Mechanism of 25-Hydroxyvitamin D_3 24-Hydroxylation: Incorporation of Oxygen-18 into the 24 Position of 25-Hydroxyvitamin D_3†

Thelma C. Madhok, Heinrich K. Schnoes, and Hector F. DeLuca*

ABSTRACT: The oxygen enzymatically inserted as a hydroxyl function by chick kidney homogenate into the 24 position of 25-hydroxyvitamin D_3 to give 24,25-dihydroxyvitamin D_3 is derived exclusively from $^{18}O_2$. Therefore, like the 25-hydroxyvitamin D_3-1α-hydroxylase system, the 25-hydroxyvitamin D_3-24-hydroxylase system is also a monooxygenase ("mixed-function oxidase").

Previous reports have established that vitamin D_3 must be metabolically activated before it can manifest its physiologic functions (DeLuca, 1974; Kodicek, 1974). The activation of vitamin D_3 primarily by the liver involves a hydroxylation reaction in the 25 position to produce 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (Horsting and DeLuca, 1969; Blunt et al., 1968; Ponchon et al., 1969). This hydroxylated derivative represents the major metabolite of vitamin D_3 circulating in blood plasma, not only in experimental rats (Lund and DeLuca, 1966) and chickens (Ponchon et al., 1969; Lawson et al., 1969), but also in man (Mawer et al., 1969; Gray et al., 1971). The 25-OH-D_3 is subsequently transported to the kidney where it is further hydroxylated on carbon 1 to yield 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Fraser and Kodicek, 1970; Holick et al., 1971; Gray et al., 1971; Norman et al., 1971) or on carbon 24 to yield 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) (Holick et al., 1972) depending on physiological circumstances. The manner by which the formation of these hydroxylated metabolites are regulated is still unknown but it is known that the production of 24,25-$(OH)_2D_3$ is generally stimulated under conditions in which the production of 1,25-$(OH)_2D_3$ is suppressed (Boyle et al., 1971; Garabedian et al., 1972; Tanaka and DeLuca, 1973). Furthermore, 1,25-$(OH)_2D_3$ stimulates the 24-hydroxylase (Tanaka and DeLuca, 1973; Tanaka et al., 1975). However, the function of 24,25-$(OH)_2D_3$ is still the subject of much debate, although it is generally agreed that 24,25-$(OH)_2D_3$ is a major metabolite of vitamin D in man and animals. Also the 24-hydroxylase also hydroxylates 1,25-$(OH)_2D_3$ to form 1,24,25-trihydroxyvitamin D_3 (1,24,25-$(OH)_3D_3$) (Tanaka et al., 1977; Kleiner-Bossaller and DeLuca, 1974). Regardless of the function of 24,25-$(OH)_2D_3$, the mechanism of 24-hydroxylation of vitamin D is of obvious importance.

The 25-OH-D_3-1-hydroxylase has been extensively studied and is in many respects similar to the monooxygenase of beef adrenal cortex mitochondria concerned with steroidogenesis (Gray et al., 1972; Sih, 1969). Hydroxylation of 25-OH-D_3 on carbon 1 takes place in isolated chick kidney mitochondria in the presence of the oxidizable substrate, molecular oxygen, and magnesium ions (Fraser and Kodicek, 1970; DeLuca, 1974; Tanaka and DeLuca, 1974; Gray et al., 1971, 1972). The reaction also requires NADPH (Ghazarian and DeLuca, 1974). Experiments with $^{18}O_2$ have revealed clearly that the oxygen, which is placed in position 1 by the hydroxylase, arises exclusively from molecular oxygen (Ghazarian et al., 1973). These findings plus the recent demonstration of a requirement for cytochrome P_{450} (Ghazarian et al., 1974) and an iron-sulfur protein (Ghazarian et al., 1974; Pedersen et al., 1976) in the mitochondria have clearly established the 1α-hydroxylase as a monooxygenase ("mixed-function oxidase").

The 24-hydroxylase is also found in chick kidney mitochondria and also requires NADPH, magnesium ions, and molecular oxygen (Knutson and DeLuca, 1974). Unlike the 1-hydroxylase, the 24-hydroxylase is not inhibited by carbon monoxide. Little is known further about this pivotal system in the metabolism of vitamin D_3.

In this paper it will be demonstrated that the oxygen enzymatically introduced as a hydroxyl function into the 24 position of 25-OH-D_3 to yield 24*R*,25-$(OH)_2D_3$ is derived exclusively from molecular oxygen providing strong evidence that the 24-hydroxylase is a monooxygenase ("mixed-function oxidase") rather than a dehydrogenase-hydratase system.

