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Feature Review

Recent Structural Insights into Cytochrome P450 Function

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Cytochrome P450 (P450) enzymes are important in the metabolism of drugs, steroids, fat-soluble vitamins, carcinogens, pesticides, and many other types of chemicals. Their catalytic activities are important issues in areas such as drug–drug interactions and endocrine function. During the past 30 years, structures of P450s have been very helpful in understanding function, particularly the mammalian P450 structures available in the past 15 years. We review recent activity in this area, focusing on the past 2 years (2014–2015). Structural work with microbial P450s includes studies related to the biosynthesis of natural products and the use of parasitic and fungal P450 structures as targets for drug discovery. Studies on mammalian P450s include the utilization of information about 'drug-metabolizing' P450s to improve drug development and also to understand the molecular bases of endocrine dysfunction.

Trends

P450s are the dominant enzymes involved in the metabolism of drugs, as well as steroids and chemical carcinogens.

In the past 15 years, X-ray crystal structures of nearly 40% of the human P450s have become available.

Structures of P450s can be utilized to understand the molecular basis of endocrine misfunction.

Structures of P450s have been of use in guiding drug development programs, avoiding problems with bioavailability and drug interactions.

Some P450s are drug targets in humans, as well as fungi, bacteria, and parasites, and P450 structures facilitate drug discovery in this area.

Importance of Cytochrome P450 Enzymes

Cytochrome P450 (P450, CYP) enzymes were discovered in the early 1960s [1]. They were implicated in a number of reactions involved in the metabolism of drugs, steroids, and carcinogens, which had already been demonstrated [2–4]. Interest in these areas has continued to fuel research in the P450 field, and it is now known that P450 enzymes are involved in oxidation/reduction of 95% of all organic chemicals for which such reactions are known [5]. Because of this, the repertoire of fields in which P450s are of interest has expanded to include bioremediation, agriculture, bacteriology, and drug development.

P450s are involved in ~75% of the enzymatic reactions that occur in the metabolism of drugs [5–7]. Of the 57 P450 genes present in humans, five are involved in the metabolism of ~90% of the small-molecule drugs in use today, with varying selectivity and overlap [5,6]. It is now possible to do *in vitro* assays to establish which P450s (and other enzymes) will be involved in the clearance of new drug candidates and thus to predict parameters of drug clearance, drug–drug interactions, and issues related to interindividual patient variation [7]i.

A long-term goal has been the development of templates for these drug-metabolizing enzymes to reduce drug design to an *in silico* process. Doing so requires accurate knowledge of the 3D structures of P450s involved in drug metabolism and a precise description of the protein interactions with the individual atoms of drugs.

Structural knowledge of P450s is not only important for understanding and predicting drug metabolism. Several microbial and human P450s are drug targets, and the design of drugs is greatly facilitated by the structures of these P450s. Microbial P450s are important in the biotechnology industry, in the context of using P450s in catalysis of biosynthetic reaction steps. The P450s involved in steroid oxidations are extremely important in endocrinology, and many genetic defects, particularly in P450s 17A1 and 21A2, dispose individual humans to various

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endocrine disorders [8]. Understanding the molecular nature of these defects should lead to a better understanding of reasons for loss of function and to prediction of the effects of new variants.

Brief History of Earlier Structural Work with P450s

Mammalian P450s were notorious to deal with in regard to early purification efforts, and developments in detergent technology were crucial [9]. Of the 57 human P450s, all are considered to be intrinsic membrane-bound proteins, with 50 in the endoplasmic reticulum and the other seven in mitochondrial membranes [7]. Although many of these membrane-bound P450s have been purified from animal and human tissues, the advent of heterologous expression methods (especially bacterial systems) has made structural and other physical studies more feasible [10].

P450s are found throughout nature, and some bacterial P450s have served as important models for work with the P450s more relevant to medical and other practical problems. First among these is $P450_{cam}$, now termed P450 101A1, isolated from a pseudomonad [11]. The first crystals of this protein were obtained in the Gunsalus laboratory in the 1970s and the structure was published in the mid-1980s[ii] (PDB 1CPP, 2CPP) [12,13]. This structure was used as a template for homology building for other P450 primary sequences, although in retrospect many of the conclusions must be considered suspect. The next structure (1993, PDB 2HPD) was the heme domain of another bacterial P450, $P450_{BM-3}$ or P450 102A1, a fatty acid hydroxylase from *Bacillus megaterium* [14].

Some more bacterial P450 structures followed $P450_{BM-3}$, for example, $P450_{eryF}$ (PDB 1OXA) [15], but a key advance was the structure of a modified rabbit P450, P450 2C5 (PDB 1DT6), by Johnson and his associates in 2000 [16]. At the time, this was a very significant development in that it showed that membrane-bound P450s could be manipulated to yield crystals and structures, and a key element was that the modified protein was still catalytically active. In 2004, the structures of human P450s important in the field of drug metabolism began to be published, for example, PDB 1R9O, 1W0E, 1W0F, 1W0G, 1TQN [17–20].

Today the Protein Data Bank (PDB) lists 812 entries with a P450 name (P450; note: CYP is the PDB designation for cyclophilins; also, ten NADPH–P450 reductase structures were found in the search). Of these entries, 590 are for microbial P450s and 218 for mammalian P450s, including 163 structures of 22 human P450s [7] (Table 1).

Overall Architecture

Cytochrome P450 enzymes, in a strict sense, are not cytochromes in that they do not transfer electrons to other proteins. They are oxygenases, in that they transfer electrons to oxygen and also catalyze the oxidation of organic chemicals. Specifically, they are generally 'monooxygenases' or 'mixed-function oxidases'. They are classified as P450s because of the signature sequence $FXXGX_bXXCXG$, in which X_b is a basic residue and the cysteine residue serves at the axial ligand to the heme and gives P450s their rather unique spectral properties, that is, an absorption band at approximately 450 nm in its cysteine thiolate form for the ferrous–carbon monoxide complex [1]. Some proteins, for example, nitric oxide synthases and some peroxidases, also have an axial thiolate ligand, similar spectral properties, and some related catalytic reactions but are not classified as P450s. They also have different 3D structures [21,22].

