Human 25-Hydroxyvitamin D_3-24-Hydroxylase, a Multicatalytic Enzyme†

Matthew J. Beckman, Praveen Tadikonda, Elizabeth Werner, Jean Prahl, Sachiko Yamada,‡ and Hector F. DeLuca*

Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, Wisconsin 53706

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ABSTRACT: Human 25-hydroxyvitamin D-24-hydroxylase has been expressed in *Spodoptera frugiperda* (*Sf21*) insect cells using the previously cloned cDNA in baculovirus (AcNPV-P450cc24). The activity of recombinant h-P450cc24 required adrenodoxin, adrenodoxin reductase, and NADPH. Incubation of this reconstituted system with 25-OH-[26,27-^{3}H]D_3 substrate produced several metabolites that were resolved on a normal-phase cyano HPLC system. These products exactly comigrated with authentic standards for 24-oxo-25-OH-D_3, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, and 24-oxo-23(*S*),25-$(OH)_2D_3$. The soluble proteins from *Sf21* cells infected with wild-type baculovirus produced neither 24,25-$(OH)_2D_3$ nor any of the other 25-OH-D_3 metabolites. The products were isolated and subjected to a normal-phase amino HPLC for further separation, purification, and characterization. Comigration on two HPLC systems, periodate cleavage reactions, and $NaBH_4$ reduction established clearly the identity of these metabolites. Incubation of recombinant h-P450cc24 with 25-OH-[3α-^{3}H]D_3 led to the isolation of an additional product that comigrated with 24,25,26,27-tetranor-23-OH-D_3. Treatment of putative 24,25,26,27-tetranor-23-OH-[3α-^{3}H]D_3 with acetic anhydride changed its migration on amino HPLC to a less polar position, indicating acetylation of a hydroxyl group(s). These data demonstrate conclusively that h-P450cc24 is a multicatalytic enzyme catalyzing most, if not all, of the reactions in the C-24/C-23 pathway of 25-OH-D_3 metabolism. It is likely that this enzyme by itself converts 25-OH-D_3 and 1,25-$(OH)_2D_3$ to one of its final excretion products.

1,25-$(OH)_2D_3$-24-hydroxylase (P450cc24) is a mitochondrial cytochrome P450 that functions in the catabolism of 1,25-$(OH)_2D_3$, the active form of vitamin D_3 (DeLuca, 1988; Pedersen et al., 1983). Vitamin D_3 is activated by C-25-hydroxylation in the liver and C-1α-hydroxylation in renal proximal tubular cells (DeLuca, 1986). Similar to P450cc24, the 25-OH-D_3-1α-hydroxylase (P450cc1α) is also a mitochondrial cytochrome P450 enzyme (Ghazarian & DeLuca, 1974).

The regulation of P450cc24 activity is tissue-specific. In all tissues where P450cc24 is located, 1,25-$(OH)_2D_3$ induces activity. However, in renal proximal tubules cells, parathyroid hormone [PTH] suppresses its activity and mRNA while stimulating the P450cc1α (Shigematsu et al., 1986; Tanaka & DeLuca, 1984). This mechanism allows for the timely secretion of 1,25-$(OH)_2D_3$, in conditions of calcium debt and without the threat of catabolism imposed by P450cc24. Thus, 1,25-$(OH)_2D_3$ induction of P450cc24 in all the target tissues thus far studied appears to ensure the degradative removal of the potent hormone 1,25-$(OH)_2D_3$ and its precursor 25-OH-D_3 (Iida et al., 1995; Shinki et al., 1992).

There are at least two specific pathways that further metabolize either 25-OH-D_3 or 1,25-$(OH)_2D_3$ substrates: (1) the C-24/C-23 catabolic pathway, and (2) the C-26/C-23 lactone pathway, with the two pathways converging at C-23-hydroxylation (Kumar, 1984). The predominant pathway is C-24/C-23 catabolism, which consists of sequential C-24-hydroxylation, C-24-ketonization, and C-23-hydroxylation followed by oxidative cleavage of the carbon−carbon bond between C-24 and C-23 to form 24,25,26,27-tetranor-23-OH-D_3 (Lohnes & Jones, 1987). The C-23 alcohol has been demonstrated to convert to a C-23 acid (calcitroic acid) (Makin et al., 1989; Reddy & Tserng, 1989), the established excretory product of 1,25-$(OH)_2D_3$ (Esvelt et al., 1979). Lactone metabolites are derivatives of sequential C-26-hydroxylation, C-23-hydroxylation, and lactonization to form C-26/C-23-lactone (DeLuca, 1986; Kumar, 1984). Both pathways have been studied in tissues or cells of the major target organs of vitamin D, kidney, intestine, and bone. In all of these tissue and cell types, the complete set of enzymatic activities exist for the formation of either calcitroic acid or lactone end-products (Esvelt & DeLuca, 1981; Napoli & Horst, 1983; Reddy & Tserng, 1989; Siu-Caldera et al., 1995).

The inducibility of P450cc24 by 1,25-$(OH)_2D_3$ led to its eventual protein purification (Ohyama et al., 1989). The properties of P450cc24 classify it as a mixed-function monooxygenase of the cytochrome P450 superfamily with a molecular mass of 55 kDa (Burgos-Trinidad et al., 1992; Ohyama & Okuda, 1991). Monoclonal antibodies generated against the purified rat P450cc24 facilitated the first cDNA cloning of P450cc24 (Ohyama et al., 1991). The isolated cDNA was 3.4 kbp and contained a 1542 bp open reading frame (orf) encoding a 514 amino acid protein (Ohyama et al., 1991). Following this discovery, Chen et al. (1993) successfully isolated the human P450cc24 cDNA from an HL-60 cell cDNA library. The h-P450cc24 cDNA consists

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* To whom correspondence should be sent: Dr. Hector F. DeLuca, Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706. Telephone: (608) 262-1620. Fax: (608) 262-3453. E-mail: deluca@biochem.wisc.edu.

‡ Present address: Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, 2-3-10 Surugadai, Kanda, Chiyo-ku, Tokyo 101, Japan.

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of 1539 bp orf, encoding a 513 amino acid protein. The two mammalian P450cc24 protein sequences share 90% homology and a 100% homology in the 21 amino acid heme-binding domain.

