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Expression, Structure-Function, and Molecular Modeling of Vitamin D P450s

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Abstract Although vitamin D_3 is a natural product of a sunlight-mediated process in the skin, the secosteroid's biological function is dependent upon specific cytochrome P450 enzymes that mediate the parent vitamin's bioactivation and inactivation. Cytochrome P450C1 (CYP27B1) is the regulatory rate-limiting enzyme that directs the bioactivation process through introduction of a C-1α hydroxyl group. The resultant 1,25-dihydroxyvitamin D_3 (1,25D) is the biologically active secosteroid hormone that directs the multitude of vitamin D-dependent actions involved with calcium homeostasis, cellular differentiation and growth, and the immune response. The circulating and cellular level of 1,25D is regulated through a coordinated process involving the hormone's synthesis and degradation. Central to the degradation and turnover of 1,25D is the regulatory multi-catalytic cytochrome P450C24 (CYP24) enzyme that directs the introduction of C-24R groups onto targeted 25-hydroxy substrates. Discussed in this article is the action of the rat CYP24 to catalyze the side-chain oxidation and cleavage of 25-hydroxylated vitamin D metabolites. Expression and characterization of purified recombinant rat CYP24 is discussed in light of mutations directed at the enzyme's active site. J. Cell. Biochem. 88: 356–362, 2003.

Key words: CYP27B1; CYP24; vitamin D; hydroxylase; structure function

HISTORICAL BACKGROUND

Vitamin D research in Hector DeLuca's laboratory during the early 1970s focused on the identification and cellular activity of the secosteroid hormone 1,25-dihydroxyvitamin D_3 (1,25D). Early in the process of characterizing the vitamin D metabolic pathway, it was determined that the enzyme responsible for 1,25D synthesis from 25-hydroxyvitami D_3 (25D) prehormone was preferentially expressed in kidney mitochondria [Gray et al., 1972]. Conducting cell-free studies with isolated chick-kidney mitochondria, it became evident that the organelle produced not only 1,25D but another major metabolite with less polar properties. This metabolite was subsequently identified as 24,25-dihydroxyvitamin D_3 (24,25D) and was determined to be the most prevalent serum metabolite of 25-hydroxyvitamin D_3 (25D). Subsequent studies of the 1-hydroxylase and 24-hdyroxylase enzymes during different vitamin D and calcium modulatory states revealed a general reciprocal enzyme-regulatory pattern. Elevated 1-hydroxylase and suppressed 24-hydroxylase activities were expressed during low-calcium and/or low-vitamin D states whereas 1-hydroxylase was suppressed and 24-hydroxylase induced during high-ambient calcium and/or high vitamin D conditions. Still fresh in my memory are the lunch time discussions in Hector's laboratory in which the physiological significance of the regulated enzyme activities were debated with mixed clarity. Most insightful was the reasoning that linked an increase in 1-hydroxylase activity during low-calcium or low-vitamin D states to the desired physiological response of increased calcium absorption in response to elevated 1,25D synthesis. However, a reasoned rationale for elevated 24-hdyrxoylase activity during high-ambient-calcium states was more confounding, since the 24,25D metabolite was envisioned during this time to promote calcium uptake in a manner approaching that of 1,25D although

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less efficiently. It would take numerous years of study in various research groups to establish a central role for the 24-hydroxylase enzyme in the vitamin D inactivation pathway [Makin et al., 1989; Reddy and Tserng, 1989]. This article will focus on current insights into these two regulatory enzymes and their seminal role in the vitamin D pathway with particular emphasis given to the 24-hydroxylase enzyme. A more extensive discussion of the vitamin D hydroxylases and their actions are presented in recent reviews [Jones et al., 1998; Omdahl et al., 2002].

