicular tissue were incubated with progesterone [5]. Inano and Tamaoki [6] subsequently demonstrated that the 16-OHPROG metabolite formed during the conversion of PROG in microsomes, prepared from testes obtained from patients with prostatic carcinoma, was not metabolized further. Furthermore, they showed that the lyase activity of CYP17 was inhibited competitively (K_i 72 μ M) by 16-OHPROG while the metabolite inhibited 20 α -hydroxysteroid dehydrogenase non-competitively (K_i 52.9 μ M). It was concluded

that 16-OHPROG specifically inhibited the 17,20 lyase reaction of 17-OHPREG in the course of androgen biosynthesis *in vitro* while the CYP17 hydroxylase, 3 β -hydroxysteroid dehydrogenase or 17 β -hydroxysteroid dehydrogenase activities were not influenced [6]. Although the physiological relevance of such high K_i values is questionable, the metabolite may impact certain processes as it was not metabolised and may thus accumulate in the tissue.

The conversion of PROG to 16-OHPROG has also been shown in the fetal adrenal [7], while Sano et al. [8] observed similar changes in 16-OHPROG and 17-OHPROG concentrations in human ovaries during the menstrual cycle. Patients presenting with natriuresis have also been shown to have an increased production of this steroid metabolite which could be correlated with ACTH secretion [9,10]. In addition, Ogawa et al. [11] demonstrated that both 16-OHPROG and 16-OHPREG plasma levels increased as the uterine sensitivity to oxytocin increased. The authors showed a significant correlation between serum and myometrial concentrations of PROG, 16-OHPREG, DHEA, conjugated DHEA, estrone and estradiol, concluding that the environment of steroid hormones may be closely related to myometrial sensitivity to oxytocin and the onset of labour. Subsequent studies have highlighted the intricate regulatory roles played by steroidogenic hormones and their metabolites. The compartmentalisation of these metabolites and the steroidogenic enzymes catalysing their biosynthesis underlies the complementary activities of steroid hormones in the fetal and placental compartments, with both 16-OHPROG and 16-OHPREG being present in the fetal compartment [12]. Adding to the complexity are the well documented steroid hormone receptors to which the steroid hormones, their metabolites as well as other ligands may bind. Quinkler et al. [13] found 16-OHPROG to have no influence on the human mineralocorticoid receptor (hMR) while 17-OHPROG was shown to be a potent antagonist.

16 α -Hydroxylase activity has been reported in other primate and mammalian species, but the levels are significantly lower than that of the human and chimp enzymes [2–4]. Arlt et al. [3] have demonstrated that the ratio of 16 α -hydroxylase:17 α -hydroxylase activity in human and chimpanzee CYP17 was eight times higher than in the baboon enzyme and twice as high as in rhesus monkey CYP17 even though the baboon and rhesus enzymes share approximately 95% sequence identity with the human and chimpanzee enzymes. Using a sensitive UPLC-APCI-MS method, Storbeck et al. [4] demonstrated that COS-1 cells, expressing angora goat CYP17, produced low, yet detectable, levels of 16-OHPROG. In a comparative study the authors showed human CYP17 metabolized PROG to 16-OHPROG and 17-OHPROG at a ratio of 1:2, while the 16-OHPROG:17-OHPROG ratios were 1:7 and 1:22 for the baboon and goat enzymes, respectively. The authors concluded that CYP17 of other species may well produce small amounts of 16-OHPROG but that the detection methods used previously were unable to detect these low levels.

The aim of this study was to investigate the 16 α -hydroxylase activity of human CYP17 which has not been the focus of recent P450 research. In addition to human CYP17 not hydroxylating PREG at C16, the distinctly different catalytic activity which the enzyme exhibits towards the Δ 5-3 β -hydroxysteroids and the Δ 4-3-ketosteroids may be attributed, in part, to the orientation of the substrate in the active pocket. The interaction of these functional groups of the substrate with specific amino acid residues of the enzyme may dictate the favorable positioning of C16 and C17 for the hydroxylation of PROG and the positioning of C17 and the C17–C20 bond for the hydroxylation and lyase reaction of PREG. Human and baboon CYP17 exhibit 96% sequence homology, with 50% of the amino acid residues differences lying in the F–G region. We previously showed that the baboon synthesizes negligible amounts of 16-OHPROG and that the rate at which PROG was metabolised to 17-OHPROG by baboon CYP17 was ~8-fold higher than that of

human CYP17 [14]. It is therefore possible that the charged residues in this region may influence the binding of PROG to the enzyme, resulting in less movement of the substrate in the active pocket, therefore not favouring both 16- and 17-hydroxylation. Sequence comparisons were thus carried out with other species and residues located at positions 195–198 in the predicted F helix, were investigated since the F–G region is generally thought to play a role in substrate recognition and binding [15]. In addition, Ala105, which is located in the predicted B' helix, was also identified as a residue possibly playing a role in the 16 α -hydroxylase activity of human CYP17. Site directed mutagenesis and subsequent expression of mutant constructs also allowed an investigation into the influence of cytochrome b_5 on the 16 α -hydroxylase activity of this enzyme. Homology modeling was carried out to further assess the contribution of Ala105 to the 16 α -hydroxylase activity of human CYP17.

2. Materials and methods

2.1. Reagents

A Gene Tailor™ site-directed mutagenesis system was purchased from Invitrogen (Carlsbad, USA). Nucleobond® AX plasmid preparation kits were purchased from Macherey-Nagel (Duren, Germany). COS-1 cells were purchased from the American Type tissue Culture Collection (Manassas, VA, USA). Penicillin–streptomycin, trypsin–EDTA, fetal calf serum and Dulbecco's PBS were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Difco Laboratories (Detroit, MI, USA), respectively. PROG, 16-OHPROG, 17-OHPROG, A4, Dulbecco's modified Eagle's medium and HEPES were purchased from Sigma Chemical Co. (St Louis, MO, USA). A Pierce BCA™ protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest analytical grade and purchased from scientific supply houses.

