Current Topics

The 50th Anniversary and New Horizons of Cytochrome P450 Research: Expanding Knowledge on the Multiplicity and Versatility of P450 and Its Industrial Applications

Diversity and Substrate Specificity in the Structures of Steroidogenic Cytochrome P450 Enzymes

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Mammalian cytochrome P450 (CYP) comprise a large group of enzymes that play many important roles in the biosynthesis of steroid hormones and vitamins. In addition, they participate in the metabolism of drugs and xenobiotics. All known mammalian CYP enzymes are membrane-associated proteins, which complicated their X-ray crystallographic analysis. In recent years, however, significant progress has been made in the X-ray crystallographic analysis of mammalian CYP enzymes involved in the steroid hormone and vitamin D₃ metabolism. The knowledge from three-dimensional structures of mammalian CYP enzymes will benefit drug discovery and development.

Key words cytochrome P450; X-ray crystallography; steroid; vitamin

INTRODUCTION

Cytochrome P450 (CYP) enzymes are heme-containing proteins found in all living things from prokaryotes (archaea, bacteria) and eukaryotes (fungi, insects, plants and animals).¹⁾ Mammalian CYP enzymes play important roles in the biosynthesis of bioactive compounds (sterols, steroid hormones, eicosanoids and vitamins) and in the metabolism of medicines. In addition, they participate in detoxification of many xenobiotics. Elucidation of the structure and function of CYP enzymes has represented a major scientific challenge since it was discovered in the early 1960s for understanding their reaction mechanism and substrate specificity.²⁾ Since the first three-dimensional (3D) structure of bacterial CYP was reported by Polous *et al.*,³⁾ more than 70 individual CYP structures have been reported, and many more will be reported in future. All known mammalian CYP enzymes are membrane-associated proteins either in the endoplasmic reticulum or the inner membrane of mitochondria, which have complicated the structural studies by X-ray crystallography. However, recent efforts have succeeded in revealing the 3D structures of several mammalian CYPs with X-ray crystallography.^{4–11)} Information of the 3D structures of mammalian CYP enzymes will provide a great impact on the pharmaceutical studies.

This review aims to describe the key features of recently revealed 3D structures of CYPs (11A1, 19, 24A1) involved in the biosynthesis of steroid hormones and activation of vitamins. Current knowledge on their substrate specificity relating to the active site structure obtained from X-ray crystallographic studies is also discussed in comparison with bacterial CYP and drug-metabolizing CYP.

1. PRESERVED STRUCTURE IN CYP

The primary structure of CYP is vastly divergent, because

CYP enzymes have evolved to catalyze the metabolism of numerous structurally diverse exogenous and endogenous molecules. Despite the diverse primary structure, CYP in many different families and many different organisms share a high degree of structural conservation in their secondary and tertiary structure levels. The overall folding comprises 12 helices (denoted as A–L) and loops¹²⁾ (Fig. 1A). CYP has a common prosthetic group, an iron-containing protoporphyrin IX (heme), which is linked to thiolate of Cys residue of apoprotein. The heme group is located between helices I and L. Comparison of the amino acid sequences between CYP and other heme-thiolate proteins such as NO synthase, chloroperoxidase and cystathionine β -synthase indicates that they have been generated independently during the evolution.^{13–15)} Variations in the positioning of secondary structure elements and the lengths of interconnecting loops of CYP contribute to the rich diversity of their active-site structures and resulting substrate specificities. The most important regions for understanding the substrate specificity within CYP is multiple small segments of the protein contacting substrates. They are the loop between the B and C helices positioned over the heme (substrate recognition site 1 or SRS1 described by Gotoh¹⁶⁾), I helix extending over the heme (SRS4), the N-terminal β -rich region (SRS5 in β 3) and β -turn at the end of β -sheet (SRS6 in β 4). In addition, variations in loop sequence and length are observed between F and G helices (between SRS2 and SRS3).

One can use the I helix as a landmark to look at the overall structure of CYP. I-helix with approximately 30 amino acid long runs in the center of the CYP molecule above the heme. It contains a highly (though not perfectly) conserved alcohol-acid pair of Thr (or Ser) and Asp (or Glu) that is essential for catalytic reaction of monooxygenase.^{17,18)} The hydrogen bond formed by these residues on I helix and water molecules supplies protons required for oxygen activation and water formation.

