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The crystal structure of CYP24A1, a mitochondrial cytochrome P450 involved in vitamin D metabolism

Andrew J. Annalora[*], **David B. Goodin**[*], **Wen-Xu Hong**[*], **Qinghai Zhang**[*], **Eric F. Johnson**[‡], and **C. David Stout**[*]

Andrew J. Annalora: annalora@scripps.edu; David B. Goodin: ; Wen-Xu Hong: ; Qinghai Zhang: ; Eric F. Johnson: ; C. David Stout: dave@scripps.edu

[*]Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

[‡]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037

Abstract

Cytochrome P450 (CYP) 24A1 catalyzes the side-chain oxidation of the hormonal form of vitamin D. Expression of CYP24A1 is up-regulated to attenuate vitamin-D signaling associated with calcium homeostasis and cellular growth processes. The development of therapeutics for disorders linked to vitamin D-insufficiency would be greatly facilitated by structural knowledge of CYP24A1. Here we report the crystal structure of rat CYP24A1 at 2.5 Å resolution. The structure exhibits an open cleft leading to the active site heme prosthetic group on the distal surface that is likely to define the path of substrate access into the active site. The entrance to the cleft is flanked by conserved hydrophobic residues on helices A′ and G′ suggesting a mode of insertion into the inner mitochondrial membrane. A docking model for 1α,25-$(OH)_2D_3$ binding in the open form of CYP24A1 is proposed that clarifies the structural determinants of secosteroid recognition and validates the predictive power of existing homology models of CYP24A1. Analysis of CYP24A1's proximal surface identifies the determinants of adrenodoxin recognition as a constellation of conserved residues from helices K, K″ and L that converge with an adjacent lysine-rich loop for binding the redox protein. Overall, the CYP24A1 structure provides the first template for understanding membrane insertion, substrate binding, and redox partner interaction in mitochondrial P450s.

Keywords

cytochrome P450; mitochondria; monotopic membrane protein; vitamin D metabolism; adrenodoxin

Correspondence to: Andrew J. Annalora, annalora@scripps.edu; C. David Stout, dave@scripps.edu.

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Introduction

Mitochondrial P450 enzymes catalyze highly specific reactions in the biosynthesis and degradation of hormones [1,2]. Mitochondrial P450s, i.e. CYP11, 24 and 27 families, cluster into a single evolutionary clan; CYP24A1 itself has less than 40% sequence identity with other P450s [3]. An N-terminal leader sequence targeting these P450s to the mitochondrion is cleaved during import; the mature enzymes bind monotopically to the matrix side of the inner membrane. Mitochondrial P450s exhibit the same monooxygenase activity as the microsomal enzymes (R-H + NADPH + H^+ + O_2 → R-OH + $NADP^+$ + H_2O), but are coupled to a two-component electron transfer system comprised of the soluble iron-sulfur ([2Fe-2S]) protein adrenodoxin (Adx), and adrenodoxin reductase (Adr), a FAD-containing flavoenzyme which binds NADPH [2,4]. In this respect, mitochondrial P450s resemble the soluble bacterial P450 CYP101 from *Pseudomonas putida*, which utilizes a comparable putidaredoxin (Pdx) and putidaredoxin reductase (Pdr) system [5]. Adrenodoxin is expected to bind to the proximal surface of the enzyme adjacent to the heme prosthetic group [6,7].

Because CYP24A1 degrades vitamin D, it is a target for inhibitor development for treatment of diseases linked to vitamin D insufficiency, such as bone disorders, kidney disease, and cancer [8-11]. Regulation of vitamin D activity is crucial to human health, as the active hormone, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$ or calcitriol), controls gene-expression and signal-transduction processes associated with calcium homeostasis, cellular growth, and the maintenance of heart, muscle, immune, and skin function [12,13]. Evidence suggests that vitamin D insufficiency, due to tissue specific CYP24A1 overexpression, contributes to development of diabetes [14] and cancers of the prostate, breast, colon and lung [15-18]. A number of promising vitamin D analogs have been developed [19], but their administration often induces hormonal resistance. Therefore, a complementary strategy for combating vitamin D insufficiency is to develop specific CYP24A1 inhibitors to retard degradation of the endogenous hormone.

The primary circulating form of vitamin D_3 (25-hydroxyvitamin D_3, 25-$(OH)D_3$) is converted to calcitriol by mitochondrial CYP27B1 (the 25D_3-1α-hydroxylase) under hypocalcemic conditions. Calcitriol elicits pleiotropic effects, in part via the vitamin D nuclear receptor (VDR). The CYP24A1 promoter contains multiple vitamin D response elements (VDREs) that respond to VDR signaling; CYP24A1 expression results in side-chain cleavage and elimination of circulating 25-$(OH)D_3$ and 1α,25-$(OH)_2D_3$ by catalyzing hydroxylation at the 23 and 24 positions [12,20]. The mechanism of CYP24A1's six step oxidation pathway for production of calcitriol remains poorly understood [21-22]. Structural knowledge of CYP24A1 would elucidate the basis for secosteroid specificity, and facilitate the rational design of inhibitors, an approach complementary to the synthetic analog design already exploiting structural studies of the VDR [23]. Here we report the crystal structure of the rat CYP24A1 determined to 2.5 Å resolution.