Extensive analysis of the promoter sequences for the two mammalian P450cc24 genes reveals there to be two 1,25-$(OH)_2D_3$ receptor (VDR) response elements separated by a stretch of approximately 93 bp (Ohyama et al., 1994; Zierold et al., 1994). Both of these response elements are necessary to confer full transcriptional expression in the presence of 1,25-$(OH)_2D_3$ as tested by chloramphenicol acetyltransferase reporter analysis (Chen & DeLuca, 1994; Zierold et al., 1995). In the absence of 1,25-$(OH)_2D_3$, the gene is silent but may still be occupied by VDR at the response element sites (Zierold et al., 1994).

The cloning of P450cc24 has expanded the opportunities for studying P450cc24. Our group has expressed functional h-P450cc24 in COS cells and in recombinant, baculovirally (AcNPV) infected *Spodoptera frugiperda* (*Sf21*) insect cells (Chen et al., 1993). Recently, Akiyoshi-Shibata et al. (1994) overproduced r-P450cc24 in a bacterial expression system. In studying C-24 hydroxylation of 25-OH-D_3 and 1,25-$(OH)_2D_3$ substrates, they discovered additional metabolism beyond 24-hydroxylation by this enzyme: the additional reactions being 24-keto-oxidation and 23-hydroxylation. Also, C-23 hydroxylation was found to occur only after C-24 keto-oxidation. In the present report we now demonstrate that P450cc24 is responsible for the catalysis of most, if not all, reactions of the C-24/C-23 pathway of 1,25-$(OH)_2D_3$ degradation to the immediate precursor of the calcitroic acid excretion product.

EXPERIMENTAL PROCEDURES

Radiolabeled Compounds. 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) and 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) were obtained from Dupont/New England Nuclear (Boston, MA). 25-OH-[3α-^{3}H]D_3 (28 Ci/mmol) was made in this laboratory (S. Yamada, H. K. Schnoes, and H. F. DeLuca, unpublished experiments). These compounds were purified on a 0.45 × 25 cm Zorbax silica HPLC column (Phenomenex, Rancho Palos Verdes, CA) before being used as substrate in enzyme assays. The HPLC systems used for purifying the radiolabeled substrates were 99/3 hexane/2-propanol and 90/10 hexane/2-propanol, respectively, for 25-OH-D_3 and 1,25-$(OH)_2$-D_3.

Chemicals. 23,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, 1,25-$(OH)_2D_3$, and 24,25,26,27-tetranor-23-COOH-D_3 were prepared in this laboratory or were purchased from Tetrionics, Inc. (Madison, WI) except for 25-OH-D_3, which was a gift from Organon, Inc. (Orange, NJ). Standards for 24-oxo-25-OH-D_3 and 24-oxo-23,25-$(OH)_2D_3$ were generously provided by one of us (S.Y). The standard for 23,24,25-$(OH)_3D_3$ was prepared in this laboratory by sodium borohydride reduction of authentic 24-oxo-23,25-$(OH)_2D_3$ and purified on cyano and amino HPLC as explained in the high-performance liquid chromatography section. The standard for 24,25,26,27-tetranor-23-OH-D_3 was prepared in this laboratory from 24,25,26,27-tetranor-23-COOH-D_3. First, 24,25,26,27-tetranor-23-COOH-D_3 was esterified using diazomethane in ether. Then the 24,25,26,27-tetranor-23-COOMe-D_3 ester was reduced using 2 equiv of DIBAL-H in ether at −78 °C to form 24,25,26,27-tetranor-23-OH-D_3. 24,25,26,27-Tetranor-23-OH-D_3 was purified on HPLC prior to use as a standard. Sodium periodate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium borohydride and sodium cholate were purchased from Sigma (St. Louis, MO). The sodium cholate was recrystallized prior to use. Dithiothreitol (DTT) was purchased from Alexis Co. (Laufelfingen, Switzerland), and phenylmethanesulfonyl fluoride (PMSF) was purchased from Sigma. Adrenodoxin and adrenodoxin reductase were purified in our laboratory from bovine adrenal glands (Burgos-Trinidad et al., 1986).

Expression of h-P450cc24 cDNA in Sf21 Cells. *Sf21* cells (Vaughn et al., 1977) were cultured in TC-100 medium (Gibco/BRL, Life Technologies, Gathersburg, MD) with 10% FBS (Hyclone Labs, Logan, UT) at 28 °C and plated at 25 × 10^6 cells per 150 mm tissue culture plate. Cells were allowed to attach in reduced serum medium for 30 min. Medium was then removed, and 3 mL of either recombinant AcNPV-h-P450cc24, containing the full orf of h-P450cc24 (Chen et al., 1993), or wild-type virus AcNPV (Granados & Williams, 1986) was titered at 2.5 × 10^8 for *Sf21* cell infection. The AcNPV and *Sf21* cells used in this study were gifts from Dr. Paul Friesen (Molecular Virology, University of Wisconsin, Madison, WI). The plates were rocked for 1 h to allow attachment of virus to the cells. An additional 12 mL of medium was added, and the incubation was continued at 28 °C for 72 h.

Sf21 Cell Mitochondria Isolation and Solubilization. The *Sf21* cells were harvested and washed with 10 mL of PBS, pH 6.2. A 20% homogenate was prepared in 0.25 M sucrose-Tris buffer, pH 7.4 (Summers & Smith, 1987) using an overhead stirrer (Wheaton Instr., Millville, NJ). The homogenate was centrifuged at 300*g* for 10 min at 4 °C. The supernatant was removed and saved. The pellet was resuspended in sucrose-Tris buffer, homogenized, and centrifuged at 300*g* for 10 min at 4 °C. The two supernatants were combined and centrifuged at 2300*g* for 20 min (4 °C) to pellet the mitochondria. The mitochondrial pellet was washed in sucrose-Tris buffer, homogenized, and centrifuged at 2300*g* for 20 min at 4 °C. The mitochondrial pellet was homogenized in 160 mM potassium phosphate buffer and centrifuged at 2300*g* for 20 min at 4 °C. A Bradford assay was run to determine protein concentration of the *Sf21* mitochondrial extract. The protein concentration was adjusted to 5 mg/mL with 160 mM potassium phosphate buffer. The mitochondrial proteins were solubilized by adding 0.63% sodium cholate, 0.1 mM DTT, and 0.25 mM PMSF, pH 7.4, and slowly mixed for 1 h at 4 °C. Then the sample was centrifuged at 100000*g* (4 °C) for 1 h using a Beckman 70.1 Ti rotor. The resulting solubilized mitochondrial protein supernatant was stored at −70 °C until use in h-P450cc24 activity assay.

