Properties of guinea-pig kidney 25-hydroxyvitamin D₃ 1 α -hydroxylase assayed by isotope dilution–mass spectrometry

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1. A highly specific and accurate method based on isotope dilution–mass spectrometry was used for characterization of the renal 25-hydroxyvitamin D₃ 1 α -hydroxylase in untreated guinea pigs with a normal vitamin D status. In previous work, the properties of the enzyme had been determined in rachitic animals only. 2. With intact mitochondria, the reaction required the presence of citric acid-cycle intermediates. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide had an inhibitory effect on the isocitrate-supported reaction, indicating that energy-dependent transhydrogenation is of importance. Mitochondrial respiratory-chain inhibitors (cyanide, rotenone, antimycin A) had no effect on the hydroxylation. CO had an inhibitory effect, suggesting participation of a species of cytochrome *P*-450 in the reaction. A fraction solubilized from mitochondria by cholate became catalytically active in 1 α -hydroxylation of 25-hydroxyvitamin D₃ after addition of ferredoxin and ferredoxin reductase. The isocitrate-supported reaction catalysed by crude mitochondria had an apparent K_m of about 1 μ M. 3. An atmosphere containing 50 % O₂ was found to be necessary for optimal activity. It is thus possible that O₂ may be a limiting factor under normal conditions *in vivo*. 4. The results demonstrate that the mammalian renal 25-hydroxyvitamin D₃ 1 α -hydroxylase is a cytochrome *P*-450-dependent mixed-function oxidase with properties similar to those previously reported for the same enzyme system in chicken. The present assay and animal system seem to be suitable for further studies on the mechanism of regulation of the mammalian renal 25-hydroxyvitamin D₃ 1 α -hydroxylase under conditions when the vitamin D status is normal.

INTRODUCTION

From a regulatory point of view, the renal 1 α -hydroxylation of 25-hydroxyvitamin D₃ (25-hydroxy-cholecalciferol) is the most important reaction in the overall metabolism of vitamin D₃. This enzyme system has been studied in detail in the chicken (Henry & Norman, 1984). It has been shown that it requires Mg²⁺, O₂ and a source of reduced nicotinamide nucleotides and that it involves participation of a cytochrome *P*-450 species (Ghazarian *et al.*, 1974; Pedersen *et al.*, 1976). Considerably less is known about the corresponding system in mammals. One explanation for this is that at least some mammalian kidney systems seem to contain an inhibitory protein that binds the substrate 25-hydroxyvitamin D₃ with high affinity (Botham *et al.*, 1974). Using high concentrations of substrate and kidney mitochondria from rachitic rats, Paulson & DeLuca (1985) reported that the 25-hydroxyvitamin D₃ 1 α -hydroxylase system in the rat, in similarity with the same system in chicken, required O₂, a source of NADPH and a species of cytochrome *P*-450. A lipid peroxide inhibitor had no effect on the reaction. In contrast with these results, Warner (1983) reported that a reconstitution system consisting only of partially purified cytochrome *P*-450 from rat kidney mitochondria had properties more like those of a peroxygenase than of a mixed-function oxidase.

The most important limiting factor in previous work with the 25-hydroxyvitamin D₃ 1 α -hydroxylase was the method of quantification of the product. Most probably

differences in methodology are responsible for the different results obtained by Paulson & DeLuca (1985) and Warner (1983). The former group used two different h.p.l.c. steps to isolate the product, whereas Warner (1983) used only one single straight-phase h.p.l.c. step. It is known that an enzymic product of 25-hydroxyvitamin D₃, 10-oxo-19-nor-25-hydroxyvitamin D₃, co-migrates with 1,25-dihydroxyvitamin D₃ in straight-phase h.p.l.c. (Brown & DeLuca, 1985). However, it is by no means excluded that small amounts of interfering products may occur also when two h.p.l.c. steps are used. Such interference may be less important in large-scale preparations designed to identify the product, but may be important and remain undetected in individual incubations.