Results

We developed a bacterial over-expression system for mature rat CYP24A1 lacking the mitochondrial import signal (Δ2-32) to enable biochemical characterization of the purified enzyme [24]. Subsequent affinity-labeling studies identified a stabilizing mutation for recombinant CYP24A1 (Ser-57-Asp) that does not alter catalytic function [25], and site-directed mutational analysis using P450 homology modeling established functional roles for conserved residues involved in substrate recognition and catalysis [26]. Recombinant preparations (Δ2-32, S57D) were solubilized from bacterial membranes and purified in the presence of CHAPS (0.8% w/v) by affinity chromatography using adrenodoxin linked to sepharose, yielding monodisperse samples that crystallize readily by vapor diffusion

(Supplemental Methods). Detergent exchange from CHAPS into CYMAL®-5 afforded crystals of CYP24A1 which diffracted to 3.0 Å resolution in space group C2 on SSRL beamline 9-2. The structure was solved by molecular replacement (MR) with Phaser [27] using an ensemble of microsomal P450 structures (Supplemental Methods). A model with two molecules in the asymmetric unit was built using COOT [28] and MiFit [29], and refined using the CCP4 suite [30]. Concurrently, an improved "mixed-detergent" method for growing CYP24A1 crystals was developed that eliminated the need for CYMAL®-5 exchange by utilizing a second detergent together with CHAPS. CYP24A1 in mixed-detergent micelles with CHAPS (0.5% w/v) and common detergents (e.g. CYMAL®-5, Foscholine®-12) at 0.5-2-fold their critical micelle concentration, grew readily, but diffracted no better than 4 Å resolution.

However, a novel class of detergents, termed facial amphiphiles (FA) [31], are effective in improving CYP24A1 crystal quality in combination with CHAPS; single, rod-like crystals grown in the presence of CHAPS and the FA, 231-chol (3α-hydroxy-7α,12α-bis[(β-D-maltopyranosyl)ethyloxy]cholane), show diffraction to 2.0 Å resolution. A 7.6-fold redundant 2.5 Å data set was collected on SSRL beamline 12-2 [32], and used to complete and refine the CYP24A1 model including residues 51-514 (Table S1). Residues 33-50 of the S57D construct were not visible in the electron density. The two copies of CYP24A1 in the asymmetric unit are very similar with a root mean square deviation between Cα atoms of 0.62 Å.

The open form structure of rat CYP24A1 displays the canonical P450 fold, including the 12 α-helices (A-L) and four β-sheet systems (β1-β4), as well as additional helices A′, B′, and G′ on the distal surface, and K′ and K″ between β2 and the conserved heme binding motif (Figs. 1A, S1). The F-helix of CYP24A1 extends for 18 residues and lacks a well defined F′-helix seen for example in structures of microsomal CYP3A4 [34,35]. The structural elements defining the substrate binding cavity, including the β1 and β4 sheets, the B-C loop, and helices E, F, G, I and K surrounding the heme, exhibit lower than average B-factors (Fig. 1A). The B-C loop contains a 3-turn B′ helix that packs closely with helices G and G′. A distinct arrangement of aromatic residues from helices B′ (Trp134, Tyr137), F (Phe249), and G (His271, Trp275, Phe279) cluster together with extensive interactions (Fig. 1A, inset), contributing to the lower B-values in this region of the structure. The interaction between residues of helix G with helix B′ entraps a water molecule hydrogen bonded with the carbonyl oxygen of Trp134 and the side chain of Arg138; the latter residue engages in a salt bridge with Glu322 on the adjacent I helix. The aromatic cluster effectively blocks access to the active site via the solvent-accessible-channel (pw2c) along the I-helix [36]. At the same time, these residues contribute significantly to the hydrophobicity of the active-site interior. The eight residues comprising the aromatic cluster and salt bridge are conserved across all mitochondrial P450s (Fig. S2). Hence, the aromatic cluster is a distinctive feature of CYP24A1, and may be a general feature of mitochondrial P450s, stabilizing the open form.

The substrate access channel apparent in CYP24A1 is occupied by detergent molecules (Figs. 1B, S4) and lies among the B-B′ segment of the B-C loop, the β1-sheet and the F-G loop (pw2a); this channel appears to partially merge with a secondary channel that also lies between the B-B′ loop and the β1-sheet, and enters from below the F-G loop (pw2b) [36]. A similar substrate access channel, oriented towards the membrane, is present in the structure of the microsomal P-450, CYP46A1, which is a cholesterol 24-hydroxylase [37]. This feature distinguishes CYP24A1 from the non-specific microsomal vitamin D 25-hydroxylase, CYP2R1, which contains a pw2c substrate binding channel typical of enzymes from the CYP2 family [38].

Among currently available structures, the overall topology of structural features that form the substrate binding cavity in CYP24A1 is most similar to that of CYP3A4 [34,35]. A comparison of the two structures suggests that the open cleft observed for CYP24A1 results from the close packing of helix F with helix G and the elevated pitch of both helices that separates helix F

from the β4 sheet. This separation is increased by the conformation of the β1-4 sheet region which is similar to that seen in CYP3A4. This conformation exposes a larger portion of the heme surface and a widening of the substrate binding cavity close to the catalytic center, relative to structures of mammalian P450s of families 1 or 2 [34]. In structures of CYP3A4, the helix F does not extend over the substrate binding cavity and the polypeptide chain exhibits a coil structure as it passes across the cavity and connects with helix F′. In contrast, the helix F of CYP24A1 extends across the structure toward helix G′ without a well defined helix F′.

Analysis of the CYP24A1 structure [39-40] identified hydrophobic surfaces of helices A′ and G′ as potential anchors to the mitochondrial inner membrane; together, these short helices flank the substrate access channel (Figs. 1A,1B). The corresponding A′-A-helix and F-G loop regions of microsomal P450s are also implicated in membrane association [42-45]. Computational prediction of CYP24A1 insertion into a simulated lipid bilayer confirms this analysis (Figs. 2A,2B). The predicted protein:membrane association transfer free-energy (-9.0 kcal/mol) is within the range of values predicted from other P450 crystal structures (e.g. CYP3A4 (1TQN) [34] = -20.7 kcal/mol; and CYP46A1 (2Q9F) [37] = -4.7 kcal/mol) [46]. This analysis also suggests that residues on helices A′ and G′ can penetrate approximately 7 Å into the alkyl chain region of the bilayer, or up to 22 Å into the outer leaflet (Fig. 2A) [47].