Enzyme Assay. The recombinant h-P450cc24 enzyme activity was determined according to the methods of Burgos-Trinidad et al. (1990). Ten micrograms of solubilized enzyme was used for these studies with 1.6 nmol of adrenodoxin, 0.1 nmol of adrenodoxin reductase, 100 nmol of NADPH, 1 μmol of glucose 6-phosphate and 0.2 units of glucose-6-phosphate dehydrogenase in a total volume of 190 μL. The reaction was initiated with the addition of 200 pmol of either 1,25-$(OH)_2$-[26,27-^{3}H]D_3 or 25-OH-[26,27-^{3}H]D_3 at 800 dpm/pmol in 10 μL of ethanol at the appropriate substrate concentration and for the appropriate time. The small scale assays were terminated by the addition of 40 μL

of 1 N acetic acid. In large scale h-P450cc24 assays where products formed were further analyzed, a modified version of the Burgos-Trinidad et al. (1990) protocol was used. Briefly, 100 μg of solubilized mitochondrial proteins prepared from *Sf21* cells infected with either wild-type or recombinant baculovirus constructs was incubated for 5, 20, 40, or 60 min at 37 °C in the presence of cofactors (16 nmol of adrenodoxin, 1 nmol of adrenodoxin reductase, 1 μmol of NADPH, 10 μmol of glucose 6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase) and 1−2 μCi of 25-OH-[26,27-^{3}H]D_3 substrate. The final reaction volume was 2 mL in a 10 mM potassium phosphate buffer, pH 7.4. All large scale reactions were terminated by the addition of 400 μL of 1 N acetic acid.

Metabolite Extraction and HPLC Analysis. Reaction mixtures were extracted three times with methylene chloride (v/v). All extracts were combined and the solvent removed under nitrogen. Each residue was dissolved in the appropriate HPLC solvent. Samples were subjected to a 0.45 × 25 cm cyano-bonded silica HPLC column (Rainin Instrument Co., Woburn, MA). The cyano system was conditioned in a quaternary solvent consisting of hexane/methylenchloride/acetonitrile/ethanol (93/5/1/1). Products were analyzed in two ways: (1) for samples in the time course, the entire 0.2 min fractions were completely dried and dissolved in scintillation fluid and counted using a Tri-CARB liquid scintillation analyzer (Packard Instr., Meriden, CT); and (2) for samples that were to be used for characterization, only 10−50 μL of each fraction was used to determine the radioactivity. The detected radioactive products were isolated, dried under N_2, and resuspended in 100 μL of column solvent for a second HPLC system. These samples were injected onto a 0.45 × 25 cm amino-bonded silica HPLC column (IBM Instruments Inc., Danbury, CT) at a flow rate of 1.5 mL/min. The amino system was conditioned in a ternary solvent consisting of methanol/isopropanol/hexane (97/2/1). Purified products from amino HPLC were monitored by counting 10 μL aliquots and comparing retention times with that of authentic standards.

Three extractions (v/v) of the sample with methylene chloride were found to extract nearly all 26,27-^{3}H from the aqueous phase recombinant and wild-type reactions carried out for 20 min. The amount of nonrecoverable radioactivity by this extraction method was assessed by counting the remaining water-soluble ^{3}H.

Periodate Cleavage. Each sample was dried under nitrogen and dissolved in 100 μL of absolute methanol. Approximately 1 mg of sodium periodate was mixed into each sample, and the tubes were capped. The reactions proceeded for 1 h at 25 °C. Afterward, the reaction mixture was dried under nitrogen and extracted in 100 μL of hexane/isopropanol/methanol (97/2/1). The extracts were injected onto amino HPLC at a flow rate of 1.5 mL/min. To demonstrate the susceptibility of each product to periodate cleavage, separate HPLC analyses were performed on equivalent portions of untreated radioactive products.

Borohydride Reduction. Amino HPLC purified products were dried under nitrogen and dissolved in absolute methanol. The reduction reaction was initiated by the addition of approximately 1 mg of sodium borohydride and allowed to proceed for 20 h at 25 °C. Reactants were extracted twice with 300 μL of methylene chloride. The methylene chloride was evaporated to dryness, and the sample was suspended in the appropriate solvent for HPLC on the amino system. Authentic standards were also run to demonstrate the effectiveness of the borohydride reduction. Standard for 23,24,25-$(OH)_3D_3$ was prepared by sodium borohydride reduction of 24-oxo-23(S),25-$(OH)_2D_3$ followed by purification on amino HPLC, hexane/isopropanol/methanol (90/7/3).

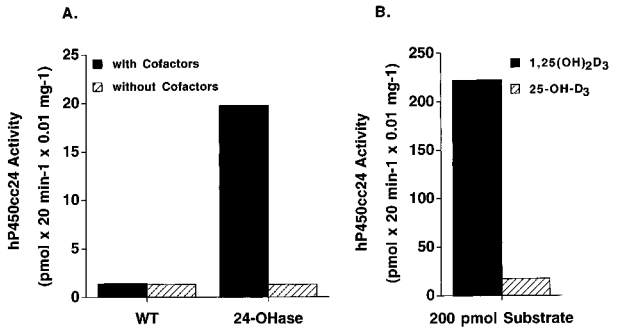

FIGURE 1: (A) Dependence of recombinant h-P450cc24 on adrenodoxin, adrenodoxin reductase, and NADPH. (B) Selectivity of h-P450cc24 for its two natural substrates [1,25-$(OH)_2D_3$ and 25-OH-D_3]. P450cc24 activity was determined by a periodate cleavage assay, using 10 μg of solubilized *Sf21* cell mitochondria obtained from either AcNPV (wild-type) or AcNPV-P450cc24 (h-P450cc24) infected *Sf21* cells. Reactions were initiated by addition of 200 pmol of appropriate [26,27-^{3}H]substrate, specific activity 800 dpm/pmol.

