RECONSTITUTION OF VITAMIN D₃ 25-HYDROXYLASE ACTIVITY WITH A CYTOCHROME P-450 PREPARATION FROM RAT LIVER MITOCHONDRIA

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1. Introduction

25-Hydroxylation of vitamin D₃ 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 D₃ 25-hydroxylase, it was shown that this enzyme had properties similar to a cytochrome P-450 monoxygenase [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 D₃ into a more polar product identified as 25-hydroxyvitamin D₃ 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 D₃ 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 $A_{450}/A_{320}$ of 0.128. Both proteins were homogenous on SDS–polyacrylamide gel electrophoresis.

Vitamin D₃ (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 D₃ was a generous gift of Professor H. F. DeLuca, University of Wisconsin, Madison. 25-Hydroxy-[26-$^{2}$H₃]vitamin D₃ was synthesized as in [11]. All other chemicals and biochemicals were standard commercial high purity materials.

Vitamin D₃ 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 \mu mol MgCl\textsubscript{2} and 0.1 \mu mol NADP. The amounts of enzymes are given in table 1. The reaction was started by the addition of 150 \mu g vitamin D\textsubscript{3} in 25 \mu l 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 \textsuperscript{3}H\textsubscript{3}-labelled 25-hydroxyvitamin D\textsubscript{3}. After extraction [5] the chloroform was evaporated under N\textsubscript{2} and the residue redissolved in 100 \mu l 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 \mu m ODS column (4.6 X 250 mm). The sample was eluted with 5% H\textsubscript{2}O in methanol at 0.8 ml/min flow rate. The fraction corresponding to eluted 25-hydroxyvitamin D\textsubscript{3} was collected. The solvent was evaporated under N\textsubscript{2} and after redissolving in 100 \mu l of the next eluting solvent the sample was rechromatographed on a Spherisorb 5 \mu m silica column (3 X 250 mm). The sample was eluted with 7.5% isopropanol in hexane at 0.8 ml/min flow rate. The fraction corresponding to eluted 25-hydroxyvitamin D\textsubscript{3} was collected. The solvent was evaporated under N\textsubscript{2} and after redissolving in 100 \mu l of the next eluting solvent the sample was rechromatographed on a Spherisorb 5 \mu m silica column (3 X 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\textsubscript{3} was collected in 0.8 ml, the solvent was removed and the trimethylsilyl/t-butyldimethylsilyl derivative formed as in [3]. The derivative of the extract was then dissolved in 20–50 \mu l hexane and aliquots were analysed by GC–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]. The specific rate of formation of 25-hydroxyvitamin D\textsubscript{3} obtained in 5 different experiments varied from 0.01–0.02 nmol nmol cytochrome P-450\textsuperscript{−1}.min\textsuperscript{−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\textsubscript{3} with the incubation mixture minus ferredoxin. A mass spectrum of the material analyzed in fig.2A showed that it consisted of a mixture of unlabelled and \textsuperscript{3}H\textsubscript{3}-labelled 25-hydroxyvitamin D\textsubscript{3} (cf. table 1). The identity of 25-hydroxyvitamin D\textsubscript{3} was further confirmed by combined GC–MS of purified material obtained from 3 separate incubations to which no \textsuperscript{3}H\textsubscript{3}-labelled 25-hydroxyvitamin D\textsubscript{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\textsubscript{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 D₃ as substrate. The incubation conditions as well as extraction and chromatography were as given in section 2. (No $^2$H₁-labelled 25-hydroxyvitamin D₃ was added to the extract.) (A) Authentic 25-hydroxyvitamin D₃ (43 ng); (B) complete incubation system; (C) incubation system without ferredoxin added.

Table 1

<table>
<thead>
<tr>
<th>Components present in the incubation medium</th>
<th>Rat liver mitochondrial cytochrome P-450 (nmol)</th>
<th>0.74</th>
<th>0.74</th>
<th>0.74</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine adrenal ferredoxin reductase (pmol)</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Bovine adrenal ferredoxin (nmol)</td>
<td>-</td>
<td>5.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Formation of 25-hydroxyvitamin D₃ (ng)</td>
<td>5±4</td>
<td>30</td>
<td>235±64</td>
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For incubation conditions see 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 D₃. 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 D₃ 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 C₂₇-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 D₃ can not yet be decided. With intact mitochondria, it was shown that the vitamin D₃ 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 D₃ to a higher extent than reported for 25- and
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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References


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.