

*Review Article (Invited)***Diverse reactions catalyzed by cytochrome P450 and biosynthesis of steroid hormone**Keisuke Fujiyama¹, Tomoya Hino^{2,3}, Shingo Nagano^{2,3}¹ *Dormancy and Adaptation Research Unit, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan*² *Center for Research on Green Sustainable Chemistry, Tottori University, Tottori 680-8552, Japan*³ *Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, Tottori 680-8552, Japan*Received March 22, 2022; Accepted May 30, 2022;
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Steroid hormones modulate numerous physiological processes in various higher organisms. Research on the physiology, biosynthesis, and metabolic degradation of steroid hormones is crucial for developing drugs, agrochemicals, and anthelmintics. Most steroid hormone biosynthetic pathways, excluding those in insects, have been elucidated, and the roles of several cytochrome P450s (CYPs, P450s), heme (iron protoporphyrin IX)-containing monooxygenases, have been identified. Specifically, P450s of the animal steroid hormone biosynthetic pathways and their three dimensional structures and reaction mechanisms have been extensively studied; however, the mechanisms of several uncommon P450 reactions involved in animal steroid hormone biosynthesis and structures and reaction mechanisms of various P450s involved in plant and insect steroid hormone biosynthesis remain unclear. Recently, we determined the crystal structure of P450 responsible for the first and rate-determining step in brassinosteroids biosynthesis and clarified the regio- and stereo-selectivity in the hydroxylation reaction mechanism. In this review, we have outlined the general catalytic cycle, reaction mechanism, and structure of P450s. Additionally, we have described the recent advances in research on the reaction mechanisms of steroid hormone biosynthesis-related P450s, some of which catalyze unusual P450 reactions including C–C bond cleavage reactions by utilizing either a heme–peroxo anion species or compound I as an active oxidizing species. This review article is an extended version of the Japanese article, Structure and mechanism of cytochrome P450s involved in steroid hormone biosynthesis, published in SEIBUTSU BUTSURI Vol. 61, p.189-191 (2021).

Key words: brassinosteroid, crystal structure, compound I, heme**◀ Significance ▶**

Steroid hormones regulate the physiologies of animals, plants, and insects, and most steroid hormone biosynthetic pathways, excluding those in insects have been clarified. Numerous cytochrome P450s (CYPs) are involved in animal steroid hormone biosynthesis, and their structures and reaction mechanisms have been extensively studied. However, those of plants and insects are yet to be fully investigated. Recently, we determined CYP90B1 structures, a key enzyme in brassinosteroid (BR) biosynthesis, and clarified the stereo-selective hydroxylation mechanism. Therefore, this review summarizes the current research progress on the P450 structure and its reaction mechanism in animal and plant steroid biosynthesis.

Introduction

Cytochrome P450s (P450s; CYPs) are heme-containing enzymes (iron protoporphyrin IX complex; Fig. 1) mainly responsible for the monooxygenation of various substrates. Since their discovery in 1962 by Omura and Sato [1], numerous P450s have been identified in animals, plants, fungi, bacteria, and viruses [2,3]. One of the most well-known functions of eukaryotic P450s is drug, agrochemicals, and xenobiotics metabolism. Drugs become more water-soluble and susceptible to further modification when monooxygenated by P450s, thereby facilitating their excretion from the human body. Furthermore, P450s are the main enzymes responsible for drug metabolism (about 75%) in humans [4–6]. Among them, CYP3A4 and CYP2C9 are the major and important contributors of drug metabolism owing to their broad substrate specificity and high expression levels. In addition to pharmacology, P450s are considered important in toxicology [6] because they metabolize and bioactivate carcinogens: 7,12-dimethylbenz[a]anthracene, a well-known carcinogen, is sequentially epoxidated and hydroxylated mainly by P450s to produce toxic, carcinogenic compounds [7–9]. Enormous numbers of P450 genes are found from bacteria, fungi, and plants, which produce numerous products with various bioactivities [10–12], and P450s found in these species are important biosynthetic enzymes that produce natural products. Furthermore, P450s are involved in the production of steroid hormones, prostanoids, vitamin D, and other bioactive compounds in higher organisms [13–20].

Animal steroid hormones are classified into three groups: sex hormones, glucocorticoids, and mineralocorticoids, which are involved in sex determination and morphological changes in maturation, energy metabolism, and maintenance of sodium/potassium balance in cells, respectively [21–24]. In addition, they produce signals in many physiological events, and are biosynthesized in reactions such as hydroxylation, epoxidation, and C–C bond cleavage mediated by P450s [13,14]. Plant steroid hormones, BRs, promote plant growth and development and also enhance environmental stress tolerance [25,26]. Interestingly, all of the enzymes identified to be involved in BR biosynthesis are P450s, excluding DET2 (DEETIOLATED2 gene product) [27,28]. Although a part of the biosynthetic pathway of ecdysteroids, an essential insect steroid hormone for metamorphosis, remains unclear, several P450s are involved in its biosynthesis [19].

In a previous study, our research group determined the crystal structures of CYP90B1, a key P450 enzyme in BR biosynthesis; the substrate- and inhibitor-bound forms allowed us to identify its regio- and stereo-selective hydroxylation mechanisms and structural plasticity, thereby providing structural and fundamental insights into plant steroid hormone biosynthesis [29]. In this review, we described the general molecular structure and mechanisms of P450s, its unique reactions involved in plant and animal steroid hormone biosynthesis, and perspectives of research on P450 in the future.

Structure and Catalytic Cycle of P450s

Since the first crystal structure of P450cam (CYP101A1) was reported by Poulos in 1985 [30], more than 1,000 structures have been deposited in Protein Data Bank. In addition, amino acid sequence similarities between P450s from distinct species are generally low and eucaryotic P450s have an extra membrane anchor helix at the N-terminal region unlike procaryotic P450s. However, almost all P450s share a common triangular prism-shaped structure known as “P450 fold” that consists of α - and β -domains (Fig. 2a). One prominent and common structural feature of P450s is the I-helix that runs through the center of the molecule and the distal side of the heme (Fig. 2b and c). In addition, the I-helix contains a highly conserved Asp/Glu-Thr motif (acid-alcohol pair), which plays important roles in providing protons required for reductive activation of heme-bound dioxygen [31,32].

