Solubilization and reconstitution of kidney 25-hydroxyvitamin D₃ 1 α - and 24-hydroxylases from vitamin D-replete pigs

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Pig kidney mitochondrial 25-hydroxyvitamin D₃ 1 α - and 24-hydroxylase activities have been solubilized with cholate/Emulgen 911 and reconstituted with NADPH, ferredoxin reductase and ferredoxin. All three of these components are required for full catalytic activity of both enzymes. Both products were identified by co-chromatography with authentic metabolites on both normal and reverse-phase h.p.l.c. using solvent systems which were shown to separate 10-oxo-19-nor-25-hydroxyvitamin D₃ from 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂-D₃]. In addition, periodate treatment of the 24,25-(OH)₂-D₃ product resulted in complete loss of the product as measured by protein-binding assay. Further purification by *p*-chloroamphetamine–Sephadex chromatography of a solubilized extract from a pig fed a normal diet increased the specific content of the cytochrome *P*-450 from 0.019 to 0.239 nmol/mg and the 1 α -hydroxylase activity from 4.75 to 268 pmol/h per mg. Activity of the 24-hydroxylase in the crude solubilized extract was 6.3 pmol/h per mg, but was undetectable after partial purification by a *p*-chloroamphetamine–Sephadex column. However, further fractionation of this material by DEAE–Sephadex chromatography resulted in a further increase in 1 α -hydroxylase activity to 430 pmol/h per mg and detection of 24-hydroxylase in a separate fraction at a level of 53 pmol/h per mg. Production of 1,25-(OH)₂-D₃ was linear with time up to 2 h and was dependent upon ferredoxin concentration as well as cytochrome *P*-450 concentration in the range of 0–40 nM. In the presence of excess ferredoxin and adequate amounts of cytochrome *P*-450, 1,25-(OH)₂-D₃ production was also dependent upon substrate concentrations in the range of 0.25 to 2.5 μ M yielding an estimated *K_m* of 1 μ M. In the presence of excess substrate and ferredoxin, the catalytic-centre activity of the enzyme was estimated to be 1 h⁻¹.

INTRODUCTION

Regulation of the renal synthesis of the Ca²⁺-regulating hormone 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂-D₃] by kidney mitochondria has been studied extensively and has been shown to be dependent upon both serum Ca²⁺ and serum phosphorus concentrations [1,2]. However, much less is known about the molecular mechanism by which 1,25-(OH)₂-D₃ is synthesized, particularly by the mammalian kidney. Studies of the avian kidney have shown 25-hydroxyvitamin D₃ (25-OH-D₃) 1 α -hydroxylase to be a mixed-function oxidase which contains cytochrome *P*-450 as its terminal component [3]. In addition, the enzyme complex also consists of a ferredoxin (iron–sulphur protein) and a ferredoxin reductase (flavoprotein) which pass electrons from NADPH to cytochrome *P*-450 in a manner similar to that previously demonstrated for steroidogenesis in adrenal-cortex mitochondria [4]. Studies in mammalian kidney have demonstrated dependence of the 1 α -hydroxylase activity upon ferredoxin and ferredoxin reductase utilizing the adrenal enzymes [5,6]. However, more recent studies utilizing solubilized and partially purified cytochrome *P*-450 preparations from rat kidney have suggested a lack of requirement for ferredoxin and ferredoxin reductase and have resulted in the proposal of a dioxygenase mechanism [7,8]. A major problem with these studies was the use of an h.p.l.c. solvent system for

product identification which may not have resolved all of the metabolites of 25-OH-D₃ from 1,25-(OH)₂-D₃, particularly a metabolite identified by Brown & DeLuca [9] as 10-oxo-19-nor-25-hydroxyvitamin D₃. Utilizing chromatographic techniques to separate the 10-oxo metabolite from 1,25-(OH)₂-D₃, the latter workers were not able to measure 1,25-(OH)₂-D₃ production by a solubilized and partially purified rat kidney mitochondrial extract. Furthermore, none of the previous studies of 1 α -hydroxylase activity in solubilized preparations from mammalian kidney have utilized h.p.l.c. systems known to separate the 10-oxo metabolite from 1,25-(OH)₂-D₃ [5,6]. This makes interpretation of these studies more difficult in light of more recent studies.