Another limiting factor in most previous work on the mammalian 25-hydroxyvitamin D₃ 1 α -hydroxylase is that rachitic animals have been used to obtain sufficient enzymic activity. Relevant information concerning the mechanism of reaction can be obtained from studies with such preparations. Studies on the mechanism of regulation, effects of different dietary and hormonal manipulations etc. would, however, require preparations from animals with a normal vitamin D status. Thus the enzyme may be maximally stimulated in the rachitic state, and the sensitivity for different regulatory factors may therefore be decreased.

It has been reported by Sommerville *et al.* (1978) and Engström *et al.* (1984) and confirmed by us (Holmberg *et al.*, 1986) that it is possible to assay the renal 25-hydroxyvitamin D₃ 1 α -hydroxylase in untreated pigs.

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Pigs are, however, less suitable as laboratory animals in large-scale experiments. Delvin & Dussault (1985) reported that it is possible to assay renal 25-hydroxyvitamin D₃ 1 α -hydroxylase activity in untreated weanling guinea pigs. From a methodological point of view, however, the assay in that work was not satisfactory. Thus only a single h.p.l.c. step was used for isolation and quantification of the product.

In the present work the properties of the mammalian renal 25-hydroxyvitamin D₃ 1 α -hydroxylase were studied in untreated guinea pigs with use of a highly accurate technique based on isotope dilution-mass spectrometry. The results support the contention that the enzyme system is a mixed-function oxidase and that cytochrome P-450 is involved in the hydroxylation.

MATERIALS AND METHODS

Chemicals

1,25-Dihydroxy[26-³H₃]vitamin D₃ was synthesized as described previously (Holmberg *et al.*, 1986). 25-Hydroxyvitamin D₃ was generously given by Dr J. Babcock (Upjohn Co). 1,25-Dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ were from Hoffmann-La Roche, Basel, Switzerland. Cofactors were obtained from Sigma Chemical Co. All other chemicals and biochemicals were standard commercial high-purity materials.

Animals

Male and female guinea pigs of the Dunkin-Hartley strain weighing 350–700 g were used. In the study concerned with sex differences, only animals weighing 370–550 g were included. Animals were kept in a controlled environment at 16–18 °C on regular illumination schedule, lights on at 06:00 h and off at 18:00 h. They were given commercial pellet diet (Ewos AB, Södertälje, Sweden) containing 0.9 % Ca, 0.5 % P and 1000 i.u. of vitamin D₃/kg.

Preparation of mitochondrial and submitochondrial fractions and incubation conditions

Animals were killed by decapitation and bled. Kidneys were rapidly removed and placed in ice-cold sucrose buffer consisting of 0.25 M-sucrose in 10 mM-Hepes/KOH buffer, pH 7.4, containing 1 mM-EGTA. The medulla was removed from the kidneys. The cortex was homogenized in 10 vol. (w/v) of the above sucrose buffer. The mitochondrial fraction was isolated by centrifugation of an 800 g supernatant fluid of a kidney homogenate at 6500 g for 20 min. The mitochondrial pellet obtained was resuspended in the initial volume of sucrose buffer and re-centrifuged as above. This procedure was repeated once. The pellet was then suspended in the incubation medium consisting of 50 mM-Tris/acetate buffer pH 7.4 at 20 °C, containing 10 mM-MgCl₂, 12 mM-isocitrate and 1.6 μ g of *NN'*-diphenyl-*p*-phenylenediamine/ml to a protein concentration of about 10 mg/ml. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

In the standard incubation procedure 10 μ g of 25-hydroxyvitamin D₃ dissolved in 10 μ l of acetone was incubated with 5–10 mg of mitochondrial protein in the incubation medium in a final volume of 1.0 ml. Incubations were performed in a water-bath shaker under air at 37 °C for 30 min and terminated by the addition of 1 ml

of acetonitrile together with 96 pmol of 1,25-dihydroxy[26-³H₃]vitamin D₃. In the study of the subcellular distribution of the 1 α -hydroxylase activity the preparations were prepared as described previously (Saarem *et al.*, 1984). The standard incubation procedure was used. Solubilization of renal mitochondrial cytochrome P-450 was performed as described previously (Oftebro *et al.*, 1981). The preparations of bovine adrenal ferredoxin and NADPH-ferredoxin reductase were performed as described previously (Pedersen & Godager, 1978; Strobel & Dignam, 1978).