P450s function as monooxygenases and use the iron(IV)-oxo porphyrin radical cation, compound I, as an active oxidizing species. The most common reaction catalyzed by P450s is hydroxylation and its mechanism is as follows: in the resting state, the hexa-coordinated low-spin ($S = 1/2$) ferric heme iron (Fe^{3+}) has a thiolate anion from Cys and a water molecule as axial ligands. Substrate binding to the heme distal pocket usually (Fig. 2c), but not always, removes a heme-bound water molecule, producing penta-coordinated high-spin state ($S = 5/2$) (Fig. 3a and b). A shift from the low to high spin-state causes upshift of redox potential of the heme, which allows efficient electron transfer to the heme from a redox

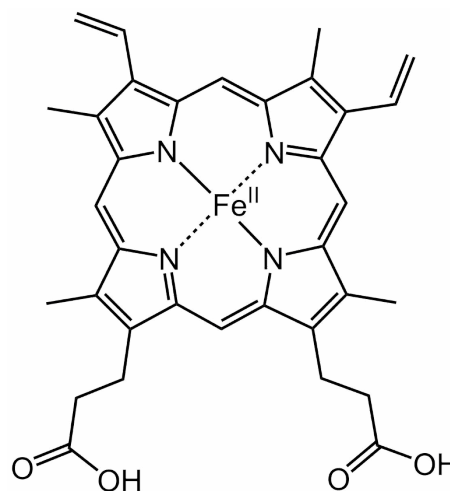


Figure 1 The structure of heme *b* (iron/protoporphyrin IX complex). Although *a* and *c*-types of hemes are found in some heme containing proteins, ‘heme’ indicates heme *b* in many heme proteins, including P450s.

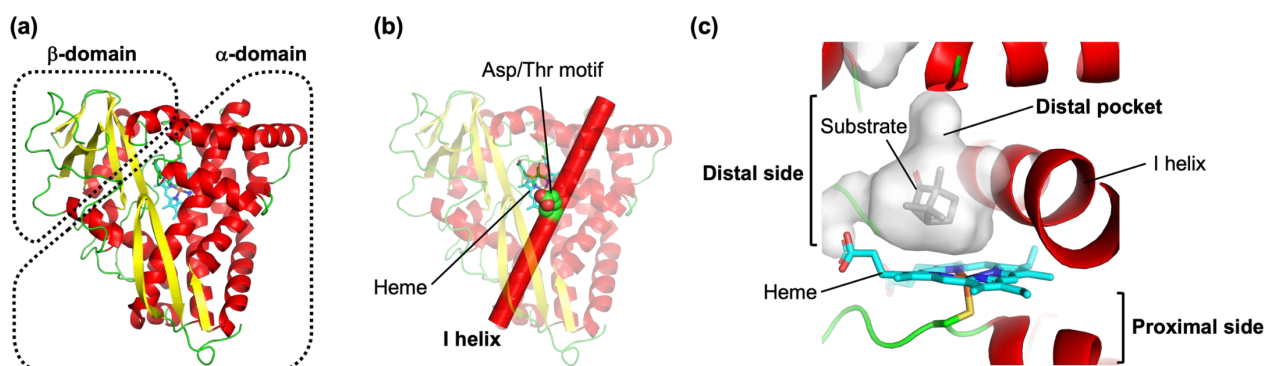


Figure 2 Crystal structure of P450cam (PDB ID: [2CPP](#)). (a) Overall structure of P450cam. (b) I-helix is indicated by the cylindrical model. Sphere models on I-helix are highly conserved acid-alcohol pair (Asp251-Thr252). (c) Close-up view of the active site of P450cam. A distal pocket is the substrate-binding site of P450.

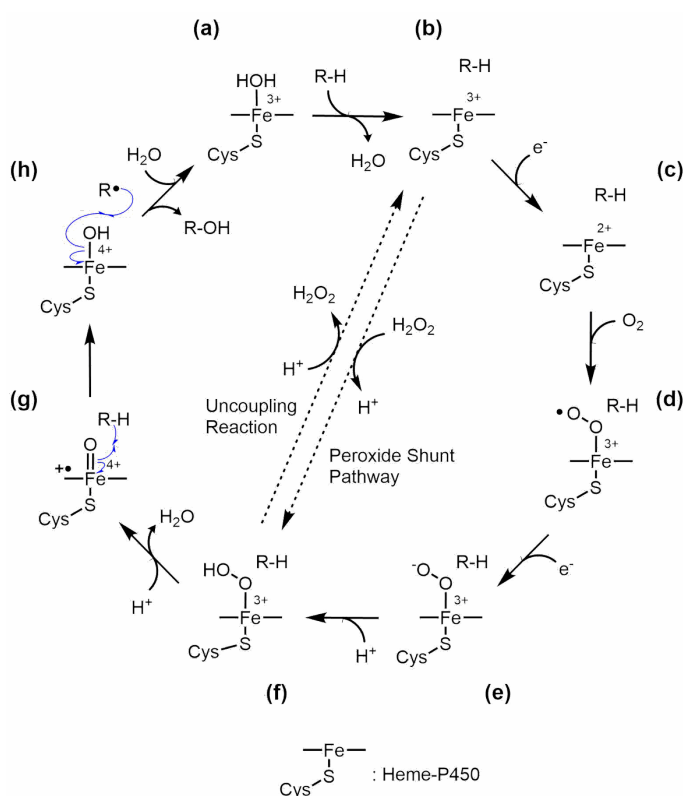


Figure 3 Catalytic cycle of P450. (a) Substrate-free and low-spin ferric resting state. (b) Substrate-bound high-spin state. (c) Substrate-bound reduced form. (d) Oxy-P450 (ternary complex of substrate/O₂/P450 complex). (e) Heme-peroxo anion species. (f) Heme-hydroperoxo species. (g) Ferryl-oxo porphyrin π -cation radical (compound I). (h) Unstable intermediates found in oxygen rebound mechanism of P450. R-H and R-OH are the substrate and hydroxylated product, respectively. Electrons are transferred from NAD(P)H via the electron transfer proteins. Asp/Glu-Thr motif on the I-helix engages in proton supply to the active site.

partner protein [33–36]. Next, one electron is transferred from NAD(P)H to heme (Fig. 3a and b) via an electron transfer protein such as membrane-anchored P450 reductases for eucaryotic membrane-bound P450s. In a bacterial and mitochondrial P450 enzyme system, an electron from NAD(P)H is transferred via an iron-sulfur protein ferredoxin/adrenodoxin and its reductase. The reduced heme then binds molecular oxygen to produce oxy-P450 (ternary complex of substrate/O₂/P450), which bears peroxy radical character (Fig. 3c and d). The second electron from the electron transfer system further reduces this species and a heme-peroxo anion species is generated (Fig. 3d and e). Protonation of the terminal oxygen atom in the heme-peroxo anion species produces a heme-hydroperoxo species, which readily breaks down via proton-assisted heterolytic O–O bond cleavage to generate a water molecule and compound I (Fig. 3e and g). Subsequently, compound I abstracts a hydrogen atom from the substrate and the oxygen atom of compound I binds to the substrate as a hydroxy radical to complete the hydroxylation reaction (oxygen rebound mechanism, Fig. 3g and h). Finally, the product is released from the distal pocket to regenerate the ferric resting state.

