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Purification of 25-hydroxyvitamin D_3 24-hydroxylase from rat kidney mitochondria

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25-Hydroxyvitamin D_3 24-hydroxylase was purified to an electrophoretically homogeneous state ($M_r = 53000$) from kidney mitochondria of female rats treated with vitamin D_3.

Vitamin D_3; Cytochrome P450; Hydroxylase, 24-; Kidney mitochondria

1. INTRODUCTION

25-Hydroxyvitamin D_2 (calcidiol) 24-hydroxylase is induced by 1,25-dihydroxyvitamin D_3 (calcitriol) and is considered to function in the catabolism of the active form of vitamin D_3 [1]. In spite of such importance the enzyme has not so far been purified, though it was suggested to be a member of the cytochrome P450 superfamily [2,3]. In this communication we report the purification of this enzyme from rat kidney mitochondria.

2. MATERIALS AND METHODS

2.1. *Materials*

25-Hydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 were a gift from Chugai Pharmaceutical Co. (Tokyo, Japan). Pentyl-Sepharose was prepared according to the method described in [4]. TSK gel DEAE-5PW was purchased from Tosoh Co. (Tokyo, Japan).

2.2. *Purification of 25-hydroxyvitamin D_3 24-hydroxylase*

All buffer solutions used in the following purification procedures contained 20% glycerol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin unless stated otherwise. Kidney mitochondria were prepared from female rats that received daily injections of 50000 IU vitamin D_3 for 1 week. Mitochondria were suspended in 15 mM Tris-HCl buffer (pH 7.4) containing 0.19 M sucrose, 0.5 mM 1,4-dithiothreitol, 1 mM EDTA, sonicated and centrifuged at 100000 × g for 1 h. The precipitate was suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 1 mM dithiothreitol. 10% cholate containing 0.08% Lubrol PX was added to the suspension under mild stirring to give a final cholate/protein ratio of 3:5 (w/w) and the solubilized suspension was centrifuged at 100000 × g for 1 h. The supernatant was applied to a pentyl-Sepharose 4B column (2 × 13 cm) equilibrated with 100 mM phosphate buffer (pH 7.4) containing 0.4% sodium cholate, 0.5 mM EDTA, and 1 mM dithiothreitol. The column was washed with equilibration buffer and then eluted with 100 mM phosphate buffer containing 0.4% sodium cholate, 0.08% Lubrol, 1 mM EDTA, and 0.5 mM dithiothreitol. The fraction with the activity was collected and dialyzed against 30 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM dithiothreitol, 0.4% sodium cholate and 0.03% Tween 20. The fraction was then applied to a hydroxylapatite column (1.2 × 6.5 cm) equilibrated with the same buffer as used for dialysis. The column was washed with 60 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM dithiothreitol, 0.4% sodium cholate and 0.05% Tween 20, and then eluted with 150 mM phosphate buffer. The fraction with the enzyme activity was collected and dialyzed against 35 mM Tris-HCl buffer (pH 7.4) containing 0.4 mM EDTA, 0.5 mM dithiothreitol, 0.1% Lubrol, 0.3% Tween 20 and 0.05% sodium cholate. From this step onwards protease inhibitors were omitted and HPLC was performed at room temperature. Aliquots were injected into a TSK-gel DEAE-5PW column (7.5 × 75 mm) equilibrated with the dialyzing buffer. The column was eluted with linear NaCl gradients (0–70 min, 0–0.1 M; 70–100 min, 0.1–0.5 M). The flow rate was 0.4 ml/min, and the effluent was monitored at 417 nm.

2.3. *Enzyme assay*

25-Hydroxyvitamin D_3 24-hydroxylase was assayed as follows. A typical mixture contained 2–50 pmol cytochrome P450, 2 nmol adrenodoxin, 0.05 U NADPH-adrenodoxin

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reductase, 0.5 μmol EDTA, 50 μmol Tris-HCl (pH 7.7), 0.5 μmol NADPH and 10 nmol 25-hydroxyvitamin D_3 dissolved in 5 μl ethanol in a final volume of 0.5 ml. The reaction was started by adding NADPH and incubated at 37°C. After 5–7 min the reaction was terminated by adding 100 μl 1 N NaOH. The products were extracted by benzene. The solvent was evaporated under reduced pressure. The residue was dissolved in a mixture of chloroform and ethyl acetate (4:1, v/v) and analyzed by HPLC using a Finepak Sil column (4.6 × 250 mm, JASCO Co. Ltd, Tokyo) with a solvent mixture of isopropanol/methanol/hexane (6:6:88, v/v) at a flow rate of 1.4 ml/min. For identification of the products reversed-phase HPLC was also performed using a Finepak Sil C18 column with a solvent mixture of methanol/water (80:20, v/v) at a flow rate of 1 ml/min.

