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SEPARATION OF THE CYTOCHROMES P-450 IN PIG KIDNEY MITOCHONDRIA CATALYZING 1α-, 24- AND 26-HYDROXYLATIONS OF 25-HYDROXYVITAMIN D_3

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The cytochromes P-450 in pig kidney mitochondria catalyzing 1α-, 24- and 26-hydroxylations of 25-hydroxyvitamin D_3 have been separated. The cytochrome P-450 fractions required NADPH, mitochondrial ferredoxin and ferredoxin reductase for catalytic activity. The present report demonstrates that different forms of cytochrome P-450 are involved in 1α-, 24- and 26-hydroxylations of 25-hydroxyvitamin D_3 and provides a basis for further purification and characterization of these enzymes. ©1990 Academic Press, Inc.

The kidney is the major site of 25-hydroxyvitamin D_3 metabolism. The most important reaction occurring is the 1α-hydroxylation of 25-hydroxyvitamin D_3 to produce 1α,25-dihydroxyvitamin D_3, a compound possessing hormone-like properties (1). 25-Hydroxyvitamin D_3 is also converted into other polar metabolites, such as 24,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3. The function of these metabolites has not been definitely established (2).

Cytochrome P-450-dependent 1α-hydroxylation of 25-hydroxyvitamin D_3 in mammals was first demonstrated by Saarem et al. (3) using pig kidney mitochondria. In 1984, Engstrom et al. (4) reported 1α- and 24-hydroxylase activities towards 25-hydroxyvitamin D_3 in pig kidney homogenates. Recently, this laboratory reported the isolation of a cytochrome P-450 fraction from pig kidney mitochondria active in 26-hydroxylation of 25-hydroxyvitamin D_3 (5). Studies with partially purified preparations from pig kidney mitochondria have not clearly demonstrated whether these hydroxylations are catalyzed by the same or by different forms of cytochrome P-450 (3,5,6). The heme content as well as the specific activities towards 25-hydroxyvitamin D_3 in the preparations have been low.

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The present communication reports methods for separation of the cytochromes P-450 in pig kidney mitochondria catalyzing the 1α-, 24- and 26-hydroxylations of 25-hydroxyvitamin D_3.

EXPERIMENTAL PROCEDURES

Materials. 24R,25-Dihydroxy [23,24(n)-^{3}H] vitamin D_3 (60-90 Ci/mmol), 25,26-dihydroxy [23,24(n)-^{3}H] vitamin D_3 (95 Ci/mmol) and 1α,25-dihydroxy [23,24(n)-^{3}H] vitamin D_3 (101 Ci/mmol) were obtained from the Radiochemical Center (Amersham, U.K.). 25-Hydroxyvitamin D_3 was obtained from Duphar (Weesp, The Netherlands). Sodium cholate (sodium 3α,7α,12α-trihydroxy-5β-cholanoate) and Lubrol PX were obtained from Sigma. Octylamine-Sepharose 4B was prepared as described previously (7) and hydroxylapatite (Bio-Gel HTP) from Bio-Rad was used in the form described by Dahlbäck and Wikvall (8). Q-Sepharose was from Pharmacia and cofactors were from Merck. The remaining chemicals were of reagent grade.
Purification procedure. Kidneys from untreated pigs were used. Kidney cortex mitochondria were prepared from 12 kg of pig kidney as described previously for preparation of rabbit liver mitochondria (9). The mitochondria were suspended in 10 mM phosphate buffer, pH 7.4, to a protein concentration of 15 mg/ml, homogenized and allowed to stand at 4°C overnight. The mitochondrial suspension was sonicated for 3 min at intervals of 15 s and centrifuged at 20,0000 x g for 30 min. The precipitate was dissolved in 0.1 M phosphate, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 1.8% (w/v) sodium cholate to a protein concentration of 6 mg/ml and homogenized. The suspension was stirred gently for 1 h at 4 C. A 50% (w/v) polyethylene glycol 6,000 solution was added to a concentration of 6% polyethylene glycol in the suspension and stirring was continued for 15 min. The precipitate between 0-6% polyethylene glycol was collected by centrifugation at 20,000 x g for 20 min and discarded. Polyethylene glycol was then added in the same way to a concentration of 17%. The precipitate between 6-17% polyethylene glycol was collected by centrifugation and was homogenized and dissolved in 0.1 M phosphate, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.8% (w/v) sodium cholate. All buffers in the following purification procedure contained 20% glycerol and 0.1 mM EDTA unless otherwise stated. Phosphate buffer was used as the potassium salt. The solution was subjected to chromatography on an octylamine-Sepharose column (4 x 40 cm), equilibrated in 0.1 M phosphate buffer, pH 7.4, containing 0.7% (w/v) sodium cholate. The column was washed with 0.1 M phosphate buffer, pH 7.4, containing 0.4% (w/v) sodium cholate and the cytochromes P-450 were eluted with addition of 0.08% (w/v) Lubrol PX to the washing buffer. Cytochrome P-450 containing fractions were diluted fivefold with 20% glycerol and subjected to chromatography on hydroxylapatite (3 x 40 cm), equilibrated in 10 mM phosphate buffer, pH 7.4. The hydroxylapatite column was washed with 25 mM phosphate buffer, pH 7.4, containing 0.2% (w/v) Lubrol PX and cytochromes P-450 were eluted with 150 mM phosphate buffer, pH 7.4, containing 0.2% (w/v) Lubrol PX. The fractions with highest concentration of cytochrome P-450 were pooled and dialyzed overnight against 20 mM Tris-acetate buffer, pH 8.0, containing 0.4% (w/v) Lubrol PX. The cytochrome P-450 fraction was then subjected to HPLC using a Q-Sepharose column (1.6 x 20 cm). The column was equilibrated in 20 mM Tris-acetate buffer, pH 8.0, containing 0.4% (w/v) Lubrol PX. The flow rate was 2 ml/min. The column was eluted in a stepwise gradient of sodium acetate in the equilibrating buffer. The 1α- and the

