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Incorporation of Oxygen-18 into the 25-Position of Cholecalciferol by Hepatic Cholecalciferol 25-Hydroxylase

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The oxygen enzymically inserted as a hydroxy function by rat liver post-mitochondrial fraction into the 25-position of cholecalciferol to give 25-hydroxycholecalciferol is derived exclusively from molecular O_2. Therefore like the other two cholecalciferol hydroxylases, i.e. 25-hydroxycholecalciferol 1α-hydroxylase and 25-hydroxycholecalciferol 24-hydroxylase, the cholecalciferol 25-hydroxylase is also a mono-oxygenase ('mixed-function oxidase').

It is well recognized that cholecalciferol must be transformed into more polar biologically active metabolites before it elicits its biological function (DeLuca, 1974; Kodicek, 1974). Thus it is first converted into 25(OH)D_3 and subsequently to 1α,25$(OH)_2D_3$ before stimulating the intestine and bone (DeLuca, 1974; Kodicek, 1974).

The 25-hydroxylation occurs in the liver (Ponchon *et al.*, 1969; Olson *et al.*, 1976; Horsting & DeLuca, 1969) and to a smaller extent in the intestine (Tucker *et al.*, 1973; Bhattacharyya & DeLuca, 1974). Studies *in vitro* have shown the cholecalciferol 25-hydroxylase of rat liver to be microsomal, requiring NADPH and a cytoplasmic protein (Bhattacharyya & DeLuca, 1974) for activity, but little else is known concerning the nature of this reaction. On the other hand it has been clearly demonstrated that 25-hydroxycholecalciferol 1α-hydroxylase is a 3-component mono-oxygenase similar to the steroidogenesis system of the adrenals (Ghazarian *et al.*, 1974*a*; Pedersen *et al.*, 1976). We have therefore examined the question of whether the cholecalciferol 25-hydroxylase is a mixed function mono-oxygenase.

In the present paper it will be demonstrated that the oxygen enzymically introduced as a hydroxy function into the 25-position of cholecalciferol to yield 25(OH)D_3 is derived exclusively from molecular O_2 providing strong evidence that the 25-hydroxylase is a mono-oxygenase rather than a dehydrogenase–hydratase system.

Methods

Animals

Some 50 male weanling albino rats (Holtzman, Madison, WI, U.S.A.) were housed individually in hanging wire cages. They were given water and fed *ad libitum* a low-calcium (0.02% calcium, w/w), cholecalciferol-deficient diet for 3 weeks.

Abbreviations used: 25(OH)D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25$(OH)_2D_3$, 24,25-dihydroxycholecalciferol.

Preparation of the enzyme

Rats were killed by decapitation and exsanguination. The livers were immediately removed, carefully separated from the adhering connective tissues and weighed. The livers were then rinsed with ice-cold 0.25M-sucrose, minced, transferred to an ice-cold Potter–Elvehjem homogenizer fitted with a Teflon pestle and homogenized in 1 vol. of the 0.25M-sucrose. The homogenate was centrifuged at 128*g* at 4°C for 10min in a Lourdes centrifuge with a 9RA rotor. The resulting supernatant was further centrifuged at 10000*g* for 30min to remove mitochondrial particles. The resulting post-mitochondrial supernatant was used for incubation.

Incubation conditions and extraction of samples

Some 25 incubations, each with a total volume of 10ml, were carried out in 21ml full-capacity serum bottles. The assay mixture consisted of 5ml of post-mitochondrial supernatant (246mg of protein), 2.5ml of phosphate–cofactor solution (0.1M-K_2HPO_4, 0.4mM-NADP, 160mM-nicotinamide, 20mM-ATP, 22.4mM-glucose 6-phosphate adjusted to pH7.4) and 2.5ml of salt solution (5mM-$MgCl_2$ and 0.1M-KCl plus 0.25 unit of glucose 6-phosphate dehydrogenase/ml; one unit as defined by Sigma Chemical Co., St. Louis, MO, U.S.A.) (Bhattacharyya & 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 N_2 passed over heated copper. Finally, 4ml of 99.3 atom%$^{18}O_2$ (Bio-Rad Laboratories, Richmond, CA,