The chemistry of P450 reactions is generally related to a role of a perferryl oxygen complex (formally FeO^{3+}) [23,24]. The general carbon hydroxylation reaction stoichiometry

$$NADPH + H^+ + RH + O_2 \rightarrow NADP^+ + H_2O + ROH$$

Table 1. Classification of Human P450s Based on Major Substrate Class [7]

Sterols	Xenobiotics	Fatty Acids	Eicosanoids	Vitamins	Unknown
1B1[a]	1A1[a]	2J2	4F2	2R1[a]	2A7
7A1[a]	1A2[a]	2U1	4F3	24A1[b]	2S1
7B1	2A6[a]	4A11	4F8	26A1	2W1
8B1	2A13[a]	4B1[b]	5A1	26B1	4A22
11A1[a]	2B6[a]	4F11	8A1[a]	26C1	4F22
11B1	2C8[a]	4F12		27B1	4X1
11B2[a]	2C9[a]	4V2		27C1	4Z1
17A1[a]	2C18				20A1
19A1[a]	2C19[a]				
21A2[a]	2D6[a]				
27A1	2E1[a]				
39A1	2F1				
46A1[a]	3A4[a]				
51A1[a]	3A5				
	3A7				
	3A43				

[a]X-ray crystal structure(s) reported (for human enzyme).
[b]Rat or rabbit X-ray crystal structure reported.

(where RH is a substrate) is often seen, but P450s also use the same basic perferryl chemistry to catalyze other reactions including desaturation, C–C bond cleavage, aryl ring couplings, heteroatom dealkylation, heteroatom oxygenation, ring formation, and rearrangements of oxygenated chemicals such as prostaglandins [24–26].

The structures of P450s are rather similar, and looking at the overall structures of several does not reveal the fine differences (Figure 1) [27–29]. The I- and L-helices contact the heme, and there is a 'β-bulge' segment of the cysteine ligand, just prior to the L-helix. There is also a basic 'patch' on the P450 surface that comprises arginines located adjacent to heme and on the same side as the catalytic cysteine (e.g., in human P450 17A1, Arg-347 and Arg-358 from helix K and Arg-449 from helix M) [30]. These basic residues are generally considered to be involved in binding the redox partner cytochrome b_5, which can play an accessory role in some (but not all) eukaryotic P450 reactions as a redox partner or as some type of allosteric modulator. The same basic region is also postulated to bind the eukaryotic accessory flavoprotein, NADPH–P450 reductase [30].

The general consensus in the P450 field is that the chemistry of P450s is relatively invariant, with some possible exceptions, and that catalytic specificity is largely due to how the individual proteins position substrates in the active sites, with the FeO^{3+} entity acting as 'nature's blowtorch' [31], so-called due to its oxidative power and ability to catalyze a great variety of different reactions [32]. Thus, the structures of individual P450s show considerable differences, largely due to the position and conformation of the B–C loop [33], and drive the catalytic selectivity.

The size of the (apparent) active site varies considerably, from ~190 $Å^3$ in human P450 2E1 (PDB 3E6I) [34] to an estimated 1385 $Å^3$ in (human) P450 3A4 (PDB 1TQN) [19] and 1438 $Å^3$ in (human) P450 2C8 (PDB 2NNI) [35]. A recent report of a bacterial P450 has a calculated active site volume of 2446 $Å^3$ [36] (all volumes as reported in the original literature) (Figure 2). The sizes

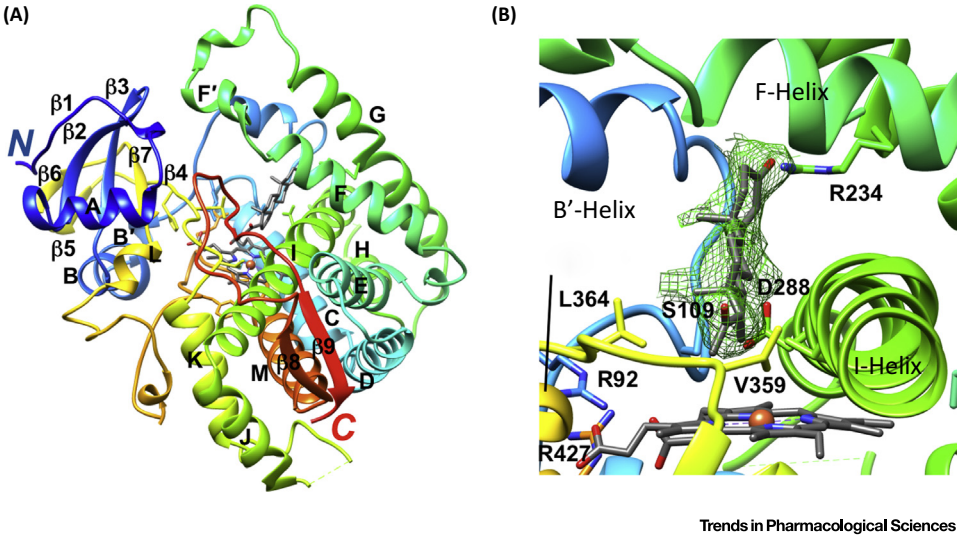

Figure 1. Tertiary Structure of the Cytochrome P450 (P450) 21A2 Progesterone Complex and Active Site Configuration (PDB 4Y8W[ii]) [28]. (A) Ribbon diagram of the overall fold of P450 21A2 with rainbow coloring from blue (N terminus) to red (C terminus). Individual secondary structural elements are labeled; carbon atoms of heme and progesterone are colored in gray; Fe^{3+} is shown as an orange sphere. Membrane P450s feature at least 12 α-helices that are designated with letters A to L and an N-terminal β-sheet domain with consecutive numbering of strands [27]. The protein core comprises helices C, D, I, K, and L and the β-sheets that make up part of the heme-binding site and the adjacent region where partner proteins dock. Substrates bind in a cleft above the heme, with several channels opening to the surface. The catalytic cysteine is located on the other side of heme, such that its thiolate moiety occupies the axial coordination site of iron opposite the bound oxygen. Helices B, C, F, and G that are situated more on the periphery of the protein and form the outer boundaries of the substrate cavity exhibit more structural variations and can be expected to display a more dynamic behavior as do the N- and C-terminal ends. (B) Close-up view of the active site with progesterone surrounded by Fourier 2Fo–Fc omit electron density drawn at the 1.2 σ threshold. Selected amino acids and α-helices wrapping around heme or forming the ceiling of the active site are labeled. All images were generated with the program UCSF Chimera [29].

of P450 active sites are congruent with known substrate selectivity. Another issue is the shape of the active site. For instance, with P450 2C8 (PDB 2NNI) the large cavity is somewhat 'L-shaped' but with P450 3A4 (PDB 1TQN) the cavity is more 'open' and explains the broader catalytic specificity. In another example, the active sites of the Family 1 P450s (1A1, 1A2, 1B1) favor the binding of flat, planar molecules, for example, α-naphthoflavone and polycyclic aromatic hydrocarbons (PDB 2HI4, 4I8V, 3PMO) [37–39]. P450s with high catalytic specificity, for example, those involved in several steroid oxidations [7], have key residues involved in making hydrogen bonds and ionic bonds to orient the substrate for the appropriate oxidation, for example, P450 19A1 Asp-309 (PDB 4KQ8) [40,41], P450 17A1 Asn-202 (PDB 4NKW, 4NKX, 4NKY, 4NKZ) [42] (hydrogen bond to 3-keto group of steroid in both cases).