Extraction and chromatography

Precipitated protein was removed by centrifugation and the supernatant was applied to a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, MA, U.S.A.), that had been washed with 3 ml of methanol followed by 3 ml of water. If re-used the cartridge was regenerated by washing with 3 ml each of methanol, chloroform, hexane, chloroform, methanol and water. The sample was washed with 3 ml of 30 % (v/v) water in methanol and eluted with 3 ml of acetonitrile. The solvent was removed under N₂ and the sample was dissolved in 100 μ l of 12 % (v/v) propan-2-ol in hexane.

Straight-phase h.p.l.c. was performed with Nucleosil silica (5 μ m particle size) (4.6 mm $\times$ 400 mm) at a flow rate of 1.5 ml/min with 12 % propan-2-ol in hexane. The fraction containing 1,25-dihydroxyvitamin D₃ (detected by absorbance at 254 nm) was collected and dried under N₂. The material was then converted into the trimethylsilyl ether (Makita & Wells, 1963) and dissolved in 20 μ l of hexane. For identification purposes, the sample was chromatographed by a reversed-phase system [Nucleosil C₁₈ (5 μ m particle size) column (4.6 mm $\times$ 250 mm), 10 % (v/v) water in methanol as solvent] before the straight-phase system.

Combined gas chromatography-mass spectrometry

The mass-fragmentographic analysis was performed by using the ions at *m/z* 452 and *m/z* 455 as described previously (Björkhem *et al.*, 1979; Holmberg *et al.*, 1986). In analyses of samples containing different amounts of 1,25-dihydroxyvitamin D₃ and a fixed amount of 26-³H₃-labelled 1,25-dihydroxyvitamin D₃, the ratio between the height of the peak at *m/z* 452 and that at *m/z* 455 was found to be linear with the amount of unlabelled 1,25-dihydroxyvitamin D₃.

Radioreceptor analysis

In some cases (see the Results section) 1,25-dihydroxyvitamin D₃ formed in incubations with 25-hydroxyvitamin D₃ was assayed also with a radioreceptor assay, with thymus receptor protein from Immuno Nuclear Corp. (Stillwater, MN, U.S.A.) under conditions previously described (Reinhardt *et al.*, 1984). In this method the 1,25-dihydroxyvitamin D₃ is purified by reversed-phase and straight-phase chromatography before the receptor assay. Recovery is evaluated with use of a recovery tracer (Reinhardt *et al.*, 1984). The coefficient of variation in this assay is 10–12 % under the conditions employed.

RESULTS

Identification of the product

The product formed from 25-hydroxyvitamin D₃ by the mitochondrial fraction of a guinea-pig kidney

Fig. 1. Multiple-ion detector recordings at m/z 452 and m/z 455 of trimethylsilyl derivative of an extract of incubation of 25-hydroxyvitamin D₃ with guinea-pig kidney mitochondria

A fixed amount of 1,25-dihydroxy[26-³H]₃vitamin D₃ (96 pmol) had been added to the incubation mixture immediately after termination of the incubation (see the Materials and methods section).