Purification of and Characterization of 24,25,26,27-Tetranor-23-OH-D_3. Two reactions of 1 μCi of 25-OH-[3α-^{3}H]D_3 each were incubated for 1 h with 10 μg of solubilized recombinant AcNPV-P450cc24 in a reaction that contained 1.6 nmol of adrenodoxin, 0.1 nmol of adrenodoxin reductase, 100 nmol of NADPH, 1 μmol of glucose 6-phosphate, and 0.2 units of glucose-6-phosphate dehydrogenase cofactors in a total volume of 200 μL. The dichloromethane extracts were subjected to cyano HPLC developed in hexane/methylene chloride/acetonitrile/ethanol (93/5/1/1). Fractions containing products that comigrated with 24-oxo-25-OH-D_3 and 24,25,26,27-tetranor-23-OH-D_3 were collected and combined. The pooled sample was dried under nitrogen, resuspended in 100 μL of hexane/isopropanol/methanol (97/2/1), and injected onto amino HPLC (Alltech Associates, Inc., Deerfield, IL) at a flow rate of 2.5 mL/min. Aliquots (500 μL) of each fraction were counted to monitor product formation and retention time with respect to authentic standards for 24-oxo-25-OH-D_3 and 24,25,26,27-tetranor-23-OH-D_3.

Acetylation Reaction. Fractions containing the putative 24,25,26,27-tetranor-23-OH-[3α-^{3}H]D_3 product were collected and combined. The sample was dried under nitrogen and dissolved in 100 μL of pyridine. Distilled acetic anhydride was added in excess and left to react overnight in a capped test tube. The reactant was dried under argon, resuspended in hexane/isopropanol/methanol (97/2/1), and reinjected onto amino HPLC.

RESULTS

Evidence for the Presence of h-P450cc24 Activity in the Solubilized Mitochondrial Fraction of Sf21 Cells. Solubilized mitochondrial protein from *Sf21* cells infected with recombinant virus AcNPV-P450cc24 cDNA expressed 24-hydroxylase activity when reconstituted with bovine adrenodoxin, adrenodoxin reductase, and NADPH (Figure 1A). Preparations from *Sf21* cells infected with wild-type virus

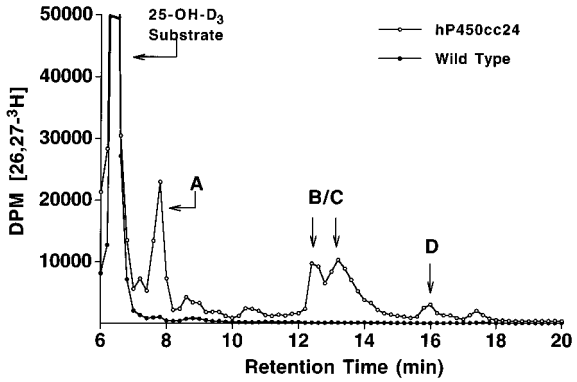

FIGURE 2: Cyano HPLC separation of metabolites in the dichloromethane extracts from 20 min incubations of both wild-type and P450cc24 (100 μg) with 25-OH-[26,27-^{3}H]D_3 (1 μCi). Peaks A−D were found to exactly comigrate with authentic standards for 24-oxo-25-OH-D_3, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, and 24-oxo-23(*S*),25-$(OH)_2D_3$, respectively. Methylene chloride extracted samples were dried under nitrogen and injected onto cyano HPLC conditioned in hexane/methylene chloride/acetonitrile/ethanol (93/5/1/1), at a flow rate of 1.5 mL/min.

AcNPV (Figure 1A) and reconstituted showed no such activity. Also, h-P450cc24 activity was not detected in the absence of adrenodoxin, adrenodoxin reductase, and NADPH. The h-P450cc24 activity was approximately 10-fold higher for 1,25-$(OH)_2D_3$ than for 25-OH-D_3 (Figure 1B). This agrees with other published findings where the apparent K_m of P450cc24 for 1,25-$(OH)_2D_3$ is an order of magnitude lower than for 25-OH-D_3 (Burgos-Trinidad & DeLuca, 1991; Inaba et al., 1991).

Metabolism of 25-OH-[26,27-^{3}H]D_3 by h-P450cc24 and Time Course of Product Formation. The incubation of h-P450cc24 with 1 μCi of 25-OH-[26,27-^{3}H]D_3 generated several metabolites separated on cyano column HPLC (Figure 2). Four of these metabolites, labeled A−D, comigrated exactly with authentic standards for 24-oxo-25-OH-D_3, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, and 24-oxo-23(*S*),25-$(OH)_2D_3$, respectively (Figure 2). A similar incubation of 25-OH-[26,27-^{3}H]D_3 substrate with cells infected with wild-type virus resulted in no observable metabolites.

Figure 3A shows the amount of 25-OH-[26,27-^{3}H]D_3 substrate recovered in cells infected with wild-type virus and from recombinant samples at 5, 20, 40, and 60 min. The disappearance of 25-OH-[26,27-^{3}H]D_3 was roughly linear from 5−40 min but then tapered off between 40 and 60 min. Products comigrating with the respective authentic standards increased to their highest levels at 40 min (Figure 3B−D). At 60 min, each product declined to a value that was less than that at 20 min. Examination of the aqueous phase postextraction revealed that the amount of nonextractable ^{3}H increased with the time of incubation in the case of the recombinant h-P450cc24 (Figure 4). There was no detectable change in the amount of nonextractable ^{3}H that correlated with the time of incubation in the case of the preparations from wild-type baculovirus.

Isolation and Identification of Metabolites A, B, C, and D. Figure 5 shows the profile of 25-OH-D_3 metabolites run on the cyano HPLC system. In panel A the full scale is provided, and in panel B the smaller product peaks are expanded for a better view. Two recombinant reactions were run to ensure that there would be enough product for the characterization experiments. Three major products formed, the second of which was presumed to be a mixture of

