Engineering Mouse P450coh to a Novel Corticosterone 15α-Hydroxylase and Modeling Steroid-binding Orientation in the Substrate Pocket

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The F209L mutation alters specificity of P450coh from coumarin 7-hydroxylation to 15α-hydroxylation of 11-deoxysteroids such as testosterone and 11-deoxycorticosterone. Neither the wild-type nor F209L exhibits activity toward 11β-hydroxysteroids including corticosterone. Mutation of Phe-209 to Asn, however, confers on mutant F209N a high corticosterone 15α-hydroxylase activity. F209V also exhibits low corticosterone 15α-hydroxylase activity; Kₘ and Vₘₐₓ are 10-fold higher and lower, respectively, than for F209N. The results are consistent with the hypothesis that direct interaction of Asn-209 with 11OH is responsible for high corticosterone 15α-hydroxylase activity. To support this hypothesis, a possible steroid-binding orientation is modeled in the substrate pocket of P450cam. Our weighted homology and constrained alignments map residue 209 of P450coh to Met-184 and Met-191 of P450cam. Energy minimization of coumarin to steroid hydroxylation (1). The type of residue 478 of P4502B1 determines the regioselectivity of steroid metabolites (2), and Arg-346 is responsible for delineating the 17α-hydroxylase activity in P45017, from the lyase activity (3).

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Expression in Yeasts—Construction of P450coh mutants used in this study was as described in our previous papers (1, 9, 10). The mutated cDNAs were ligated to yeast expression vector pAAH5 and transformed into Saccharomyces cerevisiae AH22 cells as previously described by Oeda et al. (11).

Purification of P450 and Steroid Hydroxylase Activity—We prepared microsomes from recombinant yeast and purified the P450s using previously published methods (9), except that an additional hydroxylapatite column was used to remove endogenous yeast P450. Hydroxylapatite column was equilibrated with 10 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 1 mM diethio- nitrate, and 0.2% sodium cholate. The P450 from the previous octyl- aminophosphoric acid bound was eluted from the column, washed by 100 mM potassium phosphate buffer, pH 7.25, and eluted by increasing the buffer concentration to 400 mM.

Steroid hydroxylase activity was reconstituted as described previously (12). The reconstitution system consisted of purified P450 (10 pmol), rat NADPH-cytochrome P450 reductase (30 pmol), NADPH (0.5 mM), MgCl₂ (5 mM), dilaurylphosphatidylcholine (8.0 mM), and steroid (100 µM) in 0.5 ml of 50 mM Tris-Cl buffer, pH 7.5. The substrates were [4-¹⁴C]deoxycorticosterone (58.3 mCi/mmol, Du Pont-New England Nuclear), [1,2,6,7-³H]corticosterone (88 mCi/ mmol, Amersham Corp.), and [4-¹⁴C]hydrocortisone (58.5 mCi/mmol, Du Pont-New England Nuclear). The steroid metabolites were extracted with methylene chloride, separated by thin layer chromatography with toluene/acetone (1:1) as the developing solvent, and exposed to x-ray film. Finally, spots containing the metabolites were scraped from plates and counted using liquid scintillation counting.

Proton NMR Identification of the Steroid Metabolites—Approximately 350 pmol of each P450 (F209L or F209N) and 1.4 µmol of

As a family of structurally related enzymes, P450s exhibit extreme diversity in hydroxylase activities. To understand the structural basis for the divergent activities, the substrate-binding orientation in the heme-pocket must first be delineated, and this question is currently of major interest in P450 research.

Recent site-directed mutagenesis studies indicate that the specificity of the P450s can be altered by single amino acid substitutions. Mutation of Phe-209 to Leu, for example, converts the substrate specificity of P450coh (2A5)1 from coumarin to steroid hydroxylation (1). The type of residue 478 of P4502B1 determines the regioselectivity of steroid metabolites (2), and Arg-346 is responsible for delineating the 17α-hydroxylase activity in P45017, from the lyase activity (3). Other reports have also described how amino acid substitutions affect the hydroxylase activities of P450s (4–7). Moreover, homology alignment and computer modeling based on the bacterial P450cam have provided the structural basis for some, but not all, of the observed specificities (8).

