RECONSTITUTION OF VITAMIN D_3 25-HYDROXYLASE ACTIVITY WITH A CYTOCHROME *P*-450 PREPARATION FROM RAT LIVER MITOCHONDRIA

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1. Introduction

25-Hydroxylation of vitamin D_3 has been shown catalyzed both by the microsomal [1,2] and the mitochondrial [3] fraction of rat liver. As yet, only the mitochondrial activity has been assayed under enzymological conditions involving substrate saturation [3]. At present no definitive conclusions can be drawn concerning the relative physiological importance of these two hydroxylases. Both enzymes seem to be mixed function oxidases since the oxygen incorporated in the 25-position is derived from molecular oxygen both when the mitochondrial [3] and the microsomal fraction [4] is used. In contrast to the mitochondrial activity [3], the microsomal activity seems to be affected by the vitamin D status of the animal [1]. Whether this is due to the hydroxylase per se or some other factor is unknown, however.

In a study on the mitochondrial vitamin D_3 25-hydroxylase, it was shown that this enzyme had properties similar to a cytochrome *P*-450 monooxygenase [3]. Thus the enzyme required NADPH, was inhibited by CO, and was stimulated by treatment with phenobarbital in vivo.

Recently, cytochrome *P*-450 was solubilized from rat liver mitochondria [5–7]. It is shown in the present work that this cytochrome *P*-450 preparation in the presence of NADPH, ferredoxin and ferredoxin reductase catalyzes the conversion of vitamin D_3 into a more polar product identified as 25-hydroxyvitamin D_3 by gas chromatography–mass spectrometry (GS–MS).

2. Materials and methods

Male Wistar rats were fed an ordinary pellet diet. A group of 5 animals (~200 g) was given phenobarbital (1 g/l) in the drinking water for 30 days before sacrifice. This treatment has been shown to markedly increase the content of cytochrome *P*-450 in the liver mitochondria [8] and to stimulate the mitochondrial vitamin D_3 25-hydroxylase activity [3].

The procedures for preparing liver mitochondria [9], liver mitochondrial cytochrome *P*-450 [5], adrenal ferredoxin [10] and adrenal ferredoxin reductase [9] have been reported [9]. The specific content of liver mitochondrial cytochrome *P*-450 was 0.4 nmol/mg protein. The adrenal ferredoxin exhibited an A_{414}/A_{280} of 0.71 and the ferredoxin reductase A_{450}/A_{270} of 0.128. Both proteins were homogenous on SDS–polyacrylamide gel electrophoresis.

Vitamin D_3 (cholecalciferol) from Sigma Chemical Co. (St Louis, MO) was purified on high-pressure liquid chromatography (HPLC) on a silicic acid column (see below). 25-Hydroxyvitamin D_3 was a generous gift of Professor H. F. DeLuca, University of Wisconsin, Madison. 25-Hydroxy-[26-$^2H_3$]vitamin D_3 was synthesized as in [11]. All other chemicals and biochemicals were standard commercial high purity materials.

Vitamin D_3 25-hydroxylase activity was assayed in a medium that contained the following in 1.5 ml 35 mM MOPS buffer (pH 7.7): 1 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydro-

