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Structure of cytochrome P450 2B4 with an acetate ligand and an active site hydrogen bond network similar to oxyferrous P450cam

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Graphical abstract

Superposition of the active site of acetate-bound P4502B4 and oxyferrous P450cam. Bond lengths between the heme iron, the sixth ligand, and the hydroxyl of the conserved threonine are shown. Note that different threonine rotamers form the hydrogen bonds to the acetate and oxygen.

Introduction

The cytochromes P450 (hereafter referred to as P450s) are a ubiquitous superfamily of monooxygenases which are unequaled among biological catalysts in chemical versatility. [1,2,3,4] Though distinct in enzyme reactivity and substrate specificity, soluble bacterial and membrane bound microsomal P450s share common features in their catalytic reaction pathways. Two electrons and two protons are necessary for each catalytic cycle. [5,6] P450 reductase provides the first and second electrons to microsomal P450s via its proximal surface, while cytochrome *b*₅ can only supply the second electron owing to its high redox

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potential (~25mV) relative to that of the P450s (~-245mV). [7] Proton delivery via a hydrogen-bonded water network is orchestrated and coordinated by a conserved pair of active site amino acids, one with an acidic side chain (Glu/Asp) and the second with an alcohol side chain (Thr/Ser). The exact structure and mode of action of the proton shuttle machinery has been extensively investigated by several laboratories but is still not precisely defined. [8–14] Mutation of the Asp251 to asparagine in P450_{cam} (cytochrome P450 camphor) results in an ~90% decrease in activity, which is nonetheless coupled. Coupling refers to the utilization of NAD(P)H-reducing equivalents for product formation rather than side products, such as hydrogen peroxide or superoxide. [10] Cryo-reduction studies have revealed that the P450_{cam} Asp251Asn mutant accumulates the peroxo intermediate at 77 K whereas the wild type protein forms the hydroperoxo species at this temperature. [11] These experiments were interpreted to indicate that the acidic residue is involved in delivery of the first proton to the highly anionic heme peroxo intermediates (Fe^{3+}OO)²⁻ from a preformed proton delivery network. [15] Mutation of the conserved active site P450_{cam} residue Thr252 to alanine also severely (>90%) decreases activity. In contrast to mutation of the acidic residue, Asp251, the reaction with Thr252Ala is uncoupled. NAD(P)H utilization results in a large amount of hydrogen peroxide and a small amount of product. [16] Similar results have been observed for the corresponding mutants in P450 2B4. The side chain of the conserved threonine serves as a hydrogen bond (H-bond) acceptor of the hydroperoxo species which directs protonation to the distal oxygen, and formation of compound I. [9,12] Compound I inserts an oxygen atom into a C–H bond, the product dissociates, and the cycle repeats. [17]

P450s, like most proteins, exist as an ensemble of different conformations. In the absence of substrate, the P450 residues involved in substrate binding are more dynamic than when substrate is bound. Whether substrate enters the active site via an open channel or whether the substrate “pushes” its way through a potential channel is still being investigated and debated. [18] When camphor binds to P450_{cam} in a geometrically appropriate position to undergo oxidation, it sterically displaces the water ligand of the iron (2CPP) and shifts the I-helix center away from the heme. [19] Upon binding oxygen, the conserved active site motif (A/G GX D/E T) in the I-helix of P450_{cam} partially “unwinds” for ~4 residues which lack the α -helical i to i+4 carbonyl oxygen to amide hydrogen bonds. This local I-helix “unwinding” creates a hydrophilic space that allows two water molecules to bind (1DZ8, 2A1M) and form a hydrogen bond network close to the distal, non-iron-bound oxygen with backbone atoms of the conserved acid-alcohol residues. [8,12] This closed oxyferrous conformation of P450_{cam} does not reveal how protons access the active site, since the active site is not connected to bulk solvent. The exact relationship between the open and closed conformations of P450_{cam} and hydration of the active site is controversial and is the subject of continuing investigation. However, it is generally agreed that the conserved acid residue is critical and likely transports a proton from solvent to the active site. [14,20–22] Reduction of oxyferrous P450 generates a highly anionic peroxo intermediate that is instantaneously protonated via the preformed active site proton relay network. [11,14] A second proton is subsequently donated to the distal oxygen presumably from the same or a similar proton relay network. [14,23] Following delivery of the second proton scission of the dioxygen molecule occurs, which allows catalysis to proceed.

In contrast to the well-studied but still incompletely characterized proton relay network in P450_{cam}, very little is known about the active site hydrogen-bonded water network and how it facilitates proton delivery from the solvent to the activated oxygen. In microsomal cytochromes P450, this is primarily due to the lack of a structure of an oxyferrous microsomal P450 and of high resolution structures of microsomal P450s in general. Bacterial P450s vary from microsomal P450s in two important ways that suggest their proton delivery paths may differ. Most bacterial P450s are coupled and receive electrons from an iron-sulfur protein whereas drug-metabolizing microsomal P450s tend to be highly uncoupled and receive electrons from a flavodoxin-like protein. In the course of determining the structure of a Phe429His mutant of P450 2B4, [24] it was unexpectedly observed that acetate, which was in the crystallization buffer, occupied a position that approximates the location of oxygen in oxyferrous P450_{cam}.

In view of the dearth of information about the hydrogen bonding system in the active site of oxyferrous microsomal P450s, we took this opportunity to analyze the hydrogen-bond network in the active site structure of the acetate containing microsomal P450 2B4 and compare it to the oxyferrous structure of the extensively studied P450_{cam} to understand the common and unique features of oxygen activation in the two different classes of P450. Because bacterial and microsomal proteins receive electrons from iron sulfur and flavodoxin-like proteins and because bacterial reactions tend to be highly coupled, whereas drug metabolizing microsomal P450 reactions are often very uncoupled, we considered that there might be some variation in their oxidation activation mechanism. Although a carbon atom separates the two partially negatively charged oxygen atoms in acetate, acetate can nonetheless be considered a mimic of iron-bound dioxygen whose distal oxygen in P450s also has a significant negative electrostatic potential. [20–22,24] Our analysis implies that the proton shuttle mechanisms in microsomal and bacterial P450s are very similar but less robust in microsomal P450s and illustrates the structural and functional versatility of the P450 I-helix active site motif (A/G GX E/DT).

Results and Discussion

Overview of the crystal structure of acetate and cymal-5 (5-cyclohexyl-1-pentyl-β-D-maltoside)-bound P450 2B4 Phe429His

In the course of trying to crystalize the mutant P450 2B4 in the presence of the substrate butylated hydroxytoluene (BHT), we inadvertently crystallized and determined the structure of P450 2B4 with acetate as the sixth axial ligand and two molecules of the detergent, Cymal-5. BHT was not bound to the protein. (Figure 1a) The crystal structure of acetate-bound P450 2B4 Phe429His (henceforth referred to as either P450 2B4 or P450 2B4 Phe429His) has been determined to 2.1 Å in space group *P*3₁21 with one molecule in each asymmetric unit. The structure was solved by molecular replacement using wild type P450 2B4 (1SUO) as a search model. [25] The overall structure is similar to that of the closed structure of the P450 2B4 Phe429His mutant with 4-chlorophenyl imidazole (4-CPI) bound to the iron (4MGJ) [24] and other closed P450 2B4 structures with small molecules in the active site (1SUO, 4JLT, 2Q6N) and a substrate-free structure (3MVR). [25, 26] However, due to the lack of a compound in the active site, the acetate-bound structure is most similar

to the substrate-free wild type P450 2B4 (3MVR) with a root mean square difference of 0.78 Å for the 456 Ca atoms between the two structures. The crystallographic data collection and statistics are presented in Table 1.

