Multiple steroid-binding orientations: alteration of regiospecificity of dehydroepiandrosterone 2- and 7-hydroxylase activities of cytochrome *P*-450 2a-5 by mutation of residue 209

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The mutation of Ala-117 to Val conferred dehydroepiandrosterone (DHEA) hydroxylase activity on cytochrome *P*-450 2a-4, with the production of both 2 α - and 7 α -hydroxyDHEA at similar rates. *P*-450 2a-5 which has Val at position 117, acquired high DHEA hydroxylase activity by mutation of Phe-209. Mutant F209L of *P*-450 2a-5 exhibited strong regiospecificity at the 2-position of the DHEA molecule with the production of 2 α -hydroxy DHEA as the major metabolite. On the other hand, mutant F209V of *P*-450 2a-5 showed the 7-position to be the major hydroxylation site, 7 β -hydroxyDHEA and 7 α -OHDHEA being produced. Therefore the regiospecificity of DHEA hydroxylase activity of *P*-450 2a-5

is altered between the 2- and 7- position depending on the amino acid at position 209. Modelling of the DHEA molecule in the pocket of bacterial *P*-450cam showed that the steroid can be accommodated in at least two orientations for which the 2- or 7- position is near the sixth axial position of the haem. Moreover, these two orientations, which are of similar energy, can be interconverted by a 180° rotation of the steroid molecule around its long axis. These results support the hypothesis that the steroid molecule in the pocket is in dynamic equilibrium with multiple binding orientations and that the equilibrium is apparently determined by a few critical residues including those at positions 117 and 209.

INTRODUCTION

Cytochrome *P*-450 (*P*-450) represents a large group of structurally related haem–thiolate oxygenases, which catalyse the oxidation of a large number of endogenous and exogenous chemicals. Hepatic *P*-450s play a key role in steroid metabolism as well as in metabolic detoxification and activation of environmental toxins and carcinogens. The mechanism by which these proteins provide a broad range of catalytic activity remains an intriguing question. Recent site-directed mutagenesis studies suggest that their activity is inherently versatile because the substrate pocket is geometrically flexible and can be modified structurally by minor amino acid substitutions to accommodate various substrates comfortably. The substrate specificity of *P*-450 2a-5, for example, is altered from coumarin to the Δ^4 -3-ketosteroids including testosterone, androstenedione and progesterone and then to the 11 β -hydroxy, Δ^4 -3-ketosteroids such as corticosterone by sequential amino acid mutations at position 209 [1–4]. Although *P*-450 2B1 and *P*-450 2a-4 exhibit different steroid substrate specificity and have only 50% identity in their amino acid sequences, the specificity of *P*-450 2B1 can be converted into that of *P*-450 2a-4 by amino acid mutation at only two positions (residues 114 and 206) [5]. Furthermore, homology alignment and computer modelling based on bacterial *P*-450cam have provided structural insight into the role of these residues in the determination of the observed specificities [3,5–10].

Characteristically, an hepatic *P*-450 often produces many metabolites from a single substrate. Rat *P*-450 2A2, for example, hydroxylates testosterone producing at least five main metabolites: 15 α -, 15 β -, 7 α -, 6 β -hydroxy- and possibly 12 α -hydroxy-

testosterone [11]. *P*-450 2C11 produces 2 α - and 16 α -hydroxy-testosterone at similar rates; the former exhibits A-ring hydroxylation, whereas the latter shows D-ring hydroxylation [11,12]. These examples suggest that steroid binding is flexible in the substrate pocket of *P*-450 2A2 in order for hydroxylation to occur at more than one site. Testosterone may be accommodated in at least two different binding orientations in the pocket to account for hydroxylation at the 2 α - or 16 α -position by *P*-450 2C11 [11]. In an effort to understand the mechanism by which *P*-450 produces multiple metabolites, this paper extends our previous work on dehydroepiandrosterone (DHEA) hydroxylase activity of mouse *P*-450 2a-4 and *P*-450 2a-5 [13]. We first study the regiospecificity of the DHEA hydroxylase activities of these molecules and then provide a structural basis for multiple steroid-binding orientations in the substrate–haem pocket. The discussion will focus on how different steroids may be adopted in different binding orientations of similar energy in the substrate pocket.