In the ideal stoichiometry of the P450 reaction, one NAD(P)H and one molecular oxygen are required to produce one molecule of product. In reality, this rarely happens. Molecular oxygen is also consumed for unproductive reaction, where hydrogen peroxide, superoxide, and water molecule are produced (uncoupling reaction) [37]. Hydrogen peroxide is equivalent to molecular oxygen, two protons, and two electrons. Thus, hydrogen peroxide can also drive the P450 reaction to produce compound I (peroxide shunt pathway) [38,39].

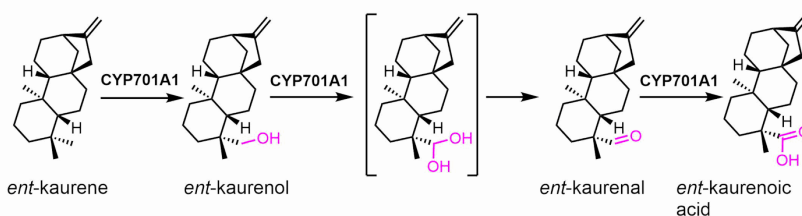
Diversity of P450 Reactions

Although hydroxylation is the most common oxidation reaction catalyzed by P450s, they can also catalyze carbonylation, epoxidation, desaturation, *O*- and *N*-dealkylation and even the C–C bond formation and cleavage. Interestingly, most of these non-hydroxylation reactions are also mediated by compound I. Carbonylation is a result of two successive hydroxylation reactions to produce geminal diol, resulting in subsequent dehydration (Fig. 4a) [40,41]. Desaturation occurs in the oxidation of valproic acid, testosterone, sterols, and several other compounds. For desaturation, after hydrogen abstraction by compound I, unlike the general hydroxylation mechanism, a vicinal hydrogen atom is abstracted by a hydroxy species and a C=C bond is formed [29,42–44]. In many such reactions catalyzed by P450s, hydroxylation products are also formed. Therefore, alteration between hydroxylation and desaturation would be dependent on subtle changes in substrate-binding conformation in enzymes (Fig. 4b). The CYP1 family enzymes catalyze epoxidation and hydroxylation of a procarcinogen, 7,12-dimethylbenz[*a*]anthracene, and DNA can be modified by the resultant epoxides owing to their electrophilicity. In addition, during olefin epoxidation, compound I attacks the substrate to form an intermediate σ -complex, and the oxygen atom makes a bond with the α -carbon to produce epoxide [45–48] (Fig. 4c). Two mechanisms have been proposed for P450-mediated *O*- and *N*-dealkylations [49,50]: in the hydrogen atom transfer (HAT) mechanism where,

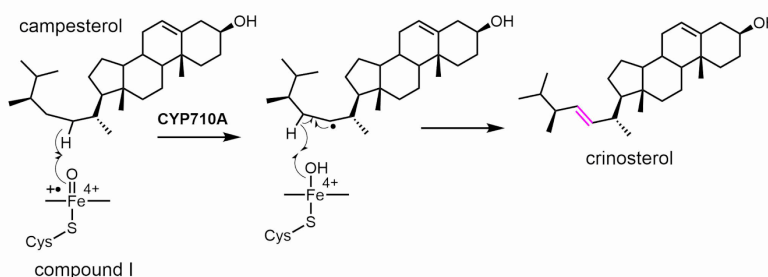
as observed in usual hydroxylation reaction by P450s, a hydrogen atom from the alpha carbon is transferred to compound I and the substrate radical, and in the single-electron transfer (SET) mechanism, one electron is transferred from the heteroatom in the substrate to compound I to form a substrate radical cation and compound II ($\text{Fe}^{4+}=\text{O}$) and the substrate subsequently is deprotonated. In either mechanism, oxygen rebound to the substrate radical produces relatively unstable hemiacetal or hemiaminal compound, and consequently, *O*- or *N*-alkyl groups are spontaneously eliminated as aldehydes (Fig. 4d). Large kinetic hydrogen isotope effects of dimethylanilines on *N*-demethylation by P450 support the HAT mechanism [51]. However, a spin-trapped radical species in the reaction of 4-alkyl-1,4-dihydropyridines with liver microsomes and several other experimental data support the SET mechanism [52–56].

Oxidative C–C coupling reactions by P450s are noted in biosynthesis of flavonoids, polyketides, and aromatic alkaloids, which are based on radical coupling or C–C bond rearrangement induced by hydrogen atom abstraction. (Fig. 5a) [57–59]. Although the radical coupling mechanisms are widely accepted, direct experimental evidence is required for further clarification of the P450-mediated C–C coupling mechanism. P450 StaP (CYP245A1) also catalyzes the oxidative C–C

(a) Hydroxylation and Carbonylation



(b) Desaturation



(c) Epoxidation

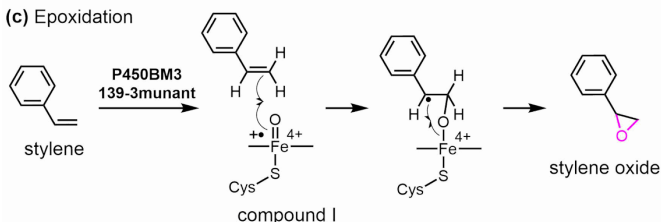
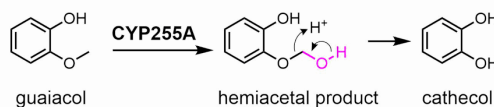
(d) *O*- and *N*-dealkylation

Figure 4 Diverse P450 reactions. (a) Three successive monooxygenation reactions to produce a phytohormone, gibberellin, by CYP701. (b) Desaturation reaction by CYP710A that is involved in phytosterol biosynthesis [42]. (c) Epoxidation catalyzed by a mutant P450BM (CYP102A1). Wild-type P450BM3 catalyzes fatty acid hydroxylation [48], while P450BM3 139-3 mutant, created by directed evolution, can catalyze alkene epoxidation [45,47]. (d) *O*-demethylation via HAT mechanism by CYP255A, which is involved in lignin metabolism in fungi

coupling reaction in the staurosporine biosynthetic pathway, wherein a C–C bond is created between the two indole rings of chromopyrrolic acid. The crystal structure of chromopyrrolic acid-bound P450 StaP, QM/MM calculations, and enzyme assay of several active site mutants suggest that the substrate is tightly bound in the distal pocket by a number of hydrogen bonds, which prevent hydroxylation of the aromatic ring; nevertheless, water diad-mediated proton-coupled