Alignment of the A′ and G′ segments with other mitochondrial P450 sequences identifies each as a *membrane insertion sequence* (MIS), implying a conserved membrane binding architecture within mitochondrial CYPs 11, 24, and 27 (Fig. 2C, 2D). In MIS-1, the conserved proline-rich (PGP) region is followed by a hydrophobic segment of 9-12 residues bracketed by aromatic amino acids (Trp64 and Trp75, Fig. 2C). MIS-2 encompasses the amphipathic G′-helix, a distinctive feature of the structure (Fig. 1A), in which polar residues are oriented back towards the protein and hydrophobic residues point outward. MIS-2 is delimited by a conserved proline in the F-G loop region (Pro256), and a tryptophan at the N-terminus of the G-helix, present in all mitochondrial P450s (Trp268, Fig. 2D). The alignment in Fig. 2D suggests that the amphipathic nature of the G′-helix is a conserved feature of mitochondrial P450s. A number of conserved, basic residues are also arrayed in the vicinity of the A′ and G′ hydrophobic regions, consistent with interactions with phospholipid head groups (Fig. S3).

Four molecules of CHAPS were observed in copy A of the 2.5 Å structure of CYP24A1. Two molecules are associated with membrane binding regions (MIS-1 & 2) and structural lattice contacts, one is bound in the substrate-access channel and a fourth is positioned above the heme in a non-productive binding orientation (Fig. 1B). Alternatively, two molecules of CYMAL®-5 occupy the pw2a access channel in the independent 3.0 Å structure of CYP24A1 (Fig. S4). The arrangement of either CHAPS or CYMAL®-5 in isomorphous CYP24A1 structures suggest that the open form of CYP24A1 is a biologically-relevant conformation that is not dependent on the specific details of detergent binding. Additionally, the presence of trapped detergent molecules is consistent with the hydrophobic nature of the access channel and binding pocket, and illustrates a potential pathway for lipophillic substrates to transit from the membrane to the active site.

There is well defined electron density for a CHAPS molecule (CPS-600, 3K9V) close to the heme within the substrate-binding pocket of CYP24A1 (Fig. 3), but density for the zwitterionic side-chain extending out from the cavity is weaker. The hydrophobic face of the cholate ring is oriented toward the heme surface and non-polar residues of helices B′ and F, whereas the polar face of CHAPS is oriented toward polar protein groups, allowing three hydrogen bonds to be formed. In solution, CHAPS does not inhibit the catalytic function of CYP24A1 and does not induce a Type-I spectral perturbation of the heme typical of a proper substrate [24-26]. So while detergent association in the active-site appears strong, it likely represents a non-productive interaction that does not promote the enzyme's closed-form. Nevertheless, CHAPS

binding is illustrative of the hydrophobic nature of the active site, and its ability to provide specific interactions with amphipathic molecules.

Residues surrounding the active site cavity are contributed from nine regions of the CYP24A1 fold: B-B′ region (Leu129, Ile131), B′-helix (Trp134), B′-C region (Met148), F-helix (Met245, Met246, Phe249), G-helix (His271, Trp275), I-helix (Leu325, Ala326, Glu329, Thr330), K-helix/β1-4 loop (Val391), β1-4 sheet (Phe393, Thr394, Thr395), and the β4-1/β4-2 turn (Gly499, Ile500) (Fig. 3). Mutational analysis of CYP24A1 based on P450 homology modeling studies have attributed roles in substrate-binding and catalysis for 13 of the residues identified in the structure: Ile131, Trp134, Met148, Met245, Met246, Phe249, Ala326, Glu329, Thr330, Val391, Thr394, Gly499, and Ile500 [26,48,49]. Structural analysis also supports predicted roles for Ala326 (I-helix) and Ile-500 (β4-1/β4-2 loop) in modulating regiospecificity of hydroxylation in CYP24A1 [50,51]. A residue from the β1-3 strand (Thr416) is also linked to this process [50], but is found outside the CHAPS binding site in the open channel. However, this location could be relevant for secosteroid binding by analogy to cholesterol-3-sulfate binding in CYP46A1 [37].

A hybrid homology model for CYP24A1 based on the structures of mammalian CYP2C5 and bacterial CYP102, successfully predicted multiple residues in the CYP24A1 binding pocket, including Met246, Phe249, Val391, Thr394, and Ile500 [26]. However, this model does retain some template bias as it predicts substrate-access and binding similar to CYP2C5, via the solvent-accessible pw2c channel that is occluded in the structure. A P450 structural-motif based method of homology model building utilized by Masuda et al. appears to be highly effective at predicting key elements of the CYP24A1 tertiary structure [48]. This 3D model has been used to make strong predictions concerning the positioning, and putative role, of conserved residues in the CYP24A1 active-site, including: Ile131, Trp134, Met148, Met246, Ala326, and Gly499. To clarify the functional role of putative substrate-binding residues in the open form, flexible ligand docking simulations with CHAPS (as a control) and 1α,25-$(OH)_2D_3$ were conducted against the rat CYP24A1 structure (Table S2) using Autodock 4.0 (see Materials and Methods) [70-72]. As illustrated in Fig. 4,1α,25-$(OH)_2D_3$ docks in the open form of CYP24A1 with computed nanomolar affinity (Ki=2.65 nM) in a reproducible conformation stabilized by two hydrogen bonds (between the 3-OH group of the vitamin D A-ring and the B-B′ loop (Leu129), and the 25-OH of the side chain and the I-helix (Leu325)) and multiple hydrophobic interactions among key conserved residues (Ile131, Trp134, Met246, Phe249, Ala326 Val391, Thr394, Thr395, Gly499, and Ile500). The docking calculations confirm that the open form of CYP24A1 is well organized to bind 1α,25-$(OH)_2D_3$, in accord with predictions made by homology modeling concerning residues likely to bind the A-C-D ring system (Leu129, Ile131, Trp134, Phe249, Thr394, Val391) or side chain (Leu325, Ala326, Met246 and Ile500) of the vitamin D hormone. In this configuration, the secosteroid is well-positioned to interact with the aromatic cluster of residues among helices B′, F and G via interactions with the B-C loop (Leu129, Ile131, Trp134, Met148) and helix F (Met246, Phe249). However, it is unlikely that this result represents the substrate's terminal binding configuration relevant for the catalytically-active enzyme; the C21 methyl group of 1α,25-$(OH)_2D_3$, and not the target C23 or C24 carbons, is positioned over the heme iron, where it is superimposed with an ordered water molecule (WAT10, 3K9V). This result implies that in the open form secosteroid substrates bind over the heme in a fashion that brings the C21-methyl group into position to perturb the water-bound, heme iron (Figs. 4,S5). Further, when WAT-10 is included in the docking simulations a similar solution for 1α,25-$(OH)_2D_3$ is obtained (Table S2,Fig. S6), but here the C21-methyl is forced to rotate away from WAT10, forming a hydrophobic interaction with the important catalytic residue Ala326 [51]; the hydrogen bonding network is also altered as the 3-OH group is repositioned to interact with the β1-4 sheet (Thr395), rather than the B-B′ loop (Leu129) (Fig. S6). These derived results suggest that electrostatic interactions between the 25-OH group and the I-helix (Leu325), and