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Fig. 1. Structures of CYP Enzymes Involved in Biosynthesis of Steroid Hormones

(A) Overall structure of CYP11A1 in a ribbon representation. Heme and cholesterol, substrate, are shown by sphere and stick models, respectively. The B' helix, I helix and F/G region is shown as blue, red and green, respectively. (B) The adrenodoxin (Adx) complex of CYP11A1. (C) Overall structure of CYP19. The substrate androstenedione is shown by yellow stick model. Color scheme is same as in (A). (D) Active site of CYP11A1 bound with the initial substrate, cholesterol (orange stick) (PDB ID: 3N9Y). The substrates for second (gray lines) and third (stick with dark gray) steps are superimposed (PDB IDs: 3N9Z and 3NA0). (E) Active site of CYP19 bound with its substrate, androstenedione (PDB ID: 3EQM).

Structures of mammalian and microbial CYP enzymes are similar but differ in detail. The largest differences are found in the positions of F and G helices and length of F/G-loop. F/G loop region of most mammalian CYP contains one or two helices (F' and G'). They form a roof over the substrate-binding cavity and play a role in substrate recognition and/or as a substrate access channel. This F/G region, as well as the B/C region, differs greatly in conformation between free and substrate/inhibitor bound structures.

2. CYP11A1 (CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME)

Several CYPs are present in the mitochondria of animal tissues. They form a unique branch in the phylogenetic tree of animal CYPs, and catalyze the oxygenation reactions of cholesterol, steroids and vitamin D.¹⁹⁾ CYP11A is bound to the inner membrane of the mitochondria and is found in all steroidogenic tissues.^{20,21)} *De novo* synthesis of all steroid hormones starts with the conversion of cholesterol to pregnenolone by CYP11A.²²⁾ The subsequent steps involve 3β -hydroxysteroid dehydrogenase (HSD), CYP17, 17β -HSD and CYP19.

CYP11A1 converts cholesterol to pregnenolone *via* the three-step reaction: $22R$ -hydroxylation, 20α -hydroxylation and C20-C22 cleavage, which occurs at a single active site on the CYP11A1 molecule.²³⁾

X-Ray crystallographic structures of CYP11A1 bound with substrate and a series of reaction intermediates were published in 2011.^{24,25)} The membrane anchor was removed for crystallization. CYP11A1 has three additional helices to the common CYP fold termed A', G' and K'' (Fig. 1A). The active site cavity is closed and cholesterol (first substrate) binds tightly with the proper geometry for hydroxylation at C22 position (Fig. 1D). Many hydrophobic residues are involved in the interaction with substrate. The 3β -OH group of substrate forms the H-bond with water but does not directly interact with the protein. The structure with the $22R$ -hydroxycholesterol that mimics the second substrate shows a small conformational change in Thr291 side chain on I helix, allowing a new water molecule that is H-bonded to the $22R$ -OH group of the second substrate. In the complex with the substrate for the third step, both of the two OH groups (20- and 22-position) are close to the heme iron but without direct interactions, leaving space for subsequent oxygen binding to the reduced heme iron.

Fig. 2. Structures of CYP Involved in Metabolism of Vitamin D

(A) Overall structure of CYP24A1. The compounds shown by stick model are the detergent (CHAPS) unexpectedly bound to the protein. Heme is shown by sphere model and B'-helix, I-helix and F/G region are colored in blue, red and green, respectively. (B) Comparison of substrate orientation of CYP2R1 (blue) and CYP105A1 (white).

Superposition of a series of substrate and intermediates reveals that the position of the sterol ring of substrate is fixed, while conformation of the side chain changes. The proposed mechanism for the final step of cholesterol side-chain cleavage reaction involves C20-peroxy intermediate, although further studies are required.

Another outstanding result in the X-ray crystallographic analysis of CYP11A1 is that the 3D structure was solved for the complex with its redox partner (adrenodoxin) that binds at the proximal surface of CYP11A1 (Fig. 1B). The authors proposed a mechanism of the initial driving force for their interaction followed by the reposition for efficient electron transfer.²⁴⁾ Conserved amino acid in the interface suggests the presence of some common mechanism for all mitochondrial CYPs.

3. CYP19 (AROMATASE)

CYP19 catalyzes the conversion of androgens into estrogens through the aromatization of the A-ring of substrates. CYP19 converts androstenedione, testosterone, and 16-hydroxytestosterone to estrone, 17-estradiol, and 17,16-estriol, respectively, through three-step oxygenations. CYP19 inhibitors such as anastrozole, vorozole, letrozole, and fadrozole are widely used as chemotherapeutic drugs for estrogen-dependent breast cancer.²⁶⁾ In 2009, the 3D structure of CYP19 was determined as the complex with its substrate androstenedione.²⁷⁾ This was the first example of the structure of CYPs involved in biosynthesis of steroid hormones. The native CYP19 isolated from human placenta, rather than a recombinant protein, was used for crystal preparation.