General Issues of Active Site Flexibility and Multiple Occupancy

Early in the study of $P450_{cam}$ it was realized that the substrate (camphor) was imbedded within the 'closed' structure (PDB 1CPP, 2CPP) [13]. Further, the structure obtained after soaking to remove the substrate (camphor) was also closed [43]. An 'open' structure of the substrate-free protein has been published (PDB 3L61) [44]. An 'open' structure has also been found in the protein crosslinked with its redox partner putidaredoxin and with camphor bound in the active site (PDB 2JWU, 2JWS) [45]. In the open structure of the camphor complex, the substrate shields the oxygen binding site from the solvent in the entrance channel, and it may not be necessary for the protein to close for efficient metabolism of camphor. However, some substrate-induced displacements seen in residual dipolar coupling NMR have not been seen in crystal structures of substrate-free and -bound $P450_{cam}$ [46]. The $P450_{cam}$ structure may be

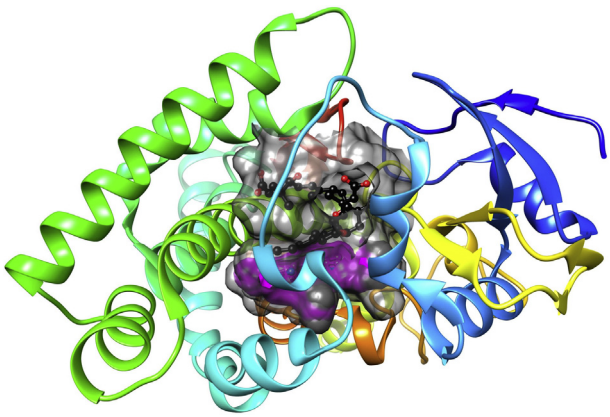

Figure 2. Crystal Structure and Active Site Volume of Bacterial $P450_{revI}$ (PDB 3WVS) [36]. Ribbon diagram of $P450_{revI}$ with rainbow coloring from blue (N terminus) to red (C terminus). The active site with a very large volume of ~2500 $Å^3$ (the difference between the gray and magenta bodies, the latter occupied by heme that is part of the enzyme) is shown as a semitransparent surface, and heme and reveromycin T are highlighted in ball-and-stick mode with carbon atoms colored in magenta and black, respectively. Abbreviation: P450, cytochrome P450.

free to sample conformational space in ways that are prevented by crystallographic packing constraints. Structural work with many P450s has led to the general view that P450s cycle between open and closed structures [33]. Because substrate binding is a fast process with many P450s [47,48], P450s must have considerable motion and be opening and closing on a rapid timescale (milliseconds).

There are some corollaries that follow. First, a structure of a P450 in the absence of a ligand is probably not particularly useful in predicting what a P450·substrate complex will look like. Another issue is that even when a structure of a P450·substrate complex is solved, it may not be instructive regarding catalysis. Although many structures are fairly consistent with function, for example, P450 17A1 (PDB 4NKW, 4NKX, 4NKY, 4NKZ) [42], P450 21A2 (PDB 4Y8W) [49], others are not. In the published structures of P450 1A1 and 1A2 complexed with α-naphthoflavone (PDB 2HI4, 4I8V) [37,38], the main site of oxidation (for 5,6-epoxidation) [50] is furthest from the heme iron (12 Å). With (human) P450 3A4, some erythromycin-, bromoergocryptine-, and progesterone-bound structures have been published [18,51–53] but only the bromoergocryptine complex (PDB 3XNU) is catalytically competent, that is, the major sites of oxidation (8′ and 9′ carbons of the proline ring) are 4.1 and 3.7 Å, respectively, away from the iron [52,54].

Another issue is that many P450s are malleable and the protein structure depends upon the specific ligand. An example of this is seen with P450 3A4 (PDB 2J0D) [51], which has internal movement of the protein in the complexes with erythromycin and ketoconazole. In addition, P450 2E1, with a small active site, can trap larger substrates such as fatty acids (which are also substrates) (PDB 3LC4) [55].

Multiple ligand occupancy was proposed more than 20 years ago to explain some of the cooperative behavior of P450 3A4, both homotropic and heterotropic [56,57]. For many years any proof offered was indirect and the results could have been rationalized in terms of other phenomena, for example, classic systems with an allosteric site distant from the catalytic ('active') site of the enzyme. However, the hypothesis that two molecules of a ligand are both present, next to each other, is difficult to prove in the absence of structural data. The hypothesis about 'dual occupancy' went unproven until 2006, when two molecules of the inhibitor (and poor substrate) ketoconazole were identified in the 'canonical' active site of P450 3A4 (PDB 2V0M) [51]. Two ligands have now been found in crystal structures of P450s 2C8 (PDB 2NNH) [35], 158A2 (PDB 1T93) [58], 2D6 (PDB 3TBG) [59], 21A2 ((PDB 3QZ1) [60], and 3A4 (PDB 2V0M, 4K9T, 4K9U) [51,61] and potentially others (Figure 3). The dual occupancy can be used to explain homotropic cooperative phenomena (e.g., 3A4), but in some cases in which the electron