Methods

Animals. Seventeen 1-day-old white Leghorn cockerel chicks were obtained from Northern Hatcheries (Beaver Dam, Wis.). They were maintained on a 3% calcium, vitamin D-deficient purified soy protein diet for 10 days (Omdahl et al., 1971) and were dosed with 1,25-$(OH)_2D_3$ (250 ng per chick, intramuscularly) 24 and 48 h before sacrifice.

Preparation of the Enzyme. The chicks were killed by cervical dislocation, and the kidneys were immediately removed and carefully separated from the adhering connective tissue. The kidneys were rinsed with ice-cold buffer (0.19 M sucrose–15 mM Tris-acetate (pH 7.4)–1.9 mM magnesium acetate), transferred to an ice-cold Potter-Elvehjem homogenizer fitted with a Teflon pestle, and homogenized in 2 volumes of the buffer. The homogenate was centrifuged at 128*g* at 4 °C for 5 min using a Lourdes centrifuge equipped with a 9RA rotor. The resulting supernatant was used for incubation.

Incubation Conditions and Extraction of Samples. Four-

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received December 27, 1976.* Supported by a grant from the National Institutes of Health No. AM-14881 and the Harry Steenbock Research Fund.

[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; HPLC, high-pressure liquid chromatography.

teen incubations, each 1.5 mL total volume, were performed in 5-mL serum bottles containing 19 mg of homogenate protein and 25 mM succinate. The incubation medium was described previously (Knutson and DeLuca, 1974). The bottles were sealed with serum bottle caps and connected through needle outlets to a vacuum train. The bottles were then evacuated repeatedly, followed by flushings with purified nitrogen passed over heated copper. Finally, 3.5 mL of 99.3 atom % $^{18}O_2$ (Bio-Rad Laboratories, Richmond, Calif.) was introduced into each of 14 incubation bottles using a gas-tight syringe. The hydroxylation reactions were initiated by introduction (with a Hamilton syringe) of 1.5 μg of 25-OH-[26,27-^{3}H]D_3 in 10 μL of 95% ethanol with a specific radioactivity of 247 703 cpm/μg. After 30 min of incubation at 37 °C and at 120 oscillations/min, the reactions were terminated by the immediate transfer of the mixtures into a 500-mL separatory funnel containing 84 mL of 2:1 CH_3OH–$CHCl_3$ mixture, and 63 mL of CH_3OH was then added. The reaction mixture was shaken and allowed to stand at room temperature for 1 h. A two-phase system at 4 °C was produced by the addition of 35 mL of deionized water and 84 mL of $CHCl_3$. The lower phase was removed and the upper phase was reextracted twice with 84-mL portions of $CHCl_3$. The combined chloroform extracts contained 6.5 × 10^6 cpm or 100% of the added radioactivity.

Chromatography. The combined chloroform extracts were evaporated to dryness using a rotary evaporator at 30 °C. The residue was dissolved in 500 μL of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 60 cm glass column containing 18 g of Sephadex LH-20 (Pharmacia Corp., Piscataway, N.J.), packed and eluted in the same solvent system (Holick and DeLuca, 1971). A total of 60 fractions (3.5 mL at a flow rate of 0.6 mL/min) were collected and 5μL of each fraction was used for tritium counting (Figure 1a) in a Packard Model 3375 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). The efficiency of counting was 35%. The relative position of the metabolites has been determined previously by chromatography of the pure metabolites under identical experimental conditions (Holick and DeLuca, 1971; Holick et al., 1973). The 24*R*,25-$(OH)_2D_3$ peak region, fractions 30–40, was combined and evaporated to dryness using a rotary evaporator at 30 °C to yield a total radioactivity of 465 666 cpm equivalent to 1.9 μg of 24,25-$(OH)_2D_3$ (based on 247 703 cpm/μg, the specific radioactivity of the substrate, 25-OH-D_3). The residue was dissolved in 100 μL of 50:50 $CHCl_3$–Skellysolve B and applied to a 1 × 60 cm glass column containing 18 g of Sephadex LH-20, packed and eluted in the same solvent system (Holick and DeLuca, 1971). A total of 80 fractions (3.5 mL at a flow rate of 0.6 mL/min) was collected and 20 μL of each fraction was used for tritium counting (Figure 1b). The product peak region, fractions 48–74, was combined and evaporated to dryness using a rotary evaporator at 30 °C to yield a total radioactivity of 433 782 cpm equivalent to 1.75 μg of 24,25-$(OH)_2D_3$. The residue was dissolved in 100 μL of CH_3OH and applied to a 1 × 90 cm glass column packed with 20 g of Sephadex LH-20 in the same solvent. The column was eluted with CH_3OH. A total of 60 fractions (2 mL at a flow rate of 0.5 mL/min) was collected and 5 μL of each fraction was used for tritium counting (Figure 1c). Peak fraction 32 (containing 125 280 cpm equivalent to 506 ng of product) was dried down under nitrogen at room temperature and applied to a 4 mm × 30 cm μPorasil column (Waters Associates) in a Du Pont 830LC apparatus fitted with a Waters U-6-K injection port. Elution was accomplished with 5% 2-propanol in hexane at 700 psi. A total of 12 fractions (2 mL