24-hydroxylating cytochromes P-450 were eluted with 75 mM sodium acetate in the equilibrating buffer whereas the 26-hydroxylating cytochrome P-450 was eluted with 0.3 M sodium acetate in the equilibrating buffer. The fraction containing 1α- and 24-hydroxylating cytochromes P-450 and the fraction containing 26-hydroxylating cytochrome P-450 were separately dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.4% (w/v) Lubrol PX and 0.1% (w/v) sodium cholate. Each fraction was subjected to chromatography on Q-Sepharose columns (1.6 x 20 cm) equilibrated in the same buffer as used for dialysis. The columns were eluted with sodium acetate gradients (0-20 min, O M; 20-40 min, 0-0.1 M; 40-60 min, 0.1 M; 60-80 min, 0.1-0.3 M; 80-100 min, 0.3 M; 101-120, 0.5 M) in the equilibrating buffer. The flow rate was 2 ml/min. The 1α-hydroxylating cytochrome P-450 was eluted with 0.1 M sodium acetate and the 24-hydroxylating cytochrome P-450 with 0.3 M sodium acetate. The 26-hydroxylating cytochrome P-450 was eluted with 0.1 M sodium acetate in the equilibrating buffer. Detergents were removed and the final cytochromes P-450 fractions were dialyzed as described by Wikvall (9). The separation procedures are summarized in Fig. 1.

Incubation procedures. Incubations were carried out for 20 min at 37°C. 25-Hydroxyvitamin D_3, 25 µg in 25 µl acetone, was incubated with 0.2 nmol of the cytochrome P-450 fraction, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase and 1 µmol of NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4. Incubations with intact mitochondria were performed with 20 mg of mitochondrial protein, 45 µmol of isocitrate, and 10 mM $MgCl_2$ in a total volume of 5 ml of 50 mM Tris-acetate buffer, pH 7.4. Before analysis, the incubation mixtures with intact mitochondria were extracted and purified as described by Holmberg et al. (10). Incubations were terminated with 5 ml of trichloroethane/ methanol

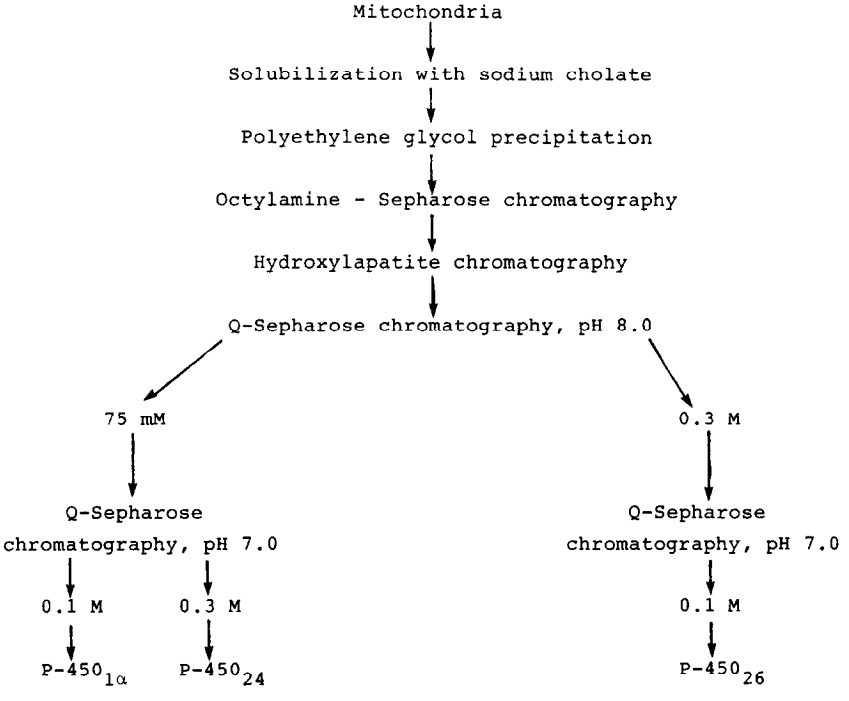

Fig. 1. Purification procedure.