One Cymal-5 is bound at the interface between the substrate binding site and the substrate access channel in a non-productive position with the C7 carbon of the cyclohexyl group ~7.4 Å away from the iron. Only one of the glucose moieties of the maltose group is visible; the second glucose moiety is presumably disordered and extends toward the molecular surface in a cleft above the first glucose moiety. Compared to the 4-CPI-bound structure (4MGJ), the substrate access channel is more rigid with the loops prior to the A-helix and the β 4-sheet at the C-terminus well defined. The second Cymal-5 molecule is observed outside of the substrate binding pocket where it inserts into a cleft between the helices F and G at the end near helix-E and is surrounded by helices-E, F, G, and I (Figure 1a).

Active site interactions of the acetate anion with the heme iron and side chain of the conserved Thr302

A key difference between our P450 2B4 structure and most other P450 structures is that an electron density similar in size and shape to acetate was bound as a weak sixth ligand (2.45 Å) to the iron using one of its oxygens (Figure 1b). Magnesium acetate was present in the crystallization solution. The size and shape of the electron density was not consistent with other possible small ligands in the crystallization solution such as water, glycerol, Cymal-5, dithiothreitol, and cacodylate.

The 4-chlorophenyl imidazole (4-CPI) bound Phe429His structure (4-MGJ) is remarkable because the histidine is a hydrogen bond donor to the axial heme thiolate. [24] As a result the mutant only retains ~5% of the wild type activity because the electron donating (“push”) effect of the iron-thiolate bond has been weakened and the trans-ligand bond has been strengthened, suggesting that acetate might bind tighter to the mutant than the wild type protein. [27,28] We do not believe the mutation significantly altered our results because a carboxylate oxygen of malonate is bound to the iron (2.2 Å) of P450_{cin} (CYP 176A1, 3BDZ) which has the normal thiolate-iron interaction. (Figure 1c) [29] The acetate ligand was an intriguing serendipitous observation that we determined warranted closer examination. Acetate has also been observed as an axial ligand in myoglobin (3SDN, 2.38 Å) and hemoglobins (2BKM 2.08 and 2.28 Å) (3S1J 1.95–2.08 Å) which have histidine as the proximal ligand. Although carboxylate anions are weak heme ligands, they are nonetheless observed when the concentration of organic anions in the crystallization buffer is high and with P450 when the substrate is absent. In substrate-free P450_{cam} the iron-water bond varied from 2.11 Å (4JX1) to 2.25 Å (3L62) while earlier studies by Poulos and coworkers reported an iron-water bond length of 2.28 Å. [30] A glycerol-iron bond length of 2.48 Å (3W93) was recorded by Ubbink and coworkers. [21] These iron-oxygen bond lengths with water, glycerol, and carboxylate are considerably longer than the 1.81 Å iron-dioxygen (2A1M) bond, presumably because they lack the ability to back bond to the iron d orbitals. Within experimental error our observation is consistent with an acetate-iron bond of 2.45 Å.

In the acetate structure the I-helix is closer to the iron by ~ 0.5 Å, occupying space formerly occupied by 4-CPI (1SUO). The side chain of Thr302 forms a hydrogen bond (2.7 Å) with the distal non-heme-bound oxygen of the acetate instead of a nitrogen of 4-CPI as in 1SUO and 4MGJ. In oxyferrous P450_{cam} (1DZ8, 2A1M), the side chain of the corresponding threonine is found in a similar position and points toward the distal oxygen. (Figure 1b and 1c) This similarity is consistent with the idea that acetate is serving as a mimic of dioxygen. Figure 1c shows a superposition of our acetate-bound structure and oxyferrous P450_{cam} (2A1M). Iron-proximal oxygen distances are 2.45 Å in the acetate structure versus 1.8 Å for P450_{cam}. The hydrogen bond lengths between the distal oxygen and threonine side chain are similar at 2.7 Å (acetate-bound) and 2.8 Å oxyferrous P450_{cam} (2A1M). Note that the conserved threonine backbones overlay well with only 0.6 Å difference between their α carbons in spite of the fact that one is substrate-free, and the other substrate-bound (2A1M). To hydrogen bond to the acetate oxygen the threonine side chain merely underwent a low-energy rotation ($\sim 109^\circ$) around the C_α - C_β carbon bond to accommodate the bulkier nonphysiological ligand. As shown in Figure 1c, the distal oxygens of acetate and dioxygen are separated by 2.4 Å, and the heme iron-distal oxygen distances are 4.35 Å, and 2.82 Å, respectively.

Hydrogen bonds and electrostatic connectivities in the active site

In the previous paragraphs we showed that the oxygens of acetate interact with the heme iron and the side chain of Thr252 (Figures 1b and 1c). This section describes the interactions of two likely critical active site water molecules. When the active site portion of the I-helix is distorted, the side chain of the conserved threonine often hydrogen bonds to the carbonyl of the i-4 residue upstream (A298), thereby obstructing solvent access to the distorted portion of the I-helix. Upon binding of a sixth heme ligand such as dioxygen or acetate with a negative charge on its distal atom the threonine side chain rotates away from the carbonyl of the i-4 residue and hydrogen bonds instead to the distal atom of the sixth ligand. A widening of the I-helix between the threonine and i-4 residue creates a space that enables two waters (Water107, Water23) in the acetate-bound enzyme and (Water1846, Water1842) in oxyferrous P450_{cam} (2A1M) to enter the active site and form robust hydrogen bonds to backbone atoms in and adjacent to the active site P450 motif (AGXET). (Figure 1b) The backbone carbonyl of the acidic residue is pointing away from the active site while the amide of the conserved threonine points into the active site creating an electrostatic environment conducive to formation of a hydrogen bonding network.

One water molecule, Water107, forms four strong interactions in the active site (Figure 1b). Its hydrogens serve as hydrogen bond donors to the carbonyl of Ala298 and the oxygen of Water23 and as hydrogen bond acceptors from the amide and side chain hydroxyl of Thr303. Water23 also forms four strong hydrogen bonds. It is a hydrogen bond donor to the carbonyls of Phe297 and Ala298, but a hydrogen bond acceptor from Water107 and the amide of Thr302. With minimal reconfiguration, a dynamic Water23 could form a hydrogen bond with the side chain of Thr302, 3.2 Å away or interact with the distal oxygen of acetate (3.4 Å). Two waters are also found in corresponding positions in the dilated I-helix in the wild type substrate-free P450 2B4 (3MVR) and paroxetine bound (4JLT) wild type P450

2B4. Both of these structures have the axial ligand binding to the side chain of the conserved threonine.