hydrophobic interactions among the hormone's C-D ring system and the B′-C loop (Met148) and the K-helix/β1-4 loop (Val391), are essential for high-affinity substrate binding in the open form. This analysis validates published CYP24A1 homology modeling studies concerning the composition of residues in the active-site, and supports our hypothesis that the open form is a biologically relevant conformation, useful for studying substrate binding in mitochondrial P450s.

The CYP24A1 structure also reveals structural elements and key residues for interacting with the [2Fe-2S] electron transfer protein, adrenodoxin (Adx) (Fig. 5A). Four basic residues from helices K (Lys378, Lys382) and L (Arg465, Arg466), involved in Adx recognition and electron transfer in CYP27B1 [52], are juxtaposed on the proximal surface. These basic residues are conserved in mitochondrial P450s, but divergent in microsomal forms (Fig. S7). Structural elements on the proximal surface include segments of helices B, C, D, and K, helices K′ and K″ flanking the meander region, and the Cys loop (Fig. 5B), all of which are implicated in redox partner binding [52-55] and circumscribe the four basic residues. Compared to microsomal P450s, all mitochondrial CYPs contain a tryptophan (Trp440 in CYP24A1) in the conserved motif PxRWL in the K″-helix (Fig. S7). Trp440 interacts with the K-helix via another conserved residue, Glu383, within the invariant KExxR motif (Figs. 5B,S7). This conserved tertiary interaction, which anchors the lysine rich loop (residues 442-446, QKEKK), and is next to Lys378 and Lys382, appears to be important for Adx interaction. For example, it links a substrate recognition sequence (SRS5, K-helix/β1-4 sheet [56]) with the lysine rich loop, which mediates redox protein interaction in P450cam [67]. It also displays the conserved residue Leu441 on the protein surface, providing a complement to electrostatic interactions in CYP24A1-Adx recognition.

Discussion

Mitochondrial CYP24A1 is a monotopic membrane protein that binds tightly to, but does not span, the lipid bilayer [47,58,59]. The CYP24A1 structure indicates that CYP24A1 associates with the membrane using hydrophobic membrane insertion sequences and conserved basic residues surrounding helices A′ and G′ (Figs. 2,S3). Prior studies of both microsomal (CYP2C2, CYP3A4) and mitochondrial (CYP11A1, CYP27A1) P450s have established roles for the N-terminal proline rich motif, A′-A-helix region, and the F-G loop in membrane binding [42-45,60]. The CYP24A1 structure is consistent with these results and clarifies the key role of the conserved membrane-insertion elements (MIS) encompassing helices A′ and G′. However, the MIS-1 and -2 features of CYP24A1 are not shared by bacterial P450-BM3 (CYP102), a fatty acid metabolizing, soluble P450 that otherwise shares common features with mitochondrial P450s [61,62]. MIS elements could also mediate protein:lipid interactions, as postulated for other monotopic membrane proteins [58], such as the C2 domain of cytosolic phospholipase A2 [63]. Penetration of CYP24A1 up to 22 Å into the bilayer could account for the roles of specific lipids, e.g. cardiolipin, in altering mitochondrial P450 function [64,65].

The open conformation of CYP24A1, compatible with substrate diffusion from within the membrane, is uniquely stabilized by a cluster of conserved aromatic amino acids from helices B′, F, and G. The aromatic cluster blocks the solvent accessible channel (pw2c) formed by helices B′, G, and I, and promotes formation of a pw2a channel directed toward the membrane [66]. Stabilization of an open conformation may also contribute to substrate specificity in CYP24A1. Active site collapse to a closed state, as required to exclude solvent during catalysis, may be triggered by solvent displacement and stabilized by the enthalpy of specific interactions with the biological substrate. Hence, the open form would disallow efficient, non-specific oxidation of improper substrates which are not able to stabilize the closed state, defining at least one mechanism by which CYP24A1, and perhaps mitochondrial P450s, may achieve their exquisite specificity. Docking analysis supports this hypothesis, as 1α,25-$(OH)_2D_3$ is

computed to bind deep within the active-site where it can interact with the heme group and presumably accommodate the cavity collapse required for catalysis. In contrast, the non-substrate CHAPS, which also docks the enzyme with computed nanomolar affinity (Ki=5.79 nM), is bound in a shallow conformation with limited access to the heme that would not be predicted to inhibit secosteroid accessibility. These studies provide new insight into the enzyme's active-site organization before substrate-binding, providing testable hypotheses concerning the determinants of secosteroid recognition. These contributions should prove useful in the evaluation and design of vitamin D analogs and CYP24A1-specific-inhibitors.