The present studies were undertaken in an effort to resolve the issue of whether the mammalian 1 α -hydroxylase requires ferredoxin and ferredoxin reductase for full catalytic activity and whether or not 24,25-(OH)₂-D₃ is also produced by that preparation as was previously reported [7]. Utilizing techniques to minimize interference of 1,25-(OH)₂-D₃ quantification by the 10-oxo metabolite, 1 α -hydroxylase activity was measured in a solubilized and partially purified cytochrome *P*-450 preparation from the renal mitochondria of pigs fed vitamin D-replete, normal or low-calcium diets. This was made possible by the use of a sensitive radioreceptor assay to measure product formation [10]. The enzyme demonstrates an absolute requirement for a ferredoxin

Abbreviations used: 1,25-(OH)₂-D₃, 1,25-dihydroxyvitamin D₃; 23,25-(OH)₂-D₃, 23,25-dihydroxyvitamin D₃; 24,25-(OH)₂-D₃, 24,25-dihydroxyvitamin D₃; 25-OH-D₃, 25-hydroxyvitamin D₃.

and ferredoxin reductase. The affinity-enriched cytochrome *P*-450 preparation does not appear to synthesize 24,25-(OH)₂-D₃ as measured by the competitive binding assay utilizing an h.p.l.c. solvent system which will resolve 24,25-(OH)₂-D₃ from other metabolites. However, production of this metabolite can be demonstrated in a crude solubilized extract as well as in a DEAE-Sepharose-purified preparation and appears also to be dependent upon ferredoxin and ferredoxin reductase as reported for the avian system [11].

EXPERIMENTAL

Materials

Activated CH-Sepharose 4B was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Cytochrome *c*, glucose 6-phosphate, NADP⁺, cholic acid, glucose-6-phosphate dehydrogenase and *p*-chloroamphetamine were from Sigma (St. Louis, MO, U.S.A.). [26,27-³H]1,25-(OH)₂-D₃ (140–160 Ci/mmol) and [26,27-³H]24,25-(OH)₂-D₃ (130–180 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Unlabelled 1,25-(OH)₂-D₃ and 24,25-(OH)₂-D₃ were gifts from Hoffmann-La Roche, and 25-OH-D₃ was a gift of the UpJohn Co. Emulgen 911 was obtained from Kao Atlas, Tokyo, Japan) and 23,25-(OH)₂-D₃ and 10-oxo-19-nor-25-OH-D₃ were gifts of Dr. R. L. Horst, National Animal Disease Center, Ames, IA, U.S.A. Organic solvents (h.p.l.c. grade) were obtained from Fisher Chemical Co., and Bond Elut extraction columns were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Animals and diets

Young [27–36 kg (60–80 lb)] pigs of either sex were obtained from a local supplier and fed either a normal diet (Purina Hi-o Hog Grower Chow no. 2124) or a synthetic vitamin D-replete low-calcium diet for 1 week. The diet was similar to that used by Engstrom *et al.* [12], except that casein was used as the sole protein source. In addition, the diet contained 1% of a 1:1 mixture of KH₂PO₄ and K₂HPO₄, 1% (w/v) of a vitamin diet fortification (ICN, Cleveland, OH, U.S.A.) and 2.5% (w/v) cod liver oil. Fresh kidneys from young normal pigs [27–45 kg (60–100 lb)] were also obtained from a local abattoir.

Solubilization and isolation of cytochrome *P*-450

Fresh kidneys were kept on ice and the cortical tissue was dissected. About 100 g of cortical tissue could be obtained from both kidneys of a 36 kg (80 lb) pig. This was minced in 4 vol. of ice-cold 30 mM-sodium phosphate buffer, pH 7.4, containing 1.15% (0.15 M)-KCl. The tissue was homogenized in 50 g aliquots in a Waring blender with 4 × 10 s bursts. The homogenate was filtered through gauze and centrifuged at 500 *g* for 10 min in a Sorvall GSA type rotor. The pellet was washed once in 3 vol. of homogenizing buffer, and the combined supernatants from the low-speed spin were centrifuged at 4800 *g* for 10 min. The resulting pellet was suspended (1 ml/g of tissue) with a glass homogenizer in a detergent-containing buffer which included 30 mM-sodium phosphate, pH 7.4, 20% (v/v) glycerol, 0.2% Emulgen 911, 0.5% sodium cholate and 0.1 mM-EDTA (solubilization buffer) [7]. The solubilized mitochondria were left overnight at 4 °C, after which the suspension was centrifuged at

30000 *g* for 20 min. In some experiments this preparation, designated 'crude extract', was stored at –80 °C for use in experiments. For further purification of the cytochrome *P*-450, the crude extract was diluted 4-fold immediately before use with 30 mM-phosphate buffer containing 20% (v/v) glycerol but no detergents.