As shown in Figures 1b and 1c, the structure of the hydrogen bond network in the active site and I-helix of our structure are modest variants of the network observed in oxyferrous P450_{cam} (2A1M) and cyanide-bound P450_{cam} (1O76) despite only a ~17% amino acid identity between the two enzymes. [31] This implies that the pattern of oxygen activation is similar in the promiscuous microsomal and specific bacterial P450s. [8,12,32] Presumably the more flexible microsomal structure is at least partly responsible for the ability of acetate to mimic oxygen binding and hydrogen-bond formation. How the two protons required for catalysis navigate the active site hydrogen bond network to reach the distal oxygen of the peroxo (Fe^{+3}OO)⁻² and hydroperoxo (Fe^{+3}OOH)⁻ intermediates is discussed later. Note that Water23 in P450 2B4 is 3.4 Å and Water1846 in P450_{cam} is 3.2 Å away from the distal oxygen of acetate and oxygen, respectively, and that a series of minimal conformational changes in a highly nucleophilic and dynamic active site protonated hydrogen bond network could provide the essential protons to the activated oxygen intermediates. Analysis of the active site structure illustrates an ingenious multifunctional design with the potential for robust but interchangeable hydrogen bonding structures among water, backbone atoms, the side chain of the conserved threonine, ultimately resulting in compound I formation.

Water chain from the active site backbone carbonyl of Gly299 to the carboxyl of Glu445 in the proximal surface L-helix

In view of the unanticipated striking parallel between the active site hydrogen-bonded waters of a ferric microsomal P450 and oxyferrous P450_{cam}, we felt it would be instructive to explore whether water chains observed in a microsomal P450 might have a counterpart in P450_{cam}. More waters with access to the active site would help explain why microsomal P450 catalysis was usually uncoupled. In addition to the tightly-bound water in the active site, a buried water chain was observed extending from the carbonyl of the active site Gly299 to the protonated carboxyl of the buried Glu445 which is in the L-helix on the proximal surface (Figure 1d). Water53 mediates a hydrogen bond between the carbonyls of Gly299 and Thr175 (helix-E) and the presumably protonated hydrogen of the buried, carboxyl of Glu445. The pK_a of the carboxyl of Glu445, which is located in a hydrophobic environment, was predicted to be 9 ± 1 using PROPKA, version 3.1. [33] An alignment of 53 P450 sequences found a corresponding glutamic acid in 42 of the sequences. [34] A water chain equivalent to the one in the acetate-bound mutant has also been observed in the open ferric substrate-free (3L61) and closed camphor-bound (1DZ8) forms of P450_{cam}, the open (1PO5) and closed forms (1SUO and 3MVR) of the wild type P450 2B4. [25,35]

Mutation of the conserved, homologous Glu366 in P450_{cam} to either methionine or glutamine only moderately decreased the activity of the P450_{cam} mutant protein indicating Glu366 was not essential for catalysis, whereas the conserved active-site acidic residue, Asp251, is critical for catalysis. [8,35,36] Currently the exact function of this conserved water chain remains elusive. One possibility suggested by Poulos is that this internal water channel anchored by Glu445 is functioning to stabilize an energetically strained disrupted α -helix conformation that is critical for successful catalysis. [37]

The conserved flexible glutamic acid 301 may shuttle protons from the solvent into the active site

To better understand how the active site hydrogen-bond network acquires protons to shuttle to the activated oxygen buried within the protein, the conformation and environment of the active site acidic residue of P450 2B4 has been examined. Since the dianionic peroxo ($\text{Fe}^{+3}\text{OO}^-$) intermediate accumulates in the P450_{cam} Asp251Asn mutant, it was concluded that the active site acidic residue functioned to transport a proton from solvent to the hydrogen bond network [10,11,13,14,23,36]. In this work we explored the likelihood that Glu301 in microsomal P450 2B4 functioned in the same manner as the acidic residue in P450_{cam}. The 22 currently (as of February 2018) available P450 2B4 structures in the Protein Data Bank, and our acetate-bound structure, demonstrate that the Glu301 side chain is very flexible and capable of alternating between the molecular surface and the active site. In the P450 2B4 1SUO structure Glu301 rotates into the active site and forms a hydrogen bond with the nitrogen of the inhibitor 4-CPI. This places one of the Glu301 side chain carboxylate oxygens in the active site in a position capable of hydrogen bonding to the conserved active site Water23 in our current structure. As mentioned previously, Water 23 is 3.4 Å from the distal acetate oxygen (Figure 1b). At the protein surface the Glu301 side chain in the acetate-bound mutant establishes hydrogen bonds with Water112, the side chains of Thr305 in the I-helix and His172 (E-helix) either directly or through water molecules 32, 144, 245 (Figure 2). A similar network connects the Glu301 side chain to the surface in substrate-free 3MVR. The calculated pK_a [33] of Glu301 in our structure is 5 consistent with its hydrophilic environment.

In the summary we speculate how the conserved acid residue transports a proton from the molecular surface to the active site water chain. Although the precise structure of the proton relay machinery remains to be elucidated, there are attractive prospects for a variety of hydrogen bonds and structural changes with only minor, low-energy rearrangements of the protein, substrates, water and oxygen, and its reactive intermediates. Undoubtedly, this structural versatility is a major factor in the abundance and prominence of P450s throughout biology.

In contrast to the highly flexible Glu301 in P450 2B4, Asp251 in the closed crystal structures of P450_{cam} maintains unique salt-bridges with the F-helix residues Arg186 and Lys178 (1DZ8). Thus it is puzzling how protons are introduced into the active site during catalysis if the side chain of the conserved acid residues is locked in salt bridges with Lys176 and Arg186. Recent crystal structures of substrate-free open conformations of P450_{cam} (3L61 and 3L62) [38] and the substrate-free and bound P450_{cam}-putidaredoxin (Pdx) complex (4JX1, 4JWS, 4JWU, 3W9C) [20,21] suggested that catalysis occurred in the open form of P450_{cam} because in the open conformation the salt bridges have been ruptured, and the Asp251 side chain is free to alternate between the solvent on the surface of the protein and active site and participate in a proton relay network. Although the hypothesis that catalysis in P450_{cam} occurs in the open structure is intriguing, it remains controversial. [14,22]

Ancillary water chains leading from or near the active site to the protein surface and solvent do not have a counterpart in P450_{cam}

The structure of acetate bound P450 2B4 was also analyzed for other possible water chains that might connect the active site and solvent and serve as a backup proton delivery pathway or some other unknown function. In addition to hydrogen bonding to the distal oxygen of acetate the side chain of Thr302 initiates a water chain to solvent by bonding to Water 140 which links to Water 165 which links to side chain of Thr305 which links to Water 144 which links to Water 32 (Figure 2). This water chain should only exist when the Thr302 side chain is hydrogen bonded to an iron ligand and close to the surface. When the Thr302 side chain forms a hydrogen bond with the carbonyl of the conserved Ala298, its side chain becomes buried, and the water chain will be disrupted. Substrate-free wild-type P450 2B4 structures (3MVR) and 4-CPI bound (1SUO) have an analogous water chain between the Thr302 and Thr305 side chains that could modulate water entering and exiting the active site, perhaps in coordination with the ingress and egress of substrate. The structures of oxyferrous P450_{cam} (1DZ8, 2A1M) do not have a comparable hydrogen bond interaction between the side chains of Thr252 and the side chain of the i+3 residue Asn255. It should be noted that the side chains of Thr302 and Glu301 are close to the protein surface and likely transiently exposed to bulk solvent by thermal fluctuations of the protein.