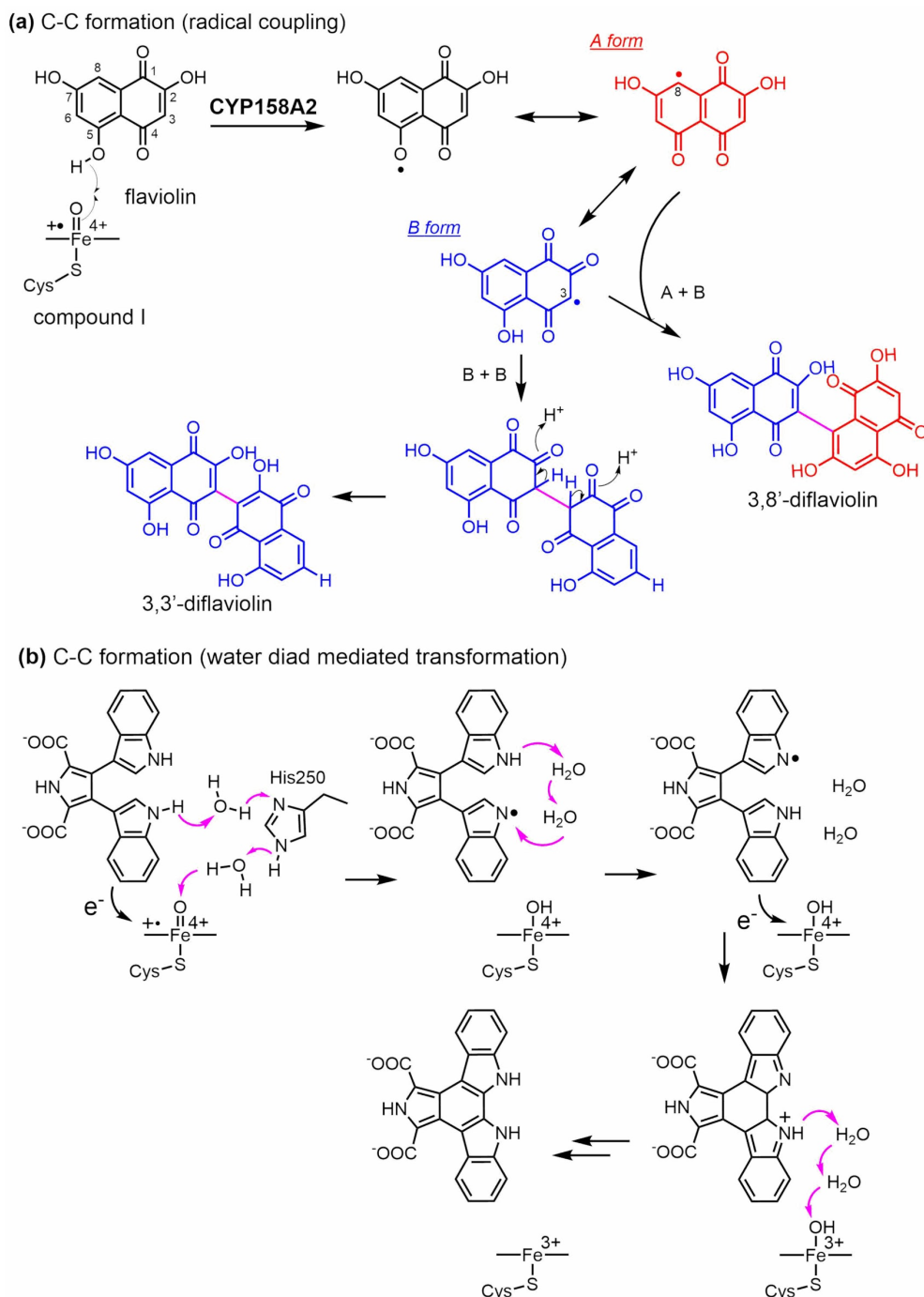


Figure 5 Mechanisms of C–C bond formation by P450s. (a) Proposed radical coupling mechanism of flaviolin by CYP158A2 [58]. The crystal structure of CYP158A2 in the complex with flaviolin indicates that the enzyme can accommodate two flaviolin molecules in the substrate-binding site, resulting in the radical coupling mechanism. (b) P450 StaP (CYP245A1) catalyzes an unusual C–C coupling reaction between the two indole rings of chromopyrrolic acid in the staurosporine biosynthetic pathway. Arrows shown in magenta represent proton or hydrogen atom transfer [60–62].

electron transfer and bond formation-coupled electron transfer result in the formation of the C–C bond between the two rings (Fig. 5b) [60–62]. P450s can also perform C–C cleavage reactions, and several examples and their detailed reaction mechanisms are discussed in the following sections.

P450s Involved in Steroid Hormone Biosynthesis

To date, numerous P450s involved in steroid hormone biosynthesis have been identified. Many typical and several unusual P450 reaction mechanisms in animal steroid hormone biosynthesis have been extensively studied and crystal structures of all human P450s involved in steroid hormone biosynthesis have been determined [63–68]. In addition, the BR biosynthesis pathway is clarified and six P450s involved in BR biosynthesis have been identified [28]. However, the crystal structures of most P450s involved in BR and ecdysteroid biosynthesis remain uncertain and their reaction mechanisms are yet to be investigated at the atomic level [29].

Plant Steroid Hormone Biosynthesis

CYP90B1

CYP90B1 catalyzes the initial and rate-limiting step of the BR biosynthesis pathway and is thus one of the most important enzymes in BR biosynthesis (Fig. 6a) [69]. CYP90B1 hydroxylates campesterol at C22 position regio- and stereo-selectively to produce (22*S*)-22-hydroxycampesterol, which is necessary for biosynthesis of BRs such as the final product brassinolide and its immediate precursor castasterone, exhibiting significant biological activity [70,71]. In a recent study, we reported the crystal structure of CYP90B1 from *Arabidopsis thaliana* in complex with cholesterol, which can be hydroxylated by this enzyme at C22, and clarified the regio- and stereo-selective hydroxylation mechanism [29].

CYP90B1 binds cholesterol with the conformation that the steroid core is roughly perpendicular to the heme plane, with the parallel alignment of the cholesterol side chain to the heme plane (Fig. 7a). Its substrate-binding conformation is in marked contrast to that of CYP11A1, which similarly catalyzes cholesterol hydroxylation at C22 but successively catalyzes C20 hydroxylation and C20–C22 bond cleavage reaction, thereby resulting in side chain removal and pregnenolone formation [63,72]. According to the crystal structure of CYP11A1 in complex with cholesterol, the steroid core is positioned with β -face towards the heme plane (Fig. 7b) [72] to locate C22 and C20 within the appropriate distance of 4.5 Å from the heme iron for P450 monooxygenation. Conversely, in the case of cholesterol-bound CYP90B1, C22 is located at a distance from the heme iron where it could be hydroxylated, whereas C20 is very distant for the reaction to take place; thus, CYP90B1 can mediate the regio-selective hydroxylation reaction.