genase, 1 μmol $MgCl_2$ and 0.1 μmol NADP. The amounts of enzymes are given in table 1. The reaction was started by the addition of 150 μg vitamin D_3 in 25 μl ethanol and allowed to continue for 60 min at 37°C under oxygen. The reaction was terminated by the addition of 10 ml methanol:chloroform (2:1) together with 145 ng $^2H_3$-labelled 25-hydroxyvitamin D_3. After extraction [5] the chloroform was evaporated under N_2 and the residue redissolved in 100 μl eluting solvent used for HPLC. The total sample was injected into a Spectra Physics HPLC instrument fitted with a Rheodyne injector, an ultraviolet detector and a Spherisorb 5 μm ODS column (4.6 × 250 mm). The sample was eluted with 5% H_2O in methanol at 0.8 ml/min flow rate. The fraction corresponding to eluted 25-hydroxyvitamin D_3 was collected. The solvent was evaporated under N_2 and after redissolving in 100 μl of the next eluting solvent the sample was rechromatographed on a Spherisorb 5 μm silica column (3 × 250 mm). The sample was eluted with 7.5% isopropanol in hexane at 0.8 ml/min flow rate. The fraction corresponding to 25-hydroxyvitamin D_3 was collected in 0.8 ml, the solvent was removed and the trimethylsilyl/*t*-butyl-dimethylsilyl derivative formed as in [3]. The derivative of the extract was then dissolved in 20–50 μl hexane and aliquots were analysed by GS–MS using an LKB 2091 instrument equipped with a multiple ion detector (MID) essentially as in [3]. The first channel of the multiple ion detector was focused on the ion at *m/e* 586 and the second at the ion *m/e* 589. The ratio between peaks at *m/e* 586 and *m/e* 589 was compared to a standard curve [3].

In order to identify the reaction product purified material from 3 incubations without added $^2H_3$-labelled 25-hydroxyvitamin D_3 was combined and analyzed by GC–MS.

3. Results

Reversed-phase HPLC on Spherisorb ODS of the chloroform extract of the complete incubation mixture (with no $^2H_3$-labelled 25-hydroxyvitamin D_3 added) revealed that vitamin D_3 had been transformed into more polar products. Rechromatography on Spherisorb silica of the fraction with retention time corresponding to 25-hydroxyvitamin D_3 disclosed that one of these products had a retention time identical to 25-hydroxyvitamin D_3 also in this system (fig.1A,B). In the absence of ferredoxin in the incubation medium, the corresponding peak was not detectable (fig.1C).

Exact determination of the amount of product formed was made after addition of $^2H_3$-labelled 25-hydroxyvitamin D_3 to the incubation mixture prior to extraction and chromatography as above. In fig.2A, MID-recordings at *m/e* 586 and *m/e* 589 are shown of the derivative of a purified extract of the complete incubation mixture. In consonance with the work in [3], two peaks were obtained, corresponding to the pyro- and isopyro-form of the derivative of vitamin D_3. Peaks were obtained in the tracing at *m/e* 589 as well as in the tracing at *m/e* 586 corresponding to a considerable formation of 25-hydroxyvitamin D_3. The amount of unlabelled 25-hydroxyvitamin D_3 could be calculated from the ratio between the peak height at *m/e* 586 and at *m/e* 589, with use of a standard curve [3]. The specific rate of formation of 25-hydroxyvitamin D_3 obtained in 5 different experiments varied from 0.01–0.02 nmol.nmol cytochrome P-450^{-1}.min^{-1} (cf. table 1). In fig.2B, MID-recordings at *m/e* 586 and *m/e* 589 are also shown of the derivative of a purified extract of an incubation of vitamin D_3 with the incubation mixture minus ferredoxin. Only very small peaks were obtained in the tracing at *m/e* 586, corresponding to a very low formation of unlabelled 25-hydroxyvitamin D_3 (cf. table 1). The small formation of 25-hydroxyvitamin D_3 seen when ferredoxin reductase was excluded from the incubation mixture (table 1) can be explained by the presence of a small amount of this reductase in the cytochrome *P*-450 preparation [8,9]. A mass spectrum of the material analyzed in fig.2A showed that it consisted of a mixture of unlabelled and $^2H_3$-labelled derivative of 25-hydroxyvitamin D_3 (cf. table 1). The identity of 25-hydroxyvitamin D_3 was further confirmed by combined GC–MS of purified material obtained from 3 separate incubations to which no $^2H_3$-labelled 25-hydroxyvitamin D_3 had been added. As shown in fig.3 prominent peaks were observed at *m/e* 586 (M), 439 (M-90-57) and *m/e* 131 (cleavage between C-24 and C-25 [3]). The same peaks with the same relative intensity were observed in the mass spectrum of the derivative of authentic 25-hydroxyvitamin D_3 (fig.3).

