Published in final edited form as:

Arch Biochem Biophys. 2007 April 15; 460(2): 262–273.

Hybrid Homology Modeling and Mutational Analysis of Cytochrome P450C24A1 (CYP24A1) of the Vitamin D Pathway: Insights into Substrate Specificity and Membrane Bound Structure-Function

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Abstract

Cytochrome P450C24A1 (CYP24A1), a peripheral inner mitochondrial membrane hemoprotein and candidate oncogene, regulates the side-chain metabolism and biological function of vitamin D and many of its related analog drugs. Rational mutational analysis of rat CYP24A1 based on hybrid (2C5/BM-3) homology modeling and affinity labeling studies clarified the role of key domains (N-terminus, A', A, and F-helices, β 3a strand, & β 5 hairpin) in substrate binding and catalysis. The scope of our study was limited by an inability to purify stable mutant enzyme targeting soluble domains (B', G, and I-helices) and suggested greater conformational flexibility among CYP24A1's membrane-associated domains. The most notable mutants developed by modeling were V391T and I500A, which displayed defective binding function and profound metabolic defects for 25-hydroxylated vitamin D₃ substrates similar to a non-functional F-helix mutant (F249T) that we previously reported. Val-391 (β 3a strand) and Ile-500 (β 5 hairpin) are modeled to interact with Phe-249 (F-helix) in a hydrophobic cluster that directs substrate binding events through interactions with the vitamin D *cis*-triene moiety. Prior affinity labeling studies identified an amino-terminal residue (Ser-57) as a putative active-site residue that interacts with the 3 β -OH group of the vitamin D A-ring. Studies with 3-epi and 3-deoxy-1,25(OH)₂D₃ analogs confirmed interactions between the 3 β -OH group and Ser-57 effect substrate recognition and trafficking while establishing that the *trans* conformation of A-ring hydroxyl groups (1 α & 3 β) is obligate for high-affinity binding to rat CYP24A1. Our work suggests that CYP24A1's amphipathic nature allows for monotopic membrane insertion, whereby a pw2d-like substrate access channel is formed to shuttle secosteroid substrate from the membrane to the active-site. We hypothesize that CYP24A1 has evolved a unique amino-terminal membrane

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DEDICATION

This manuscript is dedicated to the life and work of our friend and mentor, Dr. John L. "Jack" Omdahl.

binding motif that contributes to substrate specificity and docking through coordinated interactions with the vitamin D A-ring.

Keywords

vitamin D; calcitriol; 1,25-dihydroxyvitamin D₃; 25-hydroxyvitamin D₃ 24R-hydroxylase; 1,25-dihydroxyvitamin D₃; vitamin D inactivation; cytochrome P450; CYPs; P450C24A1; CYP24A1; site-directed mutagenesis; homology modeling; affinity labeling; monotopic membrane protein

INTRODUCTION

There is high interest in determining structures of conformationally complex cytochrome P450 enzymes (CYPs or P450s) that regulate the metabolism of vitamin D in humans [1,2]. To date only a few mammalian CYP isoforms have been characterized structurally, including human P450's 2A6 [3], 2C8 [4], 2C9 [5], and 3A4 [6,7], and rabbit P450 2B4 [8] and 2C5 [9]. This high-resolution data has greatly enhanced our understanding of the key structural elements that dictate the organization and function of mammalian CYPs. However, none of the existing structures represent the elusive mitochondrial-inner-membrane bound CYP forms that mediate key endocrine pathways, including steroidogenesis. In this regard, mitochondrial CYPs of the vitamin D pathway (CYP24A1 and CYP27B1) catalyze a complex series of anabolic and catabolic oxidation reactions that function coordinately to regulate ambient and cellular levels of vitamin D metabolites [10]. This includes the most hormonally-active form, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃ or calcitriol) that alters gene expression in numerous target tissues to regulate multiple cellular functions including calcium homeostasis, immune function an normal cellular proliferation and differentiation processes (as reviewed) [11]. Vitamin D and related drug analogs are potent inducers of cellular differentiation, making them promising targets for the treatment of antiproliferative disorders, including psoriasis and cancer [12]. In addition, there is growing evidence for a global epidemic of vitamin D deficiency underlying numerous diseases, which can be partially attributed to vitamin D resistance caused by altered regulation of CYP24A1 [13]. This observation is underscored by accumulating evidence that CYP24A1 is a candidate oncogene over-expressed in numerous human tumor types, including breast, colon, prostate, esophagus and lung [13-17]. Therefore, improved molecular understanding of CYP24A1 structure-function is essential for the biorational design of safe and efficacious vitamin D analog drugs and CYP24A1-specific inhibitors with tissue-specific antiproliferative and anticancer properties.

CYP24A1 and the closely related CYP27B1 are mitochondrial inner-membrane Class I P450 enzymes that have surface ferredoxin-binding domains that mediate electron transfer into the heme center where molecular oxygen is bound then split into a reactive oxyferryl center that is stereospecifically directed to the targeted substrate and subsequently reduced to a hydroxyl group. The other oxygen atom is reduced to H₂O (hence the term *mixed-function oxidase* for this class of P450 enzymes) [10]. Catalytic specificity for this reaction is determined by substrate orientation in the active site, whereby the target carbon atom is aligned juxtaposed to the heme-oxyferryl center. Therefore, identification of active-site residues responsible for substrate binding and positioning relative to the reactive oxygen is fundamental to understanding the catalytic process and the structure-based design of new medicines. The characterization of CYP24A1 structure and function is complicated by observations that the enzyme displays both C₂₃- and C₂₄-hydroxylase activity that depends on which side-chain carbon (C₂₃ or C₂₄) is hydroxylated first. Several studies have demonstrated that species-specific CYP24A1 isoforms express different levels of C₂₃- and C₂₄-hydroxylase activities that result in side-chain lactone formation (C₂₃ pathway) or side-chain cleavage and oxidation to a carboxylic acid (C₂₄ pathway) [18,19] Human CYP24A1 possesses both C₂₃- and C₂₄-

activity, while the rat form seems to catalyze the C₂₄ pathway exclusively. Although the structural basis of this altered specificity remains elusive, Hanamoto et al. have recently demonstrated that the C₂₃-hydroxylase activity of rat CYP24A1 could be bolstered by humanizing single residues (T416M, I500T) within the enzyme's putative active site [20] (Figure 1).