The stereoselective hydroxylation mechanism of CYP90B1 is clearly described based on the *in silico* docking simulation model of campesterol calculated from the cholesterol-bound crystal structure. Docking simulation results demonstrated that the steric hindrance between the heme plane and C24-methyl group of campesterol, which is not present in cholesterol, renders the *pro*-22*S* hydrogen atom closer to the heme iron (Fig. 8); therefore, the methyl group at C24 of campesterol is critically important for

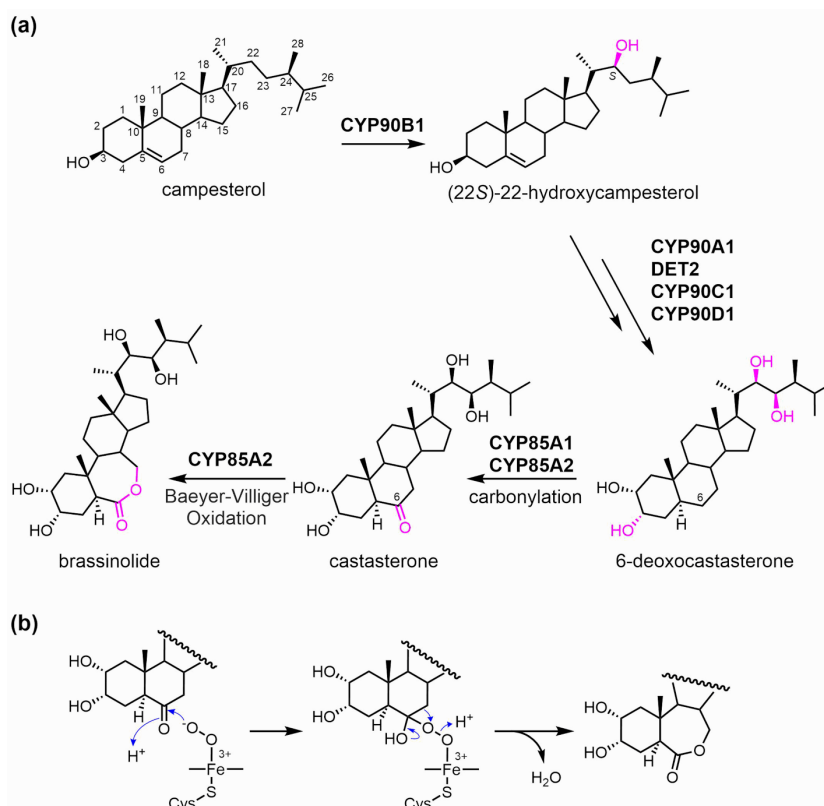


Figure 6 (a) Brassinosteroid (BR) biosynthesis pathway in *Arabidopsis thaliana*. The oxygen atoms indicated in magenta are inserted by P450s. (b) Proposed lactonization mechanism mediated by CYP85A2 with heme-peroxy species. Heme-peroxy species production requires no proton supply unlike compound I formation.

stereoselective hydroxylation by CYP90B1.

P450s involved in BR biosynthesis exhibit relatively higher amino acid sequence identity with CYP90B1 (>30 %); therefore, the structures of CYP90B1 allow us to predict reliable homology models of other P450s in the BR biosynthesis pathway. These models would help in predicting the important residues for substrate binding and docking simulations can suggest substrate-binding conformations using these models.

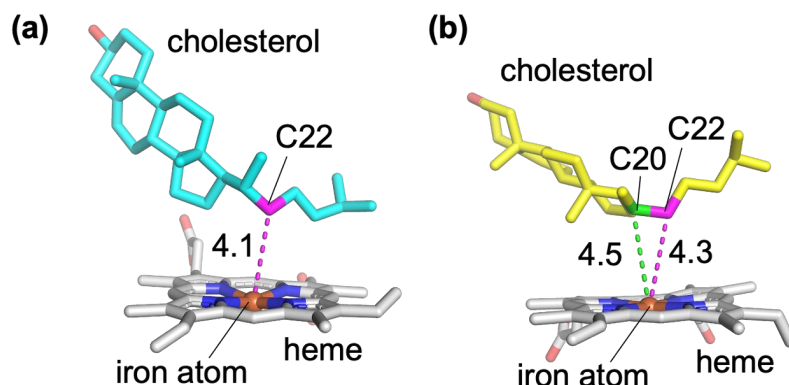


Figure 7 Cholesterol binding conformations in CYP90B1 and CYP11A1. (a) CYP90B1 (PDB ID: [6A15](#)) [29]. (b) CYP11A1 (PDB ID: [3N9Y](#)) [72]. Numbers show distances (Å) between atoms connected by dashed lines.

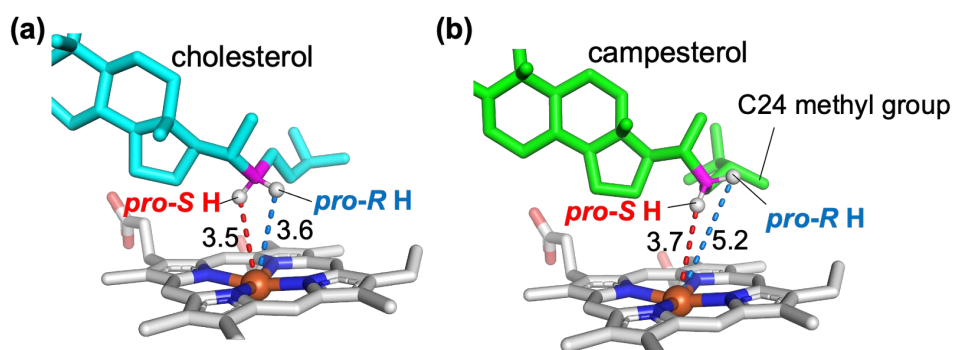


Figure 8 Comparison of binding conformations between cholesterol and campesterol in CYP90B1. (a) Cholesterol in the crystal structure of CYP90B1. (b) Campesterol binding conformation predicted by docking simulation. C22 atoms are indicated in magenta. Numbers show distances (Å) between atoms connected by dashed lines.

CYP85A2

CYP85A2 is an enzyme that catalyzes the two final steps of BR biosynthesis, in which 6-deoxocastasterone is carbonylated to form castasterone, which is subsequently lactonized to generate the final product, brassinolide. Although many intermediate products of the BR biosynthetic pathway have BR activity to promote plant growth and development, castasterone and brassinolide have substantially higher BR activity than the other intermediate products. Therefore, CYP85A2 is a key enzyme in producing highly active BR in the biosynthetic pathway [73,74]. The CYP85A2 reaction is initiated by two successive hydroxylations at C6 followed by dehydration to produce castasterone. In the second step, Baeyer–Villiger oxidation occurs, which is an uncommon reaction for P450s. As observed in a Baeyer–Villiger oxidation reaction in organic synthesis, wherein a peracid anion attacks a carbonyl group, a heme–peroxo anion species ($\text{Fe}^{3+}\text{-OO}^-$) rather than compound I is proposed as a potential oxidizing species, and the peroxo species attacks the carbonyl group of castasterone in the second step of CYP85A2 reaction [75,76]. (Fig. 6b). However, probably because of the difficulty in preparing natural substrates for CYP85A2, no experimental data that directly support the heme–peroxo anion mechanism have been reported.