EXPERIMENTAL

Site-directed mutagenesis expression of mutants and assay of hydroxylase activity

We constructed mutants of *P*-450 2a-4 and *P*-450 2a-5 using a pSELECT vector, expressed them in *Saccharomyces cerevisiae* AH22 using a pAAH5 vector and purified them from recombinant yeast microsomes as described previously [13,14]. DHEA hydroxylase activity of the wild-type and mutants was reconstituted and measured by t.l.c. and a radioactive-flow-

Abbreviations used: *P*-450, cytochrome *P*-450; COSY, shift-correlated n.m.r. spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; DHEA, dehydroepiandrosterone; 2 α -OH DHEA, 2 α -hydroxyDHEA; 7 α -OH DHEA, 7 α -hydroxyDHEA; 7 β -OH DHEA, 7 β -hydroxyDHEA.

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detector-equipped h.p.l.c. TSK-GEL ODS-80TM column as described previously [13]. The reconstitution reaction mixture contained 10 pmol of *P*-450, 1 mM rat liver cytochrome *P*-450 reductase and 5 nmol of dilauroylphosphatidylcholine in 0.5 ml of 100 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂, 1 mM NADPH and 50 μ M [4-¹⁴C]DHEA. To obtain K_m values, DHEA hydroxylase activity was determined using various DHEA concentrations (2, 5, 10, 25 and 50 μ M) and Levenberg–Marquardt's non-linear regression program. [4-¹⁴C]DHEA (51 mCi/mmol) was purchased from DuPont–New England Nuclear and diluted with non-radioactive DHEA (Sigma) to 5 mCi/mmol.

¹H-n.m.r. spectroscopy of DHEA metabolites

Preparation and n.m.r. analysis of the three DHEA metabolites formed by mutant *P*-450 2a-4 (A117V) and *P*-450 2a-5 (F209V) were performed as previously described [3]. Two-dimensional double-quantum-filtered shift-correlated n.m.r. spectroscopy (COSY) [15] and nuclear Overhauser effect spectroscopy (NOESY) [16] were performed at 500 MHz on a Bruker AMX500 spectrometer. The mixing time for cross-relaxation in the NOESY experiments was 0.5 s. Data sets consisting of $2 \times 512 \times 256$ points and F1 and F2 spectral widths of 3012 Hz were collected in the States time proportional phase incrementation mode [17] and transferred to a Silicon Graphics Personal Iris workstation for processing via Felix v2.1 (Biosym, San Diego, CA, U.S.A.) software. Before Fourier transformation to two-dimensional absorption-mode spectra, the t_2 , t_1 data were apodized with skewed Hanning windows [18] and zero-filled to 1024 points. All spectra were acquired at a sample temperature of 30 °C and the chemical shifts were referenced to tetramethylsilane as internal standard ($\delta = 0$ p.p.m.).

Modelling of DHEA-binding conformations in the substrate–haem pocket of *P*-450cam

Initially, DHEA was docked into the substrate–haem pocket in the conformation shown in a previous paper [13]. A new binding orientation was created by constraining the C-7 position of DHEA to be near the sixth axial position and the 3-hydroxy group to be near the bacterial residues Thr-101 and Ser-102 in *P*-450cam [19]. Another binding orientation was derived by subsequently rotating DHEA 180° around the long axis of the steroid molecule, which placed the C-2 position near the sixth axial ligand. We then energy-minimized these two conformations using the molecular mechanics program AMBER as described previously [3]. Briefly, the *in vacuo* minimizations were carried out in two steps, first allowing only the pocket atoms to move, and then allowing all protein and haem atoms to move independently. The AMBER 3.0a united atom force field and a distance-dependent dielectric constant were employed. The lowest energy conformation that resulted from the minimization was rotated 180° around the long axis of the steroid molecule to provide a third orientation which ultimately proved to be the lowest energy found. Similarly, several other reasonable conformations were generated by appropriate rotations. A total of six unique starting positions were employed.