2.4. *Other methods*

SDS-polyacrylamide gel electrophoresis was performed according to the method described [5] and the protein bands were visualized by silver staining [6]. Cytochrome P450 concentration was determined by a reduced CO difference spectrum using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ [7]. Protein concentration was determined by the method of Lowry et al. [8] or a modification thereof [9]. Periodate oxidation was carried out as described in [10].

3. RESULTS

3.1. *Purification of 25-hydroxyvitamin D_3 24-hydroxylase*

Table 1 summarizes the results of the purification procedure. Overall purification from solubilized fraction of kidney mitochondria was 315-fold. Fig.1 shows the separation of 25-hydroxyvitamin D_3 24-hydroxylase by HPLC. As shown in the figure the enzyme activity was observed only in one peak. An SDS-polyacrylamide gel electrophoretogram of the eluate from HPLC with the activity is shown in fig.2. The active fraction revealed a single major band. The molecular weight of the enzyme calculated from this electrophoretogram was 53000. That the major band on the electrophoretogram is the cytochrome responsible for 25-hydroxyvitamin D_3 24-hydroxylase was confirmed by Western blotting analysis with antibody raised against the purified enzyme, which inhibited the enzyme activity (data not shown).

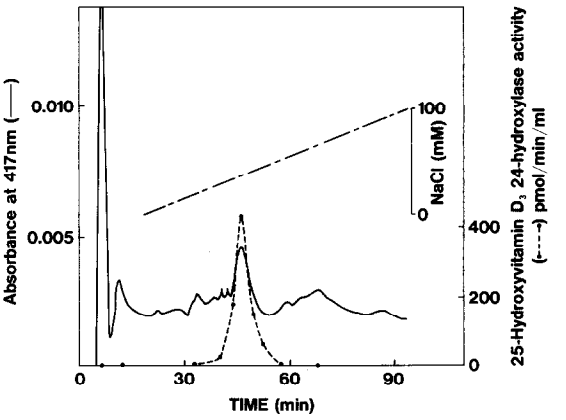

Fig.1. Separation of 25-hydroxyvitamin D_3 24-hydroxylase by high performance anion-exchange chromatography. An aliquot of the eluate from the hydroxylapatite column with the catalytic activity was injected into a TSK-gel DEAE-5PW column (7.5 × 75 mm).

3.2. *Absorption spectra of 25-hydroxyvitamin D_3 24-hydroxylase*

Absorption spectra of the oxidized form of 25-hydroxyvitamin D_3 24-hydroxylase exhibited an intense Soret absorption peak at 417 nm and 2 minor peaks at 535 and 570 nm (data not shown). The absorption maximum for the reduced CO complex was at 453 nm (fig.3).

Table 1

Purification of 25-hydroxyvitamin D_3 24-hydroxylase from rat kidney

Purification step	Protein (mg)	Total P450 (nmol)	Specific content (nmol/mg protein)	Total activity (nmol/min)	Specific activity (nmol/min per mg protein)
Mitochondria	1104				
Membrane fraction	820				
Solubilized fraction	469	16.5	0.035	81	0.173
Pentyl-Sepharose	23.4	5.0	0.21	43	1.85
Hydroxylapatite	5.6	2.1	0.37	35	6.32
DEAE-5PW	0.11	0.29	2.8	5.7	54.6

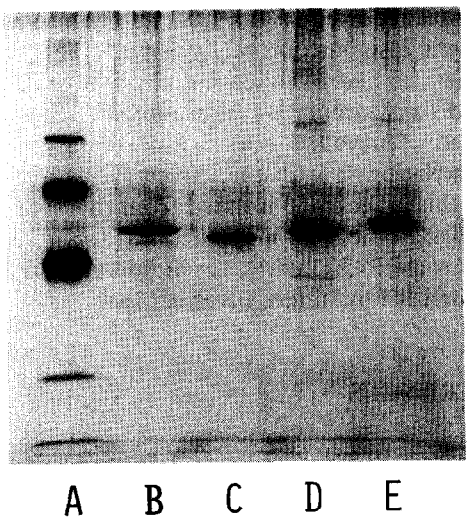

Fig.2. SDS-polyacrylamide gel electrophoretogram of the HPLC fraction showing 25-hydroxyvitamin D_3 24-hydroxylase activity. Migration was from top to bottom. The gel was stained with silver. Lane A, molecular weight standards (phosphorylase b: M_r 94000; bovine serum albumin: M_r 67000; ovalbumin: M_r 43000; carbonic anhydrase: M_r 30000); lane B, 25-hydroxyvitamin D_3 24-hydroxylase ($P450_{cc24}$); lane C, cytochrome $P450_{cc25}$ (M_r 50000, [12]); lane D, cytochrome P450b in rat (M_r 52500); lane E, P450c (M_r 56000). The amounts of cytochromes P450 applied were 0.09 μg.