Due to the lack of crystallographic data for all CYPs of the vitamin D pathway, it has not been most difficult studying the binding and catalytic features of these enzymes at the molecular level. Based upon the over-expression and purification of recombinant rat CYP24A1 (Δ 2-31) in our laboratory, it was possible to document for the first time substrate binding affinities and the role of key residues in the substrate-binding region [21]. We have demonstrated that recombinant rat CYP24A1 expressed the full C₂₄-oxidation pathway and displayed kinetic parameters similar to apparent values obtained from cellular preparations. Also, the enzyme's ability to sustain substrate side-chain oxidation to the terminal calcitroic acid product in a reconstituted system was directly related to the level of electron transfer through the flavoprotein adrenodoxin (ADX). In addition, amino-acid sequence analysis of CYP24A1 and CYP27B1 identified F-helix residues (M246, F249) whose mutation dramatically reduced substrate-affinity while decreasing the efficiency of multiple oxidation steps in the calcitroic acid synthetic pathway [21]. The current work represents a more comprehensive mapping of CYP24A1 structure/function based on P450 homology modeling and substrate-affinity labeling studies and provides insights into the structural organization of membrane-bound CYPs, while clarifying the molecular determinants that effect vitamin D substrate specificity and trafficking.

MATERIALS AND METHODS

Materials

Reagents for bacterial growth were obtained from Life Technologies (Rockville, MD). Antibiotics, inducing agents and protease inhibitors were obtained from Sigma-Aldrich (St. Louis, MO) and all other chemicals were of reagent grade and obtained from commercial sources. Quick-Change Site-Directed Mutagenesis kits were acquired from Stratagene (LaJolla, CA). All vitamin D compounds tested were obtained commercially or donated generously by our collaborators Hector DeLuca, Satya Reddy, Mark Chiu and Rahul Ray.

Homology Modeling of rat CYP24A1

The three-dimensional structural model of rat CYP24A1 was constructed using a Silicon Graphics workstation and the Modeler program from the InsightII™ suite of programs (Accelrys, Inc). The heme-domain of previously crystallized P450-BM3 (1JPZ) and cytochrome P450-2C5 (1DT6) were used as templates for threading the CYP24A1 backbone. Several structural models were generated that had different loop structures in the regions between helices and sheets. From these paradigms, the model with the least amount of buried or unpaired charges was chosen to further refine. The model was energy minimized using the steepest descent or conjugate gradient in the Discover package, carried out for 3,000 to 5,000 steps per run, until the standard deviation of the coordinates was less than 0.1 and the potential energy had reached a plateau. A water shell was added and the model was further minimized. Molecular dynamics simulations were run using the Discover package and the "cvff force-field" for 10 picoseconds and then minimized. During minimizations, the heme and its thiolate ligand were held fixed. After the model was built, the substrate 1 α ,25(OH)₂D₃ was docked into the active site above the heme such that the C₂₄ was within 2 angstroms of the oxyferryl heme center. The substrate was pivoted around this point to find an orientation that had little or no steric hindrance from the protein and did not overlay the protein. The position with the best fit to this criterion was an orientation that placed the A-ring of 1,25(OH)₂D₃ close to the

F and B' helices. Minimizations of the docked substrate and protein assembly were done with the substrate held fixed and then dynamics were carried out for another 10 picoseconds. The resulting model was subsequently analyzed at UNM for structure-function relationships using InsightII™ (Accelrys, Inc) and VMD (<http://www.ks.uiuc.edu/Research/vmd/>) [22].

Site-directed Mutagenesis of rat CYP24A1

A wild-type prCYP24-99 construct ((modified $\Delta 2-31$) [21]) was used as the template for preparing all the rat CYP24A1 mutants used in this study. The Quick-Change Site-Directed Mutagenesis Kit (Stratagene) was used to conduct all steps of the mutant cloning procedure. The oligonucleotide primers used to prepare the functional (or viable) constructs described in this study were synthesized locally (DNA Services Laboratory, University of New Mexico-SOM) and are listed in Table I. Each mutant was sequence verified across the entire coding sequence prior to expression studies in the bacterial expression system (DH5 α –FIQ).

Recombinant Expression and Purification of rat CYP24A1

Recombinant rat Cytochrome P450C24A1 (CYP24A1) enzyme used in this study were expressed and purified as described [21]. Briefly, *E. coli* (DH5 α – FIQ) transformed with mutant-specific prCYP24-99 vector were grown at 37 °C with shaking until the A_{600} reached 0.6-0.8. Protein expression was induced with IPTG (1 mM), chloramphenicol (1.0 μ g/mL) and δ -aminolevulinic acid (1.0 mM) and cells were grown at 29 °C for 40-48 h with shaking. Cells were harvested, resuspended in 10 mM potassium phosphate (KPi) buffer (pH 7.4) containing 20% glycerol and 1 mM dithiothreitol (DTT) and lysed in the presence of a EDTA-free protease inhibitor cocktail (Sigma) and 2 mM PMSF or AEBSF. CYP24A1 was extracted from bacterial membranes using CHAPS (Sigma) at 0.8% w/v. Solubilized lysates were sonicated at low temperature and the supernatant was collected via ultracentrifugation ($\sim 120,000\times g$). The viability of each CYP24 mutant was assessed by the P420:P450 ratio as measured by CO-reduced difference spectra [24]. Only enzyme with P420:P450 (r/z) ratio's exceeding 0.5 were considered stable enough for further purification and subsequent biochemical analysis.

Recombinant enzyme was purified to specific heme contents exceeding 15 nmol /mg using a two-step purification protocol, incorporating substrate affinity chromatography with co-redox agent adrenodoxin (Adx-Sepharose) and hydroxyapatite (HAP). Sample purity was analyzed by the 416/280 (R/Z) ratio in the absolute spectrum and fractions with R/Z ratios > 0.95 were considered pure enough for subsequent studies. Purified enzyme was stabilized in 500 mM KPi buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 0.1% (w/v) CHAPS, flash frozen and stored at -80°C until needed. The bovine adrenodoxin (Adx) and Adrenodoxin reductase (Adr) used in CYP24A1 reconstitution studies were expressed and purified as described [21].

Biochemical Analysis of Recombinant Rat CYP24A1 Mutants

The details of biochemical methodologies used to assess substrate recognition (spectral perturbation), binding affinity and metabolism of vitamin D substrates by the site-directed CYP24A1 mutants developed in this study are documented [21]. In summary, absolute spectral binding studies were conducted to determine the substrate (ligand)-binding constant (K_d) for various vitamin D compounds. Ligand-induced spectral shifts from ~ 416 nm to 390 nm were measured on wild-type and mutant enzyme (0.5 μ M) in degassed 100 mM phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1% (w/v) CHAPS. The corresponding spectral change from low-spin to high-spin state of the heme center was monitored in the presence of increasing ligand concentrations (~ 0.05 to 5 μ M) until saturation was achieved. The spectral perturbation is a measure of substrate binding in the heme active site; greater perturbation is correlated to more efficient binding. The rate of spectral transition determines the spectral binding constant, (K_d) that is calculated as described [21]. The concentration and stability of

fresh preparations of individual mutants was determined immediately prior to spectral studies. Deviations in total protein ([rCYP24]) among replicates were normalized to (0.5 μM) according to the difference in absolute optical absorbance ΔA_{Free} for the free enzyme.