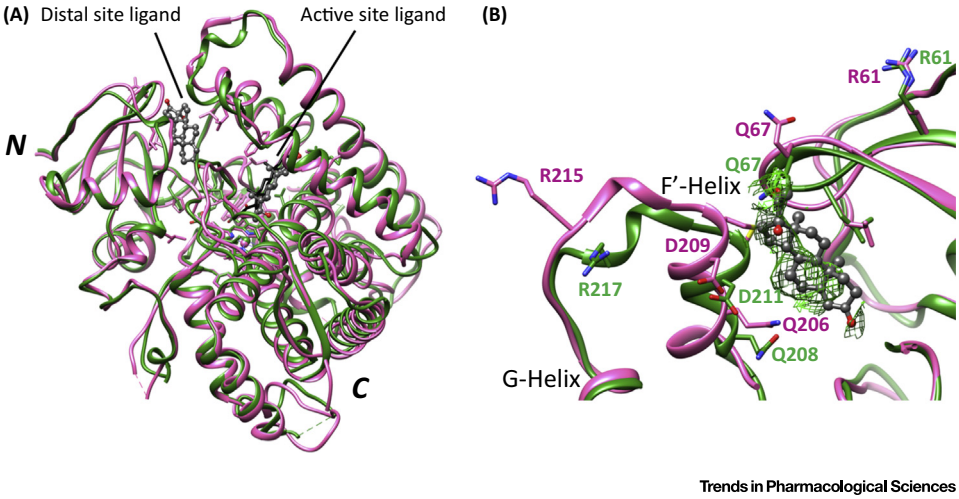

Figure 3. Binding of Two Ligands in the Crystal Structures of the Human P450:Progesterone (PDB 4Y8W) [49] and Bovine P450 21A2:17α-Hydroxyprogesterone (PDB 3QZ1) [60] Complexes. (A) Superimposition of the structures of the human (green) and bovine (pink) complexes with ligands bound at the canonical active site (progesterone, stick mode with black bonds; or 17α-hydroxyprogesterone, ball-and-stick mode with carbon atoms in gray) and a distal site (17α-hydroxyprogesterone) indicated. (B) Close-up view of the superimposed distal site regions in the human and bovine complex structures, with Fourier 2Fo–Fc sum electron density from the human structure (green meshwork, 0.7σ threshold), indicative of binding of progesterone of partial occupancy at a location that matches that of the distal site 17α-hydroxyprogesterone in the bovine structure. Selected helices and amino acids are labeled. Abbreviation: P450, cytochrome P450.

density for two molecules is very convincing, careful analyses have not revealed any evidence for homotropic cooperativity, for example, P450 2C8 [35]. Heterotropic cooperativity is known to exist in P450s (e.g., [62,63]), but the prospects of getting a P450 crystal with one of each of two different molecules present seem unlikely.

Microbial P450 Structures

The early bacterial structures of $P450_{cam}$ and $P450_{BM-3}$ were very useful in guiding later efforts in crystallography; the point can also be made that $P450_{BM-3}$ has been useful itself in the context of some biotechnology applications [64]. Since then, numerous bacterial P450 structures have been solved for various reasons.

Some of the more complex bacterial (e.g., Actinobacteria) and other microbial P450s are of interest in the context of their roles in biosynthesis of antibiotics and other drugs (e.g., vancomycin). Visualizing the structures of these P450s can be instructive in understanding the basis of catalytic specificity and in modifying the proteins for new functions.

An important area is the inhibition of sterol-metabolizing P450s in yeasts and fungi as a means of controlling infections. P450 51 is a common target (e.g., for azoles) and the first structure was published in 2001 [65]. These P450s (Family 51) are also targets for tuberculosis (*Mycobacterium tuberculosis CYP51B1*) and trypanosomiasis (Chagas disease, *Trypanosoma cruzi*) [66,67], and rational drug discovery efforts have been aided by the availability of structures. Humans also have P450 51A1, involved in cholesterol synthesis, and discrimination between the human and microbial forms is important [68].

Recent Bacterial P450 Structures

In the past 2 years (2014–2015), at least six structures of bacterial P450s have been published. One is P450 154C5 from *Nocardia farcinica*, a steroid 16α-hydroxylase [69]. This P450 binds

steroids with sub-μM K_d values, and structures of the protein with four different steroids were determined (PDB 4J6B, 4J6C, 4J6D, 4J6T). This enzyme is a candidate for biotechnology driven synthesis of novel steroids.

At least two families of P450s, P450 125 and P450 142, are involved in cholesterol degradation, a critical path in *M. tuberculosis*. Frank *et al.* [70] reported the structure of P450 142A2 in complex with cholesterol 3-sulfate (PDB 4TRI, 4UAX). The active site is much smaller than that of a P450 125A1:4-cholestene-3-one complex (PDB 2X5W) [71], and the tighter fit is proposed to help account for the more efficient oxidation of cholesterol esters, providing a source of carbon for growth.

An unusual bacterial P450 (152L1) from *Jeotgalicoccus* sp. 8456 decarboxylates fatty acids to form terminal alkenes, acting as a peroxygenase (using H_2O_2) [72]. The physiological role of this P450 is not known. The structure of P450 152L1 (obtained both in the absence and presence of a fatty acid substrate, PDB 4L40, 4L54) is similar to those of two other bacterial fatty acid-oxidizing peroxygenase P450s, $P450_{SP\alpha}$ [73] and $P450_{BS\beta}$ [74], which catalyze hydroxylations at the α- and β-carbons, respectively. An explanation emanates from the structure and the role of His-85 in the active site. His-85 is proposed to donate a proton to the Compound I form of the heme (FeO^{3+}) concomitant with reduction to Compound II ($FeOH^{3+}$) by abstraction of an electron (and addition of a proton) from the fatty acid carboxylic acid moiety [72,75]. Homolytic scission of the C–C bond is proposed to follow formation of the carboxylate radical.

Bacterial P450s are also of interest because of their roles in biosynthetic reactions, and *Streptomyces* species produce a wide variety of natural products used as drugs. One is reveromycin A, a lead compound in development with anti-osteoclastic activity. $P450_{revI}$ catalyzes C18 hydroxylation, a key biosynthetic step [36] (Figure 2). A 1.4 Å structure with the substrate reveromycin T (PDB 3WVS) is congruent with the observed site of hydroxylation. The binding of the substrate carboxylates to two $P450_{revI}$ arginines, observed in the structure, was further established by site-directed mutagenesis.

One species of *Streptomyces*, *Streptomyces avermitilis*, has 33 P450 genes. The structures of some *Streptomyces* P450s were first published several years ago [58,76]. Recently, Han *et al.* [77] reported a 2.1 Å structure (PDB 4WPZ) for P450 107W1, an enzyme involved in C12 hydroxylation in the biosynthesis of the macrolide oligomycin A. The substrate was not present in the crystal, but early in 2016 a new structure with the product oligomycin A has been published [78]. The structure (PDB 4WQ0) indicates that Trp-178, located in the open pocket of the active site, may be a critical residue for the productive binding conformation.