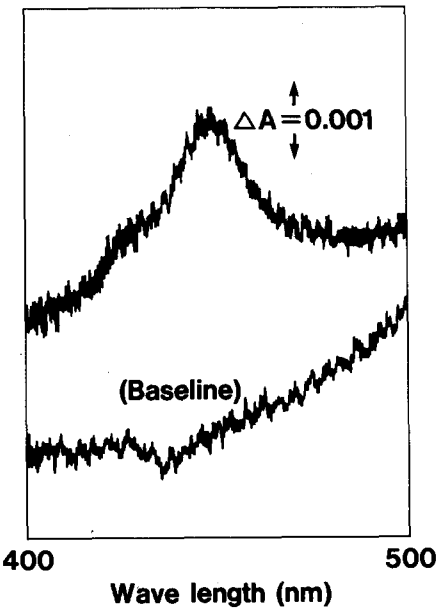

Fig.3. CO difference spectrum of the renal mitochondrial 25-hydroxyvitamin D_3 24-hydroxylase. The spectrum was measured with a sample containing 0.016 μM cytochrome $P450_{cc24}$ (5.7 μg protein/ml) in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1% Emulgen 913.

3.3. *Reconstitution of enzyme activity*

25-Hydroxyvitamin D_3 24-hydroxylase activity was reconstituted with the purified cytochrome P450, adrenodoxin, NADPH-adrenodoxin reductase, and NADPH. The omission of either cytochrome P450 or the electron-carrying system (adrenodoxin and NADPH-adrenodoxin reductase) resulted in a complete loss of activity (table 2). Liver microsomal NADPH-cytochrome P450 reductase could not be substituted for the adrenal electron-transferring system.

3.4. *Identification of the reaction product*

Fig.4 shows a typical chromatogram obtained by the straight phase HPLC of the product obtained by incubating 25-hydroxyvitamin D_3 with the purified enzyme together with adrenodoxin and NADPH-adrenodoxin reductase. As shown in the figure a peak corresponding to 24,25-dihydroxyvitamin D_3 is clearly observed, whereas no peak is seen at a retention time corresponding to 1α,25-dihydroxyvitamin D_3. A similar result was also obtained with reversed-phase HPLC (data not shown). When the product from HPLC was treated with periodate, the product could no longer be detected.

Table 2

Reconstitution of 24-hydroxylation of 25-hydroxyvitamin D_3

Reaction system	Product (nmol/min per nmol of P450)
Complete system[a]	19.7[b]
minus adrenodoxin	N.D.
minus NADPH-adrenodoxin reductase	N.D.
replacement of adrenodoxin and NADPH-adrenodoxin reductase with NADPH-cytochrome P450 reductase	N.D.
plus dilauroylglyceryl-3-phosphorylcholine	31.5

[a] The complete system contained, 2.4 pmol cytochrome P450, 2 nmol adrenodoxin, 0.05 U NADPH-adrenodoxin reductase, 0.5 μmol EDTA, 0.5 μmol NADPH, 10 nmol 25-hydroxyvitamin D_3 and 50 μmol Tris-HCl (pH 7.7) in a final volume of 0.5 ml. Incubation was conducted at 37°C for 7 min

[b] Turnover number is 22 min^{-1} when calculated from V_{max} value

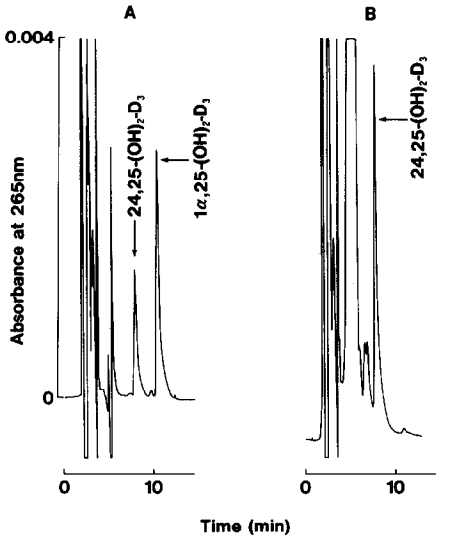

Fig.4. HPLC of the product obtained by incubating 25-hydroxyvitamin D_3 in the reconstituted system using the purified $P450_{cc24}$ (2.4 pmol). A, 24,25-dihydrovitamin D_3 (50 pmol); 1α,25-dihydroxyvitamin D_3 (100 pmol). B, the incubation products.

4. DISCUSSION

We purified kidney mitochondrial cytochrome P450 catalyzing 25-hydroxyvitamin D_3 24-hydroxylation to an electrophoretically homogeneous state based on catalytic activity, and we clearly showed the enzyme to be cytochrome P450. The turnover number of the purified enzyme is 22 min^{-1} which is several thousand-fold higher than that reported so far [10,11]. The present experiment also unequivocally solved the problem whether 24-hydroxylase and 1α-hydroxylase of 25-hydroxyvitamin D_3 are similar. Since the purified enzyme does not catalyze 1α-hydroxylation it is distinct from 1α-hydroxylase. Since this is the P450 isolated from this source for the first time, it may belong to a unique cytochrome P450 family, the amino acid structure of which is now under investigation.

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