The enzyme reconstitution system used to determine the effect of point mutations on vitamin D metabolism has also been described [21]. In the current study, reactions consisted of wild-type and mutant CYP24A1 (0.4 μM), adrenodoxin (Adx) (0.1-0.2 μM), adrenodoxin reductase (Adr) (0.1 μM), and vitamin D substrate (1-5 μM). Enzymatic reactions were initiated by addition of NADPH (1 mM) and then incubated at 37 °C until quenched by methanol. CYP24A1 Metabolites were extracted using dichloromethane and the combined extracts were dried for HPLC analysis (Hewlett Packard model 1050) on a C₁₈ reverse-phase column (YMC ODS-AQ S3, 120 angstrom, 2 $\times$ 150 mm, Waters Corp, Milford MA); the details of this procedure was described previously [21]. Retention time for products was established using standards, and area under the curve values for each metabolite were corrected for extraction efficiency using a 25(OH)D₃ internal standard added to quenched reaction just prior to extraction.

All Vitamin D ligands used in these studies were prepared in fresh propylene glycol (diethylene glycol monoethyl ether) and assayed for concentration in absolute ethanol using an O.D. extinction coefficient of 18.2 mM⁻¹cm⁻¹ at 264 nm [25].

RESULTS

Homology Modeling Analysis of Rat CYP24A1

A computer model of the rat CYP24A1 was constructed using crystallographic and alignment data for the heme domain (BMP) of cytochrome P450 BM-3 (fatty acid hydroxylase from *B. megaterium*: pdb #: 1JPZ) [27] and cytochrome P450-2C5 (CYP2C5) (eukaryotic (rabbit) enzyme: pdb #: 1DT6) [28] (Figure 2). The resulting similarity model is therefore a hybrid that resembles CYP2C5 with the exception of a hydrophobic F-G loop region that is in close proximity to a B'-helix derived from the BMP structure. Because the N-terminal regions of available CYP templates have few conserved residues, no structural data from P450BM3 and P4502C5 were used to determine the amino terminal conformation of our similarity model; the resulting model (Δ 1-58) starts near the conserved "PGP" hinge region and lacks any structural information about the amino-terminus that may alter the enzyme's tertiary structure. Second and third generation models of rat CYP24A1 were later established in other collaborations that predict N-terminal organization, but these models were not used to guide mutagenesis studies detailed herein.

The resulting 2C5/BM-3 hybrid model predicted close interactions between docked 1,25(OH)₂D₃ and conventional SRS domains [2] [32], including: SRS1 (B'-Helix, 133-145); SRS2 (F-Helix, 238-249); SRS4 (I-helix residues 322-330); SRS5 (β 3a strand, 390-394); and SRS6 (β 5 hairpin, 496-502). Highly conserved CYP24A1 residues, including Met-246 (M246), Phe-249 (F249), Val-391 (V391), Thr-394 (T394) and Ile-500 (I500) were seen positioned close to the A-ring and *cis*-diene connector link between the A- and C-rings (Figure 1). These results were consistent with the previously discussed point-mutation studies in which the mutants F249T, F249A, and F249Y displayed impaired binding and metabolism consistent with a role for F249 in directing proper alignment of the 1,25(OH)₂D₃ side-chain with binding elements in the heme-oxyferryl active center [21].

Rational Mutational Analysis of Rat CYP24A1

Using the hybrid CYP24A1 model as a guide, we probed the heme-centered substrate binding pocket and a putative substrate-access channel (now more clearly described as pw2d [33,34]).

Mutation studies targeted residues within a functional distance (~ 2 -10 Angstroms) of the docked $1,25(\text{OH})_2\text{D}_3$ substrate, or residues within a hypothesized substrate access/egress channel. Initial efforts focused on completing the mutational study of the F-G loop region modeled to interact with both amino- (A' Helix) and carboxy- ($\beta 3a$ strand and $\beta 5$ hairpin) terminal domains. We followed by preparing functional mutants for active-site residues in the adjoining B', G, and I-, helices. Site-directed mutants were cloned, expressed, purified and submitted to spectral and metabolic analysis as described [21]. Of the 20+ mutant sites chosen from the original CYP24A1 modeling work, only 8 (Y75M, Y89F, M245A, S247V, T248A, V391I, T394V and I500A) were expressed and purified to sufficient levels for biochemical analysis. A ninth mutation site at serine-57 was identified by a substrate-affinity labeling study [23] and two additional mutants (S57A, S57D) were generated (Table I).

Site-directed mutants targeting polar regions of the F-helix (M245A, S247V, T248A) caused only modest changes in binding affinity for $1,25(\text{OH})_2\text{D}_3$ compared to mutations in hydrophobic portions of the A'-helix (Y89F), $\beta 3a$ strand (V391F, T394V) and the $\beta 5$ hairpin (I500A) (Table II). Three hydrophobic mutants from the F-helix and the $\beta 3a$ strand (M245A, T248A and V391F) displayed enhanced binding affinity for $25(\text{OH})\text{D}_3$ substrate, but not $1,25(\text{OH})_2\text{D}_3$; the V391F mutant displayed similar affinities for either substrate ($K_d=0.216 \mu\text{M}$ for $25(\text{OH})\text{D}_3$ vs. $K_d=0.198 \mu\text{M}$ for $1,25(\text{OH})_2\text{D}_3$) (Table II). Mutation at residue 500 from isoleucine to alanine (I500A) had the greatest impact on lowering $1,25(\text{OH})_2\text{D}_3$ binding affinity, as the dissociation constant was over 4-fold higher ($K_d=0.235 \mu\text{M}$) than wild-type. This reduction of substrate affinity for the I500A mutant enzyme was similar to that of the F249T mutant, however, the I500A mutation did not effect substrate recognition (i.e. spectral perturbation) to the same extent as F249T (0.25 vs. 0.12) (Table II). Additionally, Y89F and I500A were the only mutants studied that decreased $25(\text{OH})\text{D}_3$ binding affinity; for I500A this defect was only half as effective at disrupting substrate binding when compared to the original F249T mutant. ($K_d=0.419 \mu\text{M}$ for I500A vs. $K_d=0.829 \mu\text{M}$ for F249T). However, most mutants had a diminished capacity to recognize $25(\text{OH})\text{D}_3$ as monitored by spectral perturbation. In general, mutations targeting the A-helix (Y89F), the $\beta 3a$ strand (V391I) and the $\beta 5$ hairpin (I500A) caused the greatest defects in substrate recognition and high-affinity binding.