The proximal surface of CYP24A1 interacts with Adx and is rich in basic residues, including the invariant arginine pair (Arg465, Arg466) at the N-terminal end of the L-helix (Fig. 5A). These side chains protrude below the heme and are within 8-10 Å of the conserved K-helix residues, Lys378 and Lys382. Mutational analysis of the arginine pair in CYP11A1 and CYP27B1 suggested that the first arginine contributes to protein folding events and/or heme binding, as mutations abolish enzyme function and promote the P-420 form [52,53]; an Arg465 mutant of CYP24A1 (R465F) showed a similar dysfunction [26]. Hence, Arg465 may play a dual role. The second arginine (Arg466) has been more clearly linked to catalytic function and is thought to mediate both electron and oxygen transfer steps [52]. In CYP24A1, Arg466 is positioned to promote electron transfer between Adx and the heme iron via main chain interactions with Cys462, as modeled for the CYP101:Pdx complex [67]. Conserved residues from the K-helix (Lys378, Lys382) and the lysine rich loop are also positioned to interact with the very acidic surface of Adx that surrounds the [2Fe-2S] cluster [5,68]. The link between the K and K″ helices provided by the conserved Glu383-Trp440 tertiary structure interaction suggests that presentation of the PxRWL motif is another key aspect of Adx recognition.

In summary, we have solved the first structure of a mitochondrial P450, CYP24A1, which defines key features for this important class of enzymes. A membrane-directed (pw2a) substrate access channel is stabilized in an open conformation by an aromatic cluster formed among conserved residues on helices B′, F, and G. Two conserved membrane insertion sequences overlap hydrophobic features on the distal surface near helices A′ and G′. Membrane insertion and orientation of the enzyme's hydrophobic channel shows how a lipophillic substrate, in this case vitamin D, can be transferred from the bilayer to the active site. Conserved surface features from helices K, K″ and L, and the lysine-rich proximal loop of the meander region, define components of the basic, adrenodoxin binding site. The composition of residues in the active site is consistent with prior biochemical, mutagenesis, and homology modeling data for CYP24A1, and related mitochondrial P450s, and docking simulations with 1α,25-$(OH)_2D_3$ clarify the structural determinants of secosteroid recognition in the enzyme's open conformation.

Materials and Methods

Recombinant rat CYP24A1 enzyme (Δ2-32, S57D mutant) was purified using established protocols [24-26]. Some modifications to our original procedure were required for crystallization (as detailed in Supplemental Methods). In short, a secondary chromatography step was added that allows CYP24A1 samples prepared in CHAPS (Anatrace) to be exchanged into alternative detergents, like CYMAL®-5 (Anatrace), as described by Wester et. al [69]. Detergent-exchange experiments identified conditions for growing a CYMAL®-5-based crystals that diffract to 2.8 Å, and allowed the CYP24A1 structure to be solved by the molecular replacement method using Phaser [27]. Later a mixed-detergent method was established for improving CYP24A1 crystal quality without the need for detergent exchange. Facial amphiphile detergents developed at Scripps [31] were found to produce high-order CYP24A1 crystals that diffracted to 2.0 Å resolution when mixed with CHAPS. Diffraction data for all

CYP24A1 crystal forms were collected at the Stanford Synchrotron Radiation Laboratory (SSRL BL9-2 and BL12-2) [32].

Detailed methods for structural determination & refinement, computational (surface and membrane binding) analysis, and flexible-ligand docking studies with Autodock 4.0 [70-72], are provided in the Supplementary materials. All sequence alignments were developed using the program CLUSTALX2 [73]; labeling and format are explained in Supplemental methods. All figures for this manuscript were generated using Pymol [74].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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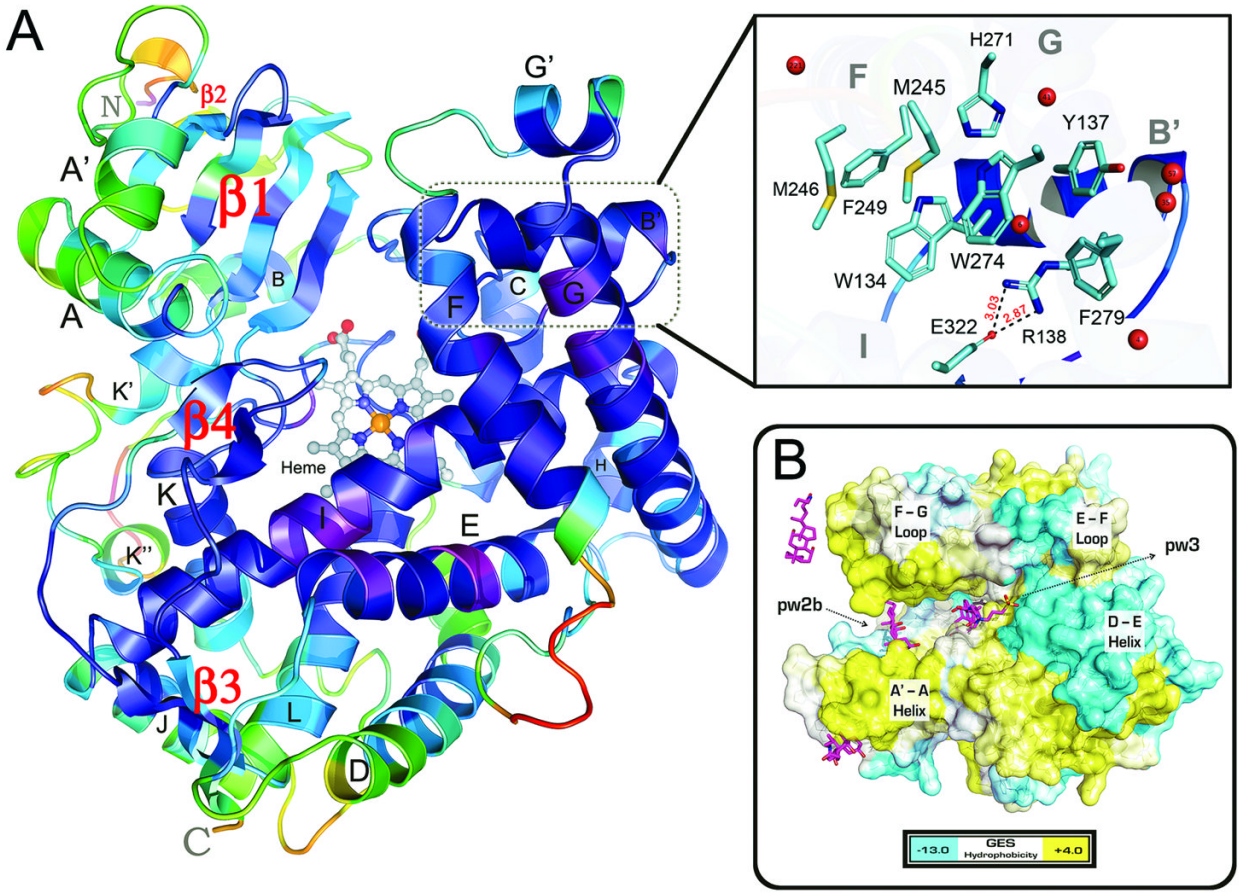