CYP19 folds in a way that is characteristic of CYP (Fig. 1C). The substrate fits tightly in the active site, as expected for a CYP that binds specifically to a small number of compounds that have closely related structures. The structure shows that the 19-methyl group of the substrate is directed toward the heme iron atom at a distance of 4.0 Å (Fig. 1E). The distance

from H atom of C-19 to the oxygen atom of reactive oxygen species (Fe(IV)=O) in the catalytic reaction is estimated as 2 Å, which is considered a proper distance with substrate orientation. The acid-alcohol pair (Asp309-Thr310) on I helix is also conserved in CYP19s. The axis of I helix is broken at Pro308, which creates the substrate-binding site specific for androstenedione. As observed in the active site of bacterial P450cam,²⁸⁾ it was suggested that the H-bond network involving the Thr310, carbonyl group of Ala306 and water molecules in the active site might have an important role in the first two hydroxylation reactions on 19-methyl group. The authors proposed that the protonation of the carbonyl oxygen atom at C-3 of the substrate by side chain of Asp309 promotes the abstraction of C-2 hydrogen by iron-oxygen moiety (Fe(III)-O₂⁻), thus inducing the A-ring aromatization in the third step.²⁷⁾

CYP19 is an important CYP necessary for the development of female characteristics, and as a target for breast cancer chemotherapy. Thus, the structural information of CYP19 has provided a great impact on the pharmaceutical science to develop next generation inhibitors.

4. CYP ENZYMES NECESSARY FOR ACTIVATION OF VITAMIN D

Vitamin D₃ (VD₃) has no hormonal activity itself, but is converted to the active form by CYP enzymes.²⁹⁾ VD₃ taken from food or produced from 7-dehydrocholesterol in the skin upon exposure to sunlight is converted to 25-hydroxyvitamin D₃ (25(OH)D₃) in liver, and stored in this form until use. 25(OH)D₃ is further hydroxylated in the kidneys to 1,25(OH)₂D₃. The former reaction is catalyzed by mitochondrial CYP27A1 or microsomal CYP2R1 in liver,^{30,31)} and the latter reaction by mitochondrial CYP27B1 in kidney.³²⁾ Only the second product, 1,25(OH)₂D₃, is a hormonally active compound binding to the vitamin D receptor (VDR).³²⁾ The fourth CYP enzyme participating to VD₃ metabolism is

CYP24A1 which inactivates the active form through the hydroxylation at 23- and 24-positions; this enzyme is considered to be an ideal target for developing the novel drugs against vitamin D insufficiency, including chronic kidney disease and cancer.³³⁾

To date, the structures of CYP2R1-vitamin D complex and CYP24A1 have been elucidated by X-ray crystallography.^{34,35)} CYP24A1 is the first mitochondrial CYP whose structure was revealed (Fig. 2A). CYP27A1, 27B1 and CYP24A1 are classified in the same branch in the phylogenetic tree of the CYP gene superfamily. Therefore, the structure of CYP24A1 is expected to provide some insights into important active site residues and interaction mode with mitochondrial membrane. However, the open form of the pocket structure and the unexpected binding of detergent from buffer in the active site pocket and access channel have limited the information regarding substrate-binding and docking calculation.

CYP2R1 contains extra helices F' and G', as in the case of other microsomal CYPs. Neither F' nor G' helix of CYP2R1 has direct contact with the substrate, but both appear to be involved in the formation of the substrate channel in the bilayer lipid membrane. A-Ring of substrate, VD₃, is fixed in the space between the B', G and I helices, mainly by hydrophobic interaction. Only one polar interaction is observed as the H-bonding between the 3 β -OH group and the carbonyl oxygen of Ala250 in the G helix. The C25 of VD₃ in the CYP2R1-VD₃ complex is located 6.4 Å from the heme iron, which is estimated to be out of reach of the activated oxygen at the heme iron.