RESULTS AND DISCUSSION

P-450 2a-5 is mouse coumarin 7-hydroxylase and exhibits little steroid hydroxylase activity. In a previous paper [13], we reported that mutation of Ala-117 to Val conferred high DHEA hydroxylase activity on *P*-450 2a-4, producing the two major

hydroxylated metabolites. Although 7 α -hydroxyDHEA (7 α -OH DHEA) was identified as the first metabolite eluted from the h.p.l.c. column, the structure of the second was unknown. We have now determined from two-dimensional n.m.r. that the second metabolite is 2 α -hydroxyDHEA (2 α -OH DHEA). The COSY spectrum indicated that the new CHOH peak at 3.66 p.p.m. is from H₃, because this line is coupled not only to H₂ (which itself is shifted upfield by 0.21 p.p.m. relative to that for the original DHEA) but also for a second pair of lines at 1.05 and 2.08 p.p.m. assigned to H₁ and H₁'. If oxidation had occurred at C-4, only a single cross-peak, namely to H₃, would have been observed. Moreover, a strong NOESY cross-peak between H₂ and the protons of the C-19 methyl group indicated that H₂ is on the β face of the steroid molecule [20], proving that this metabolite is indeed 2 α -OH DHEA. Thus we conclude that mutant A117V of *P*-450 2a-4 exhibited DHEA 2 α - and 7 α -hydroxylase activities of 5.8 ± 0.6 and 7.7 ± 0.6 nmol/min per nmol of *P*-450 respectively (see Table 1 of ref. [13]).

Mutant A117V, however, did not exhibit DHEA 15 α -hydroxylase activity. This result was surprising because the 15 α -hydroxy steroid was the major metabolite when androstenedione was used as substrate [13]. Moreover, *P*-450 2a-4 was originally characterized as an enzyme that specifically hydroxylates the 15 α -position of Δ^4 -3-ketosteroids including testosterone, androstenedione and progesterone [21,22]. Even when asparagine was present at position 209 in *P*-450 2a-4 and *P*-450 2a-5 altering the steroid substrate specificity to corticosterone (an 11 β -hydroxy, Δ^4 -3-ketosteroid), the *P*-450s retained the regiospecificity of the hydroxylation at the 15 α -position [3]. Taken together, these results suggest that the binding orientation of DHEA (a Δ^5 -3 α -hydroxysteroid) in the substrate pocket is apparently different from that of the Δ^4 -3-ketosteroids including corticosterone. Furthermore, DHEA appears to adopt at least two distinct orientations with the result that both the 2 α - and the 7 α -positions of the steroid molecule are hydroxylated by the single form of *P*-450 2a-4.

In previous modelling studies, we initially found that residue 209 and the 11 β -hydroxy group of the steroid defined corticosterone 15 α -hydroxylase activity of *P*-450 2a-4 and *P*-450 2a-5 [3]. We then showed that corticosterone can be accommodated in the substrate–haem pocket of bacterial *P*-450cam so that the bacterial residue (Met-184) that correspond to the amino acid at position 209 in mouse *P*-450 is directed toward the 11 β -hydroxy of the steroid molecule [3]. Most importantly, this model placed the C-15 hydroxylation site near the sixth axial position of the haem. In addition, we found that the C-3 position of the steroid molecule is near Thr-101 and Ser-102 of the bacterial *P*-450cam; these bacterial residues are aligned with the amino acids at positions 116 and 117 of *P*-450 2a-4 and *P*-450 2a-5. The binding model therefore provided a structural basis for why residue 117 may define DHEA hydroxylase activity of mouse *P*-450 molecules depending on the 3-hydroxy group of the steroid molecule. As subsequent site-directed mutagenesis studies supported this hypothesis, we modelled DHEA into the same binding orientation as for corticosterone [13]. Energy minimization indicated that DHEA can be accommodated in an orientation so that the 3-hydroxy group of the steroid molecule is near Thr-101 and Ser-102 of bacterial *P*-450cam. Moreover, the sixth axial position of the haem is directed toward the 7- and 15-position of the steroid, the former being one of the two major hydroxylation sites [13].

Our present findings that the mutants of *P*-450 2a-5 hydroxylate at the 2- but not the 15-position of DHEA indicate that DHEA and Δ^4 -3-ketosteroids such as androstenedione and corticosterone appear to be bound in different orientations. We

Figure 1 Energy-minimized DHEA-binding orientations II and VII in the *P*-450 cam pocket

Both binding orientations (II has C-2 near sixth axial Fe position, VII has C-7 near sixth axial Fe position) are viewed from approximately the same direction. The steroid molecule, key amino acid residues and haem are drawn with thick lines. Amino acids are indicated by the standard three-letter code. Thin lines are used to draw the backbones of the B'-C (including Thr-101 and Ser-102) and F-G loops (starting after Thr-185) and a portion of the I-helices (crossing horizontally near the observer). Steroid carbons at positions 7 and 15 are denoted by C-7 and C-15 respectively, and OH-3 and O-17 designate the oxygen of the 3-hydroxy group and the ketone at C-17 respectively. The distances from the activating oxygen to the C-2 or C-7 position are also indicated. The long axis about which rotation of the steroid takes orientation II into orientation VII is shown with the steroid molecules between the two orientation pictures.