BIOCHEMICAL CHARACTERIZATION

The vitamin D hydroxylases were determined from initial studies to require mitochondrial-derived NADPH reducing equivalents and to utilize molecular oxygen in the hydroxylation reactions [Ghazarian and DeLuca, 1974a]. These enzymes were further characterized as being inhibited by carbon monoxide and chemical inhibitors of cytochrome P450 enzymes. Additional verification that the vitamin D hydroxylase enzymes were members of the mitochondrial family of P450 enzyme was obtained from functional reconstitution studies using mitochondrial adrenodoxin and adrenodoxin-reductase electron transfer proteins [Ghazarian et al., 1973; Ghazarian et al., 1974b; Pedersen et al., 1976]. Numerous attempts over a number of years to purify the enzymes from animal tissues were unsuccessful. However, Ohyama succeeded in cloning the rat 24-hydroxylase enzyme in 1991 and determined the enzyme's derived sequence [Ohyama and Okuda, 1991]. The enzyme expressed the functional domains characteristic of mitochondrial P450 enzymes, however, its sequence was less than 40% similar to other family members, and therefore, it was given a new classification as cytochrome P450C24 (or CYP24). Subsequent investigations used homology and functional-cloning technology to clone the 1-hydroxylase enzyme [Shinki et al., 1997; St Arnaud et al., 1997; Takeyama et al., 1997]. The enzyme was verified at the sequence level to be a mitochondrial P450 and to contain greatest sequence similarity to cytochrome P450C27A1; hence, its formal classification as cytochrome P450C1 (or CYP27B1) [Shinki et al., 1997]. Both vitamin D P450s contain mitochondrial targeting sequences that direct the enzymes' uptake and localization to the inner membrane where they accept NADPH-derived electrons through a surface docking-domain for ferredoxin, a small electron-transfer iron-sulfur protein. In the reduced state, the P450C1 and P450C24 enzymes utilize molecular oxygen and catalyze the insertion of hydroxyl groups at the C1α and C24R positions in 25-hydroxyvitamin D metabolites, respectively, utilizing an activated oxyferryl-heme intermediate (Fig. 1)

TISSUE DISTRIBUTION AND REGULATION

The preferred site for P450C1 and P450C24 expression is the kidney, which functions as the major endocrine organ for 1,25D synthesis. However, the enzymes display a wide tissue distribution in which they function to regulate cellular levels of 1,25D through their autocrine/paracrine functions. The enzymes are expressed in endocrine, neurological, reproductive, and immunological cells as well as in numerous hyper-proliferative cancer cells where the hormone 1,25D functions to direct a number of cell specific functions [Jones et al., 1998; Omdahl et al., 2002]. Expression of the two vitamin D

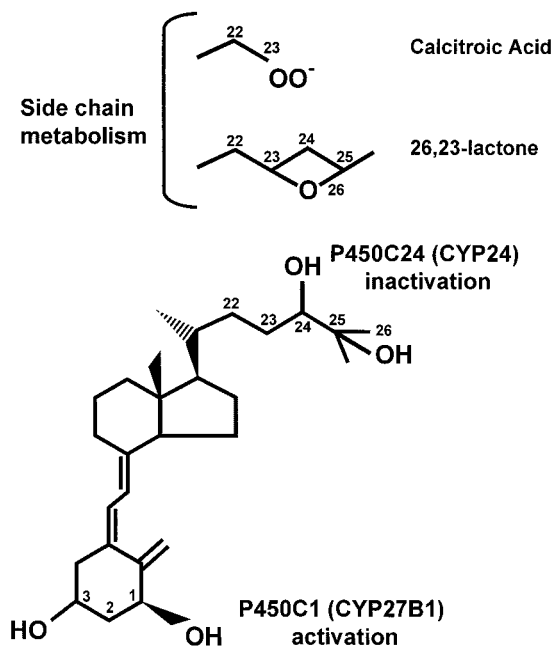

Fig. 1. Hydroxylated Vitamin D_3 Structure. The location for P450C1 and P450C24 actions are shown together with side-chain modifications catalyzed by P450C24.

P450s is modulated by a host of cell-dependent regulatory agents. Renal P450C1 expression can be upregulated by parathyroid hormone (PTH) and calcitonin (CT) and decreased ambient calcium and phosphate levels [Bland et al., 1999; Murayama et al., 1999; Yoshida et al., 2001]. The most potent down regulator of P450C1 is 1,25D with high-calcium levels also functioning as an enzyme suppressive signal [Weisinger et al., 1989; Bland et al., 1999; Murayama et al., 1999]. In contrast to the action of 1,25D to down-regulate P450C1 expression, the secosteroid hormone is the major inducer of *P450C24* gene expression [Omdahl and May, 1997; Takeyama et al., 1997] whereas PTH and low-phosphate function to suppress 24-hydroxylase expression through separate regulatory mechanisms [Wu et al., 1996; Zierold et al., 2001].

Enzyme regulation in bone osteoblast is similar to kidney but interestingly PTH has a synergistic early-phase action to augment the action of 1,25D to induce P450C24 expression [Serda and Omdahl, personal observation]. Intestinal and macrophage cells do not respond to PTH due to lack of the hormone receptor. Nevertheless, intestine is a major organ for P450C24 induction by 1,25D [Demers et al., 1997]. In contrast, INF-γ activated macrophages are refractory to the 1,25D down-regulation of P450C1 and induction of P450C24 expression [Vidal et al., 2002].

