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CYTOCHROME P450-MEDIATED METABOLISM OF VITAMIN D

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Abbreviations:

25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3;
CKD-MBD, chronic kidney disease-metabolic bone disease; CYP, cytochrome P450;
CTX, cerebrotendinous xanthomatosis; DBP, Vitamin D-binding protein;
FGF-23, fibroblast growth factor-23;
GFR, glomerular filtration rate; IIH, idiopathic infantile hypercalcemia;
PTH, parathyroid hormone; TLR, toll-like receptor;
VDDR-Type 1, vitamin D-dependent rickets type-1;
VDR, vitamin D receptor; VDRE, vitamin D responsive element;

Abstract

The vitamin D signal transduction system involves a series of cytochrome P450-containing sterol hydroxylases to generate and degrade the active hormone, 1α,25-dihydroxyvitamin D_3 which serves as a ligand for the vitamin D receptor-mediated transcriptional gene expression, described in companion chapters in this review series. This review will update our current knowledge of the specific anabolic cytochrome P450s involved in 25- and 1α-hydroxylation, as well as the catabolic cytochrome P450 involved in 24- and 23-hydroxylation steps, which are believed to initiate inactivation of the vitamin D molecule. We will focus on the biochemical properties of these enzymes; key residues in their active sites derived from crystal structures and mutagenesis studies; the physiological roles of these enzymes as determined by animal knockout studies and human genetic diseases; and the regulation of these different cytochrome P450s by extracellular ions and peptide modulators. We will highlight the importance of these cytochrome P450s in the pathogenesis of kidney disease, metabolic bone disease and hyperproliferative diseases such as psoriasis and cancer; as well as to explore potential future developments in the field.

Supplementary key words:

1,25-$(OH)_2D_3$, CYP2R1, CYP27A1, CYP27B1, CYP24A1, vitamin D-dependent rickets, chronic kidney disease, idiopathic infantile hypercalcemia, vitamin D analog, regioselectivity.

Introduction

The activation of vitamin D_3 is accomplished by sequential steps of 25-hydroxylation to produce the main circulating form, 25-hydroxyvitamin D [25-OH-D_3] followed by 1α-hydroxylation to the hormonal form, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] [1] (Figure1). The initial step of 25-hydroxylation occurs in the liver [2], while the second step occurs both in the kidney and extra-renal sites [3,4]. The fat-soluble vitamin D and its metabolites are transported from one tissue to another on the vitamin D-binding protein (DBP), DBP showing different affinity for the individual metabolites [5]. The cell-surface receptor, megalin-cubilin, is thought to facilitate the endocytosis of a DBP-bound 25-OH-D_3 into a number of cell types, especially kidney cells [6]. While it is widely believed that DBP is an essential component of the vitamin D signal transduction system, the DBP-knockout mouse is normocalcemic and exhibits normal tissue distribution of vitamin D metabolites and vitamin D action despite exhibiting very low blood levels of 1,25-$(OH)_2D_3$. This unexpected phenotype raised questions about DBP's exact role: essential transporter or buffer against toxicity? [7,8]. Work performed on the 25-hydroxylases over the past four decades in humans and a variety of animal species has revealed that several cytochrome P450 enzymes[1] (CYPs): CYP2R1, CYP27A1, CYP3A4, CYP2D25, and perhaps others, are capable of 25-hydroxylation of vitamin D_3 or related compounds and thus can be referred to as vitamin D_3-25-hydroxylases, it is CYP2R1 that is emerging as the physiologically-relevant enzyme [9]. On the other hand, there is no ambiguity over the second step of 1α-hydroxylation or the 25-OH-D_3-1α-hydroxylase enzyme responsible, which is carried by a single cytochrome P450[1] named CYP27B1 [10,11].

Footnote[1]: The nomenclature of all cytochromes P450, including those involved in vitamin D metabolism, is the responsibility of an internationally-acknowledged group headed by D. Nelson [12]. CYP names are based upon sequence similarity, function & other considerations.

The inactivation of vitamin D is carried out by the mitochondrial enzyme, 25-hydroxyvitamin D_3-24-hydroxylase first described in the early 1970s and initially believed to be involved solely in the renal 24-hydroxylation of 25-OH-D_3 [13]. Work performed over the last 35 years has shown that 24-hydroxylase enzyme activity is the result of CYP24A1 [5,14]. CYP24A1 catalyzes the conversion of both 25-OH-D_3 and 1,25-$(OH)_2D_3$ into a series of 24- and 23-hydroxylated products targeted for excretion along well-established pathways culminating in the water-soluble biliary metabolite, calcitroic acid or a 26,23-lactone.

This chapter assembles the most currently-pertinent literature on the activating and inactivating enzymes of vitamin D metabolism, in the process highlighting protein structure & enzymatic properties, crystal structures, gene organization & mutational analysis and regulation. Due to space restrictions, this overview will not cover all of the rich history which went into the early enzymology or cloning of these cytochrome P450-containing enzymes, much of which has been extensively reviewed elsewhere [15,16,5,17].

General Information regarding Vitamin D Hydroxylases

Table 1 summarizes pertinent information about all of the vitamin D-metabolizing CYPs including both the activating and inactivating enzymes. CYPs are classified into two main subtypes based upon their subcellular location: microsomal or mitochondrial; with vitamin D metabolism featuring both subtypes [14]. Both mitochondrial and microsomal CYP subtypes do not function alone but are components of electron transport chains. As with all mitochondrial CYPs, the functional enzyme activity for mitochondrial vitamin D-related CYPs (eg CYP27A1, CYP27B1, CYP24A1) requires the assistance of two additional electron-transporting proteins consisting of a general purpose ferredoxin reductase, a general purpose-ferredoxin and a highly specific CYP (Figure 2A). In contrast, microsomal CYPs (eg CYP2R1) require a single general-purpose protein NADPH-cytochrome P450 reductase (Figure 2B). All of the vitamin D-related

CYPs catalyze single or multiple hydroxylation reactions on specific carbons of the vitamin D substrate using a transient, heme-bound, Fe-O intermediate. The exact site of hydroxylation, termed regioselectivity, can be somewhat variable with vitamin D related-CYPs, human CYP24A1 being documented to hydroxylate at C23, C24, or C26.

From alignments of the vitamin D-related CYPs (Figure 3), it is immediately apparent that all CYP proteins possess around 500 amino acids and a size of 50-55 kDa, featuring abundant highly-conserved residues which suggest a common secondary structure with multiple highly-conserved helices (designated A-L) connected by loops and β-sheet structures. All CYPs possess a cysteine residue and two other residues near to the C terminus which covalently-bind and align the heme group, in addition to several other domains for interaction with the electron transferring machinery, such as ferredoxin or NADPH-cytochrome P450 reductase. The N-terminus is thought to insert into the endoplasmic reticular membrane for microsomal CYPs or the inner mitochondrial membrane for mitochondrial CYPs. The substrate-binding pocket is formed by several secondary structures folded around the distal face of the heme-group so that the substrate can be brought to within 3.2Å of the iron atom for hydroxylation. An analysis of the heme-ligand geometry of 49 substrate-bound crystal structures revealed the hydroxylation target carbons actually adopt a spatially conserved orientation to the heme iron and this can be triangulated for use in docking studies [18].

Attempts to identify the key substrate-binding residues were originally guided by homology models [18-22] based upon 10-20 available crystal structures from unrelated soluble prokaryotic CYPs. Recently, the study of the active site of vitamin D-related CYPs has been further advanced by the emergence of X-ray crystallography-derived models of CYP2R1 [23] and CYP24A1 [24] (Figure 4). In addition, two bacterial vitamin D hydroxylases capable of sequentially hydroxylating vitamin D_3 to 1,25-$(OH)_2D_3$ at production levels; CYP105A1 from Streptomyces griseolus (2zbz.pdb) [25] and P450 Vdh from Pseudonocardia autotrophica

(3a4g.pdb) [26] have been determined. Mutational analyses to pinpoint amino-acid residues involved in contact with the main functional groups (hydroxyls) or hydrophobic cis-triene of the vitamin D substrate have been largely completed. Future work to define residues closest to the hydroxylation-sensitive 1α-position in CYP27B1 or the side-chain C-23 to 27 carbons in the side-chain hydroxylases (CYP2R1, CYP24A1 and CYP27A1), are currently in progress in various laboratories.

Vitamin D_3-25-hydroxylases

As outlined above, there has been no shortage of CYPs proposed as candidates for the title of physiologically-relevant vitamin D_3-25-hydroxylase. Early work suggested that there were both mitochondrial and microsomal 25-hydroxylase enzyme activities [22,28], and experiments with the perfused rat liver suggested that these might be a low-affinity, high-capacity mitochondrial enzyme and a high-affinity, low-capacity microsomal enzyme [29; reviewed in 5]. More than three decades later we can use several criteria to decide the answer to the question: which CYP is the physiologically important 25-hydroxylase in vivo? These criteria include:
a) substrate specificity towards D_3 and D_2 substrates; b) K_m and V_{max} and enzymatic properties of the expressed enzyme; c) tissue and subcellular location; d) occurrence of natural mutations
e) disease consequence of gene deletion or mutation in human and animal models.

Currently, based upon available data for these criteria, we can conclude that the answer to the above question is still not fully resolved, since there is still insufficient evidence that deletion of any single CYP results in a rickets-phenotype in the mouse or vitamin D deficiency/rickets in humans. Indeed, it is possible that *in vivo* several CYPs could contribute to 25-hydroxylation of vitamin D and its analogs under a broad substrate concentration range. However, all available evidence suggests that CYP2R1 is probably the physiologically-relevant enzyme at normal vitamin D concentrations (low nM) but that it is possibly backed up by others

at substrate concentrations in the pharmacological range (high nM-low μM). Consequently, we have reviewed relevant information, firstly on CYP2R1 and then the other candidate CYPs.

CYP2R1

The discovery of CYP2R1 in 2003 [30] arguably ended a three decade long search for the elusive physiologically-relevant vitamin D_3-25-hydroxylase. CYP2R1 satisfies, most if not all, of the criteria listed above to describe the location and properties of the enzyme activity first defined in the early 1970s [27,28]. CYP2R1, a 501 amino acid, liver microsomal cytochrome P450, was cloned from mouse and human and shown by real-time PCR to be primarily expressed in liver and testis [30]. The full amino acid sequence of hCYP2R1 is shown in Figure 3 and alignments of all known CYP2R1 isoforms (current databases hold ~50 species) reveal that it is highly conserved in comparison to other CYP2 family members which are not highly conserved between species presumably because they are usually broad-specificity, xenobiotic-metabolizing enzymes [31]. The initial studies demonstrated that CYP2R1, unlike all other putative 25-hydroxylases, would 25-hydroxylate both vitamin D_2 and vitamin D_3 equally well at physiologically-relevant substrate concentrations [30].