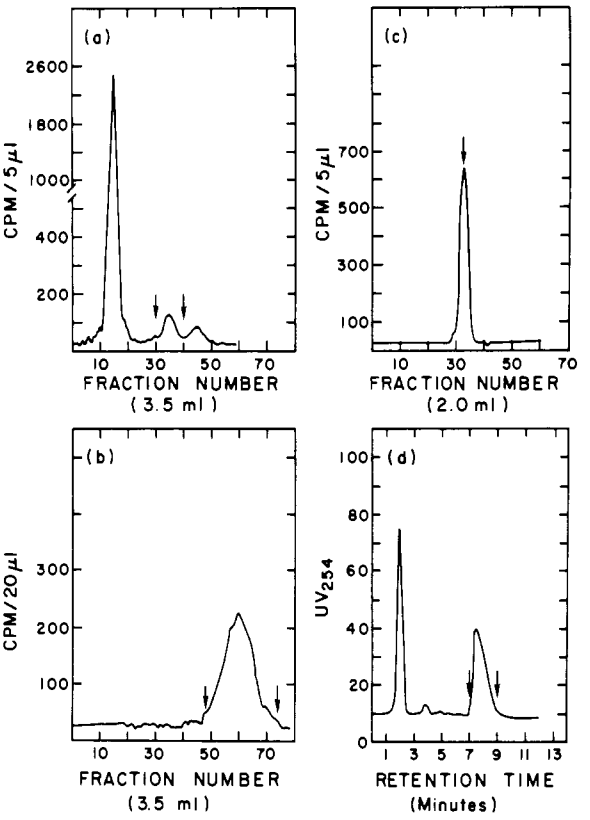

FIGURE 1: Chromatographic profiles during the isolation and purification of 24-^{18}O-24,25-$(OH)_2$-[26,27-^{3}H]D_3. (a) Sephadex LH-20 column (1 × 60 cm packed in and eluted with 65:35 $CHCl_3$–Skellysolve B). (b) Sephadex LH-20 column (1 × 60 cm packed in and eluted with 50:50 $CHCl_3$–Skellysolve B) profile of 24-^{18}O-24,25-$(OH)_2D_3$ region isolated from the Sephadex LH-20 column (65:35) $CHCl_3$–Skellysolve B shown in a. (c) Sephadex LH-20 column (1 × 90 cm packed in and eluted with CH_3OH) profile of 24-^{18}O-24,25-$(OH)_2D_3$ region isolated from the Sephadex LH-20 column (50:50) $CHCl_3$–Skellysolve B shown in b. (d) μPorasil column (4 mm × 30 cm Waters; eluted with 5% 2-propanol-hexane) profile of 24-^{18}O-24,25-$(OH)_2D_3$ region isolated from the Sephadex LH-20 column (CH_3OH) shown in c.

min^{-2} fraction^{-1}) was collected. The elution profile is shown in Figure 1d. The peak fractions 8 and 9 were combined and dried down under nitrogen to yield a total residue of 202 ng of 24,25-$(OH)_2D_3$. This material was analyzed in the mass spectrometer (Associated Electrical Industries, Ltd., Manchester, England, Model MS-902) by direct probe inlet at 120–130 °C and 70 eV ionization energy.