To study the steroid-binding orientation in the substrate pocket, we have focused on residue 209 of mouse P450coh, because our previous work suggests that this residue is close to the sixth ligand of the heme and plays a key role in altering the P450 activity (9, 10). To this end, we measured steroid hydroxylation activity of the mutants of P450coh using different steroids as substrates, and subsequently engineered a novel P450 which catalyzes corticosterone 15α-hydroxylation activity.

We then organized the biochemical information to define the steroid-binding orientation in the substrate pocket of the P450 using the three-dimensional structure of P450cam (P450 101) as the model. We propose a steroid-binding orientation in the substrate pocket of the P450.

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1 Coumarin 7-hydroxylation P450coh is a member of the mouse 2A subfamily. P450 2A5 and Cyp 2A5 are the given standard nomenclatures for the P450 and its gene. Accordingly, the standard name of P450cam is P450 101.
steroid (deoxycorticosterone or corticosterone) were incubated at 37°C for 1 h under the above described reconstitution conditions, except that the reaction volume was 4 ml. The steroid metabolites were extracted with chloroform/methanol (1:1) and applied to a silica gel column (5 mm × 3.5 cm). The column was washed with toluene and the metabolite eluted with toluene/methanol (8:2). Then the metabolite was subjected to HPLC chromatography on a TSK gel ODS-80TM (4.6 mm × 25 cm) using methanol/water (9:1). Approximately 0.2 mg of the metabolite was obtained, dried, and dissolved in 0.4 ml of "100%" chloroform-d₄ (Cambridge Isotope Laboratories). The samples were then transferred to 5-mm (outer diameter) sample tubes to obtain the 1H NMR spectrum at 500 MHz using a GN 500 spectrometer (GE Instruments) equipped with a Nicolet 1280 data system and 293E pulse programmer.

Homology Alignment and Graphic Analysis—The GCG program BESTFIT was used with default penalties for gaps and insertions to align P450coh to P450cam. To obtain a constrained alignment of P450cam against P450coh, we first defined a sphere (radius of 15 Å) around the sixth axial position in the P450cam pocket by taking the distance between the axial position and the α-carbon position of residue 209. The standard Needleman-Wunsch algorithm (13) was then modified to find the best alignment for which residue 209 mapped to some bacterial residue in the sphere. To model a steroid into the P450cam pocket, we used a Silicon Graphics workstation and the program MULTI (14) to dock corticosterone (15) into the pocket, then energy-minimized with the program AMBER 3.0a (16). Residues Phe-87, Tyr-96, Phe-98, Thr-101, Thr-185, Leu-244, Val-247, Gly-248, Val-256, Ile-395, and Val-386 all had atoms that fit within a 6.5 Å sphere centered on carboxyl bond bound to P450cam. Thus we initially minimized (using the united atom force field) only residues 86–101, 173–177, 244–250, and 395–397 by fixing (no force on their atoms) the heme, steroid, and remaining parts of the P450cam. In this alignment, C15 of the steroid molecule is 2.9 Å from the activating oxygen in the sixth axial position and no atom of the P450cam is closer than 2.3 Å to the steroid. Finally, the structure from this constrained minimization was subjected to a full minimization of all atoms in the P450cam protein and heme while freezing the position of the steroid.

RESULTS AND DISCUSSION

The metabolites of deoxycorticosterone and corticosterone were determined by the analysis and complete assignment of their 1H NMR spectra. Two-dimensional, double quantum-filtered homonuclear shift correlation spectroscopy (DQCOSY)² (17) was used to determine the scalar J-connectivity between geminal and vicinal pairs of protons. In comparison with the 1H NMR spectra of the precursors, the most significant feature in the spectra of the metabolites is the appearance of a new resonance corresponding to a single proton in the CHOH region at approximately 4.2 ppm. According to the DQCOSY spectra, this multiplet is assigned to H15 by virtue of its connectivity via strong vicinal 3Jcouplings to the 14α and 16β protons. To determine the stereospecific assignment of the H15, we used one-dimensional selective cross-relaxation or NOE spectroscopy (18). As a result, the peak assigned to H15 in the deoxycorticosterone metabolite cross-relaxes with the C18 methyl protons (Fig. 1). This indicates a β orientation for H15 and implies an α-substituted OH. Also consistent with an α-hydroxylated product is the similarity of chemical shifts for the H16α,β and H17 protons of the metabolite and for those of the closely related compound, 15α-hydroxyprogesterone (19). Similar results and conclusions concerning 15α-hydroxylation were obtained for the corticosterone metabolite. We conclude, therefore, that the metabolites formed by the mutants P450coh are 15α-hydroxydeoxycorticosterone and 15α-hydroxycorticosterone.