Mutant metabolism was also analyzed by enzyme reconstitution assay, and demonstrated the utility of the homology model to predict active-site residues involved in both substrate binding and catalysis. All mutants displayed some impaired ability to catalyze the full conversion of $1,25(\text{OH})_2\text{D}_3$ to calcitric acid (Figure 2). New F-helix mutants (M245A, S247V, T248A) displayed normal substrate binding with a lower rate of calcitric acid production associated with an elevation in the 24,25,26,27-tetranor-1,23($\text{OH})_2\text{D}_3$ (1,23) precursor. In contrast, A'-helix mutants (W75M, Y89F) showed defects earlier in the catalytic cycle as indicated by decreased side-chain oxidation of $1,24,25(\text{OH})_3\text{D}_3$ to 24-oxo- $1,25(\text{OH})_2\text{D}_3$. Mutations made to the $\beta 3a$ strand (V391I, T394V) and $\beta 5$ hairpin (I500A) regions also dramatically altered the enzyme's ability to convert 24,25,26,27-tetranor-1,23($\text{OH})_2\text{D}_3$ to calcitric acid (Figure 2). In general, mutation of residues involved in active-site organization (F-helix, $\beta 3a$ strand, and $\beta 5$ hairpin) caused defects in the terminal production of calcitric acid, while residues in the putative pw2d access channel (A'- and A-helices) showed defects earlier in the catalytic process.

An N-terminal substrate-binding residue (Ser-57) in recombinant rat CYP24A1 was identified by vitamin D affinity-labeling studies [23]. Our models positioned Ser-57 (S57) at the mouth of the substrate-access channel (pw2d) in the putative membrane-binding domain. The distant binding of the affinity probe is consistent with alignment of the C_{24} carbon of calcitriol over the heme center with the 3-OH group of the A-ring oriented away from the active site towards the protein's membrane-associated face. However, the 3-bromoester probe used to identify S57 did not perturb the heme spectra of wild-type or mutant enzyme (data not shown). It is likely

that the reactive group confounds normal trafficking and docking in the active-site. However, the specific binding of the probe to S57 implied the existence of a peripheral binding motif that provides some degree of substrate specificity on the enzyme's surface.

Site-directed mutagenesis was used to validate a role for S57 in rat CYP24A1 substrate binding [23]. The S57A mutant displayed normal binding to 25(OH)D₃ with diminished capacity to recognize and bind 1,25(OH)₂D₃. (Table III). In contrast, a charged S57D mutant displayed striking biochemical differences from wild-type enzyme. Comparing spectral binding dissociation constants (K_d) revealed that S57D had higher affinity for both 25(OH)D₃ (K_d = 0.201 μM for S57D vs. 0.311 μM for WT) and 1,25(OH)₂D₃ (K_d = 0.013 μM for S57D vs. 0.049 μM for WT) (Table III). However, in a five minute reconstitution assay, S57D was unable to keep pace with either S57A mutant or wild-type recombinant enzyme in the conversion of 1,25(OH)₂D₃ to calcitroic acid (Figure 3). This defect did not appear to be caused by altered interactions with ferredoxin (ADX) as demonstrated by similar metabolite profiles among wild-type and Ser-57 mutants under varying levels of electron transfer. This finding contradicts our previous report that demonstrated enhanced activity in S57D preparations [23]. These findings could not be reproduced and replicates with controlled enzyme preparations demonstrated that S57A and S57D displayed reduced rates of side-chain metabolism compared to wild-type enzyme but had no problem producing calcitroic acid during longer incubations (~15 min) (data not shown).

We further explored the interaction of Ser-57 and the 3-OH group of the vitamin D A ring by assessing the spectral binding properties of 1,25D analogs with altered 3-OH groups (Figure 4). A 3α-epi-1α,25(OH)₂D₃ ligand was poorly recognized and bound by both wild-type and S57D preparations (K_d = 0.421 μM for S57D and 0.460 μM for WT) (Table III). Therefore, the normal 3β orientation of the vitamin D A-ring's 3-OH group is likely a key determinant for high-affinity recognition and binding to CYP24A1, as the 3α-epimer was an extremely poor spectral agonist under all conditions tested. Studies with a 3-deoxy-1,25-(OH)₂D₃ analog clarified that the 3-OH group is important, but not the sole determinant of high affinity substrate recruitment and trafficking. The 3-deoxy analog bound wild-type and S57D preparations with similar affinity as the S57A mutant and 1,25(OH)₂D₃ (K_d = 0.136 μM and 0.145 μM for S57D and WT for 3-deoxy-1,25 vs. 0.130 μM for S57A and 1,25D) (Table III). In contrast, the S57A mutant binds the 3-deoxy analog with an affinity similar to that of wild-type enzyme for 25(OH)D₃ and the 3-deoxy compounds were better recognized in the heme center as compared to 25(OH)D₃ (Table III). This finding, while establishing the role of the 3-OH group, also supports our previous work that demonstrated the importance of the 1α carbon in high affinity binding and active-site recognition [21].

DISCUSSION

The hybrid molecular model used in this study was developed between 2002 and 2003 and is primitive when compared to contemporary motif-based models that draw from streamlined methodologies and a growing CYP structural library [38]. However, second and third generation models of rat CYP24A1 based on CYP2C5 (1NR6) and CYP2B4 (1SUO) using Swiss-Model software [43] were deemed similar with respect to positioning of the key SRS domains. We present a schematic representation of a CYP2B4-based model here to contrast the diversity of active site positioning that can be achieved using different modeling approaches and to demonstrate hypothetical folding of the N-terminus in our recombinant (Δ2-31) rat CYP24A1 construct (Figure 5); the details of the expanded 2B4-based modeling study will not be provided here.