therefore energy-minimized the additional two DHEA-binding orientations after initially directing either the 2- or 7-position toward the sixth axial position of the haem (orientations II and VII in Figure 1). In orientation II, the 2-position of the steroid is minimized to a position that is 0.361 nm (3.61 Å) from the sixth axial position. In orientation VII, the 7-position is 2.75 Å and the 15-position is 4.58 Å from the sixth axial position after minimization. These two binding orientations can be approximately interconverted by rotating DHEA 180° around the long axis of the steroid molecule. In both binding conformations, the 3-hydroxy group of the steroid molecule resides near (approx. 3.6 Å) the bacterial residue Thr-101 which is homologous to residue 116 of mouse *P*-450 2a-4 and *P*-450 2a-5 and adjacent to Ser-102. Thus the new binding orientations (II and VII) are consistent with the hypothesis that the identity of residue 117 plays a major role in defining DHEA hydroxylase activity of the *P*-450s. In addition, the implication is that DHEA must be accommodated in at least two different and interconvertible orientations so as to direct either the 2- or 7-position toward the sixth axial position of the haem.

Interestingly, Met-184 in *P*-450cam, which can be aligned with residue 209 of mouse *P*-450s, is near (approx. 5.7 Å) the C-17 position of DHEA in each of these new DHEA-binding orientations, although the Met-184 residue was directed toward the C-11 position in the previous DHEA- or corticosterone-binding orientation [13]. Even nearer to C-17 is Thr-185 (3.5 Å) and therefore it is possible that at our level of sequence comparison this residue is the appropriate homologue at position 209 in *P*-450 2a-5. We therefore examined whether the regio-specificity of DHEA hydroxylase activity in *P*-450 2a-5 was dependent on the type of residue at position 209 (Figure 2 and Table 1). As described previously [13], *P*-450 2a-5 has Val at position 117 so a mutation at position 209 would be sufficient to induce high DHEA hydroxylase activity. We measured DHEA

Figure 2 H.p.l.c. analysis of DHEA hydroxylase activity in the *P*-450 2a-5 mutants F209L and F209V

DHEA hydroxylase activity was reconstituted in the *P*-450 mutants and the metabolites were analysed by h.p.l.c. as described in the Experimental section.

Table 1 DHEA hydroxylase activity of the wild-type *P*-450 2a-5 and mutants

DHEA hydroxylase activity was measured as described in the Experimental section and previous work [13]. The substrate concentration was 50 μ M. Results are means $\pm$ S.E.M. ($n = 3$ determinations). nd, Activity not detected.

Amino acid at position 209	DHEA hydroxylase activity (nmol/min per nmol of <i>P</i> -450)		
	2 α -Hydroxylase	7 α -Hydroxylase	7 β -Hydroxylase
Phe	0.3 $\pm$ 0.01	nd	nd
Leu	2.34 $\pm$ 0.24	0.56 $\pm$ 0.04	0.1 $\pm$ 0.08
Val	2.61 $\pm$ 0.05	1.22 $\pm$ 0.02	5.5 $\pm$ 0.1

hydroxylase activity in the wild-type *P*-450 2a-5 and the mutants F209L and F209V (Figure 2 and Table 1). Collectively, these *P*-450 2a-5 systems produced three DHEA metabolites, two of which were 2 α - and 7 α -OH DHEA. We determined the structure of the remaining metabolite to be 7 β -OH DHEA again using two-dimensional n.m.r. The new CHOH peak of this metabolite appeared at 3.94 p.p.m. This peak can be assigned to H₇, as, in the COSY spectrum, it is directly coupled to the readily identified vinylic proton H₆ and, by default, to H₈. In addition, the values of the three-bond vicinyl coupling constants [$^3J(\text{H}_6\text{--H}_7)$ 2 Hz and $^3J(\text{H}_7\text{--H}_8)$ 8 Hz], derived from the COSY cross-peak intensities and the one-dimensional multiplet structure [23] indicated that H₇ is axial and therefore the metabolite is 7 β -OH DHEA. Similarly, we also confirmed by n.m.r. the previously known 7 α -OH DHEA with the unique peak at 3.96 p.p.m. Thus we conclude that the *P*-450s exhibited DHEA 2 α -, 7 α - and 7 β -hydroxylase activities.