An additional robust water chain links the carbonyl of Thr302 via the side chain of Thr306 with the surface residue, Asp361, located at the C-terminus of the K-helix near the proximal surface (Figure 2). Asp361 is the third residue after the highly conserved EXXR motif near the proximal surface. The water chain consists of four waters (98, 122, 121, 99) which are conserved in nearly all P450 2B4 structures as well as P450 2D6 (3TBG) and P450 2C9 (1OG5) but are not found in P450_{cam}. In addition to the residues anchoring the termini of the water chain (Thr302, Thr306, and Asp361), the waters also hydrogen bond with Glu357, Leu362; Ile363 and Pro429. Residues 360GDLIPFGV367 constitute substrate recognition site 5. Hence, these waters may function in the solvation and desolvation of the active site.

Figure 3 provides an overview of the interrelationship between the observed water chains in acetate-bound P450 2B4. Although these ancillary water chains external to the active site may not be critical to ordinary catalysis they may 1) help ensure the integrity and function of the proton delivery network, orchestrated by the conserved acid and alcohol amino acids, and 2) accommodate water exchange between solvent and the active site during the substrates and products entering and exiting the active site. Alternatively, the extra water chains may be utilized when the normal proton shuttle mechanism has been compromised perhaps by an extraordinarily bulky and/or uniquely shaped substrate. Except for the interaction between the active site residue Gly299 and the water chain to the buried conserved glutamic acid, P450_{cam} has no counterpart to the water chains described herein. These ancillary water chains, which appear to provide water access to the active site, may contribute to the in vitro uncoupling of the utilization of NADPH-reducing equivalents for product formation in the frequently highly uncoupled microsomal P450s. Whether the observed water chains and uncoupling of microsomal P450 reactions also occur in vivo is unknown. It may be that in the endoplasmic reticular membrane in vivo there may be more structural restraints on the membrane-bound P450s, which would result in more highly

coupled catalysis. The water chains observed in vitro might be less prominent and perhaps even absent in vivo.

Experimental Section

Materials

Yeast extract, tryptone, and glycerol were purchased from Fisher Scientific. Sodium cholate of >99% purity was obtained from Anatrace. Isopropyl- β -D-thiogalactopyranoside (IPTG), carbenicillin (disodium salt) and δ -aminolevulinic acid were from Research Products International Corp. DE52 media (preswollen diethylaminoethyl cellulose) was from Whatman. Bio-Beads SM-2 and Hydroxyapatite resins were from Bio-Rad. Dithiothreitol, Tergitol NP-10, nonaethylene glycol monododecyl ether (Brij), Cymal-5, Reactive Red 120 type 300-CL, Octyl Sepharose CL-4B, 2,6-ditertbutyl-4-methylphenol butylated hydroxytoluene (BHT), Trizma base, and lysozyme were from Sigma. DNase and the protease inhibitor cocktail were purchased from Roche Diagnostics Corp. (Indianapolis). 50% poly-ethylene glycol (PEG) 3350 solution, 10% w/v of Anapoe- $C_{10}E_9$ solution, 1 M magnesium acetate solution, 1 M sodium cacodylate solution, and 1 M HCl solution were purchased from Hampton Research (California).

Construction of Truncated Cytochrome P450 2B4 Phe429His mutant

The plasmid for the truncated cytochrome P450 (P450) 2B4 mutant was constructed from pLW01-wild type P450 2B4 plasmid. [39] 19 amino acids at the N-terminus of the plasmid (amino acids 3–21 of wild type P450 2B4) were removed using NcoI restriction sites which had been inserted at position 3 and 21 by site directed mutagenesis. After removing the plasmid between the two NcoI sites, the remainder of the plasmid was religated by T4 DNA ligase (Invitrogen). The following mutations, E2A, G22K, H23K, P24T, K25S, A26S, H27K, R29K, H226Y and Phe429His were then introduced into the 3–21 wild type P450 2B4 using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions to generate the Phe429His mutant of the truncated form of P450. The truncated P450 2B4 (lacking residues 3–21, and with mutations E2A through H226Y as indicated above) is the form which has been previously crystallized by numerous other investigators. [25] There was no histidine tag at the C-terminus.

Protein expression

Truncated rabbit pLW01 CYP450 2B4 Phe429His plasmid was transformed to *E. coli* C41 (DE3) cells (Avidis S.A.) and plated onto a Luria Broth (LB) plate containing 0.24 mM carbenicillin. Three colonies were used to inoculate 200 mL Terrific Broth (TB) medium containing 0.24 mM of carbenicillin. The medium was incubated at 30°C with shaking at 200 rpm until the cell density reached an OD at 600 nm of ~5. 10 mL of the overnight cell culture were transferred to 1 L TB medium containing 0.24 mM carbenicillin. The flasks were incubated at 21–23°C with shaking at 110 rpm until the cell density reached an OD at 600 nm of ~5. Isopropyl β -D-1 thiogalactopyranoside (IPTG) and δ -aminolevulinic acid were added to a final concentration of 0.5 mM. Incubation was continued at 20°C with shaking at 100 rpm. Thirty minutes after induction with IPTG, ethanol was added to the medium to a final concentration of 3% (v/v). The cell culture was harvested ~120 h after

induction with IPTG and an average yield of P450 was approximately 1000 nM measured using a CO-difference spectrum. [40]

Purification of Truncated Phe429His: Overview

The cell pellet was prepared by sonication, solubilization, and ultracentrifugation before loading to a series of columns including DE52, Reactive Red, Octyl Sepharose, and hydroxyapatite as previously described. [41] Since the Phe429His mutant protein is more susceptible to conversion to the inactive form, P420, butylated hydroxyl toluene (BHT) was added to all buffers (to scavenge free radicals formed by the ether containing detergent) to prevent conversion to P420 during purification. The protein was eluted from the hydroxyapatite column using buffer containing 0.4 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4 and 20% glycerol. The pure protein was diluted into a solution with a final concentration of 20% glycerol and 0.1 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4 and concentrated to ~1 mM using 50,000 MWCO VivaSpin 20. The concentrated protein was immediately used for crystallization experiments without prior freezing. Unused protein was frozen in liquid N_2 and stored at -80°C . Subsequent experiments confirmed that crystals could be successfully obtained using protein that had been frozen. The defrosted sample was refiltered prior to crystallization.