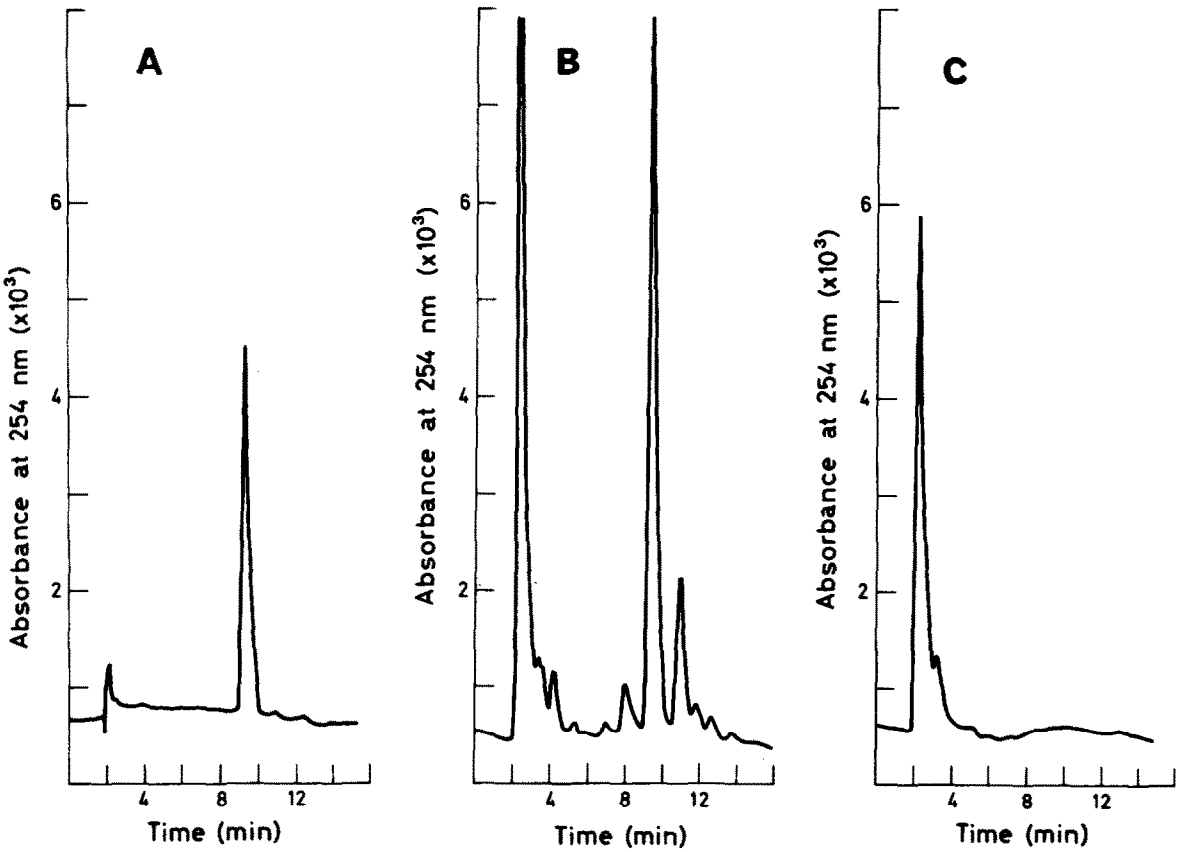

Fig.1. HPLC on Spherisorb silica after pre-purification on Spherisorb ODS of the methanol/chloroform extract of incubations containing the soluble liver mitochondrial cytochrome *P*-450 and with vitamin D_3 as substrate. The incubation conditions as well as extraction and chromatography were as given in section 2. (No $^2H_3$-labelled 25-hydroxyvitamin D_3 was added to the extract.) (A) Authentic 25-hydroxyvitamin D_3 (43 ng); (B) complete incubation system; (C) incubation system without ferredoxin added.