Subsequent work [32] using nanomolar substrate concentrations of [^{3}H]1α-OH-D_2, a vitamin D_2 analog, has reinforced the finding that transfected mouse and human CYP2R1 enzymes are able to synthesize the predominant *in vivo* metabolite 1,25-$(OH)_2D_2$, and not 1,24-$(OH)_2D_2$, the minor *in vivo* product of 1α-OH-D_2,which is also the major *in vitro* product of 1α-OH-D_2 incubated with CYP27A1. Recent work [23] using bacterially-expressed human CYP2R1 protein in a solubilized system revealed enzyme kinetic properties consistent with both of the earlier studies. hCYP2R1 showed K_m values of 4.4, 11.3 and 15.8 μM for vitamin D_3, 1α-OH-D_2 and 1α-OH-D_3 respectively, while K_{cat} values of 0.48, 0.45 and 1.17 mol/min/mol P450 were observed for the same three substrates. As defined in the associated LC analysis (Figure 5A),

the regioselectivity of hCYP2R1 was clearly confined to the C-25 position with no peaks corresponding to 24- or 26-hydroxylated products, this being in sharp contrast to the findings with CYP27A1 [23]. Furthermore, CYP2R1 failed to metabolize 25-OH-D_3, cholesterol or 7-dehydrocholesterol, thereby demonstrating a high specificity for the C-25 position on vitamin D but *not* other sterol substrates. Thus, the evidence suggests that CYP2R1 has the enzymatic properties needed for a vitamin D-25-hydroxylase capable of appropriately activating known vitamin D precursors *in vivo*.

Strushkevich et al [23] also solved the crystal structure of a functional form of CYP2R1 in complex with vitamin D_3, this representing the first crystal structure of a vitamin D-related CYP. The crystal structure generally confirmed the helical nature and binding pocket of CYP2R1 predicted from other CYPs using homology modeling [19]. Co-crystallized vitamin D_3 in the CYP2R1 occupied a position with the side chain pointing towards the heme group, but somewhat paradoxically, it was not optimally placed for hydroxylation, since the C-25 carbon was 6.5Å from the heme iron. It is unclear at this point if the substrate was trapped in an access/egress channel or if there is some other explanation for the data.

Another piece of evidence that strengthens the case for CYP2R1 being the vitamin D_3-25-hydroxylase is the finding of a human Leu99Pro mutation in a Nigerian family which results in vitamin D-dependent rickets, type 1B (VDDR Type 1B) [33]. This disease was postulated four decades ago [34] following the elucidation of vitamin D metabolism. Unfortunately, the genetic nature of the Leu99Pro mutation of CYP2R1 was determined by Cheng et al [9], a decade after the initial identification of the Nigerian rachitic patient [33], making patient and family follow-up difficult. However, subsequent genetic analysis of exon 2 of CYP2R1 [9] in 50 Nigerian individuals revealed one heterozygote with the Leu99Pro mutation suggesting that there may be a founder gene effect in the Nigerian population, and where vitamin D deficiency is quite prevalent [35]. Though the Leu99 residue is not in a region of the CYP2R1 coding for substrate-

binding domain, it is involved in water-mediated hydrogen bonding to the Arg445 amide nitrogen located three residues from the heme coordinating Cys448, and thus a Leu99Pro mutation probably results in a misfolded protein with little or no enzyme activity. Numerous attempts to bacterially-express hCYP2R1 with a Leu99Pro mutation, at the same time as the wild-type hCYP2R1, failed, leading Strushkevich et al [23] to conclude that CYP2R1 with Leu99Pro is misfolded or shows poor protein stability. Recently, DeLuca's group generated a CYP2R1 knockout mouse and preliminary studies suggest that serum 25-OH-D levels are 50% reduced compared to wild-type or heterozygous littermates [36], implying that although CYP2R1 is a major physiologically-relevant vitamin D_3-25-hydroxylase, there is some redundancy in the vitamin D_3-25-hydroxylase "family" of enzymes that can partially compensate for the deletion of CYP2R1. Furthermore, a genome-wide association study of the genetic determinants of serum 25-hydroxyvitamin D concentrations [37] concluded that variants at the chromosomal locus for CYP2R1 (11p15) was the second strongest association of only four sites; DBP (formerly known as GC), CYP24A1 and 7-dehydrocholesterol reductase being the others. Notably, variants of the other 25-hydroxylases such as CYP27A1 were not identified to be associated with serum 25-OH-D concentrations, again arguing for the fact that it plays no role in 25-hydroxylation of vitamin D at physiological substrate concentrations. Several polymorphisms of the CYP2R1 gene have now been identified in various SNP databases (Figure 3)

CYP27A1

This was the first cloned vitamin D-25-hydroxylase in the early 1990s, discovered by David Russell's group [38]. CYP27A1 is a liver mitochondrial cytochrome P450 with a homolog in >56 species, that is 531 amino acids in size in the human, and was originally cloned from rabbit but also from human [38,39]. Even the earliest claims, that CYP27A1 was a vitamin D-25-hydroxylase, were controversial as the purified liver enzyme seemed to be a better cholesterol-

26-hydroxylase than vitamin D-25-hydroxylase and thus it was proposed as a bi-functional enzyme involved in both bile acid and vitamin D metabolism [40]. Work with the recombinant protein demonstrated that CYP27A1 is a low affinity, high capacity vitamin D_3-25-hydroxylase that also 25-hydroxylates 1α-OH-D_3 but seems incapable of the 25-hydroxylation of vitamin D_2 or 1α-OH-D_2 catalyzing 24-hydroxylation to 24-OH-D_2 or 1,24S-$(OH)_2D_2$ instead (Figure 5B) [39,41]. Figure 5 shows that while CYP27A1 exhibits the ability to 24- and 26-hydroxylate 1α-OH-D_3, its primary site of hydroxylation is C25; whereas with 1α-OH-D_2, this switches to C24-hydroxylation with some 26-hydroxylation. The inability of CYP27A1 enzymatic properties to explain the formation of 25-OH-D_2 in animals *in vivo* became the main impetus for the search for an alternative 25-hydroxylase that culminated in CYP2R1 [30].

Parallel enzymatic work with bile acid substrates clearly showed that CYP27A1 could 25- and 27-hydroxylate the side chain of the cholesterol and play a role in the trimming of C-27 sterols without the secosteroid, open B-ring nucleus [42]. The same workers performed mutagenesis studies which established the important residues involved in ferredoxin interaction. Although there is currently no crystal structure of CYP27A1, numerous homology models have been proposed for the enzyme predicted from other CYPs [43,19]. Until the recent emergence of crystal structures of CYP2R1 and CYP24A1, these models offered the best structural insights into general structure & substrate binding pockets of vitamin D-related CYPs.

Several pieces of biological or clinical information argue against CYP27A1 being the physiologically-relevant vitamin D-25-hydroxylase. Firstly, the CYP27A1-null mouse phenotype does not include rickets or any other bone lesion [44]. However, this animal model is complicated by the absence of any significant bile acid defect either. Secondly, though human CYP27A1 mutations have been documented in the literature, these result in a bile acid-related condition known as cerebrotendinous xanthomatosis (CTX) rather than rickets [45] (Figure 3). Affected individuals usually have normal serum 25-OH-D, though some of these individuals can

exhibit low serum 25-OH-D and a type of osteoporosis [46]. Current opinion is that CTX is a defect in bile acid metabolism and that the bone disease is the result of malabsorption of dietary vitamin D caused by bile acid insufficiency rather than an inadequate 25-hydroxylase enzyme activity [47]. Thirdly, the genome-wide association study of the determinants of serum 25-hydroxyvitamin D concentrations [37] concluded that variants at the locus for CYP2R1 (11p15) but not CYP27A1 are associated with variations in serum 25-OH-D concentrations

A more likely possibility for an *in vivo* role for CYP27A1 in vitamin D metabolism is as a pharmacologically-relevant 25-hydroxylase for 25-hydroxylation of 1α-hydroxylated vitamin D analogs (1α-OH-D_3 and 1α-OH-D_2), popular prodrugs in the treatment of osteoporosis/ metabolic bone disease and the secondary hyperparathyroidism associated with chronic kidney disease-metabolic bone disease (CKD-MBD) [48]. It is worth noting that *in vitro* CYP27A1 synthesizes 1,25-$(OH)_2D_3$ and 1,24S-$(OH)_2D_2$, metabolites from 1α-OH-D_3 and 1α-OH-D_2 respectively, and 24-hydroxylated compounds such as 1,24S-$(OH)_2D_2$ are also observed *in vivo* following administration of pharmacological amounts of vitamin D_2 compounds [49,50,41]. It will be interesting to assess serum 25-OH-D levels, especially after administration of graded doses of vitamin D_3, in progeny of double knockouts from CYP27A-null and CYP2R1-null mice currently being generated by DeLuca's group [36]. Thus CYP27A1 may contribute to the metabolism of vitamin D compounds, including 1α-OH-D_3 and 1α-OH-D_2 when present at high concentrations, but it is unclear if it is involved in vitamin D metabolism at physiologically-relevant concentrations.

Other Potential 25-Hydroxylases

Over the past three decades, there have been numerous reports that in addition to CYP2R1 and CYP27A1, a number of other specific microsomal CYPs, partially-purified from tissues or cells, or studied in bacterial or mammalian expression systems can 25-hydroxylate a

spectrum of vitamin D substrates, but only at micromolar substrate concentrations. These include: CYP2D11, CYP2D25, CYP2J2&3 and CYP3A4 (See Table 1). Some of these are expressed in one mammalian species (eg pig or rat) and have no obvious human equivalent, show gender differences not observed for human vitamin D-25-hydroxylation *in vivo* or fail to 25-hydroxylate vitamin D_2 or 1α-OH-D_2. Again, as with CYP27A1, lack of regiospecificity for the C-25 position surfaces as an important distinguishing feature compared with CYP2R1, as many other microsomal CYPs (eg CYP3A4) catalyze the 24-hydroxylation of vitamin D_2 and D_3 compounds [51-53]. Based upon the emergence of the strong case for CYP2R1 being the vitamin D-25-hydroxylase, the pursuit of these other non-specific CYPs is becoming less urgent, but at least one of these, namely CYP3A4, deserves special mention.