Also in the realm of biosynthesis of drugs, *Rhodococcus erythropolis* JCM 6824 P450 RauA (tentatively P450 1050) has been shown to catalyze the *N*-hydroxylation of a quinolone to form aurachin RE, which has antibiotic activity [79]. The juxtaposition of the substrate in the crystal structure (PDB 3WEC) appears to be consistent with the site of oxygenation.

The sixth entry is a P450 (105AS1) from *Amycolatopsis orientalis*, which was found to be efficient in converting compactin (the first statin considered for hypercholesterolemic activity) to pravastatin, a very successful statin on the market [80]. A structure of P450 105AS1 was determined (2.05 Å) in the ligand-free state (PDB 40QS) but a co-crystal with compactin could not be obtained. Random mutagenesis of P450 105AS1 yielded a new protein, $P450_{prava}$, with better catalytic activity, as the result of five mutations. A co-crystal of $P450_{prava}$ with compactin (PDB 40QR, 1.8 Å) – compared with the P450 105AS1 open structure – showed reorientation of the BC loop region and reorganization of the FG helices. The C6 atom of compactin (which is hydroxylated) was positioned 4.7 Å from the heme iron (Figure 4). Three of the five mutations

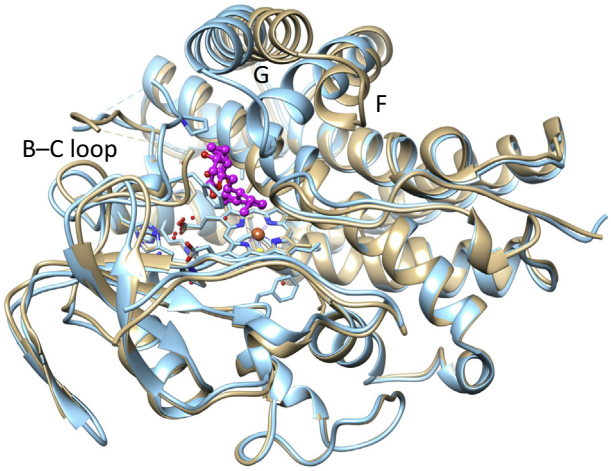

Figure 4. Crystal Structures of P450 105AS1 Ligand-Free and with Ligand-Bound (PDB 4OQR and 4OQS, respectively) [80]. Superimposition of the ligand-free structure of P450 105AS1 (beige ribbon and heme) and the structure of the complex with compactin (cyan ribbon and heme, and ligand depicted in ball-and-stick mode with carbon atoms colored in magenta). The comparison reveals the reorientation of the B–C loop region (left) and movements by the F- and G-helices (top). Abbreviation: P450, cytochrome P450.

were located within van der Waals contact of the substrate compactin. Also, the Q127R mutation was concluded to disrupt the 'open conformation' network, favoring the closed form. Thus, many of the changes that led to improved catalysis can be understood in structural terms.

Earlier this year (2016), Haslinger and Cryle [81] reported a structure (PDB 5HH3) of an *Actinoplanes teichomyceticus* P450 termed $OxyA_{tei}$, completing the gallery of P450 structures involved in the biosynthesis of the glycopeptide teicoplanin. The structure has been compared with OxyB, OxyC, and OxyE [81]. Despite the hydrophobic character of the peptide precursor, the active site of $OxyA_{tei}$ is rather polar and is conserved among the glycopeptide D-*O*-E ring forming enzymes.

Recent P450 Structural Work with Fungi and Parasites

As mentioned earlier, disruption of sterol biosynthesis has been a useful mechanism for the development of antifungals, and several structures of P450 51 enzymes are available [65–67]. A similar approach has been applied to develop inhibitors of Chagas disease caused by the protozoan parasite *T. cruzi*, for example, VNI ((*R*)-*N*-(1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl) ethyl)-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide) [82]. Structures of *T. cruzi* P450 51 have been published with three potent inhibitors (low nM IC_{50} values) (PEB 4CKA, 4CK8, 4CK9) [67].

A P450 in the worm *Schistosoma mansoni* (CYP3050A1) has been shown to be essential for both survival and egg development [83]. Its substrate(s) is unknown but this P450 forms tight complexes with many antifungal drugs. No structure is available; modeling has been done on the basis of rabbit P450 2C5 [83].

Recent P450 Structures Involving P450s Involved in Drug Metabolism

As mentioned earlier, structures are now available for all of the major human P450s involved in drug metabolism (Table 1) [7,33]. One of these is P450 2D6, which has some notoriety in that it was the first P450 shown to exhibit monogenic distribution and genetic polymorphism [84]. P450 2D6 is involved in the metabolism of 20–25% of marketed drugs [5]. In 2006, Rowland *et al.* published the first crystal structure of P450 2D6 (PDB 4F9Q) [85], although that structure involved the introduction of several amino acid substitutions and had no ligand present. Subsequently, Wang *et al.* [86] reported a structure for P450 2D6 bound to the neutral molecule prinomastat (PDB 3QM4).

More recently, Wang *et al.* [59] reported new open and closed structures of P450 2D6 (PDB 3QM4). Many of the substrates and inhibitors of P450 2D6 contain a basic nitrogen atom, and early in the history of P450 2D6 the dogma was that all ligands were basic [87,88], although exceptions are now known [86,89]. Site-directed mutagenesis studies [89–91] suggested roles for both Asp-301 and Glu-216 in binding basic atoms. The structures obtained with the ligands thioridazine, quinine, and ajmalicine showed charge-stabilized hydrogen bonds with Asp-301 and Glu-216 (PDB 4WNT, 4WNU, 4WNV, 4NVW) [59]. Open and closed complexes were observed with thioridazine (PDB 3DTA, 4WNW), and the open conformation included a second molecule of the drug bound in an expanded substrate access channel antechamber, with a charge-stabilized hydrogen bond to Glu-222 (PDB 3TBG). The authors concluded that P450 2D6 is sufficiently elastic to allow the passage of compounds between the active site and bulk solvent and to adopt a more closed form that appears to adapt to bind alternative ligands, with different degrees of closure.