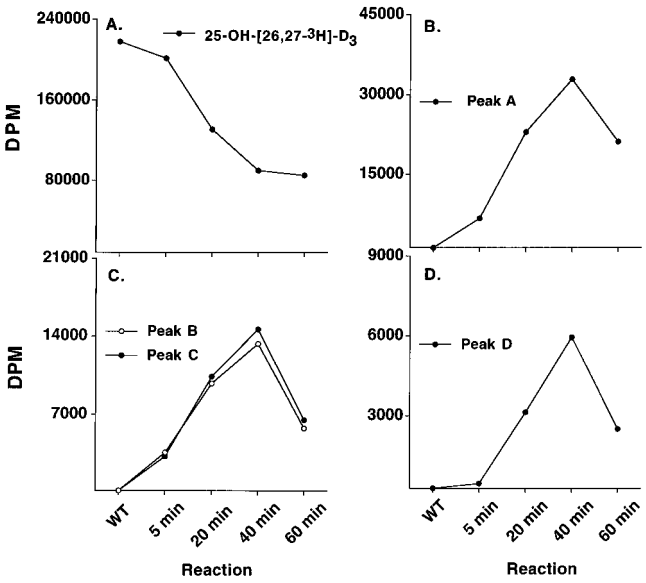

FIGURE 3: Time course of substrate utilization by h-P450cc24 (5, 20, 40, and 60 min reactions) and product formation of peaks A, B, C, and D over that of a baseline wild-type reaction incubated for 20 min. The reactions consisted of 100 μg of solubilized wild-type or h-P450cc24 protein isolated from Sf21 cells incubated with 1 μCi of 25-OH-[26,27-^{3}H]D_3 each. In each reaction, h-P450cc24 activity was reconstituted by addition of adrenodoxin, adrenodoxin reductase, and NADPH cofactors.

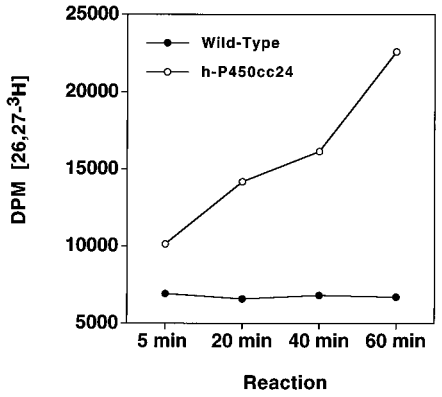

FIGURE 4: Amount of total 26,27-^{3}H retained in the aqueous fractions of incubation mixtures of wild-type and h-P450cc24 preparations versus time of incubation.

metabolites B and C. This middle peak (B/C) was first separated into two constituent peaks on an amino column HPLC system as described in Experimental Procedures (data not shown). The advantage of the amino system as a second step is the complete resolution of standards for 23,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ and a switch in the retention time positions of 24,25-$(OH)_2D_3$ and 24-oxo-23,25-$(OH)_2D_3$ standards. Figure 6 demonstrates the results of injecting each isolated product onto amino HPLC. In each case, products A−D comigrated exactly with authentic standards for 24-oxo-25-OH-D_3, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, and 24-oxo-23(*S*),25-$(OH)_2D_3$, respectively, with the positions of products in the amino HPLC system changing to A, B, D, and C.

The products were isolated from amino HPLC, dried under nitrogen, and resuspended in methanol. Treatment of portions of metabolites A, C, and D with or without $NaIO_4$ tested these compounds for either a vicinal hydroxyl or ketone group to the C-25 hydroxyl group. The presence of such a

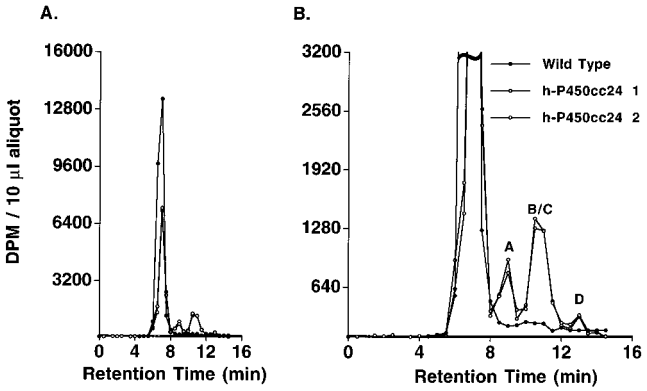

FIGURE 5: Cyano HPLC profiles of extracts from wild-type and h-P450cc24 (10 μg of solubilized protein) incubated with 1 μCi of 25-OH-[26,27^{3}H]D_3 substrate in the presence of adrenodoxin, reductase, and cofactors. Injections were made onto a cyano-bonded silica system conditioned in hexane/methylene chloride/acetonitrile/ethanol (93/5/1/1), at a flow rate of 1.5 mL/min. The fraction collector was set at 0.5 mL/min. The profile illustrated in panel A represents 10 μL aliquots of each collected fraction and depicts the full-length *y*-scale. The *y*-scale in panel B has been reduced to better represent the product formation data.

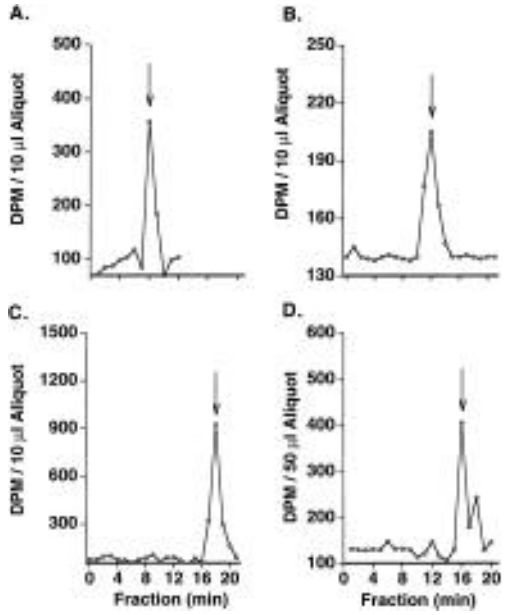

FIGURE 6: Amino HPLC characterization of samples isolated from the cyano HPLC step. Arrows represent the positions of authentic standards for (A) 24-oxo-25-OH-D_3, (B) 23(*S*),25-$(OH)_2D_3$, (C) 24-(*R*),25-$(OH)_2D_3$, and (D) 24-oxo-23(*S*),25-$(OH)_2D_3$. Isolates from cyano HPLC were injected onto an amino-bonded silica system conditioned in hexane/isopropanol/methanol (97/2/1), at a flow rate of 1.5 mL/min. The fraction collector was set at 1.0 mL/min, and the metabolites were monitored by counting 10 μL aliquots of each fraction.

reactive functional group would result in cleavage between C-24 and C-25, causing the loss of the 26,27-^{3}H. As shown in Figure 7, $NaIO_4$ treatment resulted in elimination of A, C, and D.