homogenate had the same retention time as authentic 1,25-dihydroxyvitamin D₃ in both the straight-phase and the reversed-phase h.p.l.c. systems used (see the Materials and methods section). Combined gas chromatography-mass spectrometry of the trimethylsilyl ether derivative of the product showed two peaks with retention times corresponding to those of the pyro and isopyro forms of 1,25-dihydroxyvitamin D₃ (cf. Björkhem *et al.*, 1979). The identity of the product was further established by monitoring of the characteristic ions at m/z 131 (corresponding to cleavage between C-24 and C-25), m/z 452 ($M - 2 \times 90$), m/z 542 ($M - 90$) and m/z 632 (M) (cf. Björkhem *et al.*, 1979). Peaks corresponding to the pyro and isopyro forms of the derivative of 1,25-dihydroxyvitamin D₃ were obtained in each of these tracings with relative intensities similar to those of the authentic compound.

Assay of the 25-hydroxyvitamin D₃ 1 α -hydroxylase activity

The previous assay (Holmberg *et al.*, 1986), based on isotope dilution-selected ion monitoring, was found to be suitable also for the assay of 1 α -hydroxylation of 25-hydroxyvitamin D₃ in guinea-pig mitochondria. As in the previous work, the ion at m/z 452 was used to

Table 1. Effect of different incubation media on 1 α -hydroxylation of 25-hydroxyvitamin D₃

Except for the incubation medium, standard incubation conditions were used (10 mM-MgCl₂ and 12 mM-isocitrate).

Buffer	Rate of 1 α -hydroxylation (pmol/min per mg)
Tris/acetate (50 mM)	0.41
Tris/acetate (20 mM) + sucrose (250 mM)	0.17
Mops (20 mM) + KCl (125 mM)	0.31
Mops (20 mM) + sucrose (250 mM)	0.11

monitor the trimethylsilyl derivative of the unlabelled 1,25-dihydroxyvitamin D₃ and the ion at m/z 455 to monitor the corresponding derivative of the ³H₃-labelled 1,25-dihydroxyvitamin D₃. Fig. 1 shows a typical multiple-ion detector recording obtained in connection with analysis of a purified extract from a standard incubation (see the Materials and methods section).

In a series of experiments with different amounts of enzyme protein, the amount of product formed was assayed with both the isotope-dilution-mass-spectrometric method and an established radioreceptor method similar to that used in previous work on the 25-hydroxyvitamin D₃ 1 α -hydroxylase. The latter method gave values 32–72% higher than those obtained with more specific mass-spectrometric method. The identity of the compound(s) cross-reacting in the radioreceptor method could not be established. It is evident, however, that the mass-spectrometric method has merits as compared with the radioreceptor method.

When incubated under standard incubation conditions and in accordance with the results obtained with pig mitochondria (Holmberg *et al.*, 1986), the conversion of 25-hydroxyvitamin D₃ into 1,25-dihydroxyvitamin D₃ was found to be linear with time for 30 min and with mitochondrial protein up to about 5 mg. Substrate saturation was obtained with about 5 μ g of substrate and the apparent K_m was about 1×10^{-6} M.

Optimum conditions for assay of the 25-hydroxyvitamin D₃ 1 α -hydroxylase activity

Osmolarity. As shown in Table 1, the highest conversion was obtained with an hypo-osmolar buffer (50 mM-Tris/acetate), whereas a slighter lower conversion was obtained with an iso-osmolar buffer (20 mM-Mops containing 125 mM-KCl).

Mg²⁺ concentration. Increasing the concentration of Mg²⁺ from 2 mM to 20 mM did not significantly affect the activity (results not shown). A concentration of 10 mM-Mg²⁺ was therefore used in the standard incubations.

Effect of pH. As shown in Fig. 2, the pH optimum of the 1 α -hydroxylase was in the range 7.4–8.4 under the conditions employed. A somewhat higher rate of conversion was found with potassium phosphate buffer than with a Tris/acetate buffer.

Fig. 2. Effect of pH on the 1α -hydroxylation of 25-hydroxyvitamin D_3 by guinea-pig kidney mitochondria

○, 50 mM-Potassium phosphate buffer; ●, 50 mM-Tris/acetate buffer.