Affinity chromatography

Activated CH-Sepharose 4B was obtained and coupled to *p*-chloroamphetamine (86 mg/g of gel) as directed by the manufacturer. The coupled gel was equilibrated with starting buffer, which was prepared by diluting the solubilizing buffer 4-fold with phosphate-buffered glycerol. Two Amicon columns, both 1 cm × 10 cm (8 ml), were packed with gel and eluted at a flow rate of approx. 30 ml/h as described by Warner [7]. Approx. 120–150 ml of diluted crude extract (30–40 ml crude extract) was pumped on to each column, after which the columns were washed with 50–60 ml of starting buffer containing 0.1 M-NaCl. After this, the columns were eluted with solubilizing buffer and the material eluted from the column (as monitored by absorbance at 280 nm) was collected in a single tube. The cytochrome *P*-450 and protein content were determined, and the remainder of the material was stored at –80 °C for use in assays. This material was designated 'affinity-enriched preparation'.

DEAE-Sepharose chromatography

Further fractionation of the mitochondrial cytochrome *P*-450 was accomplished using DEAE-Sepharose CL-6B. The gel was equilibrated in 10 mM-sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.2% Emulgen 911 and packed into a 1.6 cm × 8 cm (16 ml) column. The affinity-enriched cytochrome *P*-450 was diluted 3-fold with 20% glycerol containing 0.2% Emulgen 911 and was pumped on to the column at a flow rate of 0.8 ml/min. After application of the sample, the column was washed for 50 min, after which a linear KCl gradient (0–0.5 M) was applied and fractions (2.4 ml) were collected.

Removal of detergents

Before measurement of catalytic activity, detergents were removed by chromatography on hydroxyapatite, which was prepared by a Tiselius-type method [13]. Aliquots of purified cytochrome *P*-450 (1–2 nmol) or crude extract were diluted 3-fold with 0.01 M-Mops buffer, pH 7.2, and loaded on to a hydroxyapatite column (3 ml) at a flow rate of about 0.5 ml/min. After application of the sample, the column was washed with Mops buffer for 0.5 h and then eluted with 0.2 M-potassium phosphate buffer, pH 7.25. The material eluted from the column (as determined by absorbance at 280 nm) was collected and concentrated using an Amicon YM-30 membrane to a final volume of 2.5–3 ml for use in the reconstitution assays. An aliquot of this material was also taken for measurements of cytochrome *P*-450 and protein content.

Preparation of reductase components

Bovine adrenal ferredoxin (adrenodoxin) was prepared as described by Orme-Johnson and Beinert [14]. The ratio of *A*₄₁₄/*A*₂₈₀ was 0.7 and, in the presence of ferredoxin reductase, ferredoxin catalysed the reduction of cytochrome *c* at a rate of 4600 nmol/min per mg.

Ferredoxin reductase was prepared from pig kidney mitochondria as described by Pedersen *et al.* [15] and further purified by Sephadex G-200 chromatography. In the presence of adrenal ferredoxin, this preparation catalysed the reduction of cytochrome *c* at a rate of 464 nmol/min per mg. Cytochrome *c* reductase activity was measured spectrophotometrically at room temperature as previously described [15].

Enzyme assays

Reconstitution assays were carried out in a final volume of 0.5 ml and contained glucose 6-phosphate (0.5 mM), NADP⁺ (0.1 mM), glucose-6-phosphate dehydrogenase (0.5 unit), cytochrome *P*-450 fraction (as indicated in the Results section; 0–20 pmol), 0.5% normal rat serum, 25-OH-D₃ (5 μ M), adrenodoxin (30 μ g) and pig kidney ferredoxin reductase (140 μ g). Incubations were carried out at 30 °C under air with gentle shaking and were initiated by the addition of ferredoxin reductase. Reactions were stopped by the addition of 0.5 ml of 0.4 M-phosphate buffer, pH 10.6, and reaction mixtures were immediately frozen at –20 °C for subsequent measurement of vitamin D metabolite production.

Extraction and purification of vitamin D metabolites

After thawing, the reconstitution samples were 'spiked' with a small amount (1800–2000 d.p.m.) of [³H]1,25-(OH)₂-D₃ and/or [³H]24,25-(OH)₂-D₃ to monitor recovery through the extraction and purification procedures. Extraction and purification of the samples were accomplished by a slight modification of the method of Reinhardt *et al.* [16] with final purification by h.p.l.c. before the protein-binding assay. Samples were extracted with an equal volume of acetonitrile, centrifuged at 500 g for 10 min, after which another volume (equal to the original sample volume) of alkaline buffer (0.4 M-phosphate, pH 10.6) was added. This extract was applied to a C₁₈ Sep-Pak (Waters Associates) and washed with 5 ml of water, followed by 3 ml of aq. 70% (v/v) methanol. The sample was then eluted with 3 ml of acetonitrile, evaporated to dryness and dissolved in 1 ml of propan-2-ol/n-hexane (1:19, v/v) for application to a Bond-Elut silica column. After rinsing the sample with 1 ml of propan-2-ol/hexane (1:19), the column was washed with an additional 6 ml of the same solvent mixture. This removes most of the substrate, 25-OH-D₃. The column was then eluted with 8 ml of 8% propan-2-ol/hexane (2:23, v/v), and this fraction was saved for analysis of 24,25-(OH)₂-D₃. The column was then eluted with 8 ml of propan-2-ol/hexane (3:17, v/v) to collect the 1,25-(OH)₂-D₃.