Metabolites A and D were reacted with $NaBH_4$ to test for the presence of a carbonyl functional group (Figure 8). Treatment of metabolite A with $NaBH_4$ resulted in a shift of the ^{3}H to the position of 24(*R*),25-$(OH)_2D_3$ on amino HPLC, confirming the presence of a C-24 ketone on metabolite A. Likewise, a reduced form of metabolite D was confirmed by a retention time identical to the doublet of 23(*S*),24(α/β),25-$(OH)_3D_3$ isomers. Treatment of authentic 24-oxo-23(*S*),25-$(OH)_2D_3$ with $NaBH_4$ also yielded a mixture of 24 hydroxylated isomers of both α and β species.

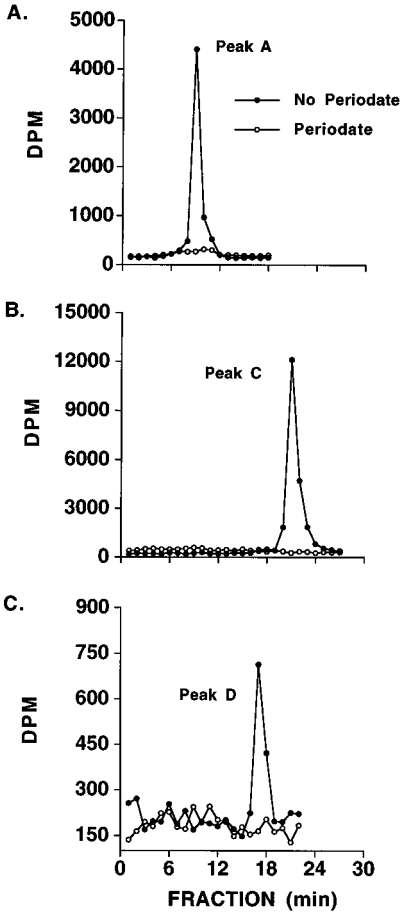

FIGURE 7: Periodate cleavage test of metabolites A, C, and D. Portions of purified isolates from amino HPLC were treated in the presence or absence of 1 mg of sodium periodate. The samples were then dried and reinjected onto the amino HPLC system [hexane/isopropanol/methanol (97/2/1), at a flow rate of 1.5 mL/min]. The analysis represents the total amount of DPM obtained by scintillation counting of each fraction (1 mL/min).

Metabolism of 25-OH-[3α-^{3}H]D_3 by h-P450cc24: Isolation of 24,25,26,27-tetranor-23-OH-D_3. The extracted organic phases from incubations of two recombinant h-P450cc24 reactions using 25-OH-[3α-^{3}H]D_3 as substrate were injected onto a cyano HPLC system as described in Experimental Procedures (data not shown). Standards for 24-oxo-25-OH-D_3 and 24,25,26,27-tetranor-23-OH-D_3 were found to comigrate on this system. Therefore, the region correlating to the position of 24,25,26,27-tetranor-23-OH-D_3 was collected, dried under nitrogen, and reinjected onto an amino (Alltech) HPLC system, which completely separated the standard, 24-oxo-25-OH-D_3, from 24,25,26,27-tetranor-23-OH-D_3. An aliquot from each collected fraction was counted. Two peaks migrating exactly in the positions of 24-oxo-25-OH-D_3 and 24,25,26,27-tetranor-23-OH-D_3 were clearly resolved (Figure 9). The second metabolite, comigrating with the 24,25,26,27-tetranor-23-OH-D_3 standard, was collected, dried under nitrogen, and redissolved in pyridine. Exposure of this sample to acetic anhydride modified the metabolite so that, when run on amino HPLC again, the retention time shifted to a less polar position (Figure 10). The shift in retention time confirmed the presence of hydroxyl groups susceptible to the acetic anhydride acetylation.

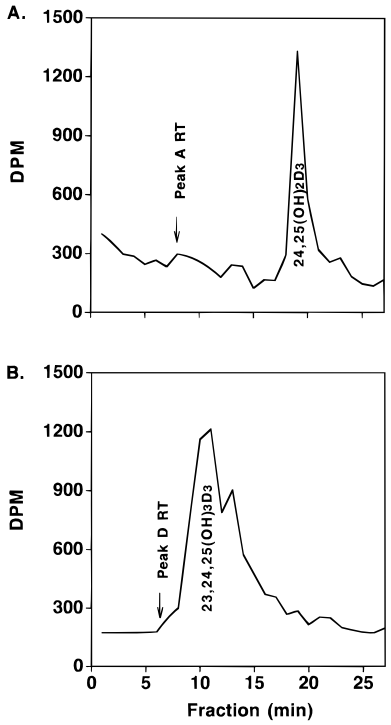

FIGURE 8: Borohydride reduction test of metabolites A and D. Portions of purified isolates from amino HPLC were treated in the presence of 1 mg of sodium borohydride. The samples were then dried and reinjected onto the amino HPLC system [hexane/isopropanol/methanol (97/2/1), at a flow rate of 1.5 mL/min]. The analysis represents the total amount of DPM obtained by scintillation counting of each fraction (1 mL/min). Retention time positions of authentic standards for 24,25-$(OH)_2D_3$ (panel A) and 23,24,25-$(OH)_3D_3$ (panel B) are marked by arrows. The retention times for peaks A and D are also provided in each respective chromatograph.

DISCUSSION

This study demonstrates that h-P450cc24 is capable of multicatalytic activity that is consistent with the conversion of 25-OH-D_3 to 24,25,26,27-tetranor-23-OH-D_3 via known intermediates of the 25-OH-D_3 catabolic pathway (C-24/C-23 pathway). Incubation of 25-OH-[26,27-^{3}H]D_3 substrate with recombinant h-P450cc24 solubilized from *Sf21* cells resulted in the formation of several products in addition to 24,25-$(OH)_2D_3$. The metabolites, labeled A, B, C, and D, migrated exactly with authentic standards for the four natural metabolites; 24-oxo-25-OH-D_3, 23(*S*),25-$(OH)_2D_3$, 24(*R*),25-$(OH)_2D_3$, and 24-oxo-23(*S*),25-$(OH)_2D_3$, respectively, on a straight phase cyano-bonded silica HPLC system. The metabolites were isolated and further characterized by a second HPLC system employing amino-bonded silica, which achieved clear resolution of metabolites B and C, and again demonstrated comigration of metabolites A, B, C, and D with respective authentic standards. The presence of a putative keto-oxidized functional group on metabolites A and D was confirmed by borohydride reduction and shifts in the HPLC migration of the reduced metabolites on amino-bonded silica. Also, the presence of an oxidized C-24 of metabolites A, C, and D was demonstrated by susceptibility to periodate cleavage. Incubation of the 25-OH-[3α-^{3}H]D_3 substrate with h-P450cc24 resulted in an additional metabolite on cyano HPLC that completely separated from 24-oxo-25-OH-D_3 on amino HPLC and comigrated with the 24,25,26,27-tetranor-23-OH-D_3 standard in both HPLC systems. Acetylation of