2.2. Site-directed mutagenesis

Site-directed mutagenesis of wild type human, Cape baboon (*Papio ursinus*), domestic pig (*Sus scrofa*) and South African angora goat (*Capra hircus*) CYP17 was carried out using the Gene Tailor™ site-directed mutagenesis system, according to the manufacturer's instructions. The primers used are shown in Table 1. The mutant constructs were confirmed by direct DNA sequencing using forward and reverse primers to analyze the complete cDNA sequences ensuring that no other mutations had occurred.

2.3. Enzymatic assays in non-steroidogenic mammalian COS-1 cells

Transient transfections with human, baboon, pig and goat wt CYP17 as well as mutant constructs were carried out in COS-1 cells as previously described [4]. Briefly, cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM), containing 0.9 g/L glucose, 0.12% NaHCO₃, 10% fetal calf serum, 1% penicillin–streptomycin and 24 h prior to transfection, plated into 12 well dishes with each well containing 1×10^5 cells in 1 ml. The medium was subsequently replaced with 1 ml medium after which 50 μ l containing 0.5 μ g plasmid DNA and 1.5 μ l Genejuice transfection reagent (Novagen, Darmstadt, Germany) was added, according to the manufacturer's instructions. Control transfection reactions were performed using the pCneo vector containing no insert. Co-transfections with CYP17 cDNA and cytochrome b_5 cDNA were carried out using 0.25 μ g of each plasmid. Cytochrome b_5 was replaced with the pCneo vector containing no insert in the control co-transfection assays. The cells were incubated for 72 h, after which steroid precursor was added to the medium. The catalytic activities of the constructs were assayed using 1 μ M PROG as

Table 1
Primer used for site directed mutagenesis of CYP17 cDNAs.

Species	Oligonucleotide sequence
Human A105L LP (sense)	5'-TCAATGGCAACTCTAGACATC CTG TCCAACAAC-3'
Human A105L RP (antisense)	5'-GATGTCTAGAGTTGCCATTTGAGGCCGCC-3'
Baboon L105A LP (sense)	5'-TCAAGTGACAACCTCTAGACATC CGG TCCAACAAC-3'
Baboon L105A RP (antisense)	5'-GATGTCTAGAGTTGTCACCTGAGGCCGCC-3'
Baboon KIVH195-198NVIQ LP (sense)	5'-TGGGGACCCCTGAGTTGAATGTCATACAGAATTACAATG-3'
Baboon KIVH195-198NVIQ RP (antisense)	5'-TTCAACTCAGGGTCCCATCTTGTAGGAG-3'
Pig L105A LP (sense)	5'-CAGACTGATGACTCTAGACATC CGG TCAGACAAC-3'
Pig L105A RP (antisense)	5'-GATGTCTAGAGTCATCACTCTGGGCCGCC-3'
Goat L105A LP (sense)	5'-CAAAGTGGCCACTCTAGACATC CGG TCAGACAAC-3'
Goat L105A RP (antisense)	5'-GATGTCTAGAGTGGCCACTTTGGGACGCC-3'

Codons for the changed amino acid are in boldface and underlined.

substrate. Medium, 500 μ l, was removed to assay substrate conversion after 8 h and the steroid metabolites were extracted with 5 ml dichloromethane and dried under a stream of nitrogen. The dried steroid residue was redissolved in 120 μ l methanol and analysed by UPLC-APCI-MS. On the completion of each experiment, the cells were collected, washed with 0.1 M phosphate buffer, pH 7.4 and homogenised prior to determining the protein concentration by the Pierce BCA method (Pierce Chemical, Rockford, USA), according to the manufacturer's instructions.

2.4. UPLC-APCI-MS steroid metabolite analyses

Steroids were analysed by UPLC-APCI-MS as previously described [4]. Briefly, steroid samples, 5 μ L, were separated and quantified by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Waters UPLC BEH C18 (2.1 mm \times 50 mm, 1.7 μ m) column. The mobile phases consisted of 1% formic acid (A) and acetonitrile (B). Steroids were eluted at a flow rate of 0.4 ml/min, using a linear gradient from 85% A to 80% B in 3.5 min, followed by a linear gradient from 80% B to 100% B in 0.1 min. An API Quattro Micro tandem mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters, Milford, USA) was used for the APCI interface in positive mode. The corona pin was set to 7 μ A, the cone voltage to 30 V and APCI probe temperature was 450 $^{\circ}$ C. All other settings were optimized to obtain the strongest signal possible. Calibration curves were constructed by using weighted (1/x²) linear least squares regression. Data was collected with the MassLynx 4.0 software program.

2.5. Homology modeling

Ten template structures with the greatest sequence homology to CYP17 were identified in the Protein Data Bank (www.rcsb.org/pdb) using the Blast server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignments of CYP17 with CYP1A2 (2HI4), CYP2A13 (2P85), CYP2R1 (3DL9), CYP2B4 (1SUO), CYP2C8 (2NNJ), CYP2A6 (3EBS), CYP2C9 (1RQO), CYP2C5 (1DT6), CYP2D6 (2F9Q) and CYP2E1 (3E4E) were generated using PROMSAL3D [16]. Homology models were constructed for each of the ten templates using the program YASARA Structure version 9.10.14. The best results were obtained using CYP1A2, CYP2R9 and CYP2E1 as templates and the NOVA force field [17]. In addition, a fourth model was built using a multiple sequence alignment containing all three templates using Modeller 9 version 7 [18].