The molecular mechanism of enzyme expression has been explored using promoter constructs for the two enzymes. Through the use of upstream-promoter constructs, it has been possible to identify basal and hormone responsive regions that are involved in the modulatory action of PTH to upregulate human *P450C1* gene expression [Brenza et al., 1998; Murayama et al., 1998; Kong et al., 1999; Gao et al., 2002]. The action of 1,25D to induce P450C24 expression has been studied in greatest detail using the rat *P450C24* upstream promoter model. Through these investigations, it has been possible to identify two vitamin D response elements involved with the 1,25D-transcriptional regulation of the enzyme expression and a non-nuclear process whereby the secosteroid hormone functions through the MAP-kinase-dependent pathways to direct the phosphorylation of specific transcription factors involved with 1,25D inductive action [Dwivedi et al., 2002].

CHARACTERIZATION AND FUNCTION OF VITAMIN D HYDROXYLASES

P450C1

Cytochrome P450C1 is a ~52 kDa hemoprotein that is anchored to the inner mitochondrial membrane and functions to direct the 1α-hydroxylation of 25-hydroxyvitamin D metabolites. Molecular oxygen is split during the catalytic reactions with one atom used in the hydroxylation step and another atom reduced to water; hence, the enzyme belongs to the class of mixed-function oxidase enzymes. It does not metabolize adrenal or sex steroids that lack the 25-hydroxy secosteroid configuration. The apparent Km for P450C1 differs between species and tissues with a broad range of 1–16 μM [Omdahl et al., 2002].

Isolation and characterization of the vitamin D 1-hydroxylase has long been regarded as a fundamental step in understanding the cellular expression and function of 1,25D. Aside from one putative success in isolating the enzyme [Hiwatashi et al., 1982] other initiatives resulted in solubilizing the active enzyme but not in its purification and molecular characterization. The non-specific low-efficiency action of CYP27A1 to1-hydroxylate 25-hydroxyvitamin D led to an early hypothesis that P540C1 activity was resident in the highly accommodating CYP27A1 enzyme. However, cloning of the *P450C1* gene and subsequent P450C1 knockout studies demonstrated with clarity that CYP27A1 does not have a significant role in the synthesis of 1,25D. Using information from cloning of the *P450C1* gene, enzyme expression constructs have been transfected in a variety of eukaryotic cells and used to express 1-hydroxylase activity [Fu et al., 1997; Kitanaka et al., 1999]. However, attempts to express P450C1 in bacteria for the purpose of isolating and purifying the enzyme has met with limited success. Membrane fractions from *E. coli* transformed with a P450C1 expression construct display 1-hydroxylase-enzyme activity, but the subsequent isolation and characterization of the enzyme has not been reported [Sakaki et al., 1999b; Sawada et al., 1999].

P450C24

The ~53 kDa P450C24 mitochondrial hemoprotein was initially identified by its ability to direct the 24R-hydryoxylation of 25D to 24,25-dihydroxyvitamin D_3 (24,25D) [Knutson and

DeLuca, 1974]. A biological role for 24,25D has been proposed with a most recent example being its action to stimulate intramembraneous bone formation. Supportive of such an action, *P450C24*-gene knockout mice display impaired intramembraneous bone formation in the presence of a very high ambient 1,25D level. However, a cross of the P450C24 null animals with VDR-knockout mice corrected the bone formation abnormality [St Arnaud et al., 2000], which indicated that inhibition of intramembraneous bone formation was due to the VDR-dependent action of 1,25D and not a lack of 24,25D.

Based upon studies in which 24,25D synthesis from 25D was assayed using intact cells and enzyme extracts, apparent Km values of 0.5–3 μM have been determined that are significantly higher than values obtained for the 24-hydroxylation of 1,25D (0.1–0.5 μM) [Burgos-Trinidad and DeLuca, 1991; Omdahl et al., 2002]. More recent studies demonstrated a multi-catalytic action for P450C24 in which the enzyme is solely responsible for the side-chain oxidative modification of 25-hydroxy secosteroids. Depending upon the species-specific isoform, the enzyme can preferentially express C-23 and C-24 hydroxylase activity [Beckman et al., 1996; Sakaki et al., 1999a]. For example, rat P450C24 expresses predominantly C-24 hydroxylase and side-chain cleavage activity whereas isoforms with mainly C-23 activity (e.g., human P450C24) catalyze reactions leading to a cyclic 26,23-lactone end-product (Fig. 1). The current work focuses on the rat P450C24 whose action sequentially entails 24-hydroxylation, 24-alcohol formation, 23-hydroxylation, side-chain cleavage, 23-alcohol generation, and 23-carboxylic acid formation that results in the loss of four carbons from the side chain [Omdahl and May, 1997] and the synthesis of a C-23 carboxylate product (e.g., calcitroic acid from the side-chain oxidative cleavage of 1,25D) (Fig. 1) that is subsequently cleared through the kidney.