A multi-functional non-specific enzyme such as CYP3A4, which is estimated to metabolize up to 50% of known drugs, would probably not attract special interest here were it not for the fact that recently it been shown to be selectively induced by 1,25-$(OH)_2D_3$ in the intestine [53-55]. CYP3A4 has been shown to 24- & 25-hydroxylate vitamin D_2 substrates more efficiently than vitamin D_3 substrates [51,52], and also 23R-and 24S-hydroxylates the already 25-hydroxylated 1,25-$(OH)_2D_3$ [53]. However, CYP3A4 is known to have K_m values for vitamin D compounds in the micromolar range, a property that questions its physiological but not pharmacological relevance. Recent work [56,57] has demonstrated that both human intestinal microsomes and recombinant CYP3A4 break down 1,25-$(OH)_2D_2$ at a significantly faster rate than 1,25-$(OH)_2D_3$ suggesting that this non-specific cytochrome P450 might limit vitamin D_2 action preferentially in selective target cells (eg intestine), where it is expressed, particularly in the pharmacological dose range. Such an observation may also offer an explanation for the well-documented lower toxicity of vitamin D_2 compounds as compared to vitamin D_3 compounds *in vivo*, the vitamin D_2 compounds not causing such severe hypercalcemia by virtue of reduced effects on intestinal calcium absorption. The same type of mechanism involving differential

induction of non-specific CYPs, such as CYP3A4, may also underlie the occasional reports of drug-drug interactions involving vitamin D, where co-administered drug classes, (eg anti-convulsants)[58,5], causing accelerated degradation of vitamin D_2 over vitamin D_3. Thus, while CYP3A4 might be occasionally considered as a vitamin D-25-hydroxylase, its main relevance to vitamin D metabolism may lie in its involvement in inactivation of vitamin D compounds at high concentrations.

25-Hydroxyvitamin D-1α-hydroxylase (CYP27B1)

The 25-hydroxyvitamin D-1α-hydroxylase has been investigated virtually from the day that its product 1,25-$(OH)_2D_3$ was discovered [3,60]. The need to define the enzyme in biochemical terms became urgent as soon as it became apparent that the 25-hydroxyvitamin D-1α-hydroxylase was a central regulatory axis of the calcium and phosphate homeostatic systems, subject to up-regulation by parathyroid hormone (PTH), low Ca^{2+} and low PO_4^{3-} levels [1,61,62]. It was quickly recognized that serum 1,25-$(OH)_2D_3$ was predominantly made in the kidney [3,63] with a PTH-regulated form located in the proximal convoluted tubule and a calcitonin-regulated form in the proximal straight tubule [64-67]. Biochemical investigations showed that the enzyme involved was a mixed-function oxidase with a cytochrome P450 component [68]. But the exact molecular description of this enzyme took another 25 years to unravel. In the meantime, there were emerging reports of so-called “extra-renal” 25-hydroxyvitamin D-1α-hydroxylase activity in several sites including placenta, bone and macrophage [69-74] which evoked the question if there was more than one or more cytochrome P450s with 25-hydroxyvitamin D-1α-hydroxylase activity. Unlike with the liver vitamin D-25-hydroxylase, this does not appear to be the case and with the cloning of CYP27B1 as a single gene, this story has become much simpler.

In 1997, several groups coincidentally cloned, sequenced and characterized CYP27B1 from rat, mouse and human species [10,11,74]. Though many of these groups used kidney libraries as the source of the enzyme, interestingly other groups reported finding the same CYP27B1mRNA in keratinocyte [76] and human colonic cell HT-29 [77] libraries, suggesting that the enzyme was identical in all locations. Subsequently, it has been confirmed that the CYP27B1 protein is identical in all locations [78,4], whether renal or extra-renal, though the regulation in these different tissue locations must involve different hormones and effectors.

hCYP27B1 is a 507 amino acid protein with a molecular mass of ~55 kDa. Best available information suggests that the enzyme 1α-hydroxylates 25-OH-D_2 and 25-OH-D_3 equally efficiently to give the active metabolite of each form of the vitamin. The genetic rachitic condition termed vitamin D dependency rickets 1A (VDDR Type 1A), in which the 1α-hydroxylase enzyme is absent or defective, presumably due to mutation of CYP27B1, had been recognized in the early 1970s by Fraser and colleagues [34,79], who showed that patients had low or absent serum 1,25-$(OH)_2D$ and they could be successfully treated using small amounts of synthetic 1,25-$(OH)_2D_3$. VDDR-Type 1A involves a resistant-rickets phenotype, characterized by hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and under-mineralized bone. It is cured by physiological (microgram) amounts of 1,25-$(OH)_2D_3$ or pharmacological (milligram) amounts of 25-OH-D_3 or vitamin D, which is consistent with a block in 1α-hydroxylation activity [34]. Subsequent work mapped the CYP27B1 gene to 12q13.1-q13.3 which is the same location established for the VDDR-Type 1A disease [10]. Human CYP27B1 mutations occur throughout the gene (Figure 3) resulting in defective and misfolded proteins with little or no activity [80-84].

At least two groups have created CYP27B1-null mice [85,86] which exhibit a lack of 1α-hydroxylated metabolites in the blood and tissues, revealing that CYP27B1 is the sole source of 1,25-$(OH)_2D$ in the body. The mouse phenotype mirrors human VDDR Type I in terms of

resistant rickets. The animals also show a reduction in CD4- and CD8-positive peripheral lymphocytes and the female mice are infertile [85]. Detailed bone histomorphometric analyses of the CYP27B1 and CYP27B1/PTH double knockout mice established that 1,25-$(OH)_2D_3$ deficiency resulted in epiphyseal dysgenesis and only minor changes in trabecular bone volume [87]. Bikle and colleagues showed that CYP27B1 is also required for optimal epidermal differentiation and permeability barrier homeostasis in the skin of mice [88]. Administration of a normal diet supplemented with either small amounts of 1,25-$(OH)_2D_3$ or use of a high calcium "rescue diet" largely corrects the mineral metabolism and bone defects seen in the CYP27B1-null mouse [89-92]. Global CYP27B1-null animals given high calcium intakes for several months do show growth plate abnormalities, probably exacerbated by secondary hyperparathyroidism and hypophosphatemia [85,87]. However, tissue-specific knockout of the mouse CYP27B1 gene in chondrocytes suggests that growth plate abnormalities are not merely the result of blood mineral ion defects and that local production of 1,25-$(OH)_2D_3$ plays a role in growth plate development [93,94].

The availability of specific CYP27B1mRNA and anti-CYP27B1 protein antibodies have allowed for a more rigorous exploration of the extra-renal expression of the enzyme. Diaz et al [95] used Northern analysis and RT-PCR to examine mRNA expression in human synctio-trophoblasts and concluded that there was CYP27B1 expression in human placenta. Using similar techniques, several groups reported low but detectable expression of CYP27B1 in a variety of cultured cell lines (eg prostate and colonic cells) [96-98]. Immunohistochemistry data from analysis of animal and human tissues has revealed the presence of the CYP27B1 protein in several tissues purported to express 1α-hydroxylase activity (eg skin, colon, macrophage, prostate, breast) [78,4]. Not all studies have supported the conclusion that CYP27B1 is expressed outside of the kidney in normal, non-pregnant animals. Using a β-galactosidase

reporter system, Vanhooke et al [92] found no evidence for expression of CYP27B1 in murine skin or primary keratinocytes, although there was expression in kidney and placenta. It is possible that the lack of detection of low abundance extra-renal CYP27B1 transcripts is due to some inherent insensitivity of the β-galactosidase reporter system, whereas it is sufficiently sensitive to detect abundant renal CYP27B1 transcripts.

Despite the fact that the existence of the extra-renal 1α-hydroxylase remains tentative, there has been much speculation about its possible role of this enzyme in health and disease [99-101]. It is now widely believed the enzyme exists in non-renal tissues to boost local production of cellular 1,25-$(OH)_2D_3$ in a paracrine/autocrine system. Such a role would suggest that cellular 1,25-$(OH)_2D_3$ concentrations in extra-renal CYP27B1 tissues might be higher than in the tissues of the classical endocrine system (eg intestine, bone, parathyroid gland) which depend entirely on renally-synthesized, blood-borne 1,25-$(OH)_2D_3$ at concentrations ~10^{-10}M. Cell differentiation and anti-proliferative genes regulated in extra-renal tissues (eg macrophage, colon, prostate, skin) may require higher 1,25-$(OH)_2D_3$ concentrations. A role for the extra-renal CYP27B1 is also consistent with the epidemiological finding that serum 25-OH-D levels are associated with various health outcomes from bone health to cardiovascular health. In particular, low serum 25-OH-D levels are associated with increased mortality for colon, breast and prostate cancer; increased auto-immune diseases and greater susceptibility to tuberculosis; increased cardiovascular diseases and hypertension. The presence of CYP27B1 in cells of the colon, breast, prostate, monocyte/macrophage and vasculature could explain why serum 25-OH-D levels are so critical to the normal functioning of these tissues.

Chronic kidney disease-metabolic bone disease (CKD-MBD, with 5 stages defined by decreasing glomerular filtration rate (GFR)) is well known to be accompanied by a gradual fall in serum 1,25-$(OH)_2D_3$ (normal range = 20-60 pg/mL), widely assumed to be due to a gradual

decline in CYP27B1 activity [102]. Whether this is in turn due to loss of the CYP27B1 protein caused by renal damage is debatable. It is possible that the fall in serum 1,25-$(OH)_2D_3$ to values below 20 pg/mL by the end of CKD Stage 2 could be due in part to increased fibroblast growth factor-23 (FGF-23) levels, a known down-regulator of CYP27B1 expression in normal kidney cells [103]. Recent reports of marked increases in FGF-23 levels in CKD Stage 5 dialysis patients with phosphate retention are consistent with FGF-23 playing a major role in vitamin D dysregulation and mortality in chronic kidney disease [104].