P450coh (25, 27) catalyzes conversion to 7α-hydroxylation but shows little steroid hydroxylase activity. The substrate specificity of

²The abbreviations used are: DQCOSY, double quantum-filtered homonuclear shift correlation spectroscopy; NOE, nuclear Overhauser effect.

³M. Iwasaki, T. A. Darden, L. G. Pedersen, D. G. Davis, R. O. Juvonen, T. Sueyoshi, and M. Negishi, unpublished observation.
Steroid-binding Model in P450

TABLE I
Steroid 15α-hydroxylase activity of residue 209 mutants P450coh

Steroid 15α-hydroxylase activity was reconstituted as described under "Experimental Procedures." In A, corticosterone 15α-hydroxylase and deoxycorticosterone 15α-hydroxylase activities were measured using the steroid concentration of 100 μM. The values reported were obtained by averaging two separate results. ND means that the activity was not detectable. In B, for the $K_m$ and $V_{\text{max}}$ values of mutants P450 for corticosterone 15α-hydroxylase activity, activity was measured at various concentrations of 2, 10, 20, 40, 100, and 200 μM. We used the Levenberg-Marquardt non-linear regression program to calculate the values.

| Mutant | $K_m$ (μM) | $V_{\text{max}}$ (nmol/min/nmol P450) |
|--------|------------|---------------------------------------|
| F209A  | 0.4        | 0.7                                   |
| F209V  | 2.2        | 14.7                                  |
| F209L  | ND         | 16.2                                  |
| F209G  | ND         | ND                                    |
| F209S  | 1.4        | 1.1                                   |
| F209N  | 16.0       | 8.0                                   |
| F209M  | ND         | ND                                    |
| F209D  | 0.4        | 0.7                                   |
| F209K  | ND         | ND                                    |

A. Steroid-binding Model in P450

B. Steroid-binding Model in P450

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4 Sequence alignments are available from the corresponding author upon written request.
In this model, corticosterone is represented in white with its oxygen molecule atom shown in red. O11 and O3 indicate the oxygen of 11β-hydroxyl and the 3-carbonyl oxygen, respectively, while C5 designates the carbon at position 5. Protein is green, although the 3 critical residues Met-191, Met-184, and Tyr-96 are yellow. A portion of helix I, which is nearest the observer, is shown without its side chains. The heme is drawn with a thicker green line.

The critical residues Met-191, Met-184, and Tyr-96 are yellow. An alignment and modeling, however, indicate that residue 209 of P450coh may be located either at the end of helix F or in the F-G loop. The helix and loop constitute part of the substrate pocket and/or substrate-access channel. Alternatively, the mutation may refold the loop structure toward the heme and bring residue 209 of P450cohc in closer contact with the 11OH of the steroid molecule. We favor the refolding hypothesis, because our studies with mutants P450cohc suggest a direct interaction between the Asn-209 and the 11OH. If residue 209 resides at the end of the F helix, on the other hand, a side-chain alteration alone would be enough to cause the direct interaction between the Asn-209 and the 11OH. Further modeling will require details of the three-dimensional structures of more closely related P450s.

In conclusion, we have engineered a novel P450, which has a high corticosterone 15a-hydroxylase activity, by mutating Leu-209 to Asn of P450cohc. Using the biochemical findings, we have then modeled a possible binding orientation in the substrate pocket of P450cohc (2A5) based on the three-dimensional structure of P450cam. Our model proposes a steroid-binding orientation in the substrate pocket, for which the C15 position of the steroid may near the sixth axial position of the heme and the 11OH of the steroid appears to interact with residue 209 of P450cohc. As a result, a structural variation of helix F and/or the F-G loop plays a key role in selecting the steroid-substrate specificity based on the group identity at the 11 position of steroid molecule.

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