Specifics

Sonication—Unless otherwise stated all procedures were conducted at 4°C . Cell pellets from 2 L of cell culture were resuspended in 200 mL buffer B containing 15% glycerol, 10 mM Tris acetate, pH 7.7 at 4°C , 1 mM EDTA, 40 mg lysozyme, 1 mg DNase, 0.1 mg/ml dithiothreitol (DTT), 2% (v/v) Brij (Nonaethylene glycol monododecyl ether), 30 μM BHT (butylated hydroxytoluene), and two tablets of protease inhibitor cocktail. The cell suspension was then sonicated on ice for 1 min on full power continuous mode for six cycles with a 20 min incubation period on ice between each cycle to minimize the heat damage to the protein.

DE52 column—The sonicated sample from 2 L of cell culture was then solubilized by adding 1 g of sodium cholate and stirred at 4°C for 3 h. The sample was then centrifuged at 35,000 rpm (147,600 g) for 45 min at 4°C . The supernatant was collected and directly loaded onto a 200 mL DE52 column (5 cm $\times$ 10 cm) pre-equilibrated with buffer C containing 20% glycerol, 20 mM Tris acetate, pH 7.7 at 4°C , 0.1 mM EDTA, 0.3% Tergitol, and 30 μM of BHT. The P450 did not bind to the DE52 column. The eluent was collected, flash frozen in liquid nitrogen, and stored at -80°C . The DE52 column was typically used with material from 2 L of cell culture while the Reactive Red column could conveniently process protein from two DE52 columns, that is, from 4 L of cell culture.

Reactive Red column—The P450 containing DE52 eluent from 4 L of cell culture was thawed and loaded onto a 70 mL Reactive Red column (2.5 cm $\times$ 14 cm) pre-equilibrated with buffer D containing 20% glycerol, 20 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4 0.1 mM EDTA, 10 μM BHT. P450 bound to the top of the Reactive Red column. The column was then washed with 300 mL of buffer D and eluted at a rate of 0.5 mL/min with buffer E containing 20% glycerol, 100 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.7, 1 M NaCl, 0.1 mM EDTA, 30 μM BHT, and 0.3% (v/v) Tergitol NP-10.

Biobeads and Octyl Sepharose column—Fractions from the Reactive Red column containing P450 were pooled and loaded onto an 80 mL Biobeads column (2.5 cm × 16 cm) to decrease the Tergitol concentration. The eluent from the Biobeads column flowed directly onto a 60 mL Octyl Sepharose column (2.5 cm × 12 cm) pre-equilibrated with buffer F containing 20% glycerol, 200 mM K₂HPO₄/KH₂PO₄, pH 7.4, 0.1 mM EDTA, 0.1% (w/v) sodium cholate, and 10 μM BHT. The columns were washed with buffer F until P450 was entirely loaded onto the Octyl Sepharose column. The P450 was eluted from the Octyl Sepharose column at a rate of 0.5 mL/min overnight with buffer G containing 20% glycerol, 10 mM K₂HPO₄/KH₂PO₄, pH 7.7, 0.1 % (v/v) Tergitol NP-10, and 10 μM BHT.

Hydroxyapatite column—The P450 containing fractions from the Octyl Sepharose column were diluted with 4× volume of buffer H containing 20% glycerol and 10 mM K₂HPO₄/KH₂PO₄, pH 7.2 and loaded onto a 30 mL hydroxyapatite column (2.5 cm × 6 cm). P450 bound tightly to the top of the hydroxyapatite column. Detergent was removed by washing the column with 500 mL of buffer H until the absorbance of the eluent at 280 nm was less than 0.005 OD. The P450 was then eluted at 0.5 mL/min with buffer I containing 20% glycerol and 400 mM K₂HPO₄/KH₂PO₄, pH 7.4.

The P450 containing protein solution was diluted 3-fold to achieve a final buffer containing 20% glycerol and 100 mM K₂HPO₄/KH₂PO₄, pH 7.4. The P450 was concentrated by centrifugation at 2900 rpm (1683 g) to ~1 mM using VivaSpin 20 with 50,000 MWCO (Sartorius Stedim Biotech). The concentrated protein was resuspended in a solution with a final concentration of ~1 mM protein, 20% glycerol, 100 mM K₂HPO₄/KH₂PO₄, 1.5 mM EDTA, 4.8 mM Cymal-5, and 0.2 mM dithiothreitol. BHT was present in the protein containing solution at a final concentration of 10 mM. The protein containing solution was incubated for 2 h on ice and then filtered by centrifugation at 13,000 rpm (17,900 g) for 1 min using a 0.45 μm Ultrafree Centrifugal Filter unit (Millipore, Co., Billerica, MA) to remove protein aggregates.

Crystallization, Data Collection, Structure Determination, and Structure refinement

Crystals of truncated P450 Phe429His were obtained using either the hanging or the sitting drop vapor diffusion method by mixing 2 μL of the previously described protein solution, 2 μL of the reservoir solution containing 0.2 M magnesium acetate, 0.1 M cacodylate, pH 6.5, 10–15% PEG 3350. To a drop of 4 μL of protein and reservoir solution, 0.4 μL of 10% (w/v) Anapoe-C₁₀E₉ was added to a final concentration of 1% (w/v). The crystallization trays were both set up and incubated at 4°C. The crystals were transferred to a cryoprotectant containing the reservoir solution and increasing concentrations of PEG 3350 up to 25% and glycerol up to 25% (while maintaining the magnesium acetate and cacodylate concentration of the reservoir solution) in ~ 30 incremental steps over the course of ~ 1 h, flash frozen, and stored in liquid nitrogen prior to data collection.

A native data set of Phe429His/Cymal-5 was collected at the Advanced Photon Source beamline 21-ID-D with a Mar 300 CCD detector to resolution of 2.1 Å in space group *P*3₂21 with cell parameters: a=b=93.185 Å, c=151.092 Å. The structure was determined by molecular replacement using 1SUO [25] as a search model. There is one molecule in

asymmetric unit. The model was refined to R_{work} 18.52% and R_{free} 20.56%. The model contains residues 28–135 and 137–491 of the truncated Phe429His. Residues 20–27 and Gly136 were not modeled as a result of poor electron densities in these regions.

The data set was processed using HKL2000 0.98.703g. [42] The structure was determined using the molecular replacement program Phaser 2.1.4 [43] in CCP4 6.1.13 suite. [44] The models were refined using AutoBuster [45] and manually rebuilt in COOT 0.6.2 [46] and O 10.0.3. [47] The ligand CIF files for heme, Cymal-5, EDTA, and acetate ion were generated using GRADE 1.1.1. [48] Structure validation was carried out in MolProbity 3.17. [49] The structure was scored at the 100% percentile among structures of similar resolution.

Summary

In spite of significant variability between the high resolution structures of the soluble P450s (camphor, eryF and cineole), and the lower resolution structures of the active sites of membrane-bound microsomal P450s, a general picture nonetheless emerges that indicates that enzymes with the conserved acid/alcohol pair and P450 motif (A/G, GXD/ET) employ a similar hydrogen-bonded water and amino acid network to accomplish dioxygen activation and catalysis. In microsomal P450s the typical resting state will have water occupying the active site, and the flexible conserved active site P450 motif (A/G GX D/ET) largely existing as an intact I-helix, with hydrogen bonds between the backbone atoms of the i to $i+4$ residues.