Using a previously developed recombinant rat CYP24A1 system [21], we employed rational mutational analysis based on homology modeling to map residues involved with trafficking

and active-site alignment of vitamin D₃ substrates. Prior sequence-alignment studies of CYP24A1 identified two key F-Helix residues (M246 and F249) likely to be influential in the delivery and positioning of correctly orientated substrate [21]. Our 2C5/BM-3-hybrid rat CYP24A1 homology model supported these findings by placing M246 and F249 within 5 angstroms (Å) of the A-ring of calcitriol when docked in the active site. The homology model allowed rapid prediction of multiple residues within the heme-centered active site and pw2a- and pw2d-like substrate access channels [33,34]

Unfortunately, stability and/or folding issues precluded the study of many mutants identified by our modeling analysis (Table I). Specifically, all mutations made to the B'-helix region of CYP24A1 yielded non-functional or catalytically inactive recombinant enzyme. Extensive efforts were made to stabilize these labile mutants, but in most cases, only the P420 form of the enzyme could be extracted from whole cells. When comparing the locations of viable and non-viable mutations superimposed on the CYP24A1 homology model, it was evident that the viability of individual mutations was domain-specific. Mutations made within distal, hydrophobic domains of the protein yielded functional enzyme, while mutations made to the proximal or mitochondrial-matrix-associated soluble domains consistently produced unstable or inactive enzyme.

The inability to express stable mutants within key hydrophilic domains (B'-helix, G-helix, I-helix, K-helix and L-helix) limited the scope of our study and underscores the discrete roles these solvent accessible regions play in the maintenance of the CYP24A1 catalytic core. This phenomenon may be an artifact of our heterologous expression system, as mutagenesis of analogous hydrophilic domains has not been precluded for other CYPs, including CYP51 [35] and the more closely related cholesterol metabolizing CYPs, CYP27A1 [36,37] and CYP7A1 [42]. Our modeling and biochemical studies suggests that proper organization of the B'-helix of CYP24A1, through contacts with the heme, F, G and I-helices, and the F-G and B'-C loops is required to maintain structural integrity of the reactive heme center. Our hybrid model predicts that the B'-helix comprises the anterior portion of the active site through interactions with the F and G helices and the F-G loop, with residues W134, A136, and Y137 directed toward the vitamin D A-ring. A second generation rat CYP24A1 model, based on rabbit CYP2B4, predicts tighter interaction among the B'-helix (and B'-C loop) with the G and I-helices, which shrinks the peripheral portion of the binding cavity and repositions the substrate-binding pocket away from pw2a substrate access channel towards the opening of the pw2d channel (Figure 5). Overall, the positioning of the B'-helix appears constrained between contacts with the F and G-helices from above and the B-C loop below. Our B'-helix mutations clearly altered this dynamic and may have flooded the heme center by altering the putative water channel that appears to form near the carboxy-terminus of the B-helix [34]. The instability and folding defects associated with mutations made to the matrix-associated domains of CYP24A1 remain puzzling, but these findings call attention to the functional importance of properly partitioning water within the enzyme's catalytic core.

In contrast, site-directed mutants targeting the hydrophobic domains of CYP24A1 were generally viable with stabilities similar to wild-type enzyme. Our mutagenesis studies probed the hydrophobic active site and a pw2d-like access channel predicted by the 2C5/BM-3 hybrid model, that included portions of the N-terminus, the A', A, F, and G helices, the β3a strand and the β5 hairpin. The accommodation of mutations in these regions suggests a higher degree of conformational flexibility among these hydrophobic domains as compared soluble domains. We speculate that the amphipathic organization of the CYP24A1 surface allows the enzyme to become deeply embedded in the inner mitochondrial membrane bilayer, where dynamic, domains recruit and traffic lipophilic substrates. Our prediction is consistent with the types of membrane interaction predicted by Zhao et al. for human CYP3A4 from data derived from the

most recent structure of mammalian CYP2B4 in complex with bifanozole, as many of our viable mutations fall within putative plastic regions (PRs) described for CYP2B4 [29].

In summary, the role of CYP24A1's A'-helix remains elusive, but studies with W75 suggests this region helps organize substrate trafficking channels. W75 is modeled to reside near the mouth of both pw2a- and pw2d-like substrate access channels in CYP24A1 (Figure 5), near a highly conserved lysine-76 (K76) residue predicted to contribute to hydrogen bonding networks that direct substrate trafficking in the closely-related P450, CYP27A1 [38]. The addition of methionine (W75M) at this position likely altered channel size and/or the proton-acceptor network required to shuttle polar metabolites in and out of the active site. W75, and portions of the A' helix are also modeled to form an apolar patch, in association with the amino-terminus, and portions of the B'-helix, F-helix and F-G loop, above the heme center that is similar to the aromatic clusters described for several CYPs, including CYP2C9[5], CYP3A4 [7] and CYP27A1 [38]. The positioning of the A'-helix in our models suggest a gatekeeper function between a pw2d (substrate-access) channel and a pw2a-like channel that could allow product channeling and/or release of polar metabolites to the matrix (Figure 5). This hypothesis coincides well with simulated studies on P450BM-3 that predict neutral substrate travels through the pw2d channel, while charged metabolites egress via channel pw2a [39].

Like the W75M mutation, the A-helix mutant Y89F also decreased substrate recognition and implies a similar role for this region in maintaining electrostatic networks that directs substrate delivery. Y89 is modeled to participate in a pw2d channel (Figure 5) and is in close proximity to the conserved leucine-86 residue postulated to contribute to the pw2a substrate access channel of CYP27A1 [38]. The altered binding and accumulation of early products in the C₂₄ pathway for both A' and A-helix mutants could result from altered access of polar metabolites through modified pw2a and pw2d channels.

Biochemical analysis of F-helix mutants (M245A, S247V, and T248A) completed mutagenesis over a helical turn of the F-helix and revealed mutants with normal substrate binding with a lower rate of calcitric acid production. Unlike M246 and F249 which likely shape the active-site, these residues (M245, S247, T248) appear to provide structural cues with the adjacent A', B', and G-helices and may help define substrate access/egress channels while playing a lesser important role in substrate docking. This was not surprising considering F-helix residues (M246 & F249) are modeled to protrude into the CYP24A1 active-site on what would be the opposite side of the F-helix.

Mutations made to the protein's β 3a strand (V391T, T394V) and β 5 hairpin (I500A) more dramatically disrupted substrate binding (Table II). Modeling studies show that these variable beta-sheet domains converge in a manner that helps shape a large portion of the secosteroid binding site. In our hybrid model, V391 and T394 are located in the middle of the CYP24A1 active site where they appear to interact with the β 5 hairpin (I500) and the secosteroid ring system (Figure 1). Mutation to polar threonine (V391T) altered the hydrophobic nature of the pocket and likely repositioned the entire β 3a strand causing disruptions to associated residues in the β 5 hairpin (I500) and F-helix (F249), which would explain why V391T expressed similar biochemical defects as F249T. Mutation of β 3a strand residues diminished substrate recognition similar to F249T, suggesting an overlapping or linked role for the β 3a strand, the β 5 hairpin and the F-helix in mediating the terminal active-site docking of calcitriol and its metabolites.