Table 1
25-Hydroxylation of vitamin D_3 by soluble rat liver mitochondrial cytochrome *P*-450

Components present in the incubation medium			
Rat liver mitochondrial cytochrome *P*-450 (nmol)	0.74	0.74	0.74
Bovine adrenal ferredoxin reductase (pmol)	26	–	26
Bovine adrenal ferredoxin (nmol)	–	5.5	5.5
Formation of 25-hydroxy-vitamin D_3 (ng) (mean ± SD, $n = 5$)	5±4	30	235±64

For incubation conditions see section 2

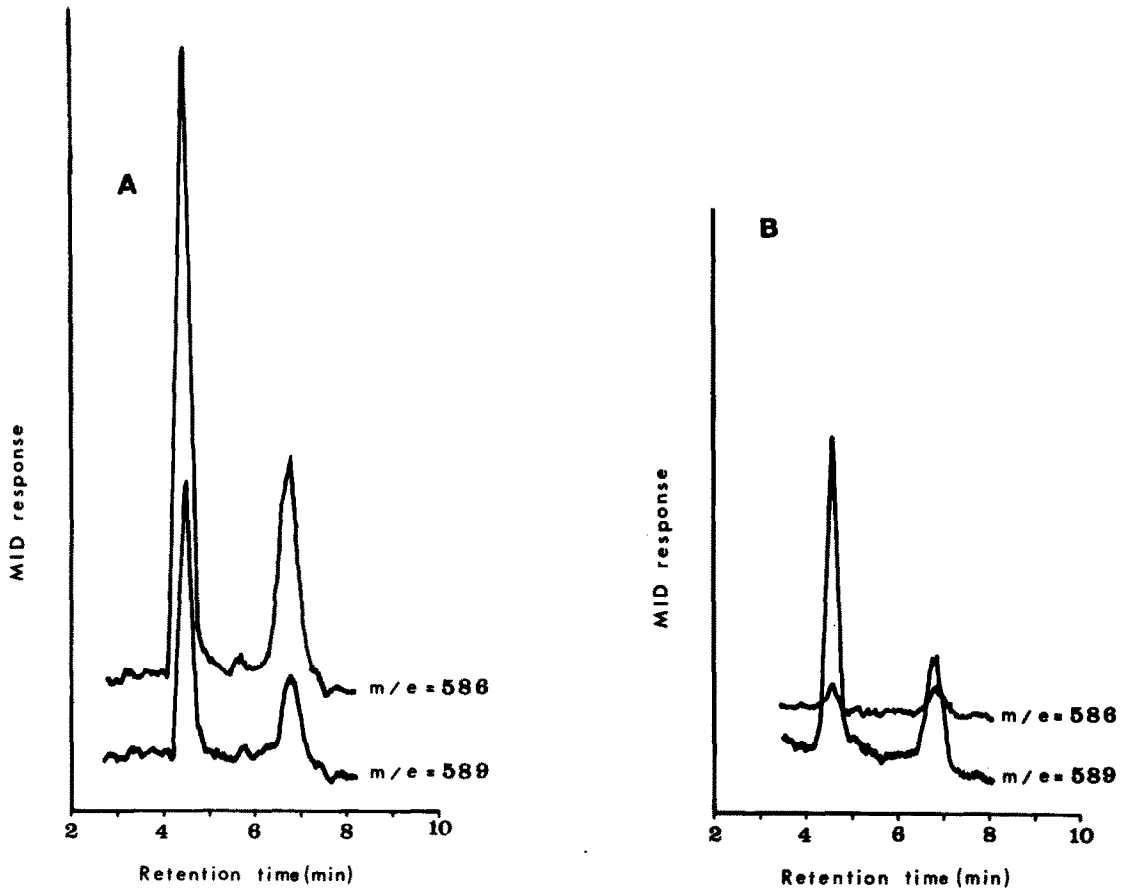

Fig.2. MID recordings at *m/e* 586 and *m/e* 589 of the trimethylsilyl/*t*-butyldimethylsilyl derivative of incubation extract of vitamin D_3 with the complete reconstituted system (A) and reconstituted system minus ferredoxin (B). A fixed amount of $^2H_3$-labelled 25-hydroxyvitamin D_3 (145 ng) had been added to the incubation mixture prior to extract (cf. section 2).