Water is displaced when substrate or inhibitor binds and, depending on the bulk of the incoming molecule, the center of the I-helix including the P450 motif is “pushed away” from the iron by ~ 1 Å to provide space for binding in an enzymatically productive conformation. At this point some P450s may retain the intact I-helix (P450_{cam}) while others, particularly the microsomal P450s may have already ruptured the regular I-helix. The breaking of the hydrogen bonds between the backbone (of typically 5) residues in the P450 motif exposes backbone amides and carbonyls which hydrogen bond with waters, thereby forming an active site relay network anchored by amino acid backbone atoms. There are also structures of microsomal enzymes that suggest that even without substrate the I-helix may exist in equilibrium between an intact and disrupted I-helix and a closed and open structure, respectively. Substrate binding triggers reduction of the heme and binding of oxygen. If the I-helix has not yet been disrupted, oxygen binding is expected to locally unwind the helix which forms a hydrogen bond network (Figure 1b) that incorporates hydrogen bonds between the superoxide-like distal oxygen (not bound to the iron) and conserved threonine side chain [8] and interactions between the newly exposed peptide backbone atoms and waters. One of the hydrogen-bonded waters is close to the distal oxygen atom (in oxyferrous P450_{cam} 3.4 Å). In spite of the increased distance, ~ 1 Å, between the distal acetate oxygen and the heme iron, the distal acetate oxygen forms hydrogen bonds, corresponding to those observed with dioxygen, with a close water (3.4 Å) and the Thr302 side chain (2.7 Å).

It is generally considered that the function of the conserved acidic side chain is to transport a proton from bulk solvent at the molecular surface to the active-site hydrogen-bonded work. The question is how does an unprotonated side chain on the molecular surface become

protonated and transfer a proton to the active site hydrogen-bonded shuttle machinery. Elucidation of the mechanism of proton-coupled electron transfer in *Azotobacter vinelandii* ferredoxin suggests a plausible mechanism. [50]

We speculate that in a highly negative electrostatic environment, such as is produced by the peroxo dianion intermediate, the pKa of the acid side chain would transiently increase and thereby promote proton capture from water. Thermal micro-picosecond acidic side chain low-energy rotations would then transport the proton to the active site hydrogen-bond network where it will exist as either a Zundel (H_5O_2^+) and/or an Eigen (H_3O^+) cation. [51,52] Protons fluctuate $\sim 10^{13} \text{ s}^{-1}$ within a highly polarizable network and, due to collective proton motions, can apparently be rapidly transported over long distances. It should be noted that the proton delivered at one of the water chains is unlikely to be the proton released at the opposite end of the chain. The ~ 2 orders of magnitude greater polarizability of protonated hydrogen-bonded networks rather than electron systems makes them ideal candidates for long-distance proton-coupled electron transfer. [51] Theoretical studies support the plausibility of the proposed mechanism of proton transfer and oxygen activation. [50,53] Delivery of two protons to the distal oxygen results in heterolytic dioxygen bond cleavage, compound I formation, oxygenation of the substrate, and, finally, product dissociation. In summary comparison of the active site structures of an acetate-bound microsomal P450 to bacterial P450s leads to the conclusion that the mechanism of proton transport from bulk solvent to the peroxo and hydroperoxo intermediates are similar.

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Abbreviation

P450	cytochrome P450
P450_{cam}	cytochrome P450 camphor
4-CPI	4-(4-chloro-phenyl) imidazole
Cymal-5	5-cyclohexyl-1-pentyl- β -D-maltoside
WT	wild type
H-bond	Hydrogen bond

References

1. Bernhardt R. Rev Physiol Biochem Pharmacol. 1996; 127:137–221. DOI: 10.1007/BFb0048267 [PubMed: 8533008]

2. Poulos, TL., Johnson, EF. Cytochrome P450: Structure, Mechanism, and Biochemistry. Ortiz de Montellano, PR., editor. Kluwer Academic/Plenum Publishers; New York, NY: 2005. p. 87-114.
3. Baldwin WS, Marko PB, Nelson DR. BMC Genomics. 2009; 10:169.doi: 10.1186/1471-2164-10-169 [PubMed: 19383150]
4. Guengerich FP, Munro AW. J Biol Chem. 2013; 288:17065–17073. DOI: 10.1074/jbc.R113.462275 [PubMed: 23632016]
5. Shaik S, Cohen S, Wang Y, Chen H, Kumar D, Thiel W. Chem Rev. 2010; 110:949–1017. DOI: 10.1021/cr900121s [PubMed: 19813749]
6. Poulos TL. Chem Rev. 2014; 114:3919–3962. DOI: 10.1021/cr400415k [PubMed: 24400737]
7. Zhang H, Gruenke L, Arscott D, Shen A, Kasper C, Harris DL, Glavanovich M, Johnson R, Waskell L. Biochem. 2003; 42:11594–11603. DOI: 10.1021/bi034968u [PubMed: 14529269]
8. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG. Science. 2000; 287:1615–1622. DOI: 10.1126/science.287.5458.1615 [PubMed: 10698731]
9. Kimata Y, Shimada H, Hirose T, Ishimura Y. Biochem and Biophys Res Comm. 1995; 208:96–102. DOI: 10.1006/bbrc.1995.1310 [PubMed: 7887971]
10. Benson DE, Suslick KS, Sligar SG. Biochem. 1997; 36:5104–5107. DOI: 10.1021/bi963170q [PubMed: 9136869]
11. Davydov R, Makris TM, Kofman V, Werst DE, Sligar SG, Hoffman BM. J Am Chem Soc. 2001; 123:1403–1415. DOI: 10.1021/ja003583l [PubMed: 11456714]
12. Nagano S, Poulos TL. J Biol Chem. 2005; 280:31659–31663. DOI: 10.1074/jbc.M505261200 [PubMed: 15994329]
13. Batabyal D, Li H, Poulos TL. Biochem. 2013; 52:5396–5402. DOI: 10.1021/bi400676d [PubMed: 23865948]
14. Batabyal D, Richards LS, Poulos TL. J Am Chem Soc. 2017; 139:13193–13199. DOI: 10.1021/jacs.7b07656 [PubMed: 28823160]
15. Petrik ID, Davydov R, Ross M, Zhao X, Hoffman B, Lu Y. J Am Chem Soc. 2016; 138:1134–1137. DOI: 10.1021/jacs.5b12004 [PubMed: 26716352]
16. Martinis SA, Atkins WM, Stayton PS, Sligar SG. J Am Chem Soc. 1989; 111:9252–9253. DOI: 10.1021/ja00208a031
17. Zaragoza JPT, Yosca TH, Siegler MA, Moenne-Loccoz P, Green MT, Goldberg DP. J Am Chem Soc. 2017; 139:13640–13643. DOI: 10.1021/jacs.7b07979 [PubMed: 28930448]
18. Wade RC, Winn PJ, Schlichting I, Sudarko. J Inorg Biochem. 2004; 98:1175–1182. DOI: 10.1016/j.jinorgbio.2004.02.007 [PubMed: 15219983]
19. Poulos TL, Finzel BC, Howard AJ. J Mol Biol. 1987; 195:687–700. DOI: 10.1016/0022-2836(87)90190-2 [PubMed: 3656428]
20. Tripathi S, Li H, Poulos TL. Science. 2013; 340:1227–1230. DOI: 10.1126/science.1235797 [PubMed: 23744947]
21. Hiruma Y, Hass MA, Kikui Y, Liu WM, Olmez B, Skinner SP, Blok A, Kloosterman A, Koteishi H, Lohr F, Schwalbe H, Nojiri M, Ubbink M. J Mol Biol. 2013; 425:4353–4365. DOI: 10.1016/j.jmb.2013.07.006 [PubMed: 23856620]
22. Skinner SP, Liu WM, Hiruma Y, Timmer M, Blok A, Hass MA, Ubbink M. Proc Natl Acad Sci U S A. 2015; 112:9022–9027. DOI: 10.1073/pnas.1502351112 [PubMed: 26130807]
23. Taraphder S, Hummer G. J Am Chem Soc. 2003; 125:3931–3940. DOI: 10.1021/ja016860c [PubMed: 12656628]
24. Yang Y, Zhang H, Usharani D, Bu W, Im S, Tarasev M, Rwere F, Pearl NM, Meagher J, Sun C, Stuckey J, Shaik S, Waskell L. Biochem. 2014; 53:5080–5091. DOI: 10.1021/bi5003794 [PubMed: 25029089]
25. Scott EE, White MA, He YA, Johnson EF, Stout CD, Halpert JR. J Biol Chem. 2004; 279:27294–27301. DOI: 10.1074/jbc.M403349200 [PubMed: 15100217]
26. Wilderman PR, Shah MB, Liu T, Li S, Hsu S, Roberts AG, Goodlett DR, Zhang Q, Woods VL Jr, Stout CD, Halpert JR. J Biol Chem. 2010; 285:38602–38611. DOI: 10.1074/jbc.M110.180646 [PubMed: 20880847]