The regulation of CYP27B1 (summarized in Figure 6A) has been a major focus ever since the enzyme's discovery in the early 1970s [1]. Ca^{2+} and PO_4^{3-} ions, probably through the hormones: PTH, calcitonin and FGF-23, regulate CYP27B1 expression through complex signal transduction processes [105,67,106,107], while 1,25-$(OH)_2D_3$, the end-product of the enzyme down-regulates its own synthesis at the transcriptional level by vitamin D receptor (VDR)-mediated action at the level of the CYP27B1 gene promoter [106,108,109]. Evidence is also accumulating to suggest that CYP27B1 expression is down-regulated through DNA methylation and up-regulated through DNA demethylation [109,110]. While it is logical to isolate CYP27B1 from the rest of the calcium/phosphate homeostatic system, in practice there is a reciprocity between CYP27B1 and CYP24A1 that suggests that the factors up-regulating one enzyme, down-regulate the other. This is evident in the isolated perfused kidney from the rat fed a low-Ca vitamin D-deficient diet, or low-PO_4 vitamin D-deficient diet which is in the 1α-hydroxylation mode, and which over a 4 hour perfusion period after being exposed to its 25-OH-D_3 substrate turns off CYP27B1 expression and 1α-hydroxylation and turns on CYP24A1 and 24-hydroxylation [111]. The vitamin D metabolic system seems ideally designed to avoid synthesis of excessive amounts of the hormone and also to degrade the hormone, or even its substrate, by super-induction of catabolic processes including CYP24A1. In the VDR-null mouse, we see a

complete breakdown of this auto-regulation process because CYP27B1 is not suppressed by excessive 1,25-$(OH)_2D_3$ production and CYP24A1 is not actively stimulated, both steps requiring VDR-mediated events.

The regulation of the extra-renal 1α-hydroxylase has also received attention over the last couple of decades. What is clear is that the renal and extra-renal enzymes are regulated by different factors: the kidney CYP27B1 by calcium/phosphate homeostatic hormones described above; while the extra-renal enzyme is regulated by tissue-specific factors, including cytokines (Figure 6B). Adams et al [112] has shown that macrophages in the granulomatous condition, sarcoidosis, are driven by pro-inflammatory cytokines, such as γ-interferon, which also stimulate extra-renal CYP27B1 activity, that can cause excessive serum 1,25-$(OH)_2D_3$, which left unchecked results in hypercalciuria and hypercalcemia. The mechanism of γ-interferon-mediated upregulation of CYP27B1 appears to involve the Janus kinase-signal transducer and activator of transcription, MAPK, and nuclear factor-kappaB pathways, with a crucial role for the transcription factor CCAAT/enhancer binding protein beta [113,114]. Also, the usual CYP24A1 counter-regulatory mechanism seems to have been replaced in the monocyte/macrophage system by an inactive splice-variant of CYP24A1 [114]. Thus, the nature of the down-regulator(s) of the extra-renal CYP27B1 in these and other cells of the immune system remains largely unknown.

Recently, the normal up-regulation of the monocyte/macrophage CYP27B1 system was elucidated [116,117,101]. Toll-like receptors (TLRs) on the cell surface respond to the presence of bacteria (eg *M. tuberculosis*) with a signal transduction process which results in upregulation of VDR and CYP27B1. Uptake of 25-OH-D bound to its blood carrier DBP, allows the cells to then manufacture 1,25-$(OH)_2D_3$, which in turn stimulates VDR-mediated gene transcription of cathelicidin. Cathelicidin is an anti-microbial peptide, which specifically kills *M. tuberculosis*.

Stubbs et al [118] have demonstrated the existence of a high VDR-high CYP27B1 sub-population of immune cells making cathelicidin that can be selected by cell-sorting techniques in CKD Stage 5 dialysis patients treated with high doses of vitamin D_3 (40,000 IU/ 2 times per week). Despite the fact that these patients are virtually devoid of circulating 1,25-$(OH)_2D_3$ at baseline because of their low renal CYP27B1 activity, vitamin D_3 supplementation causes a significant increase in serum 1,25-$(OH)_2D_3$, posing the question if this metabolite is of monocyte/macrophage extra-renal origin?

24-Hydroxylase (CYP24A1)

Though, CYP24A1 was initially referred to as the 25-hydroxyvitamin D_3-24-hydroxylase, work with the recombinant enzyme has shown that it is able to catalyze multiple hydroxylation reactions at carbons C-24 and C-23 of the side chain of both 25-OH-D_3 and its hormonal form, 1,25-$(OH)_2D_3$ [13,14]. Indeed, our view of the role of CYP24A1 has expanded greatly to suggest that this single P450, alone, is responsible for the 5-step, 24-oxidation pathway from 1,25-$(OH)_2D_3$to produce calcitroic acid, a known biliary catabolite [119,120], as well as catalyzing a similar pathway which starts with 23-hydroxylation and culminates in the 1,25-$(OH)_2D_3$-26,23-lactone (Figure 1) [121,122]. In addition, CYP24A1 also efficiently hydroxylates the vitamin D_2 side chain of 25-OH-D_2 and 1,25-$(OH)_2D_2$ to give a more limited series of polyhydroxylated products [123,124]. The 24- and 23-products of the vitamin D_3 side chain appear in a specific order, reinforcing the concept of two distinct pathways initiated by a species-dependent C-24 or a C-23 hydroxylation step. Figure 3 depicts an amino acid sequence alignment of CYP24A1 with other vitamin D-related CYPs. In fact, an alignment of CYP24A1 from >50 species shows an impressive conservation of residues for at least a good part of the protein [125]. Of particular note, is the dichotomy that exists at residue 326 where most species of CYP24A1 have Ala326 and exhibit 24-hydroxylation to a calcitroic acid product while more primitive organisms have

Gly326 and show predominantly 23-hydroxylation to give a 26,23-lactone product. The functional significance of two distinct pathways in different species is unknown [18].

In 2010, the crystal structure of the rat CYP24A1 was elucidated but in the presence of the detergents Cymal and CHAPS [24]. Although the active site of rat CYP24A1 did not contain its natural substrate, for the most part the crystal structure did confirm the predicted tertiary structure of the protein, as well as the putative active-site residues from previous homology models and mutagenesis studies [18, 20-22]. The crystal structure of rat CYP24A1 reveals a canonical cytochrome P450 structure of helices and β-sheets surrounding a prosthetic heme group and a substrate binding cavity. Virtually all of the protein is required to maintain the shape, structure, heme-binding, and function of the enzyme. The structure of the rat CYP24A1 enzyme is shown in Figure 4A with 1,25-$(OH)_2D_3$ (sticks & spheres; purple) positioned using docking software into the wide-open cleft that serves as the substrate binding cavity [24].

Even before the crystal structure of CYP24A1 was determined, mutagenesis studies were initiated based upon the remarkable conservation of structure across cytochrome P450s (Figure 4B). Sakaki and colleagues who had shown that rat CYP24A1 is primarily a C24-hydroxylase, as compared to the human enzyme which is capable of both C24- and C23-hydroxylation, performed a follow-up study in which they mutated Thr416Met and Ile500Thr in the β3b- and β5-sheets respectively to try to change the properties of the rat enzyme by substituting amino acids to those found in the human enzyme [20]. They postulated that these residues interact with A-ring and cis-triene moieties of the 1α,25-$(OH)_2D_3$ docked-substrate. The Thr416Met and Ile500Thr caused significant changes in the C24:C23 hydroxylation ratio from 100:1 to 12.5:1 or 6.3:1 respectively, whereas, in their hands, the wild-type human enzyme had a ratio of 3.7:1 [20].

A somewhat similar approach was used to study differences between the human and opossum CYP24A1 [18], the latter being representative of the orthologs that are predominantly 23-hydroxylases, with a C24:C23 hydroxylation ratio of 1:25. Using the human CYP24A1 as a starting point, they focused on mutating Ala326 to Gly326 found in the opossum and many other 'primitive' CYP24A1s (See Figure 3) because this residue occupied a critical position directly above the heme in the I-helix that abuts the side chain of 1α,25-$(OH)_2D_3$ docked within the active site of hCYP24A1(See Figure 4A). The single Ala326Gly substitution radically changed the metabolic pattern observed for the resultant enzyme by changing the enzyme properties from a 24-hydroxylase with a C24/C23 hydroxylation ratio = 8.1:1 to a 23-hydroxylase with a C24/C23 hydroxylation ratio = 1:8.3, a value that resembled opossum CYP24A1 (1:25). Thus, it appeared that residue 326 alone was responsible for much of the regioselectivity difference observed between human & opossum CYP24A1 orthologs. Docking studies comparing the positions of 1,25-$(OH)_2D_3$ for optimal C24- versus C23-hydroxylation suggested that the loss of a methyl group from the amino acid at 326 in the I-helix by substituting Gly for Ala, provides extra space for the side chain of 1,25-$(OH)_2D_3$ to slide deeper into the substrate-binding cavity in order to optimally place C23 as opposed to C24 above the heme, and committing catabolism through to 1,25-$(OH)_2D_3$-26,23-lactone. The striking impact of Ala326Gly on regioselectivity is logical, given its direct contact with the substrate side-chain directly above the heme, as compared with Ile500 & Met416, which are located in the distal substrate access channel (Figure 4C).

Mutations at other sites in human CYP24A1 that have been shown to modulate the regioselectivity of the enzyme include Ile131, Leu148, Met246, and Val391 [21]. In mutagenesis studies of residues over a single turn of the F-helix forming the top of the substrate binding cavityof rat CYP24A1 performed by Annalora et al [22,126], it was shown that mutations at sites facing away from the cavity (Met245, Ser247, Thr248) retained 1,25-$(OH)_2D_3$ binding affinity

similar to the wild-type, whereas mutations at sites Phe249 and Met 246 directly protruding into the cavity, impaired substrate binding to different degrees. Based upon this work [22,126], CYP24A1 is a 1,25-$(OH)_2D_3$-binding protein first, and a catabolic enzyme second. All of these residues including Ala326 and Ile500, originally selected on the basis of homology modeling [18, 20-22, 127] as putative substrate contact points, have been implicated in forming the CHAPS-containing substrate-binding site in the crystal structure of rat CYP24A1 [24]. A recent report [128] suggests that a Val391Leu mutation in the human CYP24A1 also changes enzymatic properties by introducing 1α-OH-D_3-25-hydroxylase activity absent in the wild-type enzyme and ascribes this to a combination of a change in the position of substrate within the active site and altered substrate binding affinity [18,126]. From homology models and mutagenesis studies of CYP24A1 we now have an unprecedented understanding of the amino-acid architecture of the substrate-binding pocket, details which have been confirmed by the availability of the crystal structure of rat CYP24A1. Our current view of the substrate-binding site is depicted in Figure 4A, where many of the residues that we have discussed are highlighted.