Table 2. Effect of different citric acid-cycle intermediates on 1α -hydroxylation of 25-hydroxyvitamin D_3

Standard incubation conditions were used (50 mM-Tris/acetate buffer, pH 7.4, and 10 mM- $MgCl_2$).

Addition	Rate of 1α -hydroxylation (pmol/min per mg)
None	< 0.06
Isocitrate (12 mM)	0.42
Citrate (12 mM)	0.38
Succinate (12 mM)	0.11
Fumarate (12 mM)	0.26
Malate (12 mM)	0.24

Effect of citric acid-cycle intermediates. As shown in Table 2, isocitrate, fumarate, malate and citrate all supported the 1α -hydroxylase activity to about the same degree, whereas succinate appeared to be less effective. Increasing the concentration of isocitrate above 12 mM did not further increase the activity. In the standard incubations, a concentration of 12 mM-isocitrate was therefore used. In the absence of any citric acid-cycle intermediate, no detectable amount of product was obtained.

Effect of inhibitors on the 1α -hydroxylase activity. The effects of various mitochondrial inhibitors and uncouplers are shown in Table 3. Rotenone, antimycin A and KCN did not effect the activity. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine, however, strongly diminished the activity. The lipid peroxidase inhibitor *NN'*-diphenyl-*p*-phenylenediamine at a concentration of 0.1 mM did not inhibit the activity (results not shown).

Table 3. Effect of mitochondrial inhibitors and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine on 1α -hydroxylation of 25-hydroxyvitamin D_3 in guinea-pig mitochondria

Standard incubation conditions were used with isocitrate (12 mM) as oxidizable substrate. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

Addition	Rate of 1α -hydroxylation (pmol/min per mg)
None	0.85
Rotenone (4 mM)	0.67
Antimycin A (4 μ g)	0.80
KCN (4 mM)	0.69
FCCP (20 μ M)	0.12

Table 4. Effect of CO and O_2 concentrations on 1α -hydroxylation of 25-hydroxyvitamin D_3

Standard incubation conditions were used. Different mitochondrial preparations were used in two experiments. Each result is a mean of two experiments. In the experiment with CO , the substrate was added to the incubation mixture immediately before the exposure to the CO -containing atmosphere.

Gas mixture	Rate of 1α -hydroxylation (pmol/min per mg)
10% O_2 + 90% N_2 (Expt. 1)	0.21
10% O_2 + 40% CO + 50% N_2 (Expt. 1)	0.14
0% O_2 + 100% N_2 (Expt. 2)	0.07
5% O_2 + 95% N_2 (Expt. 2)	0.09
10% O_2 + 90% N_2 (Expt. 2)	0.13
25% O_2 + 75% N_2 (Expt. 2)	0.62
50% O_2 + 50% N_2 (Expt. 2)	1.00
100% O_2 (Expt. 2)	0.81

Effect of CO and different concentrations of O_2 on 1α -hydroxylation of 25-hydroxyvitamin D_3

As shown in Table 4, 40% CO inhibited the activity by about 30% when the reaction was performed in an atmosphere containing 10% O_2 . A relatively high concentration of O_2 (about 50%) was found to be necessary for optimal activity. At a concentration of 10% O_2 the activity was less than 20% of that obtained in an atmosphere containing 50% O_2 (Table 4). In order to ascertain this stimulatory effect of high concentrations of O_2 , replicate incubations ($n = 6$) were performed at concentrations of 10% O_2 and 50% O_2 with a different enzyme preparation from that used in Table 4. The conversion obtained with 10% O_2 was 0.19 ± 0.03 pmol/min per mg and that with 50% O_2 was 0.58 ± 0.04 pmol/min per mg. This difference was highly significant ($P < 0.001$) by Student's *t* test.