3. Results

3.1. Enzymatic activity of CYP17 and mutated constructs

Since the F and G helices and F-G loop regions of cytochrome P450 enzymes are involved in substrate binding, this region was investigated by mutating residues Lys, Ile, Val and His at posi-

tions 195–198 in baboon CYP17 to correspond to the human CYP17 residues, Asn, Val, Ile and Gln. Expression of the mutant baboon construct in COS-1 cells showed that, although the rate of PROG to 17-OHPROG conversion was influenced significantly in both cases ($P < 0.005$) indicating that the loss of the charged Lys and His residues did not enhance the 16-hydroxylase activity of baboon CYP17 (Fig. 1).

Sequence alignments of various species (Fig. 2) showed that human and chimpanzee CYP17 are the only two species with an Ala

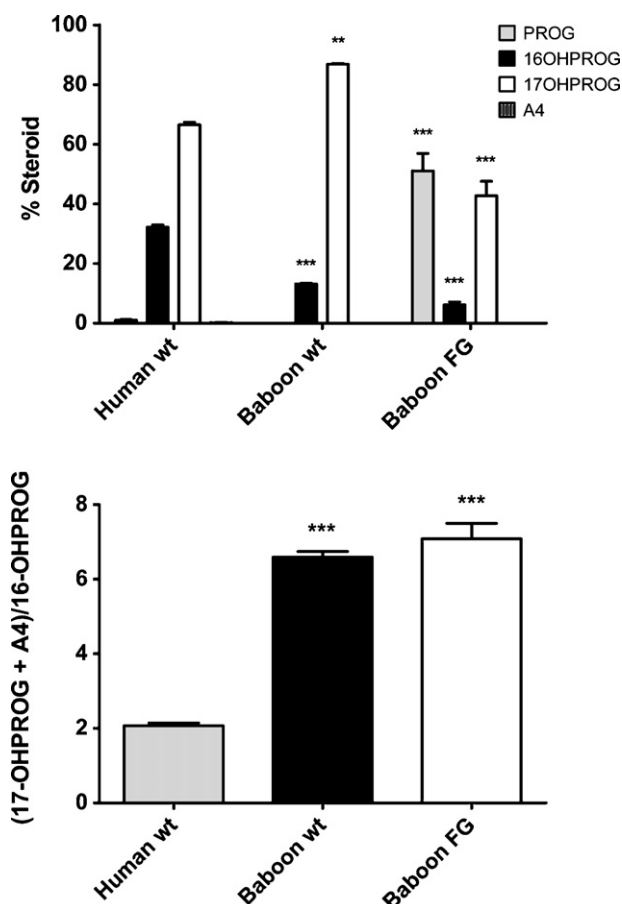


Fig. 1. PROG (1 μ M) metabolism by human wt CYP17 and baboon wt and mutant CYP17 construct, KIVH195-199NVIQ. (A) Comparison of the steroid metabolite profile of wild types CYP17 and the baboon FG mutant construct. Individual steroids were compared to those utilized and produced by wt human CYP17 by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($*P < 0.05$; $**P < 0.005$; $***P < 0.0001$). Results are representative of at least three independent experiments. (B) Ratio of 17-hydroxylated products to 16-OHPROG by wild types CYP17 and the baboon FG mutant construct. Columns were compared to wt human CYP17 by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($***P < 0.0001$). Results are representative of three independent experiments.

	80	90	100	110
<i>Homo sapiens</i>	QLAKEVLIKK	GKDFSGRPQM	ATLDI <u>AS</u> NNR	KGIAFADSGA
<i>Pan troglodytes</i>	QLAKEVLIKK	GKDFSGRPQM	ATLDI <u>AS</u> NNR	KGIAFADSGA
<i>Papio ursinus</i>	QLAKEVLIKK	GKDFSGRPQV	TTLDI <u>L</u> SNNR	KGIAFADYGA
<i>Papio cynocephalus</i>	QLAKEVLIKK	GKDFSGRPQV	TTLDI <u>L</u> SNNR	KGIAFADYGA
<i>Macaca mulatta</i>	QLAKEVLIKK	GKDFSGRPQV	TTLDI <u>L</u> SNNR	KGIAFADYGA
<i>Callithrix jacchus</i>	QLAKEVLKKK	GKDFSGRPQV	ATIDI <u>L</u> SNKG	KGIAFADYGA
<i>Equus caballus</i>	QLAKEVLIKK	GKEFSGRPQV	ATLNI <u>L</u> SDNQ	KGVAFADHGA
<i>Felis catus</i>	QLAKEVLVKK	GKEFSGRPV	VTLDI <u>L</u> SDNQ	KGIAFADHGA
<i>Capra hircus</i>	QLAREVLLKK	GKEFSGRPKV	ATLDI <u>L</u> SDNQ	KGIAFADHGA
<i>Ovis aries</i>	QLAREVLLKK	GKEFSGRPKV	ATLDI <u>L</u> SDNQ	KGIAFADHGA
<i>Bos Taurus</i>	QLAREVLLKK	GKEFSGRPKV	ATLDI <u>L</u> SDNQ	KGIAFADHGA
<i>Bison bison</i>	QLAREVLLKK	GKEFSGRPKV	ATLDI <u>L</u> SDNQ	KGIAFADHGA
<i>Cavia porcellus</i>	QLARELLIKK	GKEFSGRPLT	TTVAL <u>L</u> SDNG	KGIAFADSSA
<i>Peromyscus leucopus</i>	QLAREVLIKK	GKEFSGRPQM	VTGL <u>L</u> SDNQ	KGIAFADSD
<i>Mesocricetus auratus</i>	QLAKEVLVKK	GKEFSGRPHM	VTGL <u>L</u> SDQG	KGIAFADSGG
<i>Rattus norvegicus</i>	QLAREVLIKK	GKEFSGRPQM	VTQSL <u>L</u> SDQG	KGVAFADAGS
<i>Sus scrofa</i>	QLAKEVLLKK	GKEFSGRPV	MTLDI <u>L</u> SDNQ	KGIAFADHGT
<i>Mus musculus</i>	QLAREVLVKK	GKEFSGRPQM	VTGL <u>L</u> SDQG	KGVAFADSSS