In another study involving P450 2D6 structures, Brodney *et al.* [92] considered a candidate drug (a β-secretase inhibitor) that was almost exclusively metabolized via the P450 2D6 route (>95%). Such a situation can be unfavorable due to the genetic polymorphism of P450 2D6 and the wide interindividual variation in rates of metabolism, that is, in some individuals the drug might not be cleared appreciably [84,93]. The drug, an *N*-methylpyrazole, and its *N*-demethylated product were crystallized with P450 2D6 and the contact sites were established (PDB 4XRY, 4XRZ). With this knowledge, plus hERG channel inhibition data, an alternate lead (an isoxazole) was designed that had similar IC_{50} values to the initial lead compound but was now metabolized via multiple routes [92].

Another recent example involving P450 and drugs relates to P450 3A4, the major liver and intestinal P450 in humans, and its inhibition [94]. Currently, the inhibitor ritonavir and its derivative cobicistat are used as 'pharmacoenhancers' with drugs administered to patients with HIV. Sevrioukova and Poulos [54] have reviewed their structural and other biochemical studies with P450 3A4 and ritonavir analogs (PDB 4D6Z, 4D75, 4D78, 4D7D). Several conclusions were reached. Strong heme binding is crucial, and the most effective heterocycle was a pyridine. Removal of the backbone hydroxyl increased interactions. Two sites, termed the Phe-1 and Phe-2 pockets, were found to be important in binding of these ritonavir analogs to P450 3A4. All of these findings were utilized in the development of a new pharmacophore [54].

Recent Structural Work Related to Steroid Metabolism

P450 7A1 is the hepatic cholesterol 7α-hydroxylase, the first enzyme involved in bile acid metabolism. Tempel *et al.* [95] solved both a ligand-free structure of human P450 7A1 and also bound to cholest-4-en-3-one (the 'Δ4' homolog of cholesterol) and 7-ketocholesterol, a product of the oxidation of the alternate substrate 7-dehydrocholesterol [96]. The C7 atom is positioned for oxidation, consistent with the 7-dehydrocholesterol reaction. The structures show the rigidity of the active site, consistent with the generally high specificity of this enzyme.

Previous work yielded a structure for native P450 19A1, the steroid aromatase, and recombinant forms [40,97] (PDB 3EQM, 3S79, 3S7S, 4GL5, 4GL7). The latter work led to some new inhibitors of aromatase in 2012 [97]. More recently, there is biophysical evidence for the presence of an aspartate residue (Asp-309) in the active site with an elevated pK_a (8.2) [98]. This residue is postulated to be involved in binding to the 3-keto group of androstenedione and testosterone.

P450 21A2 is the major steroid 21-hydroxylase and forms 11-deoxycorticosterone and 11-deoxycortisol in the adrenal cortex. A structure of the bovine enzyme (PDB 3QZ1) [60] found use as a model for explaining the >100 variants identified in clinical endocrinology [99]. A structure of

human P450 21A2 (PDB 48YW), bound to progesterone, was solved (2.7 Å) and provides a more appropriate model [49]. The juxtaposition of C21 near the heme is appropriate for the observed reaction (Figure 1B) and consistent with (i) the high catalytic efficiency (10^7 M^{-1} s^{-1}) and (ii) rate-limiting C–H bond breaking. The availability of the human structure allows for more insight into the genetic disorders than was possible with models derived from the bovine structure [99]. Although the bovine structure is a reasonable guide, several conclusions about salt bridges and other contacts were probably wrong and are now superseded by the human structure (PDB 48YW) [28]. The most severe clinical cases (salt-wasting) fit into the group of variants near the active site (near the heme or substrate). The milder 'non-classical' mutations are often found near the periphery and close to the surface of the protein [28]. This phenotype is often associated with mutations having compensatory effects, for example, H-bonding replacing hydrophobic interactions.

P450 17A1 is an important enzyme, converting progesterone and pregnenolone to androgens. More than 50 variants have been identified in the clinic [100]. The enzyme is also a target for prostate cancer. DeVore and Scott first reported structures of the enzyme bound to two inhibitor drugs (PDB 3RUK, 3SWZ) [100]. More recently, the same group reported X-ray structures of P450 17A1 with all four physiological substrates – progesterone, pregnenolone, 17α-hydroxyprogesterone, and 17-hydroxypregnenolone (PDB 4NKW, 4NKX, 4NKY, 4NKZ) [42]. The binding patterns provide useful insight into the patterns of hydroxylation.

P450 17A1 is somewhat enigmatic in that it catalyzes two subsequent oxidations – 17α-hydroxylation and then a 17α,20-lyase reaction. Cytochrome b_5 stimulates, particularly the lyase reaction [101], but without electron transfer [102]. In some clinically observed variants, P450 17A1 only catalyzes the first reaction (17α-hydroxylation). Fish have two genes, P450 17A1 – with the normal two activities – and P450 17A2 – devoid of the lyase activity. Both proteins were crystallized (PDB 4R20, 4R21, 4R1Z) [103] and compared with the human enzyme (PDB 3SWZ), all with the same drug inhibitor present (Figure 5A,B). The active site regions of all three proteins, despite only ~50% overall identity, are remarkably similar. Only five residues near the heme periphery differ between zebrafish P450 17A1 and 17A2 [103] (Figure 5C).

One explanation for the differences between fish P450 17A1 and 17A2 is that the residues near the heme periphery alter the heme charge distribution and favor conversion of the ferric peroxide (FeO_2^-, Compound 0) to perferryl oxygen (FeO^{3+}, Compound I) [103]. Alternatively, P450 17A1 may have a conformation available that allows for rearrangements and the 17α,20-lyase reaction. Estrada *et al.* [104,105] have provided NMR evidence for this latter hypothesis. NMR spectroscopy has an advantage over X-ray crystallography in that it provides insight into dynamic processes and may be more useful in identifying multiple conformations that coexist. The NMR evidence is supportive of roles of NADPH–P450 reductase and cytochrome b_5 in modulating the conformations. The NMR data also argue that these two accessory proteins occupy the same site on P450 17A1 and compete for space [104–108] (Figure 6A). In light of the evidence that cytochrome b_5 does not enhance activity by electron transfer [102], the mechanism of stimulation remains enigmatic: in principle, the reductase transfers two electrons into the Fe/FeO system (generating a very reactive complex), it dissociates, cytochrome b_5 binds and changes the conformation, the reaction occurs, and then cytochrome b_5 (and the reaction product) dissociate (so that the reductase can bind again). This sequence would have to take place every cycle, so with typical rates of ~6 min^{-1} that means every 10 s. More insight into this phenomenon will be of interest.