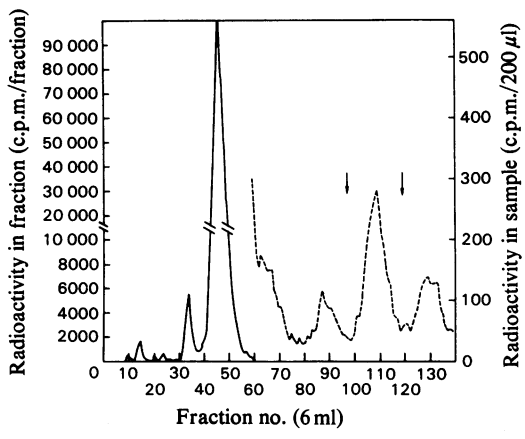

Fig. 1. *Chromatographic profile during the isolation and purification of* 25(^{18}OH)[3α-^{3}H]D_3
A Lipidex 5000 column (2cm×60cm) was used that was packed and eluted with a hexane/chloroform (9:1, v/v) solvent system. ----, Radioactivity of sample; ——, radioactivity in each fraction volume. The peak between the arrows is due to 25(OH)D_3.

U.S.A.) and 7ml of purified N_2 were introduced simultaneously into each of the 25 incubation bottles using gas-tight syringes. The hydroxylation reactions were initiated by introduction (with a Hamilton syringe) of 10µg of [3α-^{3}H]cholecalciferol (synthesized chemically by S. Yamada in this laboratory) in 10µl of 95% ethanol with a specific radioactivity of 58000 c.p.m./µg. After 4h of incubation at 37°C at 120 oscillations/min, the reactions were terminated by the immediate transfer of the mixtures into 500ml separating funnels (six incubation mixtures per funnel) each containing 60ml of alcohol and 240ml of hexane. The reaction mixture was vigorously shaken for 7min and left at 4°C overnight. The lower phase was emptied into a clean beaker and the upper phase was filtered through Shark-skin filter paper into a 500ml round-bottomed flask. The lower phase was put back into the funnel and re-extracted twice with 240ml portions of hexane. The combined hexane extracts contained 100% of the added radioactivity.

Chromatography

The combined hexane extracts were evaporated to dryness by using a rotary evaporator at 30°C. The residue was dissolved in 500µl of hexane/chloroform (9:1, v/v) and applied to a glass column (2cm×60cm) containing 100g of Lipidex 5000 (hydroxyalkoxypropyl derivative of Sephadex; Packard Instrument Co., Downers Grove, IL, U.S.A.), packed and eluted in the same solvent system. A total of 140 fractions (6ml at a flow rate of 1ml/min) was collected and 10–200µl of each fraction was used for counting (Fig. 1) in a Packard model 3255 liquid-scintillation spectrometer (Packard) with the scintillation mixture described previously (Ghazarian *et al.*, 1974*b*). The efficiency of counting for ^{3}H content was 45%. The relative elution position of the metabolites has been determined previously by chromatography of the pure metabolites under identical experimental conditions (Holick & DeLuca, 1971). The peak region 25(OH)D_3 (fractions 97–119) was evaporated to dryness by using a rotary evaporator at 30°C to yield a total radioactivity of 53000 c.p.m. equivalent to 914ng of 25(OH)D_3 (based on 58000c.p.m./µg, the specific radioactivity of the substrate cholecalciferol). The residue was dissolved in 20µl of water/methanol (1:4, v/v) and applied to a octadecylsilane-bonded microparticulate silica column (4.6mm×25cm) (Zorbax-ODS column; DuPont, Wilmington, DE, U.S.A.) in a DuPont 830 LC apparatus fitted with a Waters U-6-K injection port. Elution was accomplished with the same solvent system at 14.5MPa. A total of 55 fractions (1.5ml/min per fraction) was collected. The elution profile is shown in Fig. 2(*a*). Peak fractions 36–44 were combined, dried under N_2 and re-injected into the high-pressure liquid chromatography apparatus. The elution profile is shown in Fig. 2(*b*). The 25(OH)D_3 peak region (fractions 34–42) was dried under N_2. The residue was dissolved in 20µl of isopropanol/hexane (2.5:97.5, v/v) and applied to a microparticulate silica column (4.6mm×50cm) (PXS 1050 Partisil-10, Whatman Reeve Angel Inc., Clifton, NJ, U.S.A.) in a DuPont 830 LC apparatus. Elution was carried out with the same solvent system at 6.9MPa. A total of 36 fractions (2.6ml/min per fraction) was collected. The elution profile is shown in Fig. 2(*c*). The peak fractions 16–21 were combined and dried under N_2 to yield a total of 582ng of 25(OH)D_3. This material was analysed in the mass spectrometer (Associated Electrical Industries, Manchester, U.K.; model MS-902) by direct-probe inlet at 130–145°C above ambient temperature and 70eV ionization energy.