The CYP24A1 hybrid model depicts the β 5 hairpin running down the I'-helix where it converges with the carboxy-terminal portion of the F-helix and F-G loop to form a hydrophobic contact site that interacts with the vitamin D *cis*-triene moiety (Figure 5). An I500A mutation in this region likely compromised the hydrophobic network with the F-helix and β 3a strand

that dictate efficient substrate binding and turnover. The influential modeling studies of Prosser et al. predict similar interactions between the F-helix (F248 of CYP27A1) and the β 5-hairpin (L516 of CYP27A1) that provide side-chain regiospecificity through interactions with the secosteroid ring system [38]. Studies by Hanomoto et al. further support these findings, as Ile-500 mutants were found to alter the species-related regiospecificity of CYP24A1 [20]. In addition, they described a charged I500D mutant with altered metabolism that yielded an uncharacterized metabolite, which further establishes a role for the β 5-hairpin in directing active-site specificity [20].

Previous affinity labeling studies with recombinant rat CYP24A1 identified Ser-57 as a putative binding residue that interacts with the 3-OH group of 25(OH)D₃ [23]. A S57D mutant showed increased binding affinity for all 25-hydroxylated vitamin D compounds tested with an apparently enhanced rate of calcitroic acid production. Later, S57 mutants were actually determined to be less efficient in producing calcitroic acid (Figure 3). The original experiments were determined to be flawed by mishandling of wild-type enzyme used for comparison. In optimized reconstitution experiments, both S57 mutants (S57A, S57D) displayed defects during early stages of the catalytic process but normal processivity overall. Our models suggested that Ser-57 is located near the mouth of CYP24A1's (pw2d) putative substrate access channel on the distal face of the protein (Figure 5). Binding studies suggested that electrostatic interactions between S57 and the 3 β -hydroxyl group of secosteroids can alter substrate recruitment, trafficking and active-site recognition. We speculate that Ser-57 may participate in an N-terminal, recognition-domain that mediates the high-affinity recruitment of vitamin D₃ substrates from the mitochondrial membrane (Figure 6). We accept that the N-terminal organization of our detergent-solubilized enzyme may not be fully representative of CYP24A1 *in vivo*, but our data suggest that the enzyme's N-terminus may function in substrate binding events as well as membrane-anchoring. This is reasonable as CYP24A1 falls into a class of peripheral or "monotopic" membrane proteins (i.e. engage only one leaflet of the lipid bilayer) that have evolved unique anchoring domains that allow for membrane insertion and the trafficking of lipophilic substrates [44]. Definitive molecular insight to the mechanism of S57D's altered biochemical properties and the existence of our proposed N-terminal binding site may only be obtained from planned x-ray structural studies.

Our work also suggests that portions of the N-terminus, A'- A- B'- F- and G'-helices may organize into a hydrophobic cluster over the putative pw2a and pw2d access channels (Figure 5). This would be consistent with the type of "apolar plateau" motifs that peripherally bound enzymes use to embed within a single leaflet of the membrane [44]. CYP3A4 and CYP27A1 are predicted to contain similar aromatic clusters [38], and because both enzymes possess 24- and 25-hydroxylase activity towards the vitamin D side chain [31], a similar organization seems likely for CYP24A1. The participation of N-terminal residues in this type of a cluster, at the interface of the membrane and the active-site, would help explain both the altered binding and metabolism of Ser-57 mutants, by placing the residue in a location where it could contribute to both substrate recruitment events and active-site docking via interactions with the vitamin D A-ring.

Our studies with 3 α -epi- and 3-deoxy-1 α ,25(OH)₂D₃ analogs yielded more evidence that direct interactions between Ser-57 and the 3- β hydroxyl of the A-ring mediate substrate recruitment, trafficking and binding events. In addition, we established that a *trans* conformation of the A-ring's 1 α and 3 β hydroxyl groups (1S, 3R) is a key determinant of high-affinity binding and recognition of substrate for both wild-type and S57 mutant rat CYP24A1. This observation may help explain the extended efficacy of 3-epi-vitamin D compounds whose cellular activity has long been attributed to altered target tissue metabolism [40,41]. Our studies with the 3-deoxy analog further clarified the determinants of high affinity binding by showing that the 3-OH group is important but not essential for active-site recognition; our previous work

demonstrated a more putative role for the 1 α -hydroxyl group in this process [21]. However, the ability of 3-deoxy ligands to spectrally perturb S57A mutant enzyme at levels similar to calcitriol for the wild-type enzyme provide compelling evidence that discrete contacts (electrostatic and/or hydrophobic) between amino terminal residues and A-ring hydroxyls provide key spatial cues that direct efficient secosteroid recognition and docking.

In conclusion, our work demonstrates the utility of using homology modeling to guide biochemical analysis of unknown structures. We have clarified the functional role of several of CYP24A1's highly conserved residues (e.g. W75, Y89, F249, V391, and I500), while providing insight into the profound differences in structural flexibility among the enzyme's soluble and membrane-associated domains. We have shown that high-affinity substrate binding is mediated by interactions among the amino terminus, the F-helix and the vitamin D A-ring, where a combination of hydrophobic and electrostatic positioning cues appear to converge around the A-ring to mediate the spatial positioning of substrate required for efficient recognition and shuttling. We have also demonstrated that the β 3a strand and β 5 hairpin contribute to the CYP24A1 active-site, as these domains converge with the C-terminal portion of the F-helix to form contacts with the vitamin D *cis*-triene system that mediate recognition, binding and processing of substrate. In addition, the flexible or membrane-associated regions studied here are modeled to contribute to a pw2d like substrate-access channel that leads from the heme center to the membrane. Interestingly, W75, and portions of the A' and A-helices could form a junction between a putative pw2a and pw2d-like access channels that may contribute to a substrate/product partitioning mechanism similar to the one predicted for P450BM-3 [39]. Additionally, the unique behavior of the S57D mutant, coupled with a putative surface location, suggests that portions of the amino-terminus form a peripheral binding motif capable of directing secosteroids into the active site. This type of structure would allow CYP24A1 to sense the associated membrane for substrate, while providing an elegant mechanism for prioritizing the metabolism of calcitriol. The true role of the residues and domains probed in this study may only be fully delineated by solving the crystal structure of CYP24A1, or a related mitochondrial CYP. The refinement of purification and crystallization methods for planned x-ray crystallographic studies of rat CYP24A1 and related CYPs is the focus of current investigations.