Isolation and purification of recombinant rat P450C24 has been accomplished through expression of the recombinant enzyme in *E. coli* [Omdahl et al., 2001; Bobrovnikova and Omdahl, in preparation]. The mature part of the coding region was cloned into an IPTG-inducible vector and expressed in *E. coli* supplemented with the heme precursor δ-amino levulinic acid. Bacterial incubation at 28° resulted in a high expression level (~1.3 μmol/L) that facilitated the purification of milligram quantities of pure enzyme by use of adrenodoxin-affinity and hydroxyapatite chromatography. Characteristic cytochrome P450C24 absolute and CO-reduced-difference spectra were obtained with pure enzyme. Enzyme reconstitution reactions with adrenodoxin and adrenodoxin reductase demonstrated the enzyme's ability to express all steps involved in the side-chain oxidative cleavage of 1,25D to calcitroic acid (Fig. 2). In addition, the recombinant enzyme was functional in the metabolism of 19-nor-1,25-dihydroxyvitamin D_2 [Emery and Omdahl, personal communication]. However, purified rat P450C24 did not appear to catalyze the side-chain cleavage of 1,25-dihydroxyvitamin D_2 [Horst et al., accompanying chapter], which emphasizes the importance of the C-24 chemistry in developing susceptible vicinal hydroxyl groups (C-23/C-24) for the side-chain cleavage of 25-hydroxy secoseroids.

Substrate-induced spectral shifts with the purified rat P450C24 have been used to document relative binding affinities for natural substrate and azole-analog P450 inhibitors. Highest binding affinity was observed for 1,25D that was more than five times greater than for 25D. A number of azole-analogs (284693, 265428, VAB247, and VAB251 from Novartis Research Institute) were determined to bind the active site and function as competitive inhibitors to rat CYP24 [Chen and Omdahl, personal communication]. Dissociation of intermediates in the P450C24 reactions pathway was dependent upon the reductive kinetics of the reconstituted enzyme reactions. Therefore, Km values approaching those seen in vivo are only obtained at lower reductive rates that favor the accumulation of intermediate metabolites and their dissociation from the enzyme.

Initial active site analysis has been conducted for rat P450C24 using site-directed mutagenesis techniques [Bobrovnikova and Omdahl, personal communication]. Studies were directed to the F-helix region that is known to form part of the substrate access channel and to contain residues that are important for substrate binding and alignment with the active-site heme. Sequence alignment of known P450C24 and P450C1 enzymes were used to identify P450C24 specific regions that are associated with substrate binding (Fig. 3). These regions were identified based upon specific substrate-binding pockets for the two