Subcellular distribution of the 25-hydroxyvitamin D_3 1α -hydroxylase

A significant 1α -hydroxylase activity was detected only in the crude nuclear fraction and the heavy mitochondrial fraction (Table 5). The small activity in

Table 5. Subcellular distribution of 25-hydroxyvitamin D₃ 1 α -hydroxylase activity in guinea-pig kidney

Standard incubation conditions were used. Key to fractions: N, crude nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction.

Subcellular fraction	Rate of 1 α -hydroxylation (pmol/min per mg)
N	0.17
M	0.48
L	< 0.06
P	< 0.06
S	< 0.06

Table 6. Effect of ferredoxin and ferredoxin reductase on 25-hydroxyvitamin D₃ 1 α -hydroxylase activity catalysed by a solubilized renal mitochondrial fraction

These incubations were performed in an incubation media containing 25 mM-Mops buffer, pH 7.6, 1.5 mM-Mg²⁺, 0.5 mM-NADP⁺, 0.7 mg of solubilized mitochondrial protein/ml and an NADPH-generating system as described previously for assay of activity of solubilized liver mitochondrial cytochrome *P*-450 (Oftebro *et al.*, 1981). When added, 4 nmol of bovine adrenal ferredoxin and 30 pmol of bovine adrenal ferredoxin reductase were used.

Addition	Rate of 1 α -hydroxylation (pmol/min per mg)
Solubilized mitochondrial fraction	< 0.06
Solubilized mitochondrial fraction + bovine adrenal ferredoxin (4 μ M) + bovine adrenal ferredoxin reductase (30 mM)	0.69

the nuclear fraction may be due to a mitochondrial contamination.

Effect of sex on the 25-hydroxyvitamin D₃ 1 α -hydroxylase activity

A small apparent difference was obtained between the renal mitochondrial 25-hydroxyvitamin D₃ 1 α -hydroxylase activity in preparations from female and male guinea pigs. In the male group ($n = 6$) $V_{\max}$ was found to be 0.84 ± 0.11 pmol/min per mg (mean $\pm$ S.E.M.). In the female group ($n = 6$) $V_{\max}$ was found to be 0.55 ± 0.11 pmol/min per mg. The apparent sex difference was, however, not significant statistically ($P > 0.05$ by Student's *t* test). The K_m for 25-hydroxyvitamin D₃ was found to be about 1×10^{-6} M in both groups of animals.

Studies with a reconstituted system

Attempts were made to solubilize cytochrome *P*-450 from guinea-pig mitochondria according to previously published procedures (Oftebro *et al.*, 1981). A fraction was obtained that was completely inactive in the absence of added ferredoxin and ferredoxin reductase. After the addition of ferredoxin and ferredoxin reductase, however,

a significant activity was obtained (Table 6). Owing to the presence of small amounts of cytochrome *P*-450 in the preparation and interference from other haem proteins no accurate measurement of the amount of cytochrome *P*-450 could be performed.

DISCUSSION

Isotope dilution-mass spectrometry has a high potential for accuracy (Björkhem, 1979), and the present method for assay of the renal 25-hydroxyvitamin D₃ 1 α -hydroxylase should be more specific and accurate than previous methods. In order to interfere with the present assay, a compound must have the same retention time as 1,25-dihydroxyvitamin D₃ in the h.p.l.c. system and the gas chromatography, and in addition contain the specific ion at m/z 452 in its mass spectrum. As expected, lower values or 1,25-dihydroxyvitamin D₃ were obtained with this method than with a radioreceptor method.