ACKNOWLEDGEMENTS

We acknowledge the efforts of contributors from the Omdahl lab (Letitia Lansing, Srminvas Iyer, Peter Chen, Ana Para, Charlotte Mobarak, and Margaret Gibson) for their participation in this extended project. We also thank Tudor Oprea and Christian Bologa of the University of New Mexico, Division of Biocomputing for aiding in the development of second generation CYP24A1 homology models that aided in the interpretation of our data. We would also like to acknowledge Rahul Ray and his laboratory for their contribution to the affinity-labeling studies that identified Ser-57 as an active site residue. We would like to express deep gratitude to Dr. Mark L. Chiu (Dept. of Structural Biology, Abbott Laboratories) for his long-term support and assistance in developing functional assays for CYP24A1 and for valuable insights into enzyme preparation and handling. Additionally, discussions with Dr. Julian Peterson (University of Texas Southwestern Medical Center), Dr. Eric F. Johnson and Dr. C. David Stout (The Scripps Research Institute) were most valuable in the development and interpretation of homology models and for providing insights into data derived from our structure-function analysis.

This work was supported by United States Public Health Service Grant AR45455.

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Figure 1.

A 2C5/BM-3 hybrid homology-model based representation of 1,25(OH)₂D₃ bound in the rat CYP24A1 active-site.

Calcitriol is seen docked in the rat CYP24A1 active site with the proximity of putative binding residues (M246, F249, V391, T394 and I500) shown. Also shown are influential residues from the A' (W75) and A-helices (Y89), and the conserved catalytic threonine (T330) in the I-helix. The K'-helix residue Thr-416, not studied in the current work is also shown. Thr-416 along with Ile-500, have been reported to influence the catalytic regiospecificity of rat CYP24A1 [20]. Image was generated using VMD [22].

Figure 2.

The side-chain metabolism of $1,25(\text{OH})_2\text{D}_3$ by wild-type CYP24A1 and site-directed mutants. Wild-type (WT) CYP24A1 multi-catalytic activity with $1,25(\text{OH})_2\text{D}_3$ substrate is compared to a series of model-derived mutant enzymes using enzyme reconstitution analysis (10 min. @ 37C, 0.1 μM ADX, 0.1 μM ADR). (Abbreviations are: 1,24,25 = $1,24,25(\text{OH})_3\text{D}_3$; 24-oxo-1,25 = $24\text{-oxo-}1,25(\text{OH})_2\text{D}_3$; 24-oxo-1,23,25 = $24\text{-oxo-}1,23,25(\text{OH})_3\text{D}_3$; 1,23 = $24,25,26,27\text{-tetranor-}1,23(\text{OH})_2\text{D}_3$.)

Figure 3.

Altered metabolism of $1,25(\text{OH})_2\text{D}_3$ by CYP24A1 Ser-57 mutants.

Purified enzymes were reconstituted with ADX (0.1 (1X) and 0.2 μM (2X)) and ADR (0.1 μM) then incubated with $1,25(\text{OH})_2\text{D}_3$ for 5 min. prior to metabolite extraction. S57D displays a diminished capacity to catalyze early C24 oxidation reactions. The rate of electron flux from ADX does not appear to be involved in this phenomenon.

Figure 4. Chemical structures of calcitriol (1 α ,25-dihydroxyvitamin D $_3$) and the 3-OH analogs (3 α -epi- and 3-deoxy-1 α ,25(OH) $_2$ D $_3$) used in the present study.

Figure 5.

Schematic representations of the A.) 2C5/BM-3 Hybrid and B) CYP2B4-based homology models of rat CYP24A1.

Calcitriol (yellow) is seen docked over the heme center (black) in both models, with the active site adopting unique positions based on the positioning of individual helices (A=Gold, A'=Turquoise, B'=Orange F=Magenta; G=Blue, I=White). Both models depict close interaction among the A', B', F- and G-helices, β 3a strand and β 5 hairpin that form the top of the active-site near the opening of pw2a-like substrate access channel. Interactions between the B', F, G and I' helices appear to control the size and shape of the active-site and may control the pw2a channel that functions to shuttle polar products to the matrix. W75 is seen at the

junction of the pw2a channel and a pw2d-like channel predicted to pass out the A helix (Y89) towards the enzyme's N-terminus and the putative active-site residue Ser-57, which is fully modeled in panel B. Images in this figure were created using PyMol (www.pymol.org).

6. A model for CYP24A1 membrane binding and the proposed amino-terminal binding site. Serine-57 in rat CYP24A1 may form a contact point with the 3 β -hydroxyl group of calcitriol and that functions in substrate recruitment, trafficking and active-site docking events. Noted are the locations of viable and non-viable rat CYP24A1 mutants developed for these studies; in short, functional enzyme was limited to mutations made within membrane-associated domains implying a higher degree of conformational flexibility associated with membrane binding. The location of these flexible domains guided the hypothetical membrane insertion presented here. An N-terminal binding site, shown with a key contact point (S57), may function to recruit and guide substrate through a pw2d-like access channel. Together with portions of the F-helix, this N-terminal binding site is hypothesized to provide important spatial cues to the vitamin D A-ring that allow for efficient trafficking and docking of substrate. The CYP image was generated using VMD [22].

Table I
Oligonucleotides and locations of rat CYP24A1 mutants.

Viable Mutants	Location	Oligonucleotide
S57A	N-terminus	5'-ACTCGAAACGTCACCGCTTTGCCTGGGCCACC-3' 5'-GGTGGGCCAGGCAAAGCGGTGACGTTTCGAGT-3'
S57D	N-terminus	5'-ACTCGAAACGTCACCGACTTGCCTGGGCCACC-3' 5'-GGTGGGCCAGGCAAAGTCGGTGACGTTTCGAGT-3'
W75A	A'-Helix	5'-GAGTCTACTGGAGATTTGCCGAAAGGTGCCTGAAGAA-3' 5'-TTCTTCAGGCACCTTTCGCAAATCTCCAGTAGACTC-3'
Y89F	A'-Helix	5'-CGACACGCTGGCAGAGTTCCACAAGAAGTATGGCC-3' 5'-GGCCATACTTCTTGTGGAACTCTGCCAGCGTGTGCG-3'
M245A	F-Helix	5'-ACGGCCATCAAAACGCGCATGAGCACGTTTGGG-3' 5'-CCCAAACGTGCTCATCGCCGTTTTGATGGCCGT-3'
M246F	F-Helix	5'-ACGGCCATCAAAACGATGTTTCAGCACGTTTGGGAAGATG-3' 5'-CATCTTCCCAAACGTGCTGAACATCGTTTTGATGGCCGT-3'
S247V	F-Helix	5'-ATCAAAACGATGATGGTTACGTTTGGGAAGATG-3' 5'-CATCTTCCCAAACGTAACCATCATCGCTTTGAT-3'
T248A	F-Helix	5'-AAAACGATGATGAGCGCGTTTGGGAAGATGATG-3' 5'-CATCATCTTCCCAAACGCGCTCATCATCGTTTT-3'
F249T	F-Helix	5'-AAAACGATGATGAGCACGACTGGGAAGATGATGGTGACC-3' 5'-GGTCACCATCATCTTCCAGTCGTGCTCATCATCGTTTT-3'
V391T	β3a	5'-GAGGCTTACCCCAAGTACCCATTTACAACCTCGGACC-3' 5'-GGTCCGAGTTGTAAATGGGGTACTTGGGGTAAGCCTC-3'
T394V	β3a	5'-CCCAAGTGTCGCAATTTGTGACTCGGACCCCTTGACAAACC-3' 5'-GGTTTGTCAAGGCTCCGAGTCACAAATGGCACACTTGGG-3'
I500A	β5	5'-GGAGATGCTGCACCTTGGAGCTCTGGTACCCAGCCGGG-3' 5'-CCCGGTGGGTACCAGAGCTCAAGGTGCAGCATCTCC-3'