4. Discussion

This work clearly demonstrates that liver mitochondria contain a cytochrome *P*-450 with the ability to catalyze 25-hydroxylation of vitamin D_3. This enzyme activity was absolutely dependent upon both ferredoxin and ferredoxin reductase. The latter finding excludes that the cytochrome *P*-450 is of microsomal origin [6]. The rate of conversion obtained with the reconstituted system was of the same order of magnitude as that obtained with intact mitochondrial fraction [3]. It should be noted, however, that the in vitro conditions of the present system may not be optimal and it can not be excluded that the transfer of vitamin D_3 through the mitochondrial membranes may be rate-limiting when using intact mitochondria (cf. [8]).

The cytochrome *P*-450 preparation used in the present experiments has been shown to catalyze 25- and 26-hydroxylation of cholesterol [6,12] and 26-hydroxylation of a number of C_{27}-steroids, intermediates in the formation of bile acids [8]. Whether the same or a different species of cytochrome *P*-450 is responsible for the 25-hydroxylation of vitamin D_3 can not yet be decided. With intact mitochondria, it was shown that the vitamin D_3 25-hydroxylase had certain characteristics different from the cholesterol 25- and 26-hydroxylase [3]. Thus exogenous NADPH was found to stimulate 25-hydroxylation of vitamin D_3 to a higher extent than reported for 25- and

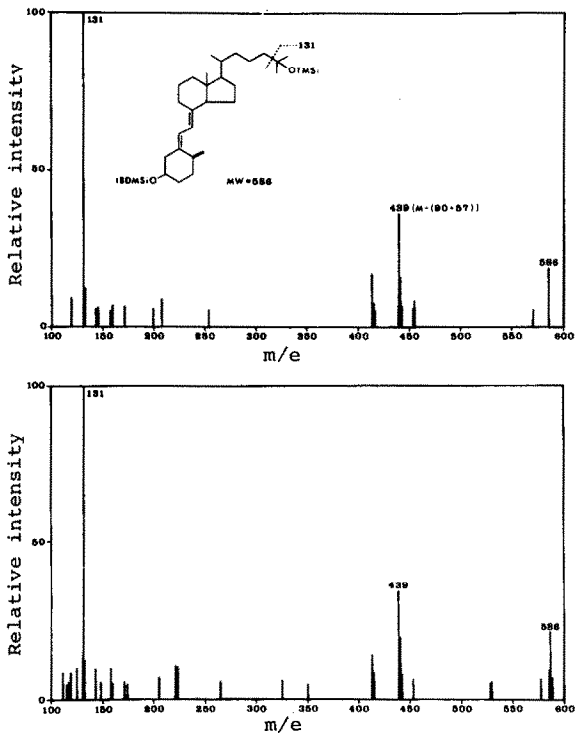

Fig.3. Mass spectrum of the pyro-form of the trimethylsilyl/*t*-butyldimethylsilyl derivative of authentic 25-hydroxyvitamin D_3 (upper spectrum) and 25-hydroxyvitamin D_3 isolated from incubations of vitamin D_3 with the reconstituted system (lower spectrum). All peaks with an intensity <2.5% of that of the base peak at *m/e* 131 were deleted.

26-hydroxylation of cholesterol [13]. Furthermore, in contrast to 25- and 26-hydroxylation of cholesterol, Mg^{2+} had little or no stimulatory effect on 25-hydroxylation of vitamin D_3 [3]. It was shown, however, that under the conditions used in [3], there is some permeability of the mitochondria towards NADH (I.B., I.H., unpublished). If non-permeable mitochondria are used, the properties of the mitochondrial 25-hydroxylase active on vitamin D_3 are more similar to the properties of the enzyme system catalyzing 25- and 26-hydroxylation of cholesterol. Inhibition studies with the reconstituted system may give further information concerning a possible heterogeneity of the present mitochondrial cytochrome *P*-450 fraction, and such studies are in progress.

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