Results

Incubation of 25-OH-D_3 with chick kidney homogenates has resulted in an overall enzymatic conversion of 9% to 24,25-$(OH)_2D_3$ (1.9 μg as calculated from the initial amount of substrate added which was 21 μg). The purification of the ^{18}O metabolite depended heavily on liquid-gel partition chromatography (Holick and DeLuca, 1971) using Sephadex LH-20 suspended in double-distilled organic solvents as indicated throughout the procedures. As a final purification step, the more refined technique of high-pressure liquid chromatography was adopted. An excellent resolution (Figure 1a) is observed of 24,25-$(OH)_2D_3$ from the unaltered, undegraded substrate 25-OH-D_3, shown as the first radioactive peak. As observed from the same figure, a third radioactive metabolite was separated. This peak cochromatographed with crystalline 1,25-$(OH)_2D_3$ on high-pressure liquid chromatography. The 1,25-$(OH)_2D_3$-biosynthesizing system, therefore, was still

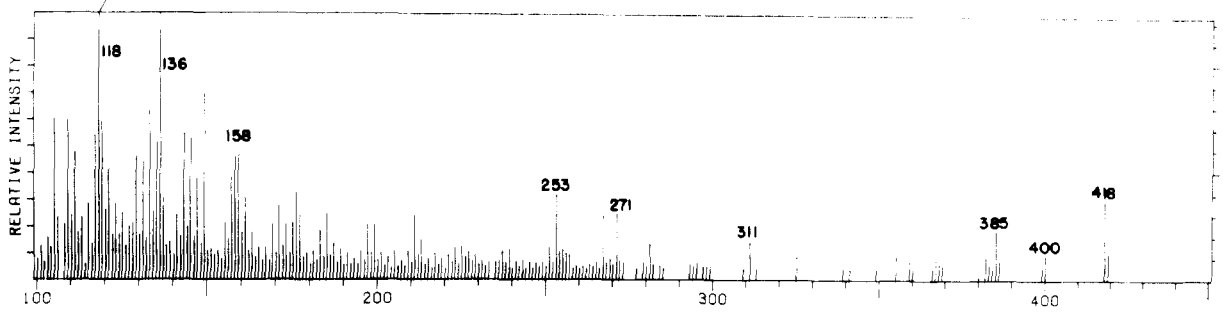

FIGURE 2: Mass spectrum of isolated 24-^{18}O-24,25-$(OH)_2$-[26,27-^{3}H]D_3. The product (202 ng) from the μPorasil column (Figure 1d) was analyzed by mass spectrometry. Note especially the molecular ion at *m/e* 418 and the absence of *m/e* 416. Note also important fragments at *m/e* 271, 136, and 118.

present in these preparations. The recovery of metabolites from the various Sephadex columns was essentially quantitative. The product obtained after HPLC was sufficiently pure to allow mass spectral analysis.

The isolation and identification of the 24,25-$(OH)_2D_3$ have been reported by Holick et al. (1972). The mass spectrum of the natural dihydroxy metabolite was shown to exhibit a molecular ion peak at *m/e* 416 and prominent fragment peaks at *m/e* 118, 136, 257, and 271.

The mass spectrum of the [^{18}O]-24-hydroxy analogue of 25-OH-D_3 (Figure 2) was compared with that of the natural metabolite (Holick et al., 1972). The molecular ion peak at *m/e* 418 and the fragment peaks at *m/e* 118, 136, 257, and 271 establish conclusively the incorporation of molecular oxygen as a hydroxyl function into the C-24 position of 25-OH-D_3 to form 24,25-$(OH)_2D_3$. Note the absence of *m/e* 416 which would arise if H_2O had provided the oxygen for the 24-hydroxyl function.

Discussion

The results of this investigation have provided strong evidence that the chick kidney (24*R*)-hydroxylase belongs to the class of "mixed-function oxygenases." This group of oxygenases catalyzes the consumption of one molecule of oxygen per molecule of substrate; one atom of the oxygen molecule appears in the product, the other is reduced to water by a hydrogen donor other than the substrate, in this case NADPH (Knutson and DeLuca, 1974). Microsomal oxygenases usually consist of at least a flavoprotein, a carbon monoxide-sensitive hemoprotein, cytochrome P_{450}, a lipid component (Lu and Coon, 1968; Strobel et al., 1970) and may contain an iron–sulfur protein (Omura and Sato, 1964; Kimura and Suzuki, 1967).

The 25-OH-D_3-1-hydroxylase is the first of the vitamin D enzymes that was shown to be a monooxygenase ("mixed-function oxidase"). It was demonstrated with $^{18}O_2$ (Ghazarian et al., 1973) that in this hydroxylation reaction all of the oxygen inserted by the chick kidney mitochondria into the 1α position of 25-OH-D_3 is derived from molecular oxygen. Furthermore, evidence was presented which demonstrated that NADPH and a carbon monoxide sensitive component are two specific intramitochondrial factors necessary for the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ (Ghazarian and DeLuca, 1974). Later experiments have established the hemoprotein component as cytochrome P_{450} and the participation of an iron–sulfur protein called renal ferredoxin (Ghazarian et al., 1974; Pedersen et al., 1976).