Wild-type *P*-450 2a-5 exhibited low DHEA 2 α -hydroxylase activity, but the activity was approx. 10-fold higher when Leu was substituted for Phe-209. 2 α -Hydroxylation was the major activity of mutant F209L, although low DHEA 7 α - and 7 β -hydroxylase activities were also observed. Mutant F209V, on the other hand, hydroxylated mainly the 7-positions of DHEA, producing 7 α - and 7 β -OH DHEA. The 7 β -hydroxylase activity was approx. 5-fold higher than the 7 α -hydroxylase activity. Although mutant F209V also had 2 α -hydroxylase activity, it was approx. 3-fold lower than the combined 7 α - and 7 β -hydroxylase activities. We measured DHEA hydroxylase activity in other 209 mutants (F209A, F209G, F209S, F209N, F209M, F209D and F209K). None of these exhibited DHEA hydroxylase activity, except for F209N which showed a similar regiospecificity to that of mutant F209L, i.e. 2 α -hydroxylation was the major activity (see Table 1 of ref. [13]). Although its V_{max} values differed (1.0 $\pm$ 0.08, 0.8 $\pm$ 0.017 and 2.2 $\pm$ 0.15 mmol/min per mg respectively) mutant F209V exhibited very similar K_m values for DHEA 2 α -, 7 α - and 7 β -hydroxylase activity (10.8 $\pm$ 2.3, 16.4 $\pm$ 0.8 and 13.1 $\pm$ 2.4 μ M respectively). V_{max}/K_m ratios further confirmed that mutant F209V catalysed 7-hydroxylation approx. 3-fold more efficiently than the 2-hydroxylation. Moreover, these similar K_m values provide additional evidence that the different DHEA-binding orientations may occur at similar energies.

Our site-directed mutagenesis and modelling studies indicate that the broad range of *P*-450 activity is probably due to the ability of the substrate pocket to accommodate the steroid in multiple binding orientations. Moreover, as suggested from the binding orientation derived from modelling, the major hydroxylation site of *P*-450 2a-5 can be altered from the 2- to 7-

position of DHEA by a single mutation (F209V). Given the caveat that it is the binding pocket of bacterial *P*-450cam that provides our fundamental model, DHEA can nevertheless be adopted in two different orientations (II and VII) which are derived from a 180° rotation around the long axis of the steroid molecule. Our present model suggests that the residue at position 209 of *P*-450 2a-5 (homologues Met-184 and Thr-185 in *P*-450cam) may be near the steroid so that different side chains on residue 209 may lead to different hydroxylation sites. The model thus concerns only the regiospecificity of DHEA hydroxylase activity and is not sufficiently detailed to explain the stereospecificity of DHEA 7 α - and 7 β -hydroxylase activity. Other directional alterations in addition to the long-axis rotation would be required to obtain binding orientations so that the stereospecificity as well as the regiospecificity could be explained. Moreover, the reactivity of the hydroxylation site may not be determined solely by its distance from the sixth axial position. The C-6 position of DHEA in the minimized VII orientation, for example, is 0.1 Å closer to the position that the C-7 position, although 6-hydroxylase activity is not observed. This may be due to the substantially different chemical environments at the 6 and 7 positions of the steroid or it may reflect the limitations of modelling the mammalian system from the bacterial system. Our binding model, however, should be taken as reasonable support for our site-directed mutagenesis experiments until the three-dimensional structures of these *P*-450s are determined. The model does provide a simple basis for understanding the steroid substrate specificity of *P*-450 2a-4 and *P*-450 2a-5 and also other mammalian *P*-450s.

In conclusion, *P*-450 appears to adopt multiple substrate-binding orientations that are in dynamic equilibrium. The equilibrium may be determined by a small number of critical residues, in the case of DHEA those at positions 117 and 209. The concept of a flexible substrate pocket which can accommodate various substrates in equilibrium with multiple binding orientations provides a structural basis for understanding not only the remarkable diversity of *P*-450 activity but also the role of *P*-450 in the defence against toxic and carcinogenic chemicals.

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