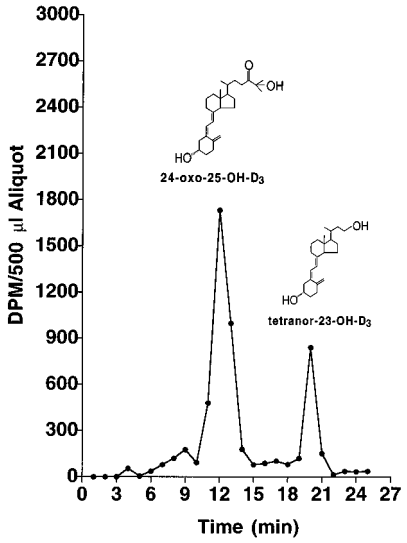

FIGURE 9: Amino HPLC isolation and purification of putative 24,25,26,27-tetranor-23-OH-[3α^{3}H]D_3. Two reactions of 10 μg of h-P450cc24 incubated with 1 μCi each of 25-OH-[3α^{3}H]D_3 for 40 min were used. The sample was first isolated from cyano HPLC (93/5/1/1) in the region where the comigrating authentic standards for 24-oxo-25-OH-D_3 and 24,25,26,27-tetranor-23-OH-D_3 eluted (data not shown) at a retention time of 7−9 min. These fractions were combined and injected as a single sample onto an Alltech amino HPLC system, hexane/isopropanol/methanol (97/2/1), at a flow rate of 2.5 mL/min. The positions of authentic standards are labeled. One min fractions were collected, and 500 μL of each fraction was used for measuring ^{3}H.

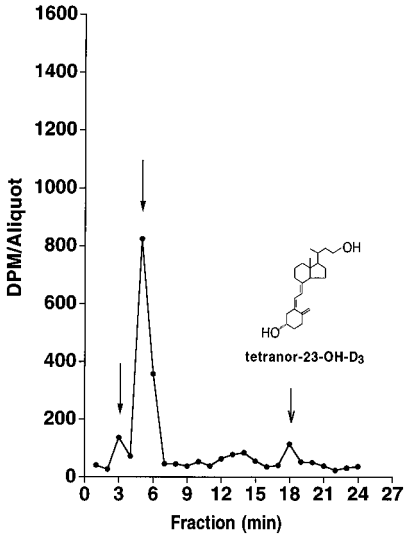

FIGURE 10: Acetylation test of putative 24,25,26,27-tetranor-23-OH-[3α^{3}H]D_3. The putative 24,25,26,27-tetranor-23-OH-D_3 metabolite isolated from amino HPLC was dried and redissolved in pyridine. Excess acetic anhydride was reacted with the sample overnight. The reaction mixture was dried by a steady stream of argon gas, and the sample was reinjection onto amino HPLC, hexane/isopropanol/methanol (97/2/1), at a flow rate of 2.5 mL/min. One minute fractions were collected and scintillation counted for monitoring constituent peaks in the sample. The position of authentic 24,25,26,27-tetranor-23-OH-D_3 standard is labeled. Arrows mark the trace in positions where the modified metabolite eluted.

the two putative hydroxyls of this metabolite by acetic anhydride caused the metabolite to shift to a less polar position on amino HPLC. These data collectively illustrate the potential of h-P450cc24 to perform several well characterized reactions of the C-24/C-23 catabolic pathway including side-chain cleavage to a C-23-alcohol product.

Early experiments demonstrated that none of the metabolites detected in the recombinant system could be produced from the system infected with the wild-type AcNPV vector. Also, h-P450cc24 activity was not detected in reconstituted mitochondrial protein obtained from wild-type infected *Sf21* cells nor was any h-P450cc24 activity detected from recombinant h-P450cc24 incubated without adrenodoxin and adrenodoxin reductase. Therefore, the formation of products was not the result of a mitochondrial P450 found in *Sf21* cells.

There is the question of whether *Sf21* cells can further metabolize 24-hydroxylated derivatives of 25-OH-D_3. However, we did not have [^{3}H]24,25-$(OH)_2D_3$ of sufficiently high specific activity available to us to address this question. In the study of Akiyoshi-Shibata et al. (1994), r-P450cc24 further oxidized 24,25-$(OH)_2D_3$ or 1,24,25-$(OH)_3D_3$ to additional products, while membrane fractions from control bacteria could not.

A major difference between our work versus that done with r-P450cc24 is the formation of 23,25-$(OH)_2D_3$ by h-P450cc24 as a major product. This may represent a species difference since the formation of 23,25-$(OH)_2D_3$ in rats has been shown to be a quantitatively minor pathway (Jones et al., 1983; Napoli & Horst, 1983). Similarly, work done in cell culture using rat cells has demonstrated C-23 oxidation primarily on C-24 oxidized derivatives (Lohnes & Jones, 1987; Makin et al., 1989). However, C-23 oxidation of vitamin D analogs independent of a prior C-24 oxidation was demonstrated in keratinocytes of human origin but not in cells of rat origin (Masuda et al., 1994). An interesting model for study of 23-hydroxylation is the guinea pig, which in a study by Pedersen et al. (1988) was found to form 23,25-$(OH)_2D_3$ as a major product of 25-OH-D_3 relative to 24,25-$(OH)_2D_3$.