Fig. 2. Sequence alignment of the amino acids from position 80–120 of mammalian CYP17 enzymes. Ala105 and Ser106 are in boldface and underlined. GenBank accession numbers: *Homo sapiens*, **NP000093**; *Pan troglodytes*, **NP001009052**; *Papio ursinus*, **AY034635**; *Papio cynocephalus*, **Q8HYNO**; *Macaca mulatta*, **NP001035322**; *Callithrix jacchus*, **AY746982**; *Equus caballus*, **NP001075992**; *Felis catus*, **NP001009371**; *Capra hircus*, **EF524063**; *Ovis aries*, **NP001009483**; *Bos Taurus*, **NP776729**; *Bison bison*, **Q9GMC7**; *Cavia porcellus*, **Q64410**; *Peromyscus leucopus*, **Q91285**; *Mesocricetus auratus*, **P70687**; *Rattus norvegicus*, **NP036885**; *Sus scrofa*, **P19100**; *Mus musculus*, **AAH64793**.

residue at position 105. All other species have a Leu residue at this alignment position in the predicted B' helix. Adjacent to this residue are residues Ile104 and Ser106 which are also highly conserved, with the latter being conserved in all mammalian species for which CYP17 sequences have been determined to date. In birds, fish, amphibians and reptiles Ser106 is substituted with a Thr residue. The Ala105Leu mutation was introduced into human CYP17 as the

increased 16 α -hydroxylase activity observed in the human and chimp CYP17 may be due to the smaller residue at this alignment position. Expression of the mutant human construct in COS-1 cells showed that the enzyme could no longer produce significant levels of 16-OHPROG (Fig. 3A).

An Leu105Ala substitution was subsequently introduced in baboon CYP17 and increased the production of 16-OHPROG signif-

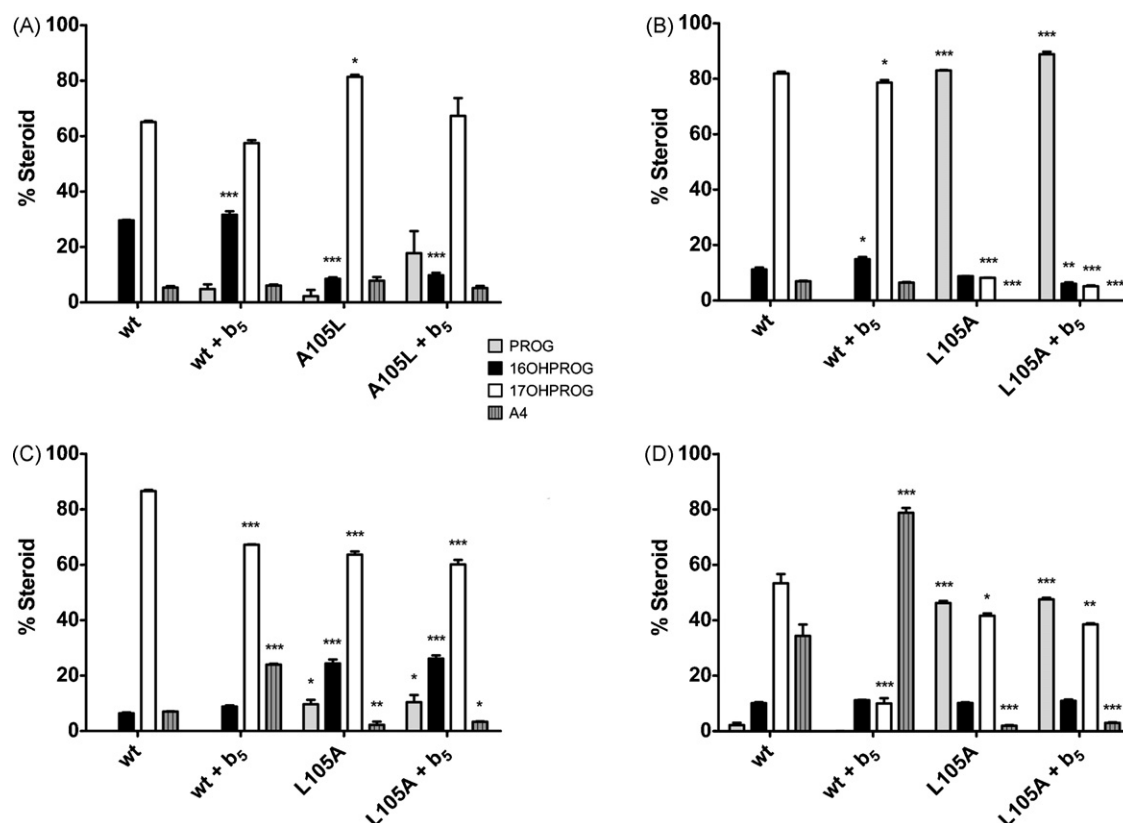


Fig. 3. Steroid profile of PROG (1 μ M) metabolism without and in the presence of cytochrome b₅. Steroid metabolites produced by wild types CYP17 and mutant constructs: (A) human, (B) baboon, (C) goat and (D) pig. Individual steroids were compared to those utilized and produced by wt human CYP17 by a one-way ANOVA, followed by Bonferroni's multiple comparison test (* P < 0.05; ** P < 0.005; *** P < 0.0001). Results are representative of three independent experiments.