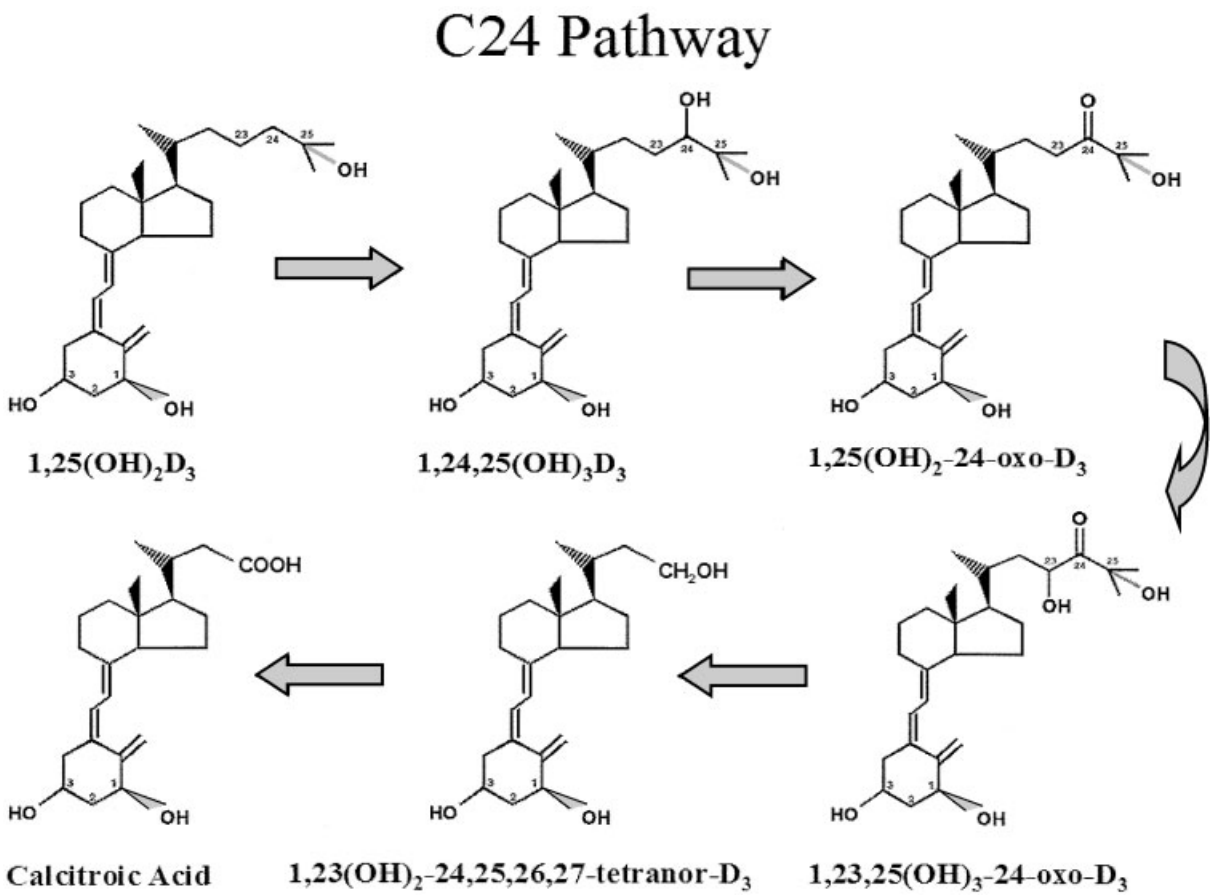

Fig. 2. C24 Pathway for 1,25-dihydroxyvitmain D_3 Metabolism. Sequence for side-chain oxidative metabolism and cleavage is shown for 1,25-dihydroxyvitamin D_3 with recombinant rat P450C24 enzyme.

F-helix Domain

R-24	m	**m**	g	t	**f**	g	k	m	**N**
M-24	m	**m**	g	t	**f**	g	k	m	**N**
H-24	m	**m**	g	t	**f**	g	k	m	**N**
R-1	v	**f**	v	a	**t**	l	l	t	**N**
M-1	v	**f**	v	a	**t**	l	l	t	**N**
H-1	v	**f**	v	a	**t**	l	l	t	**N**
		246			**249**				

Fig. 3. Active-site alignment for domain associated with substrate-binding. A segment of the F-helix domain is shown for P45024 and P450C1 isoforms. Sequence alignment is shown for rat (R-24 & R-1), mouse (M-24 & M-1), and human (H-24 & H-1) enzymes with the mutated sites shown in bold and lower case and a residue common to all six isoforms is capitalized and shown in bold.

enzymes that involve binding of the A-ring regions (i.e., P450C1) or the diametric 25-hydroxyl side-chain (i.e., P450C24). Based upon the sequence-alignment difference analysis, met 246 and phe 249 were identified as residues that could participate in binding to the side-chain of 25-hydroxy secosteroid substrates and were mutated to contain residues present in P450C1. To evaluate this hypothesis, met 246 was mutated to phe (M246F) and phe 249 was mutated to thr (F249T). The mutant enzymes were subsequently expressed in *E. coli*, purified and tested for function in enzyme-reconstitution reactions. Mutant M246F displayed increased 24-hydroxylase activity in metabolizing 25D to 24,25D but showed lower activity in the subsequent oxidation steps when compared to control wild-type enzyme [Bobrovnikova K and Omdahl, personal communication]. In contrast,

the F249T mutant displayed low 24-hydroxylase activity with an even lower rate of formation for subsequent oxidation products. For either mutation, the dissociation of 24,25D from CYP24 was greater than for the subsequent oxidation products. Both mutants enzymes expressed higher 24-hydroxylase activity when metabolizing 1,25D (i.e., greater 1,24,25D synthesis), which was coupled with lower calcitroic acid synthesis in both instances. Therefore, it would appear from these initial mutational-analysis studies that M246 and F249 within the F-helix region participate in the substrate side-chain alignment with the oxidative heme center that is required for the multi-functional action of CYP24 to oxidize the side chain of 25D and 1,25D. At present, a more complete investigation is in progress to utilize similarity modeling and substrate-docking analysis to identify the full complement of active-site residues that are involved in the orientation and binding of 25-hydroxy vitamin D metabolites during their side-chain oxidation and cleavage.

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