Results

Incubation of cholecalciferol with rat liver post-mitochondrial fractions has resulted in an overall enzymic conversion of 0.4% into 25(OH)D_3 (914ng as calculated from the initial amount of substrate added, which was 250µg). Previous reports have shown that unlike the other two cholecalciferol hydroxylases, rat liver 25-hydroxylase exhibits lower activity when assayed *in vitro*. Besides, addition of saturating concentrations of cholecalciferol would be expected to decrease the percentage conversion of the substrate into the product. We have utilized such large amounts of cholecalciferol for isolation of sufficient amounts of the metabolite for analysis.

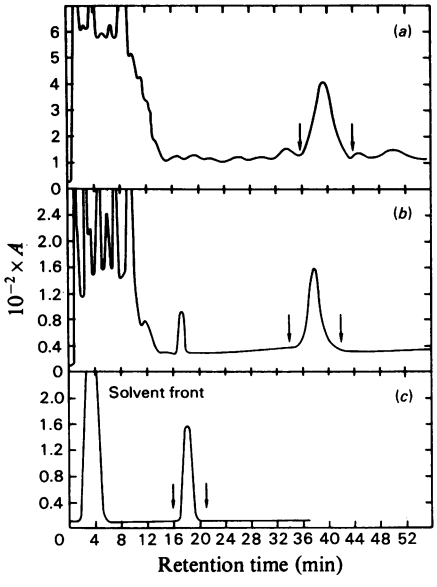

Fig. 2. *High-pressure liquid chromatography of* 25-(^{18}OH)[3α-^{3}H]D_3
(*a*) Zorbax ODS (Dupont) column [4.6mm×25cm, eluted with water/methanol (1:4, v/v)] profile of the 25(^{18}OH)D_3 region isolated from the Lipidex 5000 column shown in Fig. 1. The peaks on the left are due to impurities. (*b*) Zorbax-ODS (Dupont) column [4.6mm×25cm, eluted with water/methanol (1:4, v/v)] profile of 25(^{18}OH)D_3 region isolated from (*a*). The peaks on the left are due to impurities. (*c*) Partisil-10 (Whatman) column [4.6mm×50cm; eluted with isopropanol/hexane (2.5:97.5, v/v)] profile of 25(^{18}OH)D_3 region isolated from (*b*). The peak on the left is due to the solvent front. The peaks between the arrows in (*a*), (*b*) and (*c*) are due to 25(OH)D_3

The purification of the ^{18}O metabolite depended on liquid/gel partition chromatography using Lipidex 5000 suspended in double-distilled organic solvents. The more refined technique of high-pressure liquid chromatography was used for further purification of the metabolite. An excellent resolution (Fig. 1) of 25(OH)D_3 was observed from the unaltered undegraded substrate cholecalciferol, which is the major radioactive peak from the Lipidex column. As observed from the same Figure, several other radioactive peaks were obtained. Fractions 30–40 may well be esters of cholecalciferol or precholecalciferol. The other radioactive peaks, fractions 62–75 and appearing as shoulder peak of cholecalciferol (*a*) fractions 80–96 (*b*) and fractions 120–140 (*c*) are unknown metabolites. The 25(OH)D_3 obtained after three passes on high-pressure liquid chromatography was sufficiently pure to allow mass-spectral analysis (Fig. 3).