Fig 1. The Crystal Structure of CYP24A1

A) The structure of CYP24A1 refined at 2.5 Å resolution (R=0.206, R_{free}=0.252) shown colored by B-factor from high (red) to low (violet) temperature. P450 structural elements (α-helices, β-sheets) are labeled. Conserved residues from helices F (M245, F249), G (H271, W275, F279) and B′ (W134, Y137, R138) participate in an "aromatic cluster" centered on a water-molecule bound to R138 that promotes a membrane-directed (pw2a) substrate access channel. B) The distal surface of CYP24A1 is shown colored from negative (cyan) to positive (yellow) hydrophobicity (GES scale [41]) revealing the open channel between the A′-A helix & F-G loop regions. CHAPS molecules (pink sticks) from the crystal structure are overlaid to illustrate a model for substrate diffusion from the membrane-associated channel (pw2a) to a putative exit channel (pw3) between the D-E helix region & E-F loop [36].

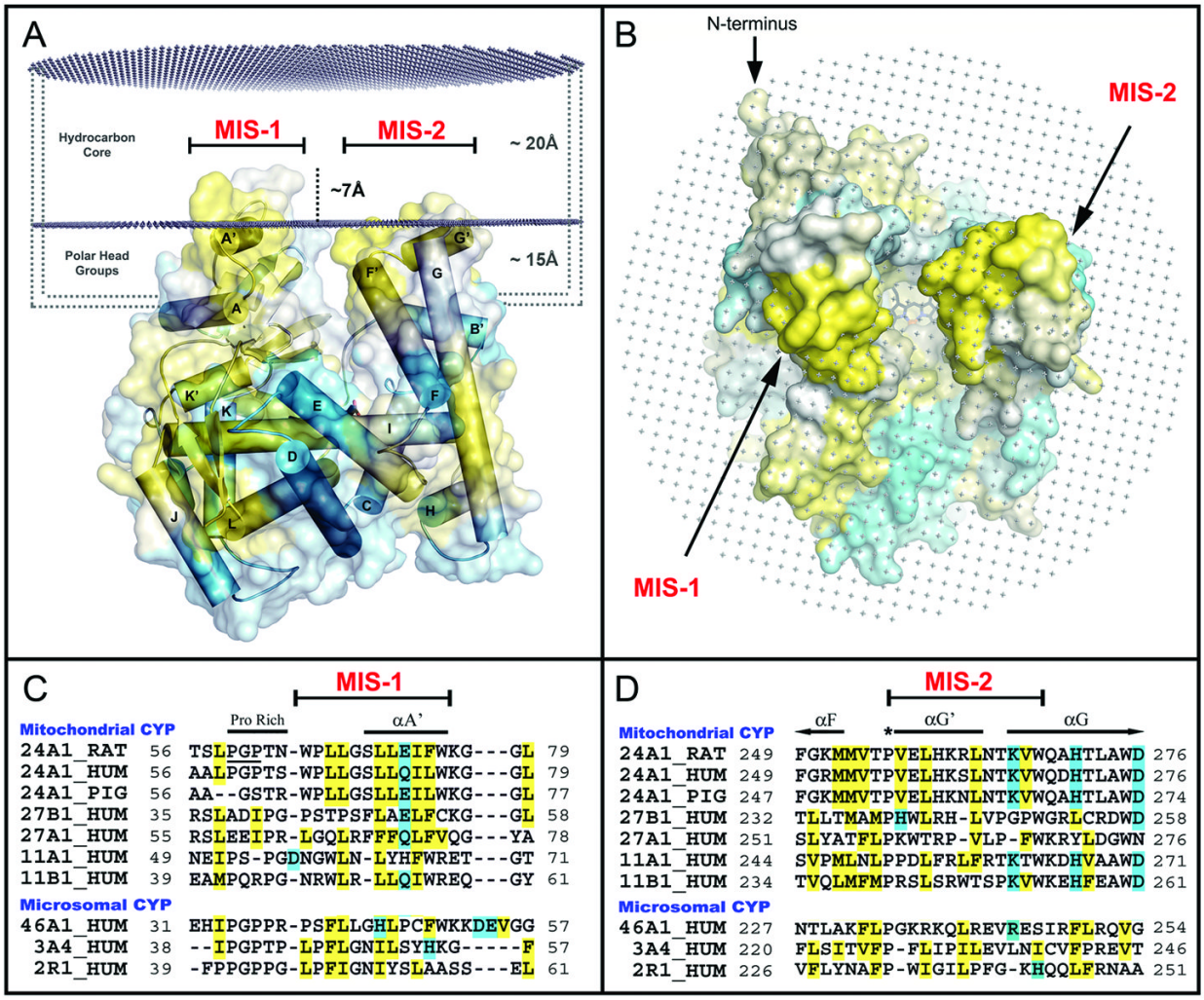

Fig 2. Adaptations for Monotopic Membrane Binding

A) Interactions between CYP24A1 and the lipid-bilayer were studied computationally with the OPM server [46] and our model for membrane-binding is shown colored by hydrophobicity (GES [41]). Two *membrane-insertion-sequences* (MIS) are predicted for CYP24A1 that correspond to hydrophobic surface regions (helices A′ and G′) that are modeled to penetrate into the