During the same period of time in which the role of CYP24A1 in multi-step hydroxylation of the side-chain of vitamin D was being elucidated, it was also shown that the enzyme is expressed in many, if not all, target cells containing the vitamin D receptor (VDR), including kidney, bone, intestine, etc. and is strongly inducible by vitamin D receptor agonists in such tissues [125]. This led some to propose that the role of CYP24A1 is primarily to limit or attenuate the action of 1,25-$(OH)_2D_3$ on target cells after an initial round of transcriptional activation in a negative feedback loop [129] (Figure 6A). The cloning of CYP24A1 in the early 1990s [130] confirmed both the target cell pattern of CYP24A1 expression and its inducibility by its substrate, 1,25-$(OH)_2D_3$. Moreover, analysis of the CYP24A1 gene revealed the presence of a strong positive vitamin D response element (VDRE) element in the upstream promoter that mediates this induction at the transcriptional level [131]. This concept has been extended using CHIP

technology that shows that there are multiple VDR-binding response elements in the CYP24A1 gene, and regulation involves downstream elements as well [132]. This suggested that raising 1,25-$(OH)_2D_3$ in target cells could trigger CYP24A1-mediated catabolism and thus protect cells from excess VDR pathway activation. Work with the CYP24A1-null mouse also added support to a catabolic role for CYP24A1, since the clearance of 1,25-$(OH)_2D_3$ is dramatically reduced and the plasma half-life of the hormone increases 10-fold from ~6 hours to ~60 hours when CYP24A1 is absent [133,134]. Thus, there is abundant evidence that CYP24A1 exists in normal physiology to catabolize 25-OH-D_3 to prevent its eventual activation to 1,25-$(OH)_2D_3$ and/or degrade the hormone, 1,25-$(OH)_2D_3$ within its target cells to terminate its biological activity.

Recent work by St-Arnaud *et al* [135] has challenged this solely catabolic role for CYP24A1 by noting the accelerated healing of bone fractures in laboratory animals after the administration of 24-hydroxylated metabolites of vitamin D. The CYP24A1-null mouse exhibits an “intramembranous bone” lesion originally thought to be due to the absence of a bone-specific 24-hydroxylated metabolite. However, it was later noted that the bone lesion was similar to that observed after excessive 1,25-$(OH)_2D_3$ administration and the lesion is absent when a double CYP24A1/VDR-null mouse is engineered, implying that it is caused by excessive VDR-mediated gene expression [136]. However, in more recent studies of bone fracture healing in the CYP24A1-null mouse, 24-hydroxylated metabolites appear to accelerate the rate of repair [135]. Acceptance of a unique anabolic role for 24,25-$(OH)_2D_3$ in bone healing would seem to depend heavily on the demonstration of a 24,25-$(OH)_2D_3$ receptor and elucidation of the signal transduction pathway mediating the effect. Reports indicate that a putative 24,25-$(OH)_2D_3$ receptor has been cloned, and attempts are being made to engineer a receptor-knockout mouse [137]. It will be also important to show that this 24,25-$(OH)_2D_3$ receptor-knockout mouse has a defective bone fracture-healing phenotype.

While CYP24A1 has been clearly established as the key enzyme responsible for vitamin D catabolism, it has become evident that CYP24A1 works in balance with CYP27B1, which is the cytochrome P450 enzyme responsible for converting 25-OH-D_3 to 1,25-$(OH)_2D_3$ both in the kidney where its role in vitamin D hormone activation was first established as well as in extra-renal tissues where its specific purpose remains to be elucidated. The emergence of the extra-renal 1α-hydroxylase (CYP27B1) as a mechanism for raising the cellular concentration of 1,25-$(OH)_2D_3$ [99,100] has refocused our attention on the crucial role of target-cell CYP24A1 as a fine-tuning mechanism to attenuate and eventually reduce its level after gene expression has been modulated. While the renal CYP24A1 enzyme may function to balance systemic 25-OH-D_3 and 1,25-$(OH)_2D_3$ levels, target-cell extra-renal enzyme probably acts in conjunction with CYP27B1 to "fine-tune" target tissue exposure to 1,25-$(OH)_2D_3$ hormone [138] (Figure 6B).

Vitamin D signaling plays a critical role in regulating bone and mineral homeostasis and consequently, enzymes such as CYP24A1 which control vitamin D levels are regulated by hormones which are integral to mineral metabolism [5] (see Figure 6A). In addition to the self-induced regulation of CYP24A1 by 1,25-$(OH)_2D_3$ itself, the enzyme is regulated by such key factors such as PTH and FGF-23.1,25-$(OH)_2D_3$-mediated induction of CYP24A1 expression is significantly attenuated by PTH [139-141],due to destabilization and increased degradation of CYP24A1mRNA [142]. As with PTH, FGF-23 also plays a central role in the regulation of mineral homeostasis affecting both expression of genes regulating serum phosphate, as well as those controlling vitamin D metabolism [143-145]. Induction of FGF-23 expression in osteocytes and osteoblasts follows rising serum phosphate levels; subsequently, FGF-23 reduces renal phosphate reabsorption by inhibiting Na/Pi co-transporter activity [146,147] and indirectly suppresses intestinal phosphate absorption by suppressing renal expression of CYP27B1 thus lowering blood 1,25-$(OH)_2D_3$ [147-149]. FGF-23 also controls 1,25-$(OH)_2D_3$ levels by inducing expression of CYP24A1 mRNA in the kidney [148-152].

The initial demonstration that 1,25-$(OH)_2D_3$ is an anti-proliferative, pro-differentiating agent for certain cell types *in vivo* and many cell lines *in vitro* [153], coupled with the fact that cancer cell studies have showed decreased CYP27B1 and increased CYP24A1 expression in prostatic, colonic and breast cell lines as they progress towards a more tumorigenic phenotype [154-158] has caused some researchers to speculate that cancer progression involves dysfunctional vitamin D metabolism (Figure 6B) [158]. But the hypotheses that vitamin D deficiency contributes to cancer incidence or that supplemental vitamin D_3 might prevent cancer are difficult to test because of the duration of clinical trials or the multiple confounding factors that accompany vitamin D deficiency. The VDR-knockout mouse which lacks vitamin D-mediated signaling altogether is more susceptible to chemically-induced cancers arguing that vitamin D plays a role in cancer prevention [159]. Although elevated CYP24A1 expression and reduced CYP24A1 gene silencing has been reported in specific tumors [160-162], proof that it is a causative agent in cancer development is still lacking. Nevertheless, there are many claims that CYP24A1 is a candidate oncogene [163-165].

Mining of several genomic databases reveals that a number of polymorphisms of CYP24A1 have been identified in recent years and the list is growing rapidly (Figure 7). Though little is known of the effects of these polymorphisms on CYP24A1 enzyme activity, inactivating mutations would be expected to give rise to a hypercalcemic phenotype. Hypercalcemic conditions are not uncommon in the pediatric literature but they appear to be a heterogeneous group of diseases including: Williams-Beuren Syndrome and Idiopathic Infantile Hypercalcemia (IIH); all characterized by transient hypercalcemia and other features. Of these, only IIH has unknown etiology and until recently, had no gene locus assigned to it [166,167]. In 2011, Schlingmann et al [168] demonstrated loss-of-function mutations of CYP24A1 are an underlying cause of IIH in 9 families of German, Turkish and Russian origin. This work recently confirmed

by other US-based groups [169-171] reinforced the important conclusion drawn from the CYP24A1-null mouse studies that CYP24A1 is primarily a catabolic enzyme.

Dysfunctional CYP24A1 activity has also been implicated in a number of acquired diseases including metabolic bone disease, chronic kidney disease and several types of cancer; as well as being involved in genetically-linked hypophosphatemia due underlying defects in FGF-23 signaling [172,173]. A detailed description of the connection between CYP24A1 and these diseases is beyond the scope of this review [see 172, 173].

Future Perspectives

This study of the cytochrome P450s involved in vitamin D metabolism has come of age with the cloning and structural elucidation of several of the family members. Just as the crystal structure of the VDR has opened the door to new families of vitamin D analogs which more precisely position the vitamin D ligand in the ligand-binding pocket (See companion review on VDR), the substrate-binding pockets of the vitamin D-related CYPs, especially CYP24A1, will allow us to design “metabolism-resistant” or “metabolism-sensitive” vitamin D analogs as well as a second generation of CYP24A1 or CYP27B1 inhibitors using rational drug design [174]. From a biochemical perspective such information will also allow us to better understand the mechanism of multiple hydroxylation reactions executed by these enzymes

As was pointed out throughout this review, the number of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 polymorphisms in the genomic databases is expanding at an exponential pace. Undoubtedly, the recent discovery of inactivating CYP24A1 mutations in IIH patients [168] will also drive clinical interest in CYP24A1 research. One would expect that more of these polymorphisms may be loss-of-function mutations associated with mild and more severe diseases in the hypercalcemic constellation, including IIH, but it remains to be seen whether CYP24A1 dysregulation can be connected with other disease states e.g. nephrolithiasis. There

is no doubt that the CYP24A1-knockout mouse [133-137] still has much more to reveal about the roles of CYP24A1 *in vivo*. Likewise the development of the CYP2R1-null mouse [36] and its crossing with the CYP27A1-null mouse should lead to a much better understanding of the vitamin D-25-hydroxylase. Lastly, and perhaps most importantly, the exact role of the extra-renal CYP27B1 should also be clarified over the next few years. This is an exciting time to be involved in the study of vitamin D-related cytochromes P450 and vitamin D metabolomics.

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Figure Legends

Figure 1: Important Steps in Vitamin D Metabolism.

The main cytochrome P450-mediated steps involved in vitamin D metabolism are depicted along with the main metabolites of vitamin D. Two other proteins: DBP and VDR play essential roles in the transport of metabolites from one tissue to another and the key signal transduction events involved in target cell action, respectively.

Figure 2: Electron transport chains & protein components of the vitamin D hydroxylases. A) In mitochondria, NADPH is oxidized by the flavoprotein, ferredoxin reductase, which transfers single electrons through a pool of ferredoxin iron-sulphur proteins to the mitochondrial P450s on the inner membrane.; B) In the endoplasmic reticulum, electron equivalents from NADPH are captured by the NADPH P450 reductase (also known as P450 oxidoreductase, POR). The two electrons from NADPH are transferred sequentially to the microsomal P450 (e.g. CYP2R1). (From Reference 14 with permission).