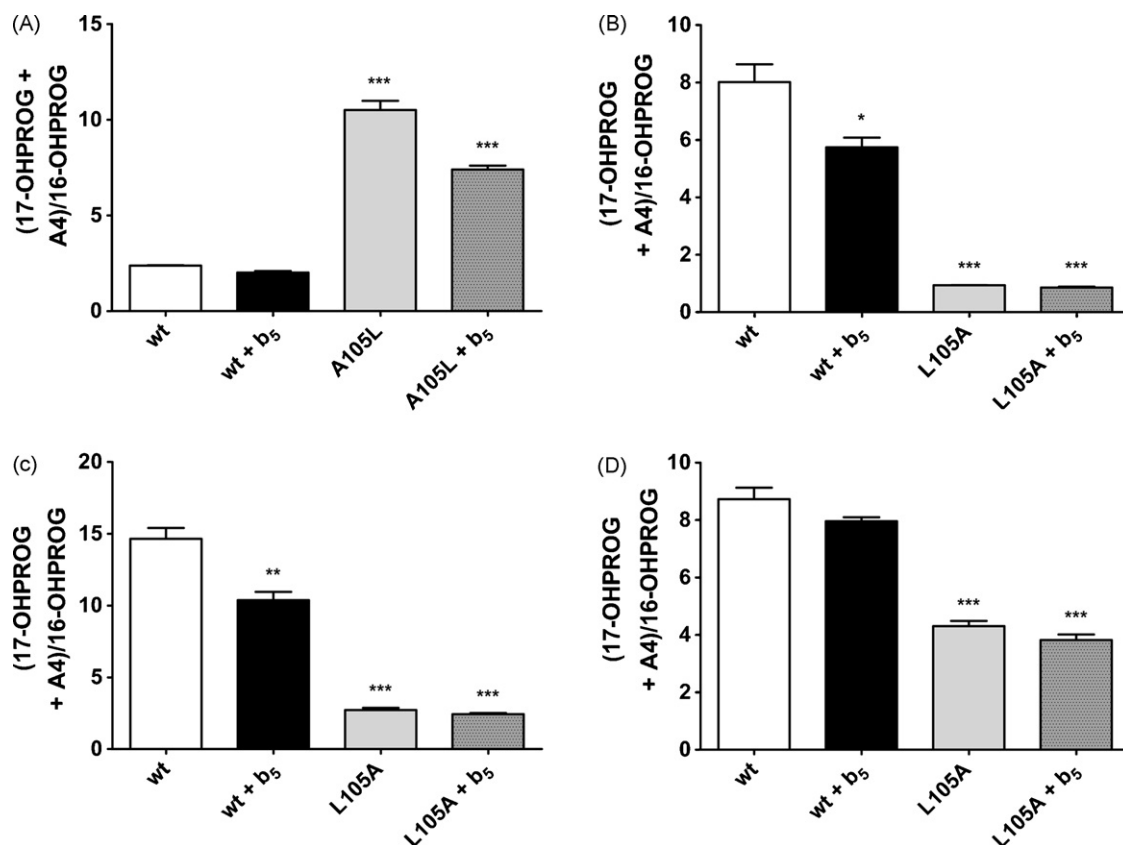


Fig. 4. Ratio of 17-hydroxylated products to 16-OHPROG produced in the metabolism of PROG (1 μ M) without and in the presence of cytochrome b₅. Wild types CYP17 and mutant constructs: (A) human, (B) baboon, (C) goat and (D) pig. Columns were compared to the respective wild types by a one-way ANOVA, followed by Bonferroni's multiple comparison test (* P < 0.05; ** P < 0.005; *** P < 0.0001). Results are representative of three independent experiments.

icantly (P < 0.0001), exhibiting a similar ratio of 17-hydroxylated products to 16-hydroxylated product as the wild type human enzyme (Fig. 4B). The Leu105Ala mutation was subsequently introduced into different mammalian species other than primates, namely goat and pig. These share 72% and 66% sequence identity with the human enzyme respectively and exhibit low 16 α -hydroxylase activity. All constructs were expressed in COS-1 cells, in the presence or absence of their corresponding cytochrome b₅, and assayed for activity with PROG as substrate. PROG was converted to 16-OHPROG, 17-OHPROG and A4 in the transfected COS-1 cells (Fig. 3). After 8 h, cells expressing human CYP17 converted substantially more PROG to 16-OHPROG (\approx 30%) than the wild types of the other three species viz. baboon (\approx 11%), goat (\approx 6%) and pig (\approx 10%). While the substitution of Ala105 for a Leu residue reduced the production of 16-OHPROG by human CYP17 to levels similar to those observed in the other three species (\approx 9%), there was a concurrent increase of 17-OHPROG production. The introduction of an Ala at position 105 into the baboon, goat and pig enzymes resulted in a relative increase of 16-OHPROG production as can be seen in by the decreased ratio of 17-hydroxylated products to 16-OHPROG (Fig. 4). In addition, the introduction of the Leu105Ala into the baboon and pig enzymes significantly (P < 0.0001) decreased the overall catalytic activity of the enzyme.