membrane's carbonyl core (~17-22 Å) with polar lipid head groups reaching deep into the substrate-access channel between the A′-A helix and F-G loop regions. B) An orthogonal view of the OPM model illustrates the width and depth of the hydrophobic substrate access leading to the heme center. C.) Primary sequence alignments of predicted MIS-1 and D) MIS-2 binding regions demonstrate the conservation of membrane binding features across key membrane-bound P-450 forms.

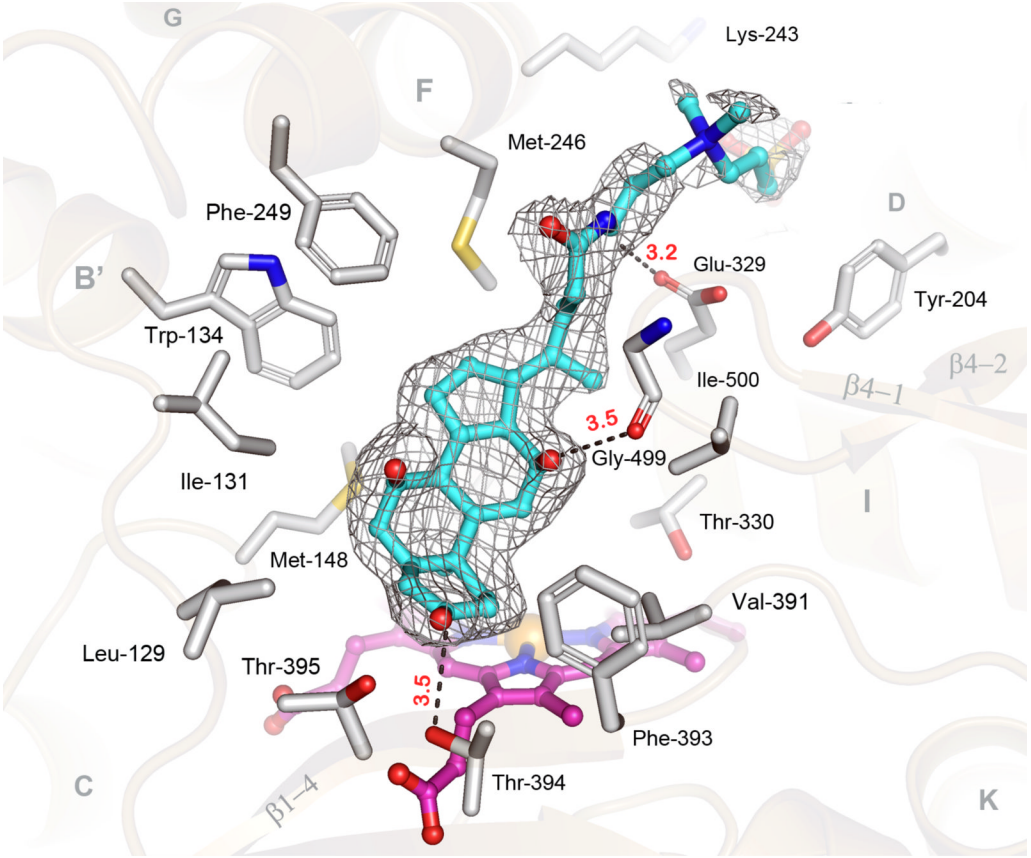

Fig 3. The Active Site of Rat CYP24A1 Bound to CHAPS

A) The electron density for CHAPS (cyan carbons) (σ_A−weighted 2|F_o−F_c|-composite-omit map, 1.0σ) is shown in the heme-centered active-site of CYP24A1; important secondary structural elements (tan), CHAPS binding residues (grey carbons), the heme prosthetic group (pink carbons), and notable bond distances (Å, red) are noted. Amino acid residues from the B-C loop (I131, M148) and Helices B′, F, G & I (W134, M246, F249 & T330) the K-helix/β1-4 loop (V391, F393) and the β4-1/β4-2 turn (Ile-500) mediate hydrophobic interactions with CHAPS. Polar/charged residues from the I-helix (E329), β1-4 sheet (T394, T395) and the β4-1/β4-2 turn (G499) mediated bonds to the polar face of CHAPS. The negatively charged tail of CHAPS is bound outside the active site and interacts with residues from the helices D (Y204) and F (K243).