General Phenomena of P450 Reactions Studied by Structural Methods

Two papers published in 2015 by leading groups in the field are of interest in terms of general issues raised about P450s.

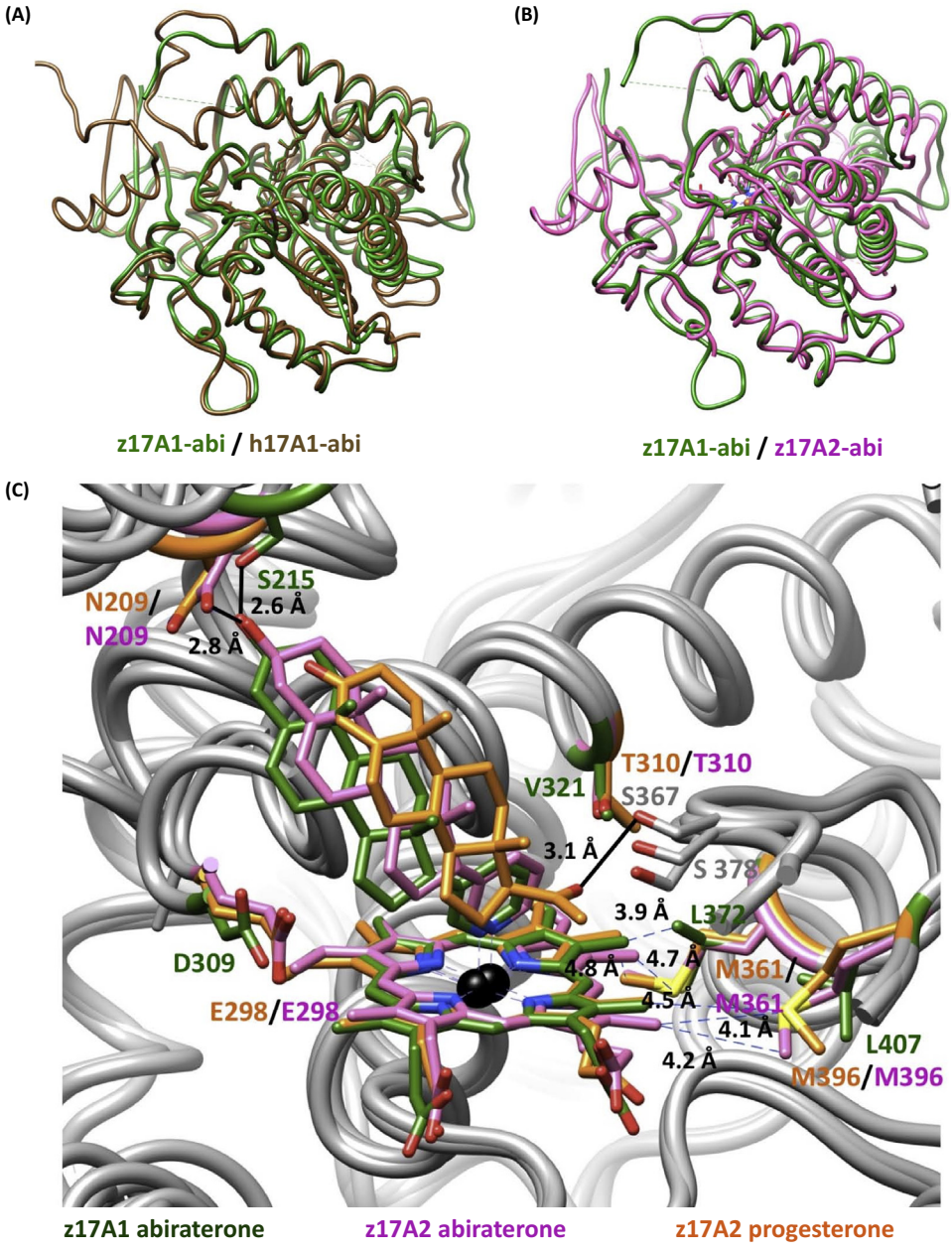

Figure 5. Similar Tertiary Structure Folds of Human P450 17A1 and Zebrafish P450 17A1/A2 and Subtle Differences in the Active Sites of Zebrafish P450 17A1 and 17A2 [103]. (A) Overlay of the abiraterone (abi) complexes of human P450 17A1 (PDB 3RUK; brown) [84] and zebrafish 17A1 (PDB 4R1Z; green). (B) Overlay of the abiraterone complexes of zebrafish P450 17A1 (green) and P450 17A2 (PDB 4R20; pink). (C) Comparison between the active site configurations of the zebrafish P450 17A1:abiraterone (green), 17A2:abiraterone (pink), and 17A2:progesterone (PDB 4R21; orange) complexes reveals five differences in the amino acid compositions of 17A1 (from human or zebrafish) and 17A2 (from zebrafish) in the vicinity of the heme moiety and near the ceiling of the active site. Color labels of residues match the corresponding structures. Abbreviation: P450, cytochrome P450.

In one paper, Sevrioukova and Poulos [53] studied several structures of P450 3A4, an enzyme that has been rather enigmatic in terms of its allosteric behavior. As in one of the first published structures of P450 (PDB 1W0E, 1W0F, 1W0G) [18], a progesterone molecule (added during crystallization) was found in the same peripheral region (PDB 51AP). The authors argue that the