(2:1, v/v) and extracted and analyzed by HPLC as described previously (11). The retention times for 1α,25-, 24,25- and 25,26-dihydroxyvitamins D_3 were 12, 6 and 10 min on straight phase, respectively, and 6.9, 6.2 and 7.5 min respectively, on reversed phase HPLC. Total recovery was 50-60%. The identity of the 1α,25-dihydroxyvitamin D_3 and the 25,26-dihydroxyvitamin D_3 was confirmed by combined gas chromatography-mass spectrometry of the trimethylsilyl ether derivatives.

Other methods. Ferredoxin and ferredoxin reductase from adrenal mitochondria were prepared as described previously (9). Protein was determined by the method of Lowry et al. (12) and cytochrome P-450 as described by Omura and Sato (13).

RESULTS AND DISCUSSION

Solubilization of pig kidney mitochondria with sodium cholate, followed by polyethylene glycol fractionation and chromatography on octylamine-Sepharose, hydroxylapatite and Q-Sepharose in two steps resulted in the isolation of cytochromes $P\text{-}450_{1\alpha}$, $P\text{-}450_{24}$ and $P\text{-}450_{26}$ in separate fractions. The preparations contained, respectively, 2.0, 3.5 and 2.2 nmol of cytochrome P-450 per mg of protein. The specific cytochrome P-450 content was 20-35-fold higher in the partially purified enzyme fractions than in mitochondria (3,14). The cytochrome P-450 fractions, in the presence of ferredoxin, ferredoxin reductase and NADPH, catalyzed 1α-, 24- and 26-hydroxylations of 25-hydroxyvitamin D_3 at rates of 76, 155 and 50 pmol of product formed x nmol of cytochrome $P\text{-}450^{-1}$ x min^{-1} (Table 1). When expressed as pmol x mg of $protein^{-1}$ x

Table 1. Hydroxylase activities in mitochondria and partially purified cytochrome P-450 fractions from pig kidney

Fraction	Specific content (nmol/ mg protein)	Hydroxylation of 25-hydroxyvitamin D_3 (pmol/mg of protein/min) 1α	24	26	(pmol/nmol of P-450/min) 1α	24	26
Mitochondria	N.D.*	0.08	≤0.05	0.15	N.D.	N.D.	N.D.
$P\text{-}450_{1\alpha}$	2.0	153	≤0.05	≤0.05	76	≤1	≤1
$P\text{-}450_{24}$	3.5	≤0.05	543	≤0.05	≤1	155	≤1
$P\text{-}450_{26}$	2.2	≤0.05	≤0.05	111	<1	<1	50

* ND, not determined

min^{-1}, these activities represented a 1,900-fold purification of the 1α-hydroxylase, a 21,000-fold purification of the 24-hydroxylase and a 700-fold purification of the 26-hydroxylase.

Partial purification of 25-hydroxyvitamin D_3 1α-hydroxylase and 24-hydroxylase from both vitamin D-repleted and rachitic pigs has been previously reported (3,6). However, the cytochrome P-450 content as well as the hydroxylase activities in these partially purified enzyme fractions were several times lower than in the present report. The 1α-hydroxylase and the 24-hydroxylase activities have not been previously separated into distinct enzyme fractions from one and the same preparation of kidney mitochondria.

Hiwatashi et al. (15) have reported the isolation of an electrophoretically homogeneous enzyme fraction from bovine kidney mitochondria catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3 but not 24-hydroxylation. Although the specific content of cytochrome P-450 reported by Hiwatashi et al. (15) was six times higher than in the present 1α-hydroxylating enzyme fraction from pig kidney mitochondria, the rate of 1α-hydroxylation was almost ten times lower. Ohyama et al. (16) reported recently the purification of 25-hydroxyvitamin D_3 24-hydroxylase from kidney mitochondria of female rats treated with vitamin D_3. The cytochrome P-450 content was not higher than that of the present 24-hydroxylating enzyme fraction. However, the 24-hydroxylase activity towards 25-hydroxyvitamin D_3 was 100 times higher in the preparation described by Ohyama et al. (16). No 1α-hydroxylase activity was detected in the enzyme fraction.

In the present work, a complete separation of the 1α- and 24-hydroxylases was achieved. In addition, the 26-hydroxylase active on 25-hydroxyvitamin D_3, recently shown to be a renal mitochondrial cytochrome P-450 (5), was isolated free from 1α- and 24-hydroxylase activities. Thus, the reported purification procedure clearly demonstrates that it is possible to separate the 1α-, 24- and 26-hydroxylating cytochromes P-450 and provides a basis for their further purification and characterization.

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