Animal Steroid Hormone Biosynthesis

CYP17A1

Similar to the lactonization by CYP85A2 in BR biosynthesis, several uncommon P450 reactions have been noted in

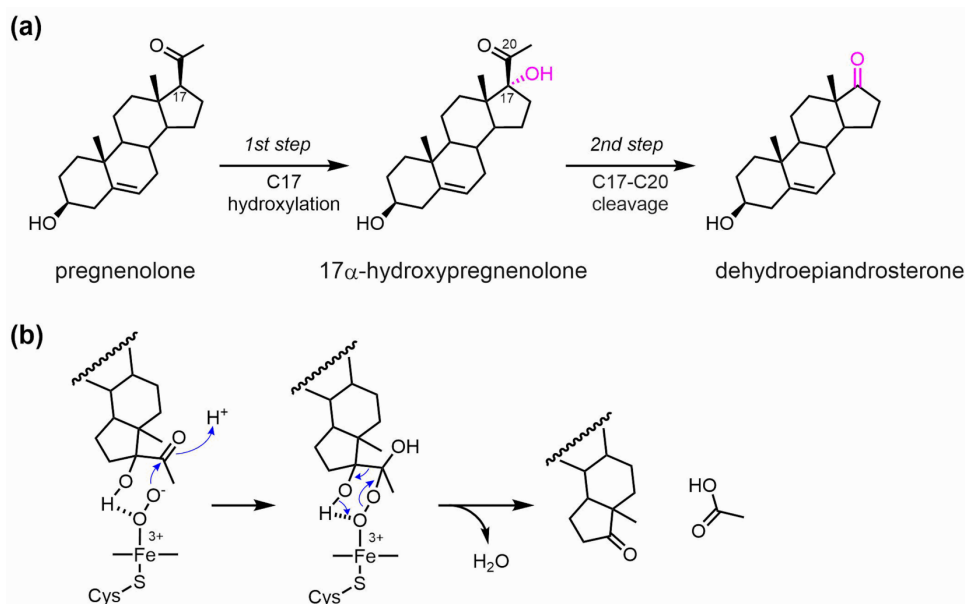


Figure 9 C–C bond cleavage reaction catalyzed by CYP17A1. (a) Two successive hydroxylation reactions followed by dehydration to produce dehydroepiandrosterone. (b) Heme–peroxo anion catalyzed C–C bond cleavage reaction mechanism.

animal steroid hormone biosynthesis. CYP17A1 converts pregnenolone and progesterone to dehydroepiandrosterone and androsterone, respectively, via a C17–C20 cleavage through a two-step reaction [64]. Therefore, CYP17A1 is an essential enzyme for androgen production and a target for prostate cancer drugs. The first step of the reaction by CYP17A1 is C17 hydroxylation in pregnenolone and progesterone to produce hydroxylated intermediates, which are precursors of glucocorticoids. The second step is the C17–C20 cleavage reaction of 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone (Fig. 9a). Almost all P450 reactions including the C17 hydroxylation by CYP17A1 utilize compound I as the active oxidizing species. Production of compound I from ferrous oxy-P450 requires supply of two protons, which is assisted by the highly conserved Asp/Glu-Thr motif (Asp251-Thr252 and Glu305-Thr306 in P450cam and CYP17A1, respectively) on the I-helix [49,52]. Therefore, the T252A mutant of P450cam, where the native proton transfer mechanism is perturbed, cannot catalyze hydroxylation of the substrate and hence, hydrogen peroxide is produced as an uncoupled product. Similarly, hydroxylation activities of T306A mutant CYP17A1 for pregnenolone and progesterone are drastically reduced (6–8 % of wild type). Conversely, substantial C–C bond cleavage reaction activities of this mutant for 17-hydroxypregnenolone and 17-hydroxyprogesterone were observed (~50 % of wild type) [77]. A low-temperature resonance Raman spectroscopic study indicated that heme–peroxo anion species are produced by cryoreduction of the ternary complex of 17-hydroxypregnenolone/dioxygen/CYP17A1 (oxy-CYP17A1) [78,79]. Furthermore, the spectroscopic studies detected the plausible intermediate where the substrate is attacked by the heme–peroxo anion species, resulting in C17–C20 bond cleavage in 17-hydroxypregnenolone. Based on these biochemical and spectroscopic studies, catalytic mechanism of C17–C20 bond cleavage by CYP17A1 is proposed to be mediated by the heme–peroxo species, as shown in Fig 9b.

CYP19A1

CYP19A1 mediates conversion from androgens to estrogens to maintain steroid hormone balance in animals [68,80,81]. CYP19A1 (P450arom; aromatase) catalyzes conversion of androgen to estrogen via a three-step reaction (Fig. 10a). The first and second steps are typical compound I-mediated hydroxylation of C19 of androgen to produce unstable 19-dihydroxyandrogen, while the third step is a C10–C19 cleavage reaction followed by aromatization, which is an unusual reaction for P450s. Therefore, this enzyme attracted much attention from scientists, and whether the aromatization by CYP19A1 is mediated by compound I as observed in typical hydroxylation or by the heme–peroxo anion species is a long-standing question. Isotope labeling and various experiments and theoretical studies have been performed [59,82], and the heme–peroxo anion species-mediated mechanism is the most popular one (Fig. 10b, pathway A), in which nucleophilic attack of the heme–peroxo species on 19-oxo androgen and subsequent C–C bond cleavage and aromatization produce estrogen.

In 2014, Yoshimoto and Guengerich performed an enzyme assay of the third step reaction of CYP19A1 using a labeled substrate and ¹⁸O₂ and they confirmed that no labeled oxygen (¹⁸O) from molecular oxygen was incorporated into the

formic acid product [81]. Furthermore, they identified androgen 19-carboxylic acid as a previously unknown product. Based on these and other kinetic experimental results, the study concluded that aromatization catalyzed by CYP19A1 is best explained by compound I-mediated mechanism. The proposed compound I-mediated aromatization mechanism is explained as follows (Fig. 10b, pathway B): compound I abstracts a hydrogen atom from C1 of a C19 diol intermediate; subsequently, one electron is transferred to the heme to produce a substrate cation, as proposed by DFT calculation [83]. Finally, $\text{Fe}^{3+}\text{-OH}$ abstracts a proton from the diol moiety to produce water, formic acid, and $\text{C}=\text{C}$ bond in the A ring of the steroid core of the substrate, leading to aromatization and formation of estrogen. Moreover, the kinetic solvent isotope effect on the C–C bond cleavage activity of CYP19A1 [84] and resonance Raman study on oxy-CYP19A1 [85] support the compound I-mediated mechanism.