27. Davydov R, Im S, Shanmugam M, Gunderson WA, Pearl NM, Hoffman BM, Waskell L. *Biochem.* 2016; 55:869–883. DOI: 10.1021/acs.biochem.5b00744 [PubMed: 26750753]
28. Mak PJ, Yang Y, Im S, Waskell LA, Kincaid JR. *Angew Chem.* 2012; 51:10403–10407. DOI: 10.1002/anie.201205912 [PubMed: 22968976]
29. Meharena Y, Slessor KE, Cavaignac SM, Poulos TL, De Voss JJ. *J Biol Chem.* 2008; 283:10804–10812. DOI: 10.1074/jbc.M709722200 [PubMed: 18270198]
30. Poulos TL, Finzel BC, Howard AJ. *Biochem.* 1986; 25:5314–5322. DOI: 10.1021/bi00366a049 [PubMed: 3768350]
31. Fedorov R, Ghosh DK, Schlichting I. *Arch Biochem Biophys.* 2003; 409:25–31. DOI: 10.1016/S0003-9861(02)00555-6 [PubMed: 12464241]
32. Madrona Y, Tripathi S, Li H, Poulos TL. *Biochem.* 2012; 51:6623–6631. DOI: 10.1021/bi300666u [PubMed: 22775403]
33. Olsson MHM, Sondergaard CR, Rostkowski M, Jensen JH. *J Chem Theory Comput.* 2011; 7:525–537. DOI: 10.1021/ct100578z [PubMed: 26596171]
34. Nelson DR, Strobel HW. *Biochem.* 1989; 28:656–660. DOI: 10.1021/bi00428a036 [PubMed: 2713336]
35. Raag R, Poulos TL. *Biochem.* 1991; 30:2674–2684. DOI: 10.1021/bi00224a016 [PubMed: 2001355]
36. Vidakovic M, Sligar SG, Li H, Poulos TL. *Biochem.* 1998; 37:9211–9219. DOI: 10.1021/bi980189f [PubMed: 9649301]
37. Raag R, Martinis SA, Sligar SG, Poulos TL. *Biochem.* 1991; 30:11420–11429. DOI: 10.1021/bi00112a008 [PubMed: 1742281]
38. Lee YT, Wilson RF, Rupniewski I, Goodin DB. *Biochem.* 2010; 49:3412–3419. DOI: 10.1021/bi100183g [PubMed: 20297780]
39. Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, Waskell L. *J Biol Chem.* 1998; 273:17036–17049. DOI: 10.1074/jbc.273.27.17036 [PubMed: 9642268]
40. Omura, T., Sato, R. *Methods Enzymol.* Estabrook, RW., Pullman, ME., editors. Vol. 10. Academic Press; 1967. p. 556–561.
41. Saribas AS, Gruenke L, Waskell L. *Protein Expr Purif.* 2001; 21:303–309. DOI: 10.1006/prep.2000.1377 [PubMed: 11237692]
42. Otwinowski, Z., Minor, W. *Macromolecular Crystallography, Part A.* In: Carter, CWJ., Sweet, RM., editors. *Methods Enzymol.* Vol. 276. Academic Press; New York: 1997. p. 307–326.
43. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. *J Appl Crystallogr.* 2007; 40:658–674. DOI: 10.1107/S0021889807021206 [PubMed: 19461840]
44. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AG, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS. *Acta Crystallogr D Biol Crystallogr.* 2011; 67:235–242. DOI: 10.1107/S0907444910045749 [PubMed: 21460441]
45. Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Roversi, P., Sharff, A., Smart, OS., Vornrhein, C., Womack, TO. *BUSTER version 2.10.0.* Global Phasing Ltd; Cambridge, United Kingdom: 2011. <https://www.globalphasing.com/buster/>
46. Emsley P, Lohkamp B, Scott WG, Cowtan K. *Acta Crystallogr D Biol Crystallogr.* 2010; 66:486–501. DOI: 10.1107/S0907444910007493 [PubMed: 20383002]
47. Kleywegt GJ. *Methods Mol Biol.* 2007; 364:255–272. DOI: 10.1385/1-59745-266-1:255 [PubMed: 17172770]
48. Smart, OS., Womack, TO., Sharff, A., Flensburg, C., Keller, P., Paciorek, W., Vornrhein, C., Bricogne, G. *Grade 1.1.1.* Global Phasing Limited (GPhL); Cambridge, United Kingdom: 2011. <http://www.globalphasing.com>
49. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB 3rd, Snoeyink J, Richardson JS, Richardson DC. *Nucleic Acids Res.* 2007; 35:W375–383. DOI: 10.1093/nar/gkm216 [PubMed: 17452350]
50. Chen K, Hirst J, Camba R, Bonagura CA, Stout CD, Burgess BK, Armstrong FA. *Nature.* 2000; 405:814–817. DOI: 10.1038/35015610 [PubMed: 10866206]

51. Zundel G. *J Mol Struct.* 1994; 322:33–42. DOI: 10.1016/0022-2860(94)87019-5
52. Xantheas SS. *Science.* 2016; 354:1101.doi: 10.1126/science.aal1413 [PubMed: 27934720]
53. Harris DL. *J Inorg Biochem.* 2002; 91:568–585. DOI: 10.1016/s0162-0134(02)00477-4 [PubMed: 12237223]

Highlights

- Crystal structure of cytochrome P450 2B4 in complex with acetate
- Two water molecules insert in the I-helix of the cytochrome P450 2B4 mutant active site
- In cytochrome P450 2B4 acetate binds to the heme and side chain of Thr302
- The acetate bound active site of ferric cytochrome P450 2B4 resembles that of the oxygen bound P450_{cam}

Figure 1.