The time course experiments of the present study revealed a decrease in the amount of detectable product formed after 40 min, which led to the finding that 26,27-^{3}H was accumulating over time in the aqueous phase. The isolation of 24,25,26,27-tetranor-23-OH-[3α-^{3}H]D_3 confirmed our assumption of side-chain cleavage by recombinant P450cc24. Examination of the aqueous phase partitions from incubations with 25-OH-[3α-^{3}H]D_3 revealed a lower total accumulation of nonextractable 3α-^{3}H in the aqueous phase compared with incubations where 25-OH-[26,27-^{3}H]D_3 was used (data not included). In addition, an HPLC analysis of the aqueous partition following a drying step failed to reveal if a C-23-[3α-^{3}H]acid product had formed. This would suggest that 24,25,26,27-tetranor-23-OH-D_3 might be the final end-product of 25-OH-D_3 metabolism by h-P450cc24. In the rat, however, it is well established both *in vivo* (Esvelt et al., 1979; Onisko et al., 1980) and using cell culture or kidney perfusion systems (Jones et al., 1984; Makin et al., 1989; Reddy & Tserng, 1989) that calcitroic acid is the major catabolic end-product of 1,25-$(OH)_2D_3$. Perhaps the conversion of 24,25,26,27-tetranor-23-OH-D_3 to a C-23 acid requires a C-1α hydroxylation. It also is possible that a separate enzyme system within target cells is required for C-23 acid formation. Another possibility is that the incubation conditions used for the reactions of the present study or the isolation procedure may not have been optimal for detecting the formation of a C-23 acid product. We are presently addressing these possibilities.

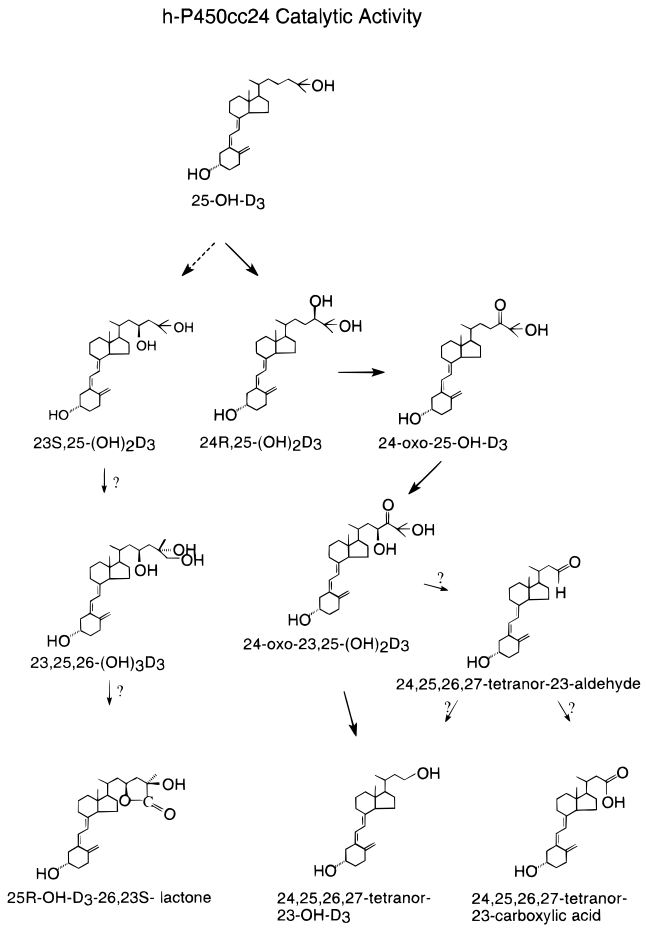

FIGURE 11: Proposed multicatalytic function of h-P450cc24 in the catabolism of 25-OH-D_3. The large arrows represent catalytic steps identified in the present study, whereas small arrows depict additional steps in the catabolism of 25-OH-D_3.

This study and the study of Akiyoshi-Shibata et al. (1994) suggest that the C-24/C-23 catabolic pathway is carried out entirely by h-P450cc24. This would explain why other enzymes in this pathway have not been isolated to date. It is also clear that all the major target tissues are capable of carrying out the complete set of reactions reported in the present study (Makin et al., 1989; Napoli & Horst, 1983), in conditions where P450cc24 is induced. These reactions are dependent upon 1,25-$(OH)_2D_3$-induction (Napoli & Martin, 1984), and they do not exist in vitamin D-deficient conditions (Engstrom et al., 1984).

It is intriguing, but perhaps not surprising, that P450cc24 is capable of catalyzing several reactions in sequence. Many other cytochrome P450 enzymes also exhibit multicatalytic activity. The liver cytochrome mitochondrial P450, CYP27, was shown to 27-hydroxylate cholesterol and catalyze 25-hydroxylation of vitamin D_3 and 1α-hydroxylation of 25-OH-D_3 (Axen et al., 1994). In addition, cholesterol side chain cleavage enzyme (SCC) catalyzes sequential C-22 and C-20 hydroxylations and oxidative cleavage of the 22,20 carbon−carbon bond to form pregnenolone (Miller, 1987). In rats, steroid microsomal 17α-hydroxysteroid catalyzes the conversion of progesterone to 17α-hydroxyprogesterone and catalyzes oxidative cleavage of the 17,20 carbon−carbon bond to form androstenedione (Miller, 1987). Another example is mitochondrial 11β-hydroxylase, in which the 11B2 isoform 18-hydroxylates and then 18-keto-oxidizes corticosterone to aldosterone and cortisol to 18-oxo-cortisol,

in addition to 11β-hydroxylating 11-deoxycortisol to cortisol (Miller, 1987). It is also noteworthy that, at the amino acid level, r-P450cc24 shares considerable identity with r-P450cc25 (32%), r-SCC (26%) and r-11β-hydroxylase (28%), which may relate to catalytic domains that participate in the similar reactions carried out by these respective enzyme systems.

The postulated physiological role of P450cc24 (DeLuca & Schnoes, 1983; Haussler et al., 1988) is to initiate the catabolism of 1,25-$(OH)_2D_3$ via C-24 hydroxylation. It was envisioned that other P450 enzyme systems, also induced by 1,25-$(OH)_2D_3$, would further catabolize 1,24,25-$(OH)_3D_3$ to a side-chain cleaved C-23 alcohol or C-23 acid end-product. The present results and those of Akiyoshi-Shibata et al. (1994) greatly simplify the catabolism of 1,25-$(OH)_2D_3$ and 25-OH-D_3 by providing evidence that a single enzyme carries out the reactions previously thought to be carried out by several enzymes (Figure 11).

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