Coexpression of the CYP17 constructs with the corresponding cytochrome b₅ significantly increased A4 production in the case of wt pig (P < 0.0001) and wt goat CYP17 (P < 0.0001) (Fig. 3) while the mutant constructs produced negligible A4 and was not stimulated by the addition of cytochrome b₅. The presence of cytochrome b₅ did not influence the production of A4 by either the wt or mutated human and baboon enzymes. Interestingly, coexpression of cytochrome b₅ also significantly decreased the

ratio of 17-hydroxylated products to 16-OHPROG in the constructs with a Leu residue at position 105, while little effect was observed in the constructs with an Ala at this alignment position (Fig. 4).

3.2. Homology modeling of CYP17

Previous published models of CYP17 have relied on the solved crystal structures of bacterial cytochromes P450 [19–23]. The recent availability of the crystal structures of more closely related mammalian cytochromes P450 has contributed towards the reliability of homology models for cytochromes P450 [24,25]. In this study homology models of human CYP17 were built using three different mammalian templates, CYP1A2, CYP2R9 and CYP2E1, individually and in combination.

It has previously been shown that the substitutions His373Leu [26] and Pro409Arg [27] both abolished CYP17 enzymatic activity as a result of the inability to incorporate the haem prosthetic group correctly. The substitution, Arg440His, which causes complete enzymatic deficiency, is located very close to Cys442, which is the fifth ligand of the haem iron. This substitution is therefore thought to interfere with the orientation of this vital haem binding domain [28]. Furthermore, previous models have shown that this residue interacts directly with one of the propionate groups of haem [29]. Previous modeling studies have predicted that His373 does not lie near the haem itself, but that the His373Leu mutation causes a substantial structural change elsewhere in the protein that subsequently abolishes haem binding [23]. However, analysis of our models reveals that His373 interacts directly with the second haem propionate group, thereby stabilizing haem binding. This clearly demonstrates that the use of more closely related mam-

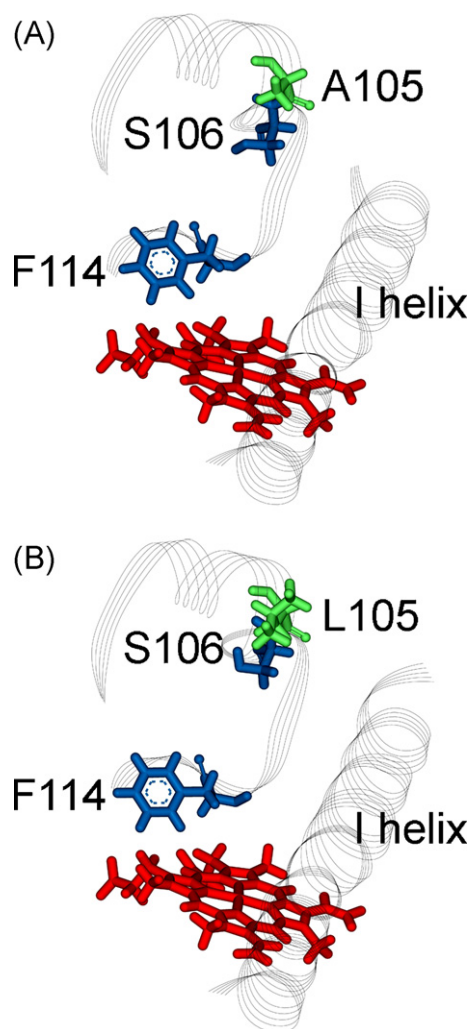


Fig. 5. Putative positions of residues 105, 106 and 114 in the B'-C domain of human CYP17. (A) Wild type CYP17 and (B) Ala105Leu mutant construct.

malian cytochromes P450 as templates can yield more accurate homology models.

It should, however, be noted that there are still limitations to these models especially in predicting the conformation of the active pocket. While the overall structural fold is conserved in all cytochromes P450, it is the regions with the greatest structural difference that influence substrate recognition and binding [30]. Two of the most variable domains amongst the cytochromes P450 include the F and G helices together with the F-G loop and the B'-C domain [31–33]. It is the structural differences in the SRS's that allow the cytochromes P450 to metabolise such a plethora of substrates. This is demonstrated in the four constructed models, while the overall structural core is very similar, the structure of the B'-C domain varies greatly depending on the template used. Homology modelling of this loop therefore remains challenging with the exact orientation and positioning of the side-chains remaining uncertain.

Nevertheless, in all of the constructed models, residues in this domain which have been identified experimentally to be critical to catalysis all form part of the active pocket [34,35]. Furthermore, all the models reveal that Ala105 forms part of this SRS and faces towards the active pocket (Fig. 5). Ala105 in the human enzyme therefore creates more space in the active pocket when compared to the leucine in the other three species. This allows for the repositioning of the PROG substrate with the C16 aligned with the iron-oxygen complex for the 16 α -hydroxylase reaction. The large

Leu residue at this position would sterically hinder the repositioning of the substrate.

4. Discussion

Since researchers first studied the catalytic activity of CYP17 in the early seventies, the enzyme has been shown to be one of the most versatile enzymes in mammalian steroidogenesis. This hemo-protein, which is dependent on a complex electron transfer system for its catalytic integrity, not only accepts a number of substrates but also exhibits different activities in adrenal and gonadal tissue which differ from specie to specie. Given the multifaceted nature of this catalysis, it is therefore not surprising that a number of steroid products are produced of which 16OH-progesterone is one. Since none of the mammalian steroidogenic P450 enzymes have, to date, been crystallised the challenging question remains as to how this enzyme catalyses these reactions. Consequently gene mutations which manifest in clinical conditions, site directed mutagenesis and homology modeling have contributed immensely towards understanding the complex nature of CYP17. Clinical conditions characterized by mineralocorticoid excess and decreased androgen biosynthesis have led to the identification of novel mutations in the CYP17 gene, resulting in either the 17 α -hydroxylase/17,20-lyase activity being influenced or the lyase activity being deficient [36–39]. In addition, the engineering of mutations by site directed mutagenesis enabled researchers to identify amino acid residues of catalytic importance in the hydroxylase as well as the lyase capabilities of the enzyme, together with the enzyme's interaction with its redox partner and cytochrome b₅ [40–42]. These data together with homology models, based on the crystal structures of mammalian and bacterial P450 enzymes have contributed towards structure predictions, identifying catalytic key residues and domains impacting on the activity of the enzyme.