The following mutants were cloned, expressed and purified but determined to be non-viable (excess P420 form) for biochemical analysis in this study: **Surface Cysteines** (C44A, C194A, C303A); **B'-Helix** (P133L, W134S, A136E&D, Y137H, R138A, H140R, R141V, Y145A); **F-Helix** (F238S, I242D); **G-Helix** (W268S, W274S); **I-Helix** (E322A, L325D, A326S, T330V); **K-Helix** (K378A, K382A); β3a (V392I); **L-Helix** (R465F, R466F); β5 (L496A, I500D, V502F).

Table II
Substrate binding properties of recombinant rat CYP24A1 wild-type and site-directed mutants.

Enzyme	Location	Spectral Perturbation ^a _{1,25D} (ΔAbs _{Max} -ΔAbs _{Free})	Dissociation Constant ^a _{1,25D} Kd (μM)	Spectral Perturbation ^a _{25D} (ΔAbs _{Max} -ΔAbs _{Free})	Dissociation Constant ^a _{25D} Kd (μM)
Wild-Type	---				
S57D	N-terminus	0.044 ± 0.003 ^c	0.049 ± 0.006 ^c	0.025 ± 0.002	0.311 ± 0.014
W75M	A-helix	0.048 ± 0.002	0.013 ± 0.002 ^e	0.029 ± 0.001	0.201 ± 0.008 ^e
Y89F	A-helix	0.050 ± 0.001	0.075 ± 0.006 ^d	0.028 ± 0.002	0.283 ± 0.011
M245A	F-helix	0.028 ± 0.002 ^e	0.165 ± 0.011 ^e	0.015 ± 0.001 ^e	0.353 ± 0.020 ^d
M246F	F-helix	0.035 ± 0.001 ^d	0.058 ± 0.002	0.017 ± 0.002 ^e	0.207 ± 0.025 ^e
S247V	F-helix	0.056 ± 0.001 ^d	0.123 ± 0.021 ^e	0.027 ± 0.001	0.346 ± 0.023
T248A	F-helix	0.049 ± 0.001	0.036 ± 0.002 ^d	0.029 ± 0.001	0.279 ± 0.015
F249T	F-helix	0.048 ± 0.001	0.066 ± 0.003 ^d	0.014 ± 0.001 ^e	0.253 ± 0.007 ^e
V391T	β3a	0.012 ± 0.003 ^e	0.278 ± 0.025 ^e	0.006 ± 0.001 ^e	0.829 ± 0.009 ^e
T394V	β3a	0.026 ± 0.001 ^e	0.198 ± 0.017 ^e	0.014 ± 0.001 ^e	0.216 ± 0.003 ^e
I500A	β5	0.039 ± 0.003	0.161 ± 0.027 ^e	0.015 ± 0.001 ^e	0.286 ± 0.032
		0.025 ± 0.001 ^e	0.235 ± 0.015 ^e	0.012 ± 0.001 ^e	0.419 ± 0.016 ^e

^a ΔAbsFree represents the initial difference in optical absorbance between 415 nm and 390 nm for the free enzyme prior to each titration.

^b ΔAbsMax represents the maximal change in optical absorbance between 415 nm and 390 nm during each titration.

^c Mean ± SD for wild-type (WT) experiments (N=4 or greater). Values for mutants (N=3 or greater).

^d p < 0.05, compared to corresponding WT mean.

^e p < 0.001, compared to corresponding WT mean.

* Measure of binding efficiency in the substrate pocket as reflected by perturbation of the heme spectra in the enzyme's active site for 1,25D (1α,25-dihydroxyvitamin D3 and 25D (25-hydroxyvitamin D3).

Table III

Substrate binding properties of recombinant rat CY24A1 wild-type and serine-57 mutants for vitamin D3 metabolites and 3-OH analogs.

Substrate	Enzyme	Spectral Perturbation* ($\Delta\text{Abs}_{\text{Max}} - \Delta\text{Abs}_{\text{Free}}$)	Dissociation Constant Kd (μM)
25-(OH)D ₃	WT	0.025 ± 0.002	0.311 ± 0.014
	S57D	0.029 ± 0.001	0.201 ± 0.008 ^e
	S57A	0.017 ± 0.003 ^e	0.320 ± 0.025
1,25-(OH) ₂ D ₃	WT	0.044 ± 0.003	0.049 ± 0.006
	S57D	0.048 ± 0.002	0.013 ± 0.002 ^e
	S57A	0.031 ± 0.003 ^e	0.130 ± 0.008 ^e
3-epi-1,25-(OH) ₂ D ₃	WT	0.011 ± 0.001	0.460 ± 0.013
	S57D	0.015 ± 0.001	0.421 ± 0.029
	S57A	0.012 ± 0.002	0.394 ± 0.024 ^d
3-deoxy-1,25-(OH) ₂ D ₃	WT	0.032 ± 0.002	0.145 ± 0.008
	S57D	0.029 ± 0.001	0.136 ± 0.028
	S57A	0.039 ± 0.003 ^d	0.315 ± 0.016 ^e

^a $\Delta\text{Abs}_{\text{Free}}$ represents the initial difference in optical absorbance between 415 nm and 390 nm for the free enzyme prior to each titration.

^b $\Delta\text{Abs}_{\text{Max}}$ represents the maximal change in optical absorbance between 415 nm and 390 nm during each titration.

^c Mean ± SD for wild-type (WT) experiments (N=4 or greater). Values for mutants (N=3 or greater).

^d p < 0.05, compared to corresponding WT mean.

^e p < 0.001, compared to corresponding WT mean.

* Measure of binding efficiency in the substrate pocket as reflected by perturbation of the heme spectra in the enzyme's active site.