The results of the present work support the contention that the mammalian 25-hydroxyvitamin D₃ 1 α -hydroxylase is a cytochrome *P*-450-dependent mixed-function oxidase (Paulson & DeLuca, 1985), and not a dioxygenase as suggested by Warner (1983). Although the degree of inhibition by CO was relatively low, there was a significant inhibition. In similarity with the hepatic and adrenal hydroxylases, solubilization by cholate gave a fraction that became enzymically active after reconstitution with ferredoxin and ferredoxin reductase. The hydroxylation by intact mitochondria was dependent upon NADPH generated intramitochondrially by citric acid-cycle intermediates. Since the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone had an inhibitory effect, it may be concluded that energy-dependent transhydrogenation is of importance. In similarity with this, Paulson & DeLuca (1985) found an inhibitory effect of the uncoupler 2,4-dinitrophenol on the 25-hydroxyvitamin D₃ 1 α -hydroxylase when the hydroxylation was supported by succinate or malate (Paulson & DeLuca, 1985). Mitochondrial respiratory-chain inhibitors had no effect on the hydroxylation. Under the same conditions also the renal mitochondrial 25-hydroxyvitamin D₃ 23-hydroxylase in guinea pigs is unaffected or even slightly stimulated by such inhibitors (Pedersen *et al.*, 1988).

Under the conditions used the O₂ affinity appeared to be relatively low for the 25-hydroxyvitamin D₃ 1 α -hydroxylase. Thus optimal activity was obtained in an atmosphere containing 50% O₂. Most other cytochrome *P*-450-dependent mixed-function oxidases are saturated when exposed to 5–10% O₂. In a situation with diminished blood flow and decreased O₂ concentration in the kidney, the activity of the 25-hydroxyvitamin D₃ 1 α -hydroxylase may thus be depressed more than other O₂-dependent biochemical systems.

The apparent K_m of the renal 25-hydroxyvitamin D₃ 1 α -hydroxylase was found to be very low as compared with most other cytochrome *P*-450-dependent mixed-function oxidases, about 1 μ M. Because of methodological difficulties and also possibly the presence of small amounts of vitamin D-binding protein in the mitochondrial preparations, no more precise measurement of the K_m could be performed. It can thus not be excluded that the true K_m may be even lower than 1 μ M. Delvin & Dussault (1985) reported a K_m of 0.6 μ M for the renal 25-hydroxyvitamin D₃ 1 α -hydroxylase in weanling guinea

pigs, and Engström *et al.* (1984) reported a K_m of $0.5 \mu\text{M}$ for the same enzyme in pigs. In the work by Paulson & DeLuca (1985) on the renal 25-hydroxyvitamin D_3 1α -hydroxylase in rachitic rats, very large amounts of substrate had to be added to the mitochondrial preparations in order to overcome the inhibitory effect of vitamin D-binding proteins. It was therefore not possible to determine the K_m for the reaction. The V_{max} obtained with our preparations was similar to that reported by Delvin & Dussault (1985) for weanling guinea pigs (about 1 pmol/min per mg of protein) but markedly lower than that reported by Paulson & DeLuca (1985) for rachitic rats (about 500 pmol/min per mg).

Most of the 25-hydroxyvitamin D_3 1α -hydroxylase activity was found in the mitochondrial fraction. The small amount of activity in the nuclear fraction may represent mitochondrial contaminations. Almost identical results were obtained by Paulson & DeLuca (1985), who showed that the 1α -hydroxylase activity had the same distribution as the mitochondrial enzyme succinate dehydrogenase. The possible influence of sex hormones, in particular oestrogen, on the 25-hydroxyvitamin D_3 1α -hydroxylase is uncertain (Henry & Norman, 1984). There was a tendency to a higher renal 25-hydroxyvitamin D_3 1α -hydroxylase activity in preparations from male than from female guinea pigs. Preliminary studies in our laboratory on the effect of gonadectomy and treatment with oestrogen and testosterone support a stimulatory effect of testosterone.

To summarize, the results of the present work demonstrate that the mammalian renal 25-hydroxyvitamin D_3 1α -hydroxylase is a cytochrome *P*-450-dependent mixed-function oxidase with properties similar to those previously reported for the same enzyme system in chicken (Henry & Norman, 1984). The present assay and animal model seem to be suitable for further studies on the mechanism of regulation of this enzyme system.

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