Figure 3: Sequence alignments of structurally determined or predicted secondary structures for vitamin D hydroxylases. Residues conserved in both mitochondrial & microsomal P450s (shaded) are structurally or functionally important, although elements of substrate recognition, binding, and specificity are inherently less conserved. Heme-binding residues and ERR-triad residues are also indicated. Locations of missense mutations leading to CYP2R1 deficiency (Vitamin D-dependent rickets-type IB); CYP27A1 deficiency (cerebrotendinous xanthomatosis, CTX); CYP27B1 deficiency (Vitamin D-dependent rickets-type IA) and CYP24A1 deficiency (idiopathic infantile hypercalcemia, IIH) are indicated by the red shading. Single nucleotide polymorphisms from dbSNP, Ensembl, Sanger Cosmic, and 1000 Genomes are shown in blue.

Figure 4: Structural analysis of CYP24A1. (With Permission from Annalora et al. [24])

Panel A depicts a stereographic view of the CYP24A1 crystal structure (3k9v.pdb) with labeled secondary structures. An analysis of heme-ligand geometry in cytochrome P450s permitted docking of the substrate 1,25-$(OH)_2D_3$ (yellow) into the heme distal cavity active site.

Panel B shows degree of amino acid conservation in CYP24A1 observed across approximately 50 species orthologs: green (>95%), yellow (>85%), orange (>60%) and blue (<60%). The black curve indicates a possible membrane-binding surface.

Panel C shows an open active site cavity/cleft (white mesh) and an earlier model of a closed cavity (black mesh). Various access/egress channel trajectories are indicated.

Figure 5: Comparison of the enzymatic properties of two vitamin D-25-hydroxylases: CYP2R1 and CYP27A1. The substrates used are the prodrugs, 1α-OH-D_2 and 1α-OH-D_3 to gauge the site and efficiency of the two vitamin D 25-hydroxylases towards D_2 and D_3 family members. Chromatograms of metabolites from A) *in vitro* reconstituted CYP2R1 enzyme [23] and B) transiently-transfected CYP27A1 in COS-1 cells [39]. The lack of CYP27A1-mediated 25-hydroxylation towards 1α-OH-D_2 is evident, although 1α,24$(OH)_2D_2$ metabolite is detectable in the serum of animals given large doses of vitamin D_2 [41] & is a VDR agonist.

Figure 6A: Physiological Roles of Renal CYP27B1 and CYP24A1 in Calcium and Phosphate Homeostasis. (Reproduced from Schlingmann et al. [168])

Ca and PO_4 ions through PTH, FGF-23 and the hormone 1,25-$(OH)_2D_3$ play key roles in determining the balance between the synthesis and degradation of 25-OH-D_3 and 1α,25-$(OH)_2D_3$ by regulating renal CYP27B1 and CYP24A1 respectively.

Figure 6B: Putative Roles of Extra-Renal CYP27B1 and CYP24A1 in establishing the optimal target cell concentration of 1,25-$(OH)_2D_3$ for regulation of gene expression in non-calcemic functions. Cytokines are believed to regulate these extra-renal/target cell enzymes. Normal cells balance synthesis & degradation to generate optimal levels of 1,25-$(OH)_2D_3$. Cancer cells show reduced CYP27B1 & increased CYP24A1 expression.

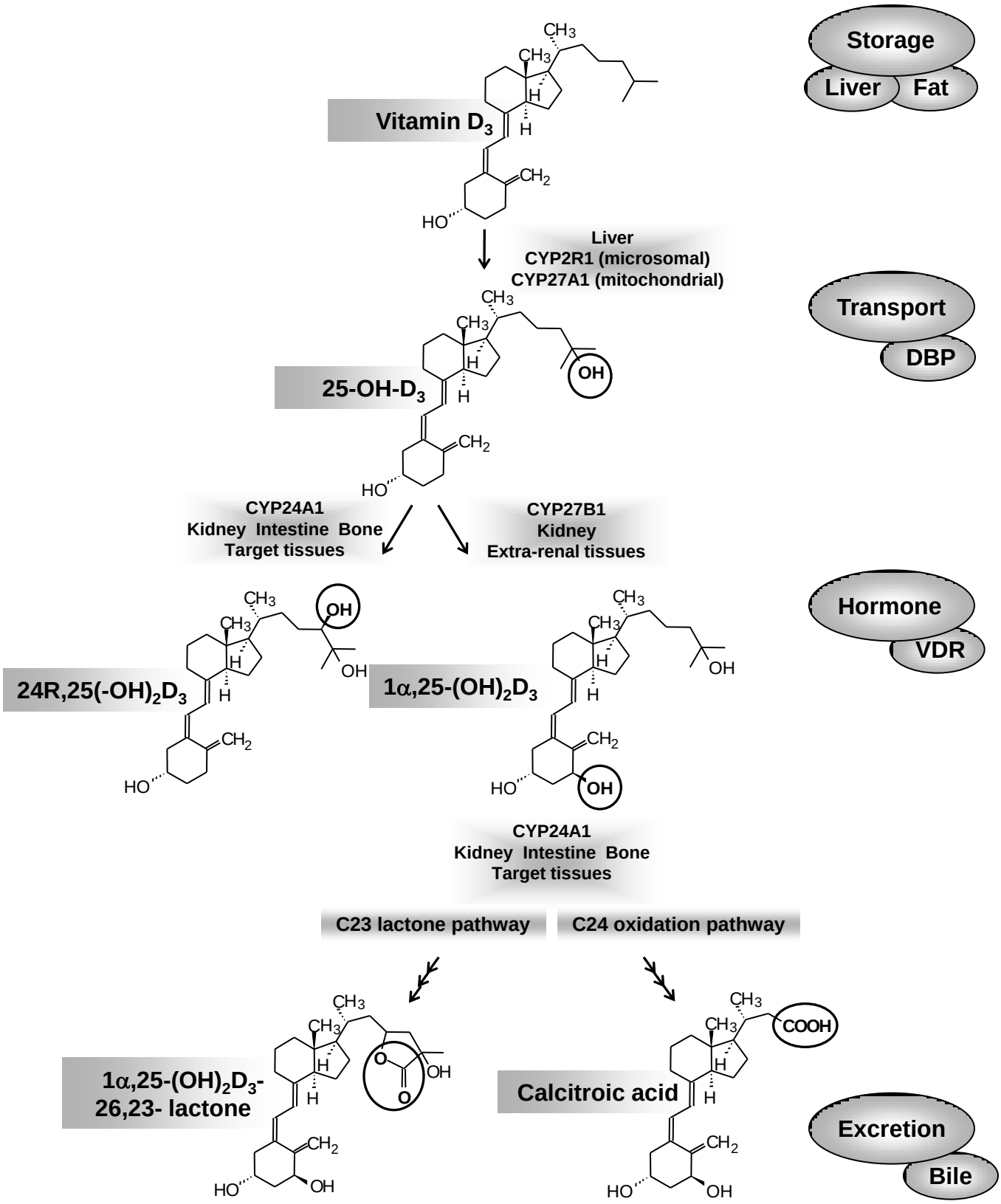
Figure 7: Location of CYP24A1 polymorphisms (in SNP databases) and CYP24A1 missense mutations identified in patients with Idiopathic Infantile Hypercalcemia (IIH). The relative positions of the conserved α-helices and β-strands in the CYP24A1 protein are indicated in yellow and blue respectively. Secondary structures positioning substrate contact residues are located in the β-1, A-helix, B'-helix, B'/C-loop, F/G-loop, I-helix, β-3a, β-3b &β-5 structures; heme-binding & ERR-triad residues stabilize protein structure& are involved in ferredoxin binding & electron transfer to the heme iron.

Table 1: Vitamin D metabolizing CYPs*

P450	Species	Tissue location	Subcellular location	Size (aa)	Human Gene Locus	Enzyme Activity	Disease State Human or (Mouse XO)	Function
CYP2R1	human >47 species	Liver	micro.	501	11p15.2	25-hydroxylation of D_3 25-hydroxylation of D_2	VDDR-1B (unknown)	Physiological 25-hydroxylase
CYP27A1	human >56 species	Liver Macrophage	mito.	531	2q33-qter	25-hydroxylation of D_3 24-hydroxylation of D_2	CTX	Pharmacological 25-hydroxylase
CYP2C11	rat	Liver (male)	micro.	500		25-hydroxylation of D_3 25-hydroxylation of D_2		
CYP2D25	pig	Liver	micro.	500		25-hydroxylation of D_3		
CYP2J2 CYP2J3	human rat	Liver	micro.	502	1p31.3-p31.2	25-hydroxylation of D_2 25-hydroxylation of D_3		
CYP3A4	human	Liver Intestine	micro.	503	7q22.1	25-hydroxylation of D_2		
CYP27B1	human >39 species	Kidney	mito.	508	12q13.1-q13.3	1α-hydroxylation of D_3 1α-hydroxylation of D_2	VDDR-1A (Rickets)	1α-hydroxylase
CYP24A1	human >51 species	Target tissue	mito.	514	20q13.2-q13.3	23- & 24-hydroxylation of 25(OH)D/1,25$(OH)_2$D		24-hydroxylase

Reproduced with permission from reference 15. Jones, G., and D.E. Prosser. 2011. Chapter 3: The activating enzymes of vitamin D metabolism (25- and 1α-hydroxylases). *In*: "Vitamin D 3rd Edition". D. Feldman, J.W. Pike and J.S. Adams, editors. Academic Press, San Diego CA. pp 23-42.