It should be noted that a bacterial CYP (CYP105A1 or CYP107BR1) catalyzes the 25 and 1 α -hydroxylation of VD₃.^{36,37)} Comparison of the main chain folds between CYP2R1 and CYP105A1 shows a large difference in the F-G loop region where CYP105A1 lacks extra helices F' and G', as do other bacterial CYPs.³⁸⁾ The 1 α ,25(OH)₂D₃-binding manner of CYP105A1 shows some similarities to the VD₃-binding site of CYP2R1 (Fig. 2B). Note that Arg193 in G helix of CYP105A1 corresponds, in both sequence and structural alignment, to Ala250 of CYP2R1. Thus, similar orientation of 1 α ,25(OH)₂D₃ in CYP105A1 and VD₃ in CYP2R1 suggest a general mechanism for the recognition of vitamin D by the CYP enzymes, including mitochondrial CYP27A1, since the substrate preference and regio-specificity of CYP27A1 closely resemble those of CYP105A1. This structural information of bacterial CYP is useful for industrial application, because production of the active form of VD₃ by microorganism has already been put to practical use.³⁶⁾

5. DIVERSITY AND COMMON FEATURE IN CYP STRUCTURES AND REACTION MECHANISM

The available structural information on CYP enzymes show that the overall fold, comprising 12 helices and loops denoted as A–L, is preserved during evolution. Alignments of currently-available folding structures for CYPs suggest that most significant variations occur in three regions: B region (B'-helix, B-helix and B/C-loop), the F/G region (C-terminal half of F-helix, the F–G loop and N-terminal half of G-helix) and the b4 region (SRS6). These variations in backbone structure are considered to contribute to the diversification of substrate specificity of CYP enzymes. For example, CYP3A4 and 2C9 have the flexibility and adaptability for substrate-binding by a

Fig. 3. Comparison of Substrate-Binding Cavities of CYP3A4 and CYP19

The erythromycin complex of CYP3A4 (A) and androstenedione complex of CYP19 (B). The cavity was calculated by VOIDOO program³⁹⁾ using the probe radius 1.4 Å.

large substrate-binding cavity (Fig. 3A), and this reflects the binding of a variety of structurally different drug substrates. In contrast, the CYP enzymes in this review catalyze specific reactions in the metabolism of bioactive compounds, such as steroid hormones. These CYPs bind specifically with a small number of structurally-related compounds to prevent the production of undesired bioactive compounds. As expected, active site structures of their substrate complexes reveal the close fitting of substrate to active site mainly by hydrophobic interaction (Fig. 3B). Very few water molecules locate inside the active site cavity other than the catalytically essential water molecules.^{24,25,27,35)}

In addition to the structural diversity associated with recognition and binding, the structures responsible for the interaction with redox partners are also multiple, because the redox partners of microsomal, mitochondrial and bacterial CYPs differ.³⁸⁾ Microsomal CYPs tightly bound to the membrane of the endoplasmic reticulum by anchoring α -helix in N-terminus, and the redox partner is a flavoprotein

called reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent P450 reductase, which is also bound to the membrane. Mitochondrial CYPs do not contain N-terminal anchoring helix in its mature form, while they associate with the inner membrane of mitochondria, and the redox partner is a ferredoxin-type 2Fe–2S protein, which does not bind to the inner membrane. In the bacterial system, neither CYP and its redox partner (a ferredoxin-type 2Fe–2S protein) bind to the membrane.

6. CONCLUSION

Recent progress in structural studies filled significant gaps between our understanding on the structure and function of CYP enzymes. They also provide novel information useful for drug design to expand efficacy and to reduce adverse reactions. This review has discussed recent findings on the structure of CYP enzymes involved in the metabolic pathway of steroid and vitamin D. The substrate fits tightly in the active site of steroidogenic CYP11A1 or CYP19, as expected for such physiologically important CYPs showing strict substrate specificity. This structural feature of steroidogenic CYPs presents a sharp contrast to the drug-metabolizing CYPs such as CYP3A4 and CYP2C9 that bind loosely with many structurally different compounds to accommodate various drug substrates. Determination of the 3D structure mitochondrial CYPs such as CYP24A1 and CYP11A1 allows us to propose a more precise model of their interactions with mitochondrial inner membrane and a redox partner. Currently obtained X-ray crystallographic structure is a snapshot of protein. Therefore, it is challenging and important to capture the enzyme molecule at the intermediate step in the multi-step reaction of CYP11A1 or CYP19. More precise structural studies of CYPs with their substrate or specific inhibitor would be useful, and novel compounds for this purpose should be explored. X-Ray crystallographic analysis with higher resolution is also important, because, in some cases, the water molecules inside the substrate-binding pocket or substrate-access channels often play essential roles in the catalytic mechanism.

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