That the 24-hydroxylase also incorporates molecular ^{18}O into 25-OH-D_3 to form 24*R*,25-$(OH)_2D_3$ suggests that this system is also a monooxygenase ("mixed-function oxidase"). However, it was shown (Knutson and DeLuca, 1974) that, in contrast to the 1-hydroxylase, the 24-hydroxylase is not carbon monoxide sensitive which casts doubt on the involvement of cytochrome P_{450} in the 24-hydroxylation reaction. Yet mitochondria containing 24-hydroxylase have the same level of cytochrome P_{450} as the mitochondria containing 1-hydroxylase (Ghazarian et al., 1974). It is possible that a cytochrome P_{450} is involved in the 24-hydroxylation but may be resistant to carbon monoxide inhibition. On the other hand, the 24-hydroxylase system may be a monooxygenase ("mixed-function oxidase") not requiring cytochrome P_{450} as electron acceptor (Knutson and DeLuca, 1974). This question can only be resolved by reconstitution experiments similar to those carried out by Ghazarian et al. (1974) and Pedersen et al. (1976) for the 1α-hydroxylase. However, the present investigation permits the exclusion of a mechanism involving dehydrogenation and hydration.

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Protein-Catalyzed Exchange of Phosphatidylcholine between Sonicated Liposomes and Multilamellar Vesicles†

Paul E. DiCorleto‡ and Donald B. Zilversmit*,§

ABSTRACT: Phospholipid exchange protein from beef heart or beef liver does not catalyze the transfer of phosphatidylcholine from multilamellar vesicles of phosphatidylcholine. Certain combinations of phospholipids, however, do yield multilamellar vesicles that will exchange phosphatidylcholine with liposomes in the presence of exchange protein. Multilamellar vesicles of phosphatidylcholine:phosphatidylethanolamine:cardiolipin (70:25:5, mol %) can be used in place of mitochondria or erythrocyte ghosts as an improved acceptor particle in the study of liposome structure with phospholipid exchange proteins. These multilamellar vesicles act as a well-defined reservoir of unlabeled phosphatidylcholine with 7% exchangeable phospholipid. When the distribution of phosphatidylcholine in liposomes is studied by the exchange protein technique, results can be influenced by the choice of phospholipid acceptor particle. With mitochondria as acceptor particle, the percentage of phosphatidylcholine in the outer monolayer of a liposome appears to be 60%, whereas a value of 70% is obtained when multilamellar vesicles are the acceptor. The discrepancy can be explained by a heterogeneity in liposomes prepared by sonication. A size-dependent fusion or adsorption process occurs between liposomes and mitochondria; the very small liposomal vesicles, obtained by gel filtration, combine nearly quantitatively with the natural membrane. This phenomenon is not seen with multilamellar vesicles. Thus by using multilamellar vesicles one obtains a less biased estimate of phospholipid distribution between inner and outer layers of liposomes.

Proteins that catalyze the transfer of phospholipid molecules between membranes have been isolated and characterized from several sources (for review, see Zilversmit and Hughes, 1976). Recently, these phospholipid exchange proteins have been employed in several laboratories as membrane probes in the study of phospholipid distribution and motion in liposomes—sonicated, unilamellar vesicles (Johnson et al., 1975; Rothman and Dawidowicz, 1975) and natural membranes (Bloj and Zilversmit, 1976; Rothman et al., 1976; Rousselet et al., 1976). The value of the technique lies in the high degree of sensitivity of radioisotope monitoring and the apparently minimal perturbation of the bilayer. The transposition (flip-flop) rate of PC[1] in liposomes has been determined with this technique in two laboratories by the use of radioactive PC liposomes as donor particles and mitochondria (Johnson et al., 1975) or erythrocyte ghosts (Rothman and Dawidowicz, 1975) as acceptor particles. The kinetics of catalyzed replacement of donor

† From the Division of Nutritional Sciences and Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received September 16, 1976.* This research was supported in part by Public Health Service Research Grant HL 10940 from the National Heart and Lung Institute, United States Public Health Service.
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[1] Abbreviations used: PC, phosphatidylcholine; CL, cardiolipin; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.