gure 1



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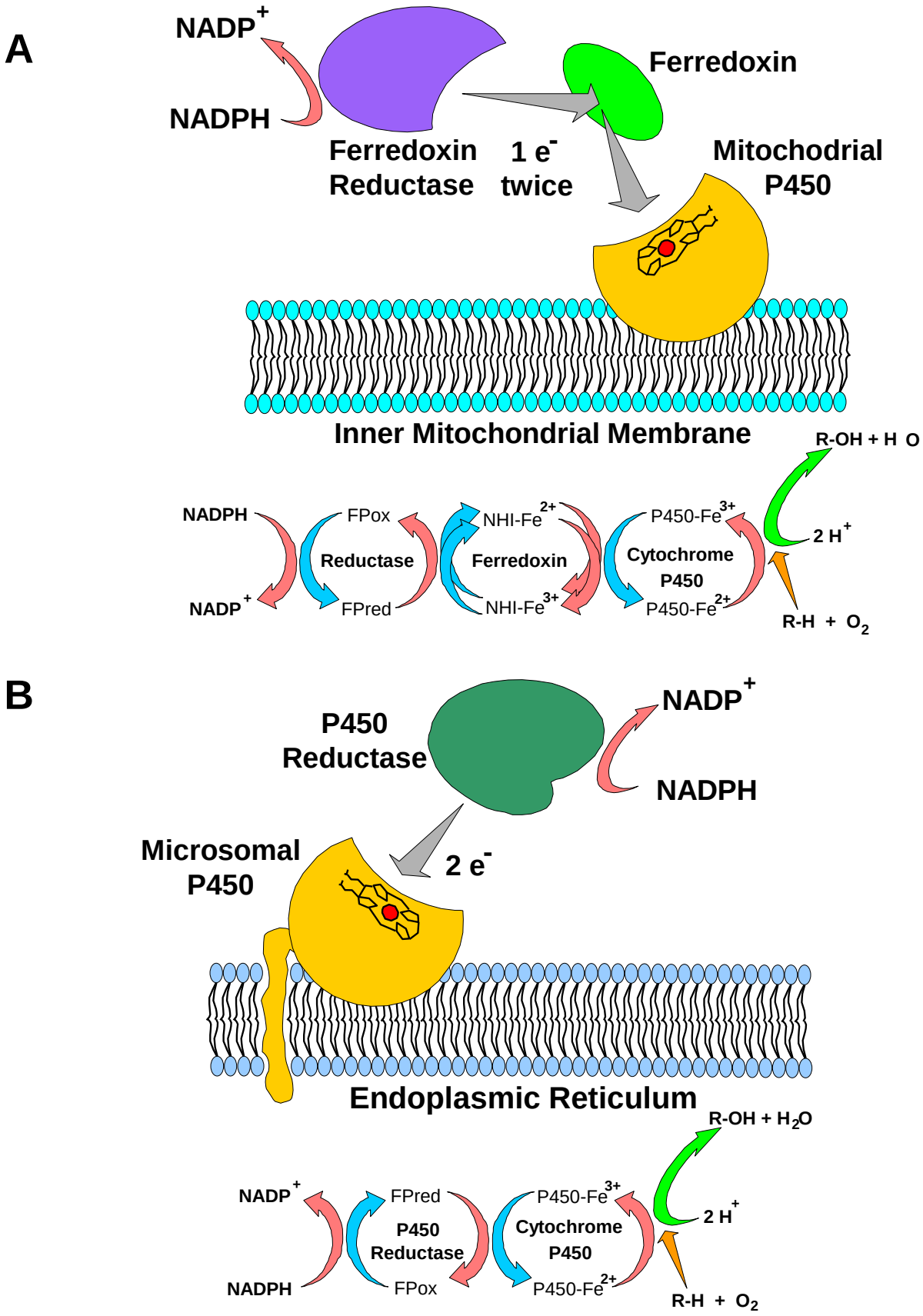

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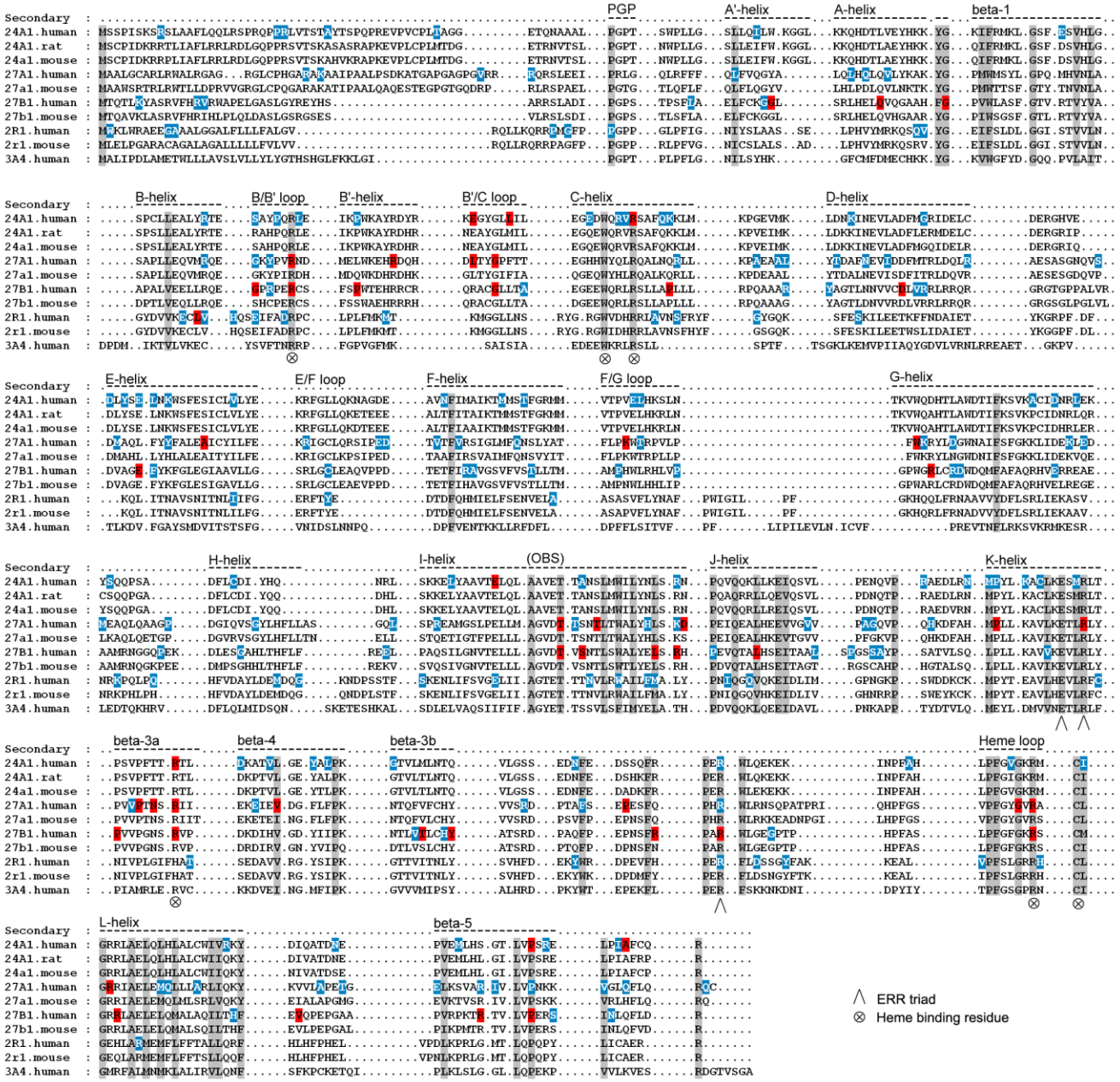

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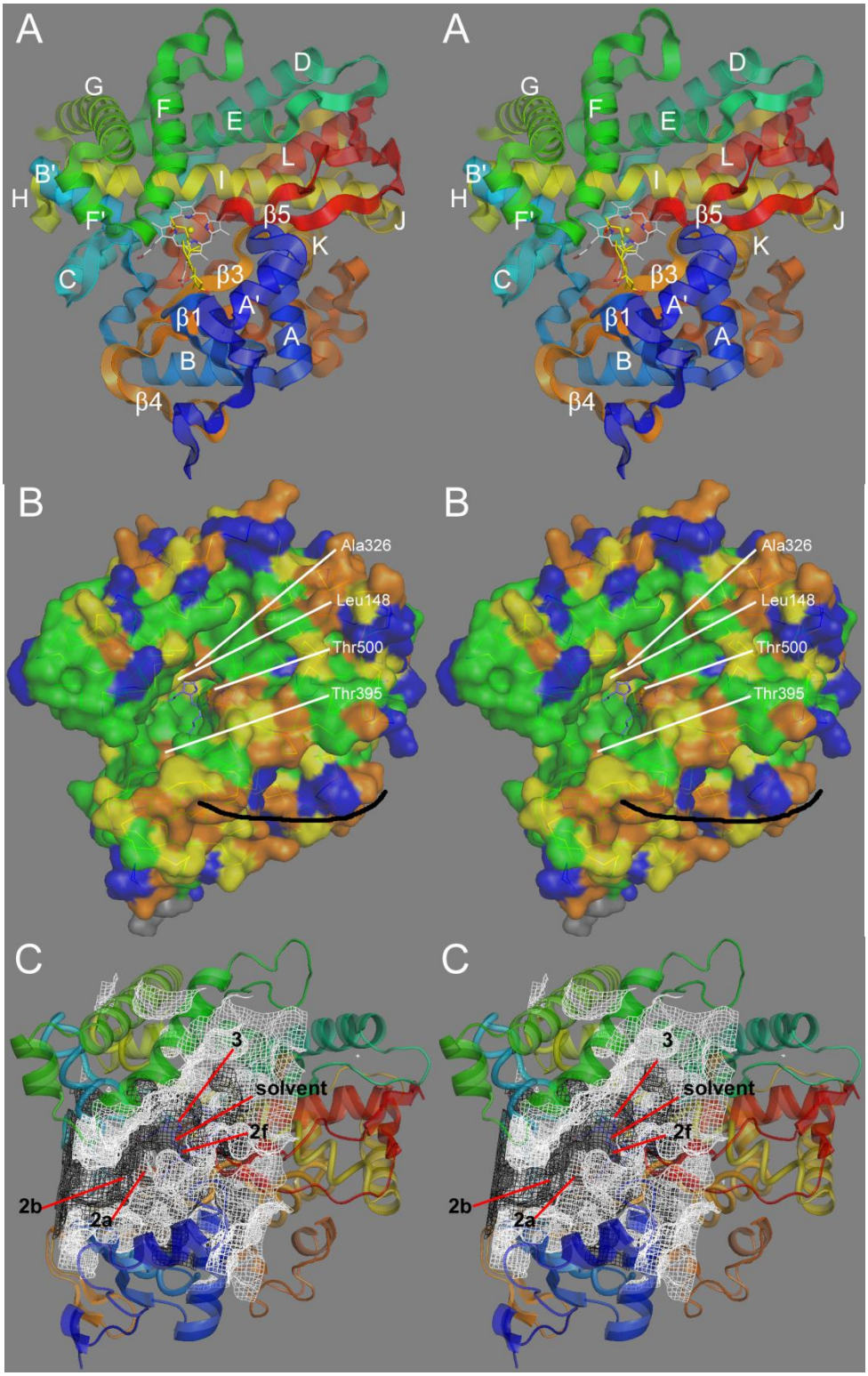