Figure 1a. The overall structure of acetate bound P450 2B4 in a closed conformation.

An acetate, shown in cyan, is bound to the iron as the axial ligand. One molecule of Cymal-5, shown in green, is bound in a non-productive position in the substrate binding pocket of P450 2B4 with the C7 carbon of the cyclohexyl group ~ 7.4 Å away from the iron. The second Cymal-5 is located outside of the substrate binding pocket and in a depression formed by helices E, F and G.

Figure 1b. Comparison of active site water molecules in ferric P450 2B4 and oxyferric P450 camphor. Note the acetate bound to the iron in P450 2B4 where oxygen is bound in P450cam (2A1M). A sigmaA-weighted $mF_o - DF_c$ map contoured at 3σ is shown for the acetate. The map was calculated after extensive refinements with acetate removed from the model. It provides strong evidence that the acetate is indeed bound to the heme. Thr302 forms a H-bond of 2.7 Å with the distal oxygen of the acetate, similarly in P450cam, Thr252

forms a H-bond of 2.8 Å with the distal oxygen. Two water molecules 23 and 107 insert into the I-helix between residues Phe297 and Thr303. The corresponding waters in oxyferrous P450cam are 1846 and 1842. Water 1846 in P450cam ($\text{H}_2\text{O}-\text{O}_2$ 3.2 Å) has the potential to protonate the distal oxygen of the peroxo state of P450cam. The inset presents a better view for the acetate electron density.

Figure 1c. Superposition of the active sites of P450 2B4 with those of oxyferrous P450cam (2A1M) and ferric P450cin (3BDZ). All the superpositions were performed by superimposing the non-H atoms of the heme.

Left This figure shows key bond lengths between the heme, the sixth ligand and the conserved threonine. Note the different threonine rotamers utilized to form H-bonds with the distal oxygen of the acetate and the distal oxygen.

Right Superposition of the acetate bound P450 2B4 and the malonate bound P450cin N242A (3BDZ). Only one of the two disordered malonate positions is shown. The distal malonate oxygen points in a different direction than the acetate since the corresponding threonine is lacking. The proximal malonate oxygen–iron bond is 2.2 Å compared to 2.45 Å in the acetate bound P450 2B4.

Figure 1d. Hydrogen bond network between the carbonyl of Gly299 and the buried side chain of Glu445. The carbonyl of Gly299 is hydrogen bonded to Water 53 (cyan) which is hydrogen-bonded to the protonated and buried side chain of the conserved Glu445. For clarity, some residues and side chains on the I-helix are omitted.

Figure 2. Illustration of the two water chains connecting acetate to solvent and also the solvent connectivity of Glu301 on the molecular surface

Intriguingly, the iron bound acetate participates via the side chain of Thr302, Water 140, and Water 165 (in green), the side chain of Thr305 and Water 32, 144, and 245 (in red) in a hydrogen bonding network to solvent. A similar water chain is observed in wild type P450 2B4 (3MVR).

A second conserved hydrogen bond network (magenta) is shown connecting Thr306 near the active site to a surface residue Asp361. The first hydrogen bond of the network is the side chain of Thr306, which is a hydrogen bond acceptor from water 98 and hydrogen bond donor to the carbonyl of Thr302. The side chain of Thr306 hydrogen bonds to Water 98, then Water 122, 121 and 99 which interacts with the side chain of Asp361 on the surface. The carbonyl oxygen of Pro428 which forms a hydrogen bond with Water 121 is represented as a grey sphere with the rest of the residue omitted for clarity. The proximity and interactions of these waters adjacent to the active site and the uncoupling of catalysis in mutants lacking the threonine suggests that waters from these chains likely gain access to the active site. Also illustrated are the interactions of the side chain of Glu301 on the molecular surface include hydrogen bonds with water 112, the ϵ nitrogen of His172, and side chain of Thr305.

Figure 3. Demonstration of the electron densities on the waters of the four networks in and near the active site of P450 2B4

1) The active site water molecules 23 and 107 are shown in yellow. 2) Intriguingly the side chain of Thr302 (green sticks) is bound not only to the acetate but also initiates a hydrogen bonding network to waters 140 and 165 (green) on the protein surface. Water 165 also links to the Thr305 side chain (grey lines) which links to both solvent Water 32 and the side chain of Glu301. 3) The carbonyl of Thr302 (green sticks) hydrogen bonds to the side chain of Thr306 (grey lines) which proceeds via Water 98, 122, 121, and 99 (magenta) to the surface side chain of Asp361 (pink sticks). 4) The carbonyl of Gly299 (yellow sticks) binds Water 53 (cyan) which hydrogen bonds to the buried protonated side chain of Glu445 (L-helix) and the carbonyl of Thr175. For clarity, only iron and part of the heme is shown. Side chains of Phe297, Leu362, and Ile363 are also omitted. Not all residues on the I-helix are shown. The acetate is shown in cyan. The $2mF_o-DF_c$ map, contoured at 1σ , is displayed for the water molecules.

Table 1

Crystallographic data collection and refinement statistics

Data Reduction	Phe429His/Cymal-5
PDB code	6BWW
beamline	APS ID21-D
Wavelength, Å	0.97852
Space group	P3 ₁ 21
Cell a, b, c, Å	93.185*93.185*151.092
Cell α , β , γ , °	90*90*120
Resolution range, Å	80.7–2.1
No. of unique reflections	44827
Completeness (high ¹), %	99.6 (99.5)
Redundancy (high)	6.2 (6.3)
I/ σ (high)	32.3 (6.0)
R _{merge} (high), %	0.062 (0.41)
Structure Refinement	
Resolution range, Å	46.6–2.1
No. Reflections (high ²)	44799(3231)
Reflections in test set (high), %	4477 (319)
R _{work} /R _{free} (high) (%)	18.52/20.56(20.26/24.18)
No. of molecules/asymmetric unit	1
No. protein atoms	7519
No. hetero atoms	654
No. Solvent atoms	312
Avg. B factor, Å ²	
protein	39.19
heme	22.50
acetate that binds the iron	47.95
Cymal5 near the active site	103.48
Cymal5 outside the active site	47.86
waters	47.55
Ramachandran Plot (%)	
Preferred	97.6
Allowed	2.4
Outliers	0.0
RMSD bond length, Å	0.01
RMSD bond angles, °	0.97

¹ Highest resolution shell in the data reduction is from 2.14–2.10 Å² Highest resolution shell in the structure refinement is from 2.15–2.10 Å