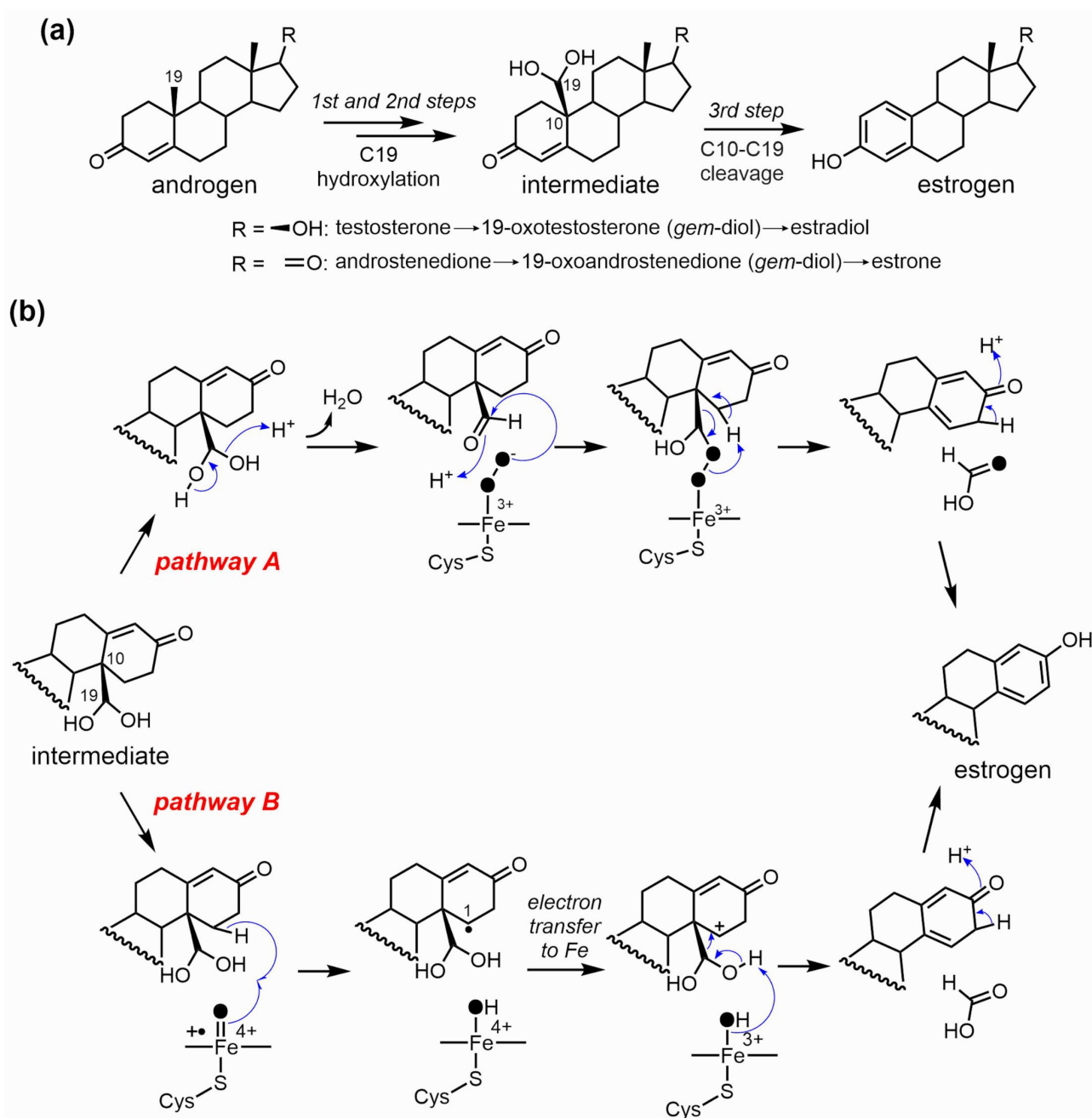


Figure 10 Conversion of androgens to estrogens by CYP19A1. (a) Three-step reactions of CYP19A1. Both testosterone and androstenedione are androgens and substrates of CYP19A1. (b) Proposed aromatization mechanisms of CYP19A1. Pathway A shows a reaction mechanism with nucleophilic attack by heme-peroxo anion species. Pathway B shows a reaction scheme via hydrogen abstraction by compound I.

CYP51

CYP51 enzyme is the most conserved P450 among the biological kingdoms and the enzyme catalyzes 14 α -demethylation of various sterols, which is an essential step to produce animal, fungal, and plant sterols [86,87]. Pathogenic fungi also have CYP51 as an essential enzyme to produce membrane sterol and, thus, the enzyme has been a target for antifungal drugs such as fluconazole that has higher affinity for fungal CYP51 than its animal counterpart [88]. As observed in CYP85A2, CYP17A1, and CYP19A1, CYP51 mediates multi-oxidation steps including C14-aldehyde derivative production by two successive hydroxylation reactions of the C14 methyl group, followed by C14–C32 bond cleavage with formic acid formation to introduce C14=C15 bond in the third oxidation step (Fig. 11a) [89,90]. Isotope-labeling experiments, QM/MM analysis, and DFT calculations suggested that the C–C bond cleavage step is mediated by the heme–peroxy anion species (Fig. 11b) [90]. However, Guengerich and Lepsheva proposed that the C–C bond cleavage reaction in the third step of CYP51 is mediated by compound I, since D231A/H314A mutant CYP51, which cannot produce compound I owing to the crippled proton delivery system, cannot perform the C–C bond cleavage step with appreciable activity (Fig. 11c) [89]. To elucidate the mechanism and identify an active oxidizing species of the C–C bond cleavage reaction by CYP51 as well as Baeyer–Villiger oxidation by CYP85A2 unambiguously, spectroscopic studies such as resonance Raman spectra of the ternary complex of substrate/O₂/P450 or its cryo-reduced form to detect heme–peroxy species and theoretical calculation of the heme–peroxy species-mediated C–C bond cleavage reaction are required.