Despite the low degree of homology shared between the cytochromes P450, all classes of these enzymes share a common structural fold. These highly conserved structures play a vital role in protein folding and haem binding. However, it is the regions of greatest structural difference that influence substrate recognition and binding [15]. Six such domains, substrate recognition sites (SRS), have been identified and are generally accepted for all cytochromes P450. Studies have shown that missense mutations are more frequent within the SRS than outside of these sites [43]. SRS1 is located in the B'-C domain and meanders in and out of the haem pocket [15]. In our model and in those of other groups, alignments of CYP17 with solved mammalian cytochromes P450 place Ala105 in the B'-C domain. It is within the B'-C domain that specific residues have been shown to contribute towards substrate specificity and hydroxylation regioselectivity in both bacterial [15,44–46] and mammalian cytochromes P450 [43,47–54]. Halpert and He [54] observed that the mutations Ile114Val and Ile114Ala resulted in a decrease in 16 β -OH:16 α -OH and an increase in 15 α -OH:16-OH androgen ratios in rat CYP2B1 for both mutations. In rabbit CYP2C4 and CYP2C5, residue 113 (Val113Ala) is responsible for the >10-fold difference in Km for progesterone 21-hydroxylation [49]. Lindberg and Negishi [51] have shown that the mutation Val117Ala is one of three mutations that confer coumarin 7-hydroxylase activity to mouse CYP2A4, a steroid 15 α -hydroxylase.

The flexibility of the B'-C domain, together with the F-G loop, has been shown to be important in allowing substrates access to the buried active pocket of the cytochrome P450 enzymes. Crystal structures show that inhibitor-free CYP2B4 demonstrates a large open substrate access channel, formed by the repositioning of helices B' to C and F through G, while the enzyme bound to a phenylimidazole inhibitor shows a closed conformation [55,56].

Our investigation into the influence of the Lys and His residues, which are located in the predicted F helix, indicated that while these residues were not involved in the 16-hydroxylation of PROG they did, however, influence the rate at which the substrate was converted to 17-OHPROG. While it is interesting to note that Negishi et al. [57] concluded that charged residues at key positions in the SRSs in general tend to decrease P450 enzyme activities, our data shows that the mutation of the charged residues to uncharged polar residues decreased the hydroxylase activity of baboon CYP17.

In CYP2C5, the B'-C domain adopts different orientations when bound with the substrates diclofenac and 4-methyl-N-methyl-N-benzenesulfonamide, further illustrating the importance of this domain [58]. Mutations in close proximity to Ala105 in the B'-C domain of CYP17 have further confirmed the importance of the B'-C structural domain. A Ser106Pro substitution abolished both the hydroxylase and lyase activity of CYP17 [34]. A homology model of CYP17, constructed using the soluble bacterial cytochrome P450, P450cam, as a template [19] revealed that Ser106 lies in close proximity to two putative regions (amino acids 101–102 and 111–116) that form part of the steroid binding pocket [34]. Mutating Ser106 to either Thr or Ala resulted in a substantial loss in both 17 α -hydroxylase and 17,20-lyase activity, suggesting that this residue forms part of the catalytic site, with the position of the hydroxyl group of Ser being critical [59]. In addition, this group ruled out the possibility that Ser106 participates in the cytochrome P450 oxidoreductase docking site. Ser106 is conserved in all mammalian species for which sequences are available on GenBank (Fig. 1). All other species, which include frog, fish, turtle and bird species, have a Thr at this alignment position.

Within this domain, conserved residues 93, 96, 114, and 116 have been linked to substrate recognition and binding with mutations impacting on the catalytic activity of CYP17. Residues 93–97 are perfectly conserved in CYP17 of many species and Di Cerbo et al. [60] suggested that this domain is critical to the function of CYP17. The substitution Phe93Cys was shown to result in a mutant CYP17 protein which retains merely 10% of both 17 α -hydroxylase and 17,20-lyase activities [60]. The substitution Arg96Trp, another residue which is highly conserved across species, almost completely abolished the activity of the mutant protein [61]. Brooke et al. [62] later identified a novel point mutation, Arg96Gln, to cause a combined hydroxylase/lyase deficiency. Two substitutions at positions 114 and 116 in the putative steroid-binding domain, was shown by van der Akker et al. [35] to cause complete (Phe114Val), or partial (Asp116Val) 17 α -hydroxylase and 17,20-lyase deficiencies.

Our homology models confirm that the above residues form part of the B'-C domain with Ser106 and Phe114 facing directly into the active pocket (Fig. 5). Experimental data shows that the hydroxyl group of Ser106 almost certainly interacts directly with the bound substrate [59]. Phe114 occupies a position close to the haem moiety, where the aromatic residue could have a major steric influence on the orientation of substrates in the active pocket. Phe120 at a similar alignment position in CYP2D6 has been shown to play a role in controlling the regioselectivity of substrate oxidation through its steric influence on the orientation of molecules in the active site [63]. Although Asp116 does not form part of the active pocket directly, it may play an indirect role in stabilizing the haem through an interaction with Arg440. Similarly, Arg96 is situated between the haem propionate groups and may be critical for haem binding.