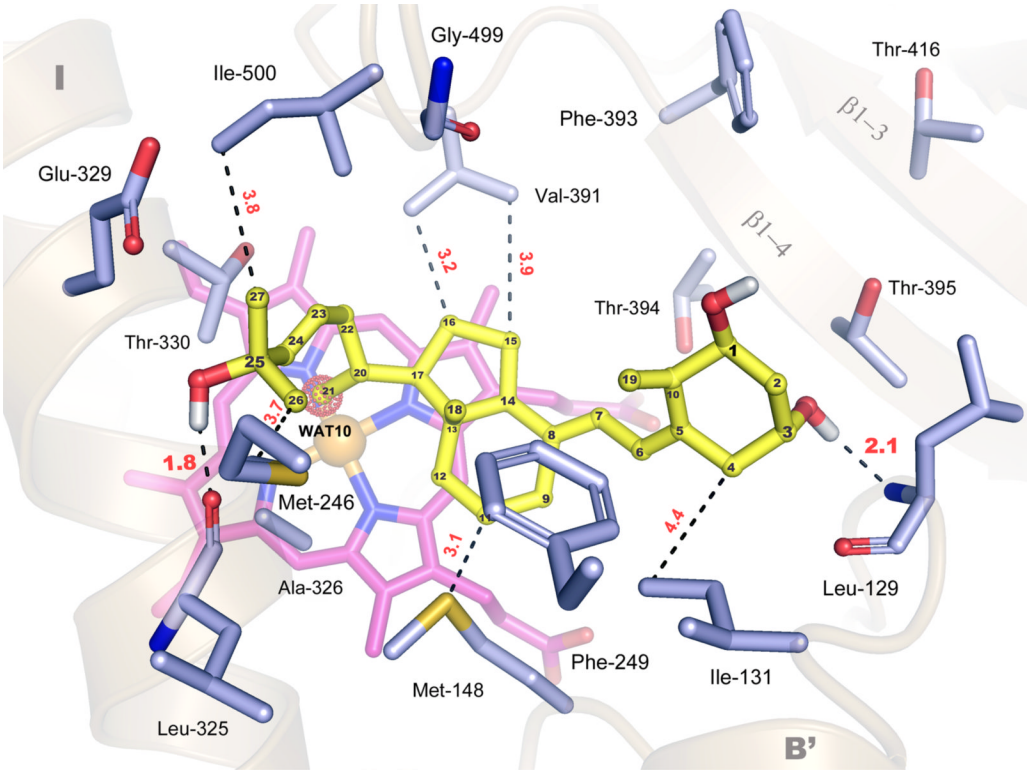

Fig 4. Secosteroid Docking in the Open Form of Rat CYP24A1

A model for 1α,25-$(OH)_2D_3$ (yellow) binding in the heme-centered (pink) active-site of CYP24A1 was developed with a crystal structure-calibrated docking protocol, using Autodock 4.0 (see methods). Amino acid residues that flank the secosteroid docking site are shown from the B-B′ loop (L129, I131) B′-C loop (M148), helix F (M246, F249), helix I (L325, A326, E329, T330), the K-helix/β1-4 loop (V391, F393), the β1-4 sheet (T394, T395), the β1-3 sheet (T416), and the β4-1/β4-2 turn (G499, I500), and notable hydrogen bond distances (Å, red) are given with respect to the computed H atom positions. Individual carbon atoms on 1α,25-$(OH)_2D_3$ are labeled for reference. Multiple hydrophobic interactions and two hydrogen bonds between the 3-OH group and the B-B′ loop (L129) and the 25-OH group and helix I (L325) are predicted to stabilize secosteroid binding in the open form. In this configuration, the C21-methyl group superimposes with a structural water (WAT-10) bound to the heme (shown as red-dotted sphere); this water molecule was excluded from the calibrated docking experiment shown here (Table S2, Fig. S5). An alternative docking model derived using control parameters, which include WAT-10, is presented in Supplemental Fig. S6.

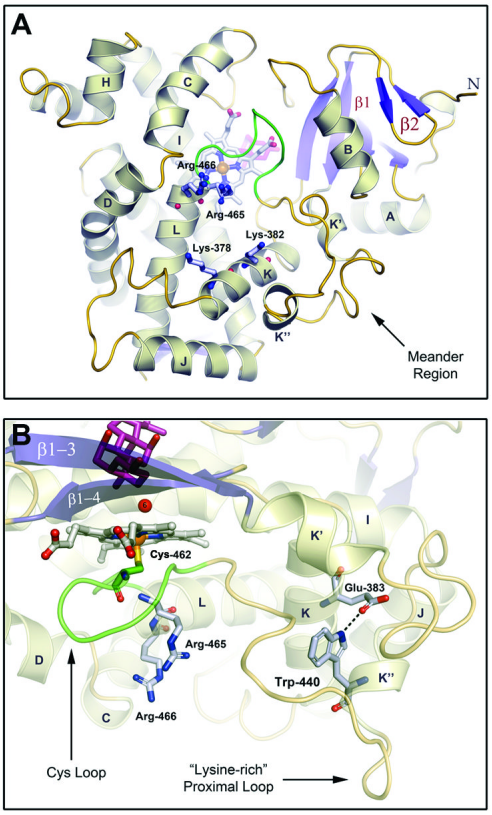

Fig 5. Adaptations for Adrenodoxin Binding

A) CYP24A1's proximal surface is shown below the heme prosthetic group with key structural elements implicated in adrenodoxin binding noted. Basic residues from helices B, C, D, J, K and L, the Cys-Loop and the (bacterial) meander region line the positively-charged Adx binding. Fully-conserved residues from helices K (K378,K382) and L (R465,R466) known to mediate adrenodoxin binding and electron transfer in related P450s are labeled [52-55]. B) A conserved tryptophan residue (W440), from the K″ helix of mitochondrial P450s, forms a salt-bridge to the K-helix via a fully-conserved glutamate residue (E383) that may contribute to the display of the meander's lysine-rich, proximal loop, that is associated with Adx binding [57]. Residues from the L-helix (R465,R466) and Cys-loop (M462), implicated in the electron shuttle process are shown below the heme in close proximity to the K-helix. The lower portion of the active-site is also shown with CHAPS (pink) positioned above the water (WAT6) bound heme iron.