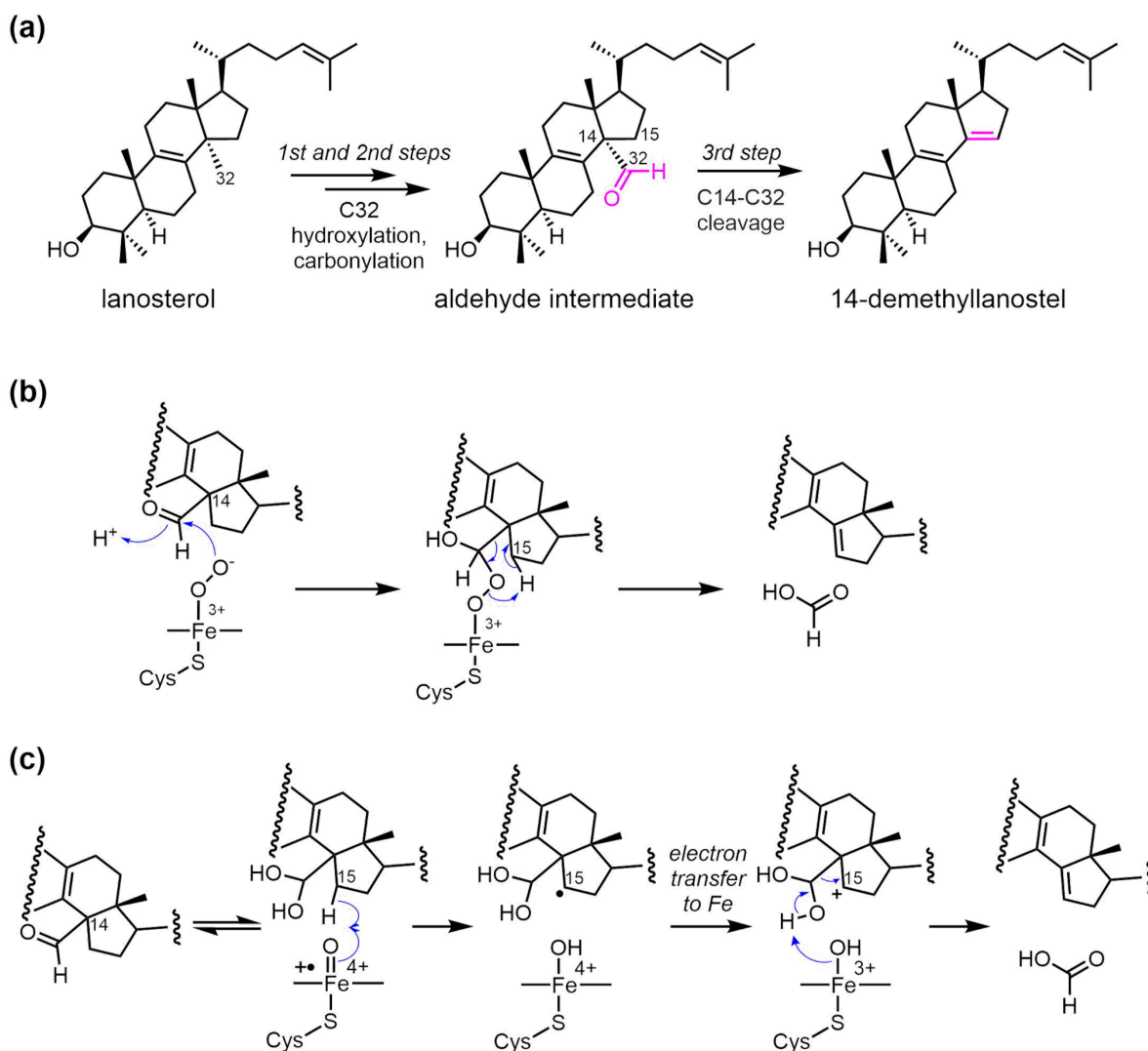


Figure 11 Reaction catalyzed by CYP51. (a) Three-step reaction to remove the methyl group at C14 of lanosterol by CYP51. (b) Proposed C–C cleavage mechanism with a heme–peroxy anion species. This reaction also accompanies formic acid molecule elimination as in the lyase reaction by CYP17A1 and CYP19A1. (c) Potential reaction mechanism mediated by compound I.

Sligar's and Kincaid's research groups proposed that a hydrogen bonding interaction with the Fe-O-O fragment in ferrous oxy-P450 determines the reactivity of heme-peroxo species. Hydrogen bond donation to the *terminal* O atom of the Fe-O-O fragment in the ternary complex of progesterone/O₂/CYP17A1 was observed in the resonance Raman studies [91–96]; this hydrogen bond donation facilitates O-O bond cleavage to form compound I. Indeed, progesterone is hydroxylated by CYP17A1 compound I. Contrarily, 17-OH-pregnenolone undergoes the heme-peroxo species-mediated lyase reaction. A hydrogen bonding interaction with the *proximal* O atom of the Fe-O-O fragment in the ternary complex of 17-OH-pregnenolone/O₂/CYP17A1 was observed in the resonance Raman study [97]. This indicates that the hydrogen bonding pattern in the substrate/O₂/P450 potentially regulates and controls the tendency of heterolytic O-O bond cleavage and nucleophilicity of the heme-peroxo species.

Summary, Conclusions, and Future Perspectives

In this review, we overviewed the general function, catalytic cycle, hydroxylation mechanism, and structure of P450s. Reductive activation of dioxygen to produce general active oxidizing species, such as compound I, is the core of P450 chemistry, and compound I mediates diverse chemical reactions rather than just hydroxylation. Numerous P450s are found in animal, plant, and insect steroid hormone biosynthesis. Our group determined the crystal structure of CYP90B1, which is the first and rate-determining enzyme in BR biosynthesis, and the structure clearly explained the regio- and stereo-selective hydroxylation mechanism. Several P450s mainly identified in animal and plant steroid hormone biosynthesis pathways catalyze unusual reactions, which are important for production and functionalization of the steroid hormone. Extensive exploration of the reaction mechanism of the C–C bond cleavage reactions clarified that many of these reactions are mediated by compound I. However, CYP17A1 cleaves the C–C bond in pregnenolone by utilizing the heme-peroxo anion as a plausible oxidizing species. Moreover, resonance Raman studies on this enzyme suggests that the hydrogen bonding pattern observed in oxy-CYP17A1 would control the heme peroxo species stability and tendency toward heterolytic cleavage to form compound I.

Recently, deep learning-based structure modeling programs AlphaFold2 and RosettaFold have been developed and user-friendly structure prediction applications of these programs are available online. In addition, greatly accelerated and inexpensive whole genome sequencing provides us a vast number of amino acid sequences of previously unexplored proteins including P450s. Thus, it is now possible to predict the structures of unexplored P450s and a method to predict their substrates and reaction mechanisms based on these model structures is necessary. Furthermore, time-resolved serial femtosecond x-ray crystallography (TR-SFX) using an x-ray-free electron laser enables recording a movie of enzyme reaction in real-time. Combining these state-of-the-art bioinformatics, structural biology, and molecular simulation methods would help in unveiling the reaction mechanisms of unusual P450 reactions such as the heme-peroxo anion-based lyase reaction, and would even help in creating a new P450 chemistry to produce materials and pharmaceutical products.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

K.F., T.H., and S.N. wrote the manuscript.

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