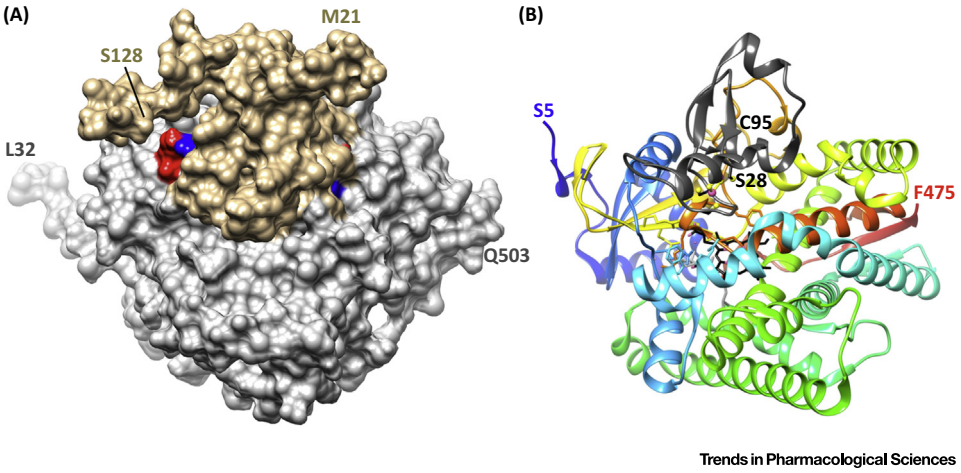

Figure 6. 3D Structures of Cytochrome P450 (P450) Complexes. (A) Surface representation of the complex between human P450 17A1 (gray) and cytochrome b_5 (beige) based on crosslinking and mass spectrometric analysis [106]. The model was generated with the program HADDOCK [108], using the X-ray coordinates of human 17A1 (PDB 3RUK) [100] and NMR coordinates of human b_5 (PDB 2I96). Two salt bridges identified by XLMS are highlighted in blue (lysine) and red (glutamate), and N- and C-terminal residues of both proteins are labeled. (B) Crystal structure of the complex between P450 11A1 and adrenodoxin (Adx, PDB 3N9Y) [107]. P450 11A1 is shown in cartoon mode with rainbow coloring from blue (N terminus) to red (C terminus), adrenodoxin is depicted as a dark gray ribbon (only a portion of Adx, residues Ser-28 to Cys-95, was visible in the crystal structure), carbon atoms of heme and cholesterol are colored in black and gray, respectively, and iron (P450 Fe^{3+} and Adx [2Fe–2S] cluster) and sulfur atoms are highlighted as pink and yellow spheres, respectively. The complexes depicted in the two panels are shown in similar orientations.

position of the steroid makes it unlikely that it is either a substrate (in this position) or acting as an effector. Other steroids were also found to be in this region (testosterone, androstenedione, cholesterol). The other point of interest is that citrate, added in the buffer, was present in the structure (PDB 51AP). Subsequent biochemical assays showed that citrate and numerous other anions could affect catalytic activities, acting at multiple points in the mechanistic cycle. This behavior is reminiscent of stimulation previously reported with anionic phospholipids [109] and glutathione [110]. The physiological relevance is still unclear.

Finally, work with bacterial P450 119 is of interest [111]. Crystal structures of P450 119 are known. One general issue with P450s is whether adding a ligand induces a conformational change ('induced fit' [112]) or whether the addition of a ligand leads to binding and the selection of one of multiple pre-existing conformational states. This is a general issue in protein chemistry [113,114]. The difference is subtle, in that in a 'thermodynamic box' analysis [115], the free energy changes are identical (i.e., the overall energy is the same regardless of whether a conformational change occurs before or after ligand binding). Basudhar *et al.* [111] used a series of azole ligands and both X-ray crystallography and NMR with P450 119. They concluded that some azoles bound to the open form and that some substituted phenylimidazoles gave rise to two closed conformations that depended on the size of the *para*-substituent (PDB 4TT5, 4TUV, 4WPD, 4WQJ). Thus, at least with the model P450 119, ligands selectively stabilize discreet populations of conformational states.

Concluding Remarks and Future Needs in P450 Structural Work

We have provided a general background on P450 research and focused on new structural insights in the past 2 years (2014–2015). Suffice it to say that considerable progress is being made (e.g., Table 1), on a variety of fronts. What remains to be done? Several items can be listed.

One problem is that almost all of the P450 structural work has been done on single proteins, but we know that almost all of them operate with redox partners. Only a few reports have appeared

Outstanding Questions

When multiple structures of a P450 are seen with different ligands, can we accurately predict what the structure will be with a new drug or another ligand?

How do accessory proteins such as NADPH–P450 reductase and cytochrome b_5 bind to P450s? High-resolution structures of their complexes with P450s are needed to accurately map the binding interfaces.

How do the structures of P450s change when bound to their accessory proteins (e.g., NADPH–P450 reductase and cytochrome b_5)? Do the changes explain catalytic behavior?

How different are the structures of P450 variants with low activity that have been identified in the clinics? Can we rationalize the low activity in terms of poor substrate fit, or is it gross deformation of the protein?

How does heterotropic cooperativity occur? Can we catch a P450 with one of each of two ligands present?

How much does the structure change as a P450 moves through the catalytic cycle? Currently, we only have (some of) this information with $P450_{cam}$.

Are there really separate entry and egress ports on a P450, or do P450s just open and close to bind and release ligands?

of structures of complexes [45,107,116] (Figure 6). Some NMR studies have been done to identify parts of proteins involved in complexation [104,117], but many questions still remain (see Outstanding Questions), as discussed under the description of P450 17A1.

Another issue is that through modern genetic approaches it has become almost routine to identify single nucleotide variants with human P450s that are seen in clinics and affect catalytic activity. However, there is almost no structural information about such variants, usually only modeling. More activity in this area would be very useful with P450s that are involved in the metabolism of both steroids and drugs. One concern with single nucleotide variants of 'drug-metabolizing P450s' is that what is learned with one drug may not apply to the next.

This caveat leads to the last major issue we will present. One example has been presented of a metabolism problem with a drug that could be addressed with structural information [92]. Ideally, many drug candidates could be examined in this way if issues were defined empirically. However, the pool of chemical candidates for any drug project is large ($\sim 10^4$ chemicals for each drug that eventually reaches the market), and the time allocated to preliminary analysis of an individual compound is short in each program (generally ~2 weeks). This is too short for the pace at which X-ray crystal structures can be obtained. Recall the discussions about malleability of P450s and the evidence that different ligands can yield different protein structures, making predictability difficult for new chemical entities. What is the answer? Will there be breakthroughs in the development of new higher throughput crystallography technology? Can NMR systems such as residual dipolar coupling be applied after crystal structures are solved [46,118]? Will we find that most P450s can be defined in the context of a small number of different states that can be associated with all ligands, and can we utilize this in modeling?

These are questions for current and new scientists in the P450 field. The answers will require more work, just as in all other types of science.

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Resources

[i] medicine.iupui.edu/clinpharm/ddis/clinical-table

[ii] www.rcsb.org

[iii] http://medicine.iupui.edu/clinpharm/ddis/clinical-table

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