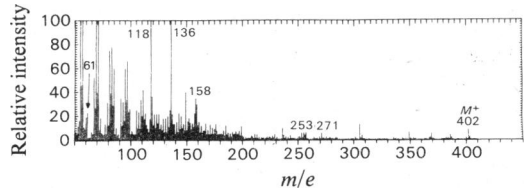

Fig. 3. *Mass spectrum of isolated* 25(^{18}OH)[3α-^{3}H]D_3
The product (582ng) from the Partisil-10 column (Fig. 2*c*) was analysed by mass spectrometry. The molecular ion at *m/e* 402 and the absence of one at *m/e* 400 are especially noteworthy, as are the fragments at *m/e* 271, 253, 136 and 118.

The isolation and identification of the 25(OH)D_3 have been reported by Blunt *et al.* (1968). The mass spectrum of the natural hydroxymetabolite was shown to exhibit a molecular ion peak at *m/e* 400 and characteristic fragment peaks at *m/e* 367 ($M-CH_3-H_2O$), 349 ($367-H_2O$), 341 ($M-59$, loss of C_3H_7O from ring A), 271 ($M-$side chain), 253 ($271-H_2O$), 158 (ring A+4 carbon atoms), 136 (ring A+C-6 and C-7), 118 ($136-H_2O$) and 59 [$(CH_3)_2C{=}OH^+$, including C-25, C-26 and C-27 of side chain].

The mass spectrum of the ^{18}O-labelled 25-hydroxy analogue of cholecalciferol (Fig. 3) was compared with that of the natural metabolite (Blunt *et al.*, 1968). The molecular-ion peak at *m/e* 402 instead of 400 and the fragment peaks at *m/e* 369 ($M-CH_3-H_2O$), 349 ($369-H_2{}^{18}O$), 343 ($M-59$), 271, 253, 158, 136, 118 and 61 [$(CH_3{}^{2}C{=}{}^{18}OH$) establish the structure of the metabolite as [^{18}O]25(OH)D_3. The incorporation of molecular O_2 as a hydroxy function into the C-25 position is shown by the peak at *m/e* 61 and the fact that all fragments do not include the side chain (e.g. *m/e* 271, 158, 136) and also do not contain the ^{18}O-isotope. The absence of *m/e* 400, which would arise if water had provided the oxygen for the 25-hydroxy function, is noteworthy.

Discussion

The 25-hydroxycholecalciferol 1α-hydroxylase is the first of the cholecalciferol enzymes that was shown to be a mono-oxygenase ('mixed-function oxidase'). Ghazarian *et al.* (1973) demonstrated with $^{18}O_2$ that in this hydroxylation all of the oxygen inserted by chick kidney mitochondria into the 1α-position of 25(OH)D_3 is derived from molecular O_2. The chick kidney mitochondrial 24-hydroxylase also incorporates molecular $^{18}O_2$ into 25(OH)D_3 to form 24,25-$(OH)_2$[^{18}O]D_3, which suggests that this system is also a mixed-function oxidase (Madhok *et al.*, 1977). That the 25-hydroxylase also incorporates molecular $^{18}O_2$ into cholecalciferol to form 25(OH)[^{18}O]D_3 demonstrates that this system is also a mixed-function

oxidase. Thus investigations can now be directed to the exact enzymic machinery involved in this mixed-function mono-oxygenase system.

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