In our model and in all the other constructed models, Ala105 is pointed directly into the active pocket, with the adjacent Ser at 106 believed to interact directly with the substrate [59]. The 16 α -hydroxylase reaction requires the repositioning of the steroid substrate with the C16 aligned with the iron-oxygen complex, which an Ala in this position may allow more readily than a Leu residue. Although low levels of 16 α -hydroxylase activity towards PROG have been reported for CYP17, significant 16 α -hydroxylation

of PROG has only been observed with the human and chimp enzymes [2,3]. It was postulated by Arlt et al. [3] that the position of the steroid substrate is altered by residues in the region spanning helices E through H, reducing the accessibility of the C16 α hydrogen atom to the haem iron. Our mutation of the residues in the predicted F helix of baboon CYP17, Lys196, Ile197, Val198 and His199, to correspond with those of the human sequence (Asn, Val, Ile and Gln) show that although these mutations lowered the rate of PROG metabolism, they had no influence on the 16 α -hydroxylase activity (Fig. 2). Ala105 therefore appears to be the primary cause of 16 α -hydroxylase activity towards PROG.

It is possible that the Ala residue at position 105 in the human and chimp enzyme may provide less steric hindrance for the repositioning of the substrate. Creating a steric hindrance with the substitution of the Ala residue for the bulkier Leu residue, may thus prevent the repositioning of PROG, reducing the amount of 16 α -hydroxylase activity as seen for the human Ala105Leu mutant (Figs. 3 and 4). Replacing the Leu residue in the baboon, goat and pig enzymes by Ala resulted in a significant increase in 16 α -hydroxylase activity ($P < 0.001$). However, although the 16 α -hydroxylase activity was increased relative to that of the 17 α -hydroxylase and 17,20-lyase activities, the overall metabolism of PROG by the baboon and pig wt enzymes after 8 h was reduced to 13% and 54% respectively (Fig. 3). It is likely that the substitution of the Leu residue at position 105 affects the position of the adjacent Ser residue. Lin et al. [59] have shown that both the Ser106Ala and Ser106Thr substitutions reduced the 17 α -hydroxylation of PROG to less than 30% of the wt, while reducing the 16 α -hydroxylation of PROG to only ~60%. The authors concluded that the hydroxyl group of the Ser residue may therefore play an important role in positioning the substrate for the 17 α -hydroxylation and 17,20-lyase reactions, but not for the 16 α -hydroxylation reaction. These data correlate with the increased 16 α -hydroxylase and decreased 17 α -hydroxylase and 17,20-lyase activities observed for the Leu 105Ala substitutions in the baboon and pig enzyme. Interestingly, the Leu105Ala mutant of goat CYP17 maintained 90% of the activity of the wt (Fig. 3), suggesting that the rest of the B-C region plays an important role in the positioning of the substrate for catalysis. Human Ala105Leu and wt goat CYP17 have similar 17-hydroxylated products to 16-OHPROG ratios, while baboon and pig CYP17 have similar ratios. This suggests that the structural fold of SRS1 in the human enzyme may be more comparable to that of goat CYP17 rather than to that of the other two species. One similarity is that the human and goat enzymes have an Ala residue at position 100 while at this alignment position the baboon and pig enzymes have larger Thr and Met residues, respectively.

Interestingly, cytochrome b_5 promoted the 16 α -hydroxylase activity in assays carried out with the CYP17 constructs containing a Leu at position 105 (human Ala105Leu, wild types baboon, goat and pig CYP17) resulting in a significant increase in 16-OHPROG (Fig. 3). Cytochrome b_5 has been reported to promote only the 17,20 lyase reaction while having no influence on the 17 α -hydroxylase activity. This allosteric interaction promotes the association of CYP17 with P450 oxidoreductase, thus increasing the efficiency of electron transfer required for the 17,20 lyase reaction [64–66]. Since additional electrons from P450 oxidoreductase are not required for the 16 α -hydroxylase reaction, the increased 16 α -hydroxylase activity must be exclusively due to the allosteric interaction between CYP17 and cytochrome b_5 . We have previously proposed that this interaction alters the three-dimensional structure of CYP17 in such a way as to promote the repositioning of the substrate in an orientation that is more favourable for the 16 α -hydroxylase reaction [67]. It follows that a cytochrome b_5 induced change in the three-dimensional structure of CYP17 can result in the optimal repositioning of the steroid substrate for the 17,20 lyase reaction as the 17,20-lyase reaction requires the C20 of the steroid substrate, rather than the

C17, to align with the iron–oxygen complex. Cytochrome b₅ had no significant effect on the catalytic activity of the constructs with an Ala residue at position 105 (human CYP17, baboon Leu105Ala, goat Leu105Ala and pig Leu105Ala), suggesting that the smaller Ala residue provides more space for the repositioning of PROG for the 16 α -hydroxylase reaction to occur. The subsequent structural changes brought about by the allosteric interaction with cytochrome b₅ cannot further enhance the binding of the substrate.

This data has provided evidence that the 16 α -hydroxylase activity observed in human CYP17 is mediated by Ala 105. The Leu residue in this position in other species creates a sterical hinderance, preventing the repositioning of the PROG substrate required for the 16 α -hydroxylase reaction. Furthermore, we have also shown that cytochrome b₅ is able to increase 16 α -hydroxylase activity in CYP17 enzymes with a Leu residue in position 105, bringing about a structural change that relieves the steric hinderance caused by the bulkier Leu residue.

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