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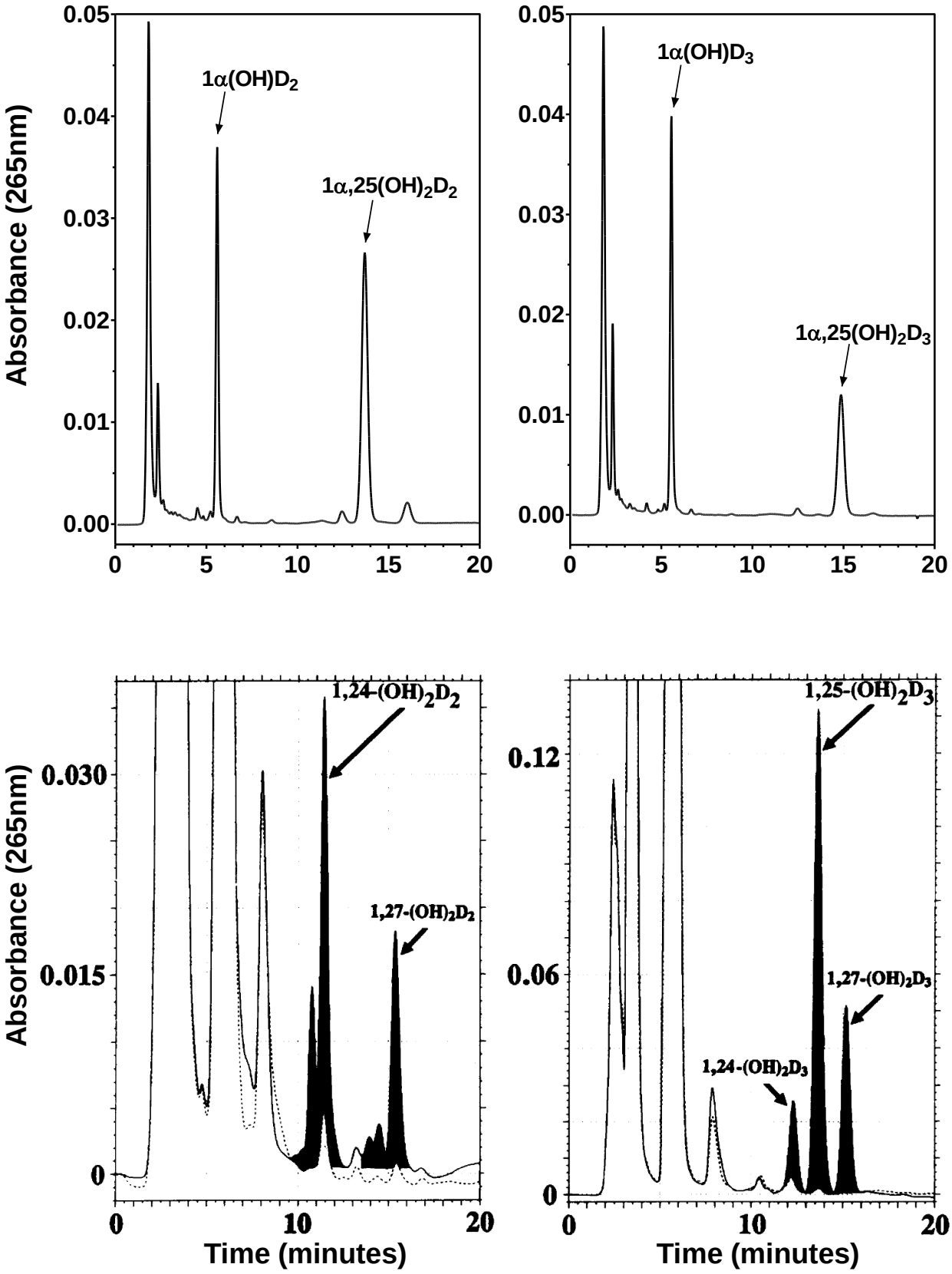

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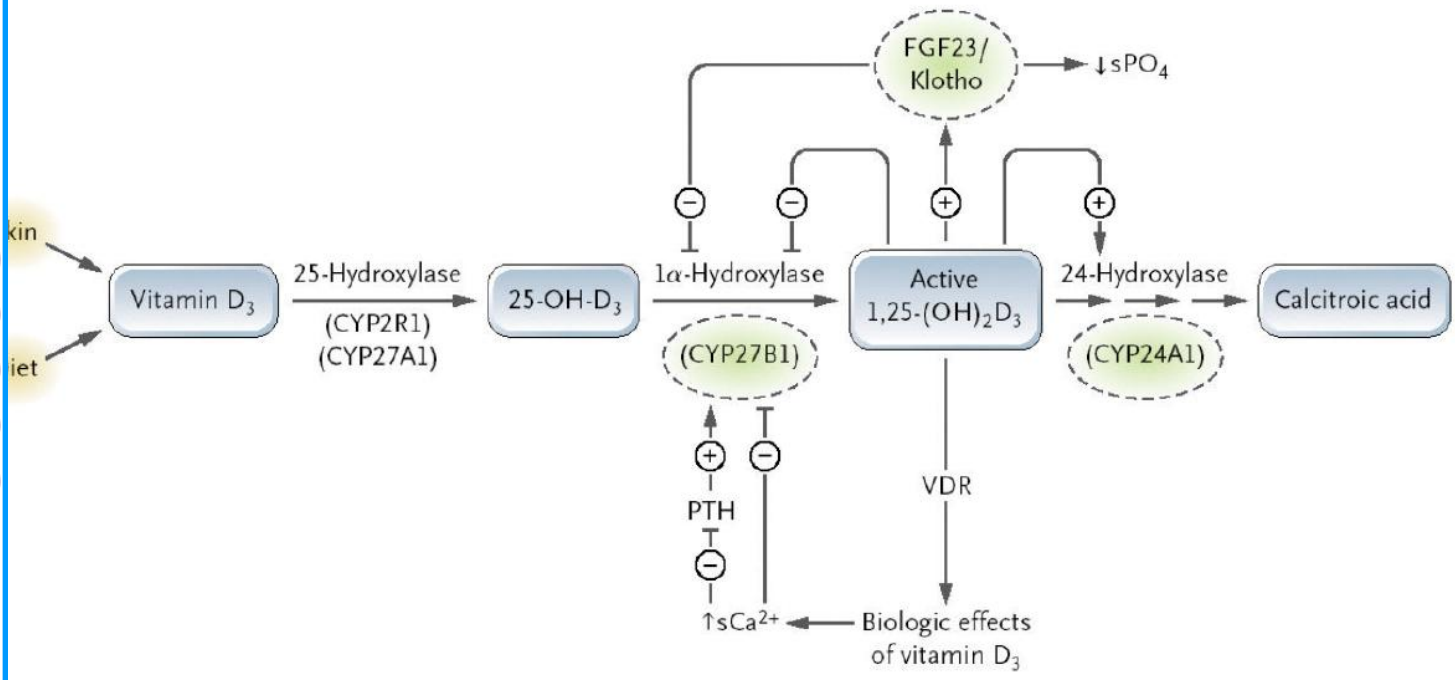
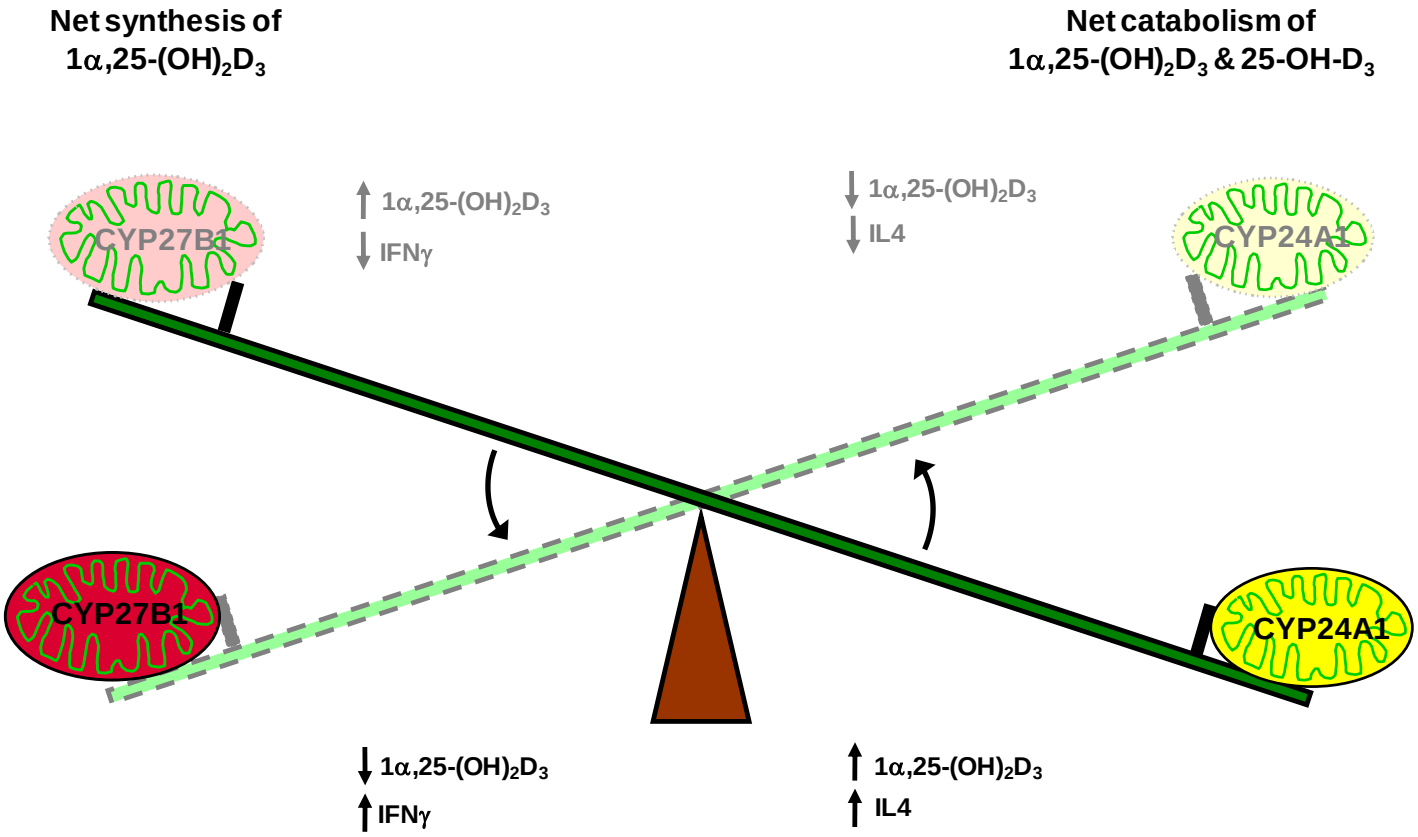


Figure 6B



igure 7