Final purification of the samples before the protein-binding assay was accomplished by using h.p.l.c. For routine purification of samples, a normal-phase silica column was used (du Pont Zorbax, Golden Series) employing a four-solvent system containing hexane/propan-2-ol/methylene chloride/methanol in the proportions 163:7:28:2 (by vol.) for 1,25-(OH)₂-D₃ and 162:5:32:1 (by vol.) for 24,25-(OH)₂-D₃ [15]. These solvent systems had been shown previously to separate 10-oxo-19-nor-25-OH-D₃ and to separate the lactone and other metabolites from 24,25-(OH)₂-D₃ [18]. In some experiments, further identification of the products was carried out using a reverse-phase column (Zorbax ODS, Golden Series) and a solvent system containing water/methanol (1:3, v/v) for 1,25-(OH)₂-D₃ and 24,25-

(OH)₂-D₃ as previously described [11]. A Waters Associates model-204 liquid chromatograph was used equipped with a u.v. detector (model 440) capable of monitoring absorbance at either 254 nm or 313 nm. Flow rates ranged from 1.5 to 2.0 ml/min.

Analytical methods

Serum levels of 1,25-(OH)₂-D₃ were measured by radioreceptor assay using the calf thymus receptor as described previously [16]. Serum 24,25-(OH)₂-D₃ levels were measured by competitive binding assay as described previously [20]. Assay of 1,25-(OH)₂-D₃ and/or 24,25-(OH)₂-D₃ production in reconstitution samples was the same as above, except that both metabolites were purified by h.p.l.c. before their assay. In the case of 1,25-(OH)₂-D₃, duplicate aliquots of the sample were run at different dilutions to confirm parallel displacement of the product with the authentic standard.

Other analytical methods

Spectral measurements of cytochrome *P*-450 content were made using either a Shimadzu UV265 spectrophotometer or an Aminco DW2. Cytochrome *P*-450 content was determined from the CO-reduced difference spectrum after reduction with dithionite as described by Omura & Sato [21], an absorption coefficient of 91 mM⁻¹·cm⁻¹ being used. Protein was measured by the Bradford method [22], with bovine serum albumin as standard. Serum metabolite levels are presented as means $\pm$ s.d., and statistical comparisons were made using Student's *t* test for unpaired data. Regression analysis was performed by the method of least squares.

RESULTS

Preparation of mitochondrial cytochrome *P*-450

Solubilization of pig kidney mitochondria with cholate and Emulgen 911 and chromatography of the extract on *p*-chloroamphetamine-Sepharose yielded affinity-enriched cytochrome *P*-450 preparations similar to those obtained by Warner [7]. Fig. 1 shows representative absorbance spectra from such a preparation. The reduced CO difference spectrum is nearly identical with that reported by Warner [7]. However, inspection of the reduced-minus-oxidized spectrum (A) reveals the presence of additional absorbance maxima at 556, 526 nm and 426 nm, indicating the presence of cytochrome *b*₅ in this preparation [23]. The specific content of this partially purified cytochrome *P*-450 in eight preparations from pigs fed a low-calcium diet averaged 0.208 $\pm$ 0.035 nmol/mg of protein, a value slightly higher than, but not significantly different from, an average of 0.162 $\pm$ 0.105 nmol/mg of protein in six preparations from pigs fed a normal diet. Yields of cytochrome *P*-450 from pig kidney cortex ranged from 0.11 to 0.13 nmol/g of tissue and were also similar in pigs fed either diet.

Reconstitution of catalytic activity and identity of the product

Upon removal of detergents with hydroxyapatite, affinity-enriched cytochrome *P*-450 preparations catalysed the production of a metabolite of 25-OH-D₃ which co-migrated with authentic 1,25-(OH)₂-D₃ using both normal- and reverse-phase h.p.l.c. Fig. 2 shows a re-chromatography of the isolated product on either the same normal-phase h.p.l.c. system originally used to

Fig. 1. Absorbance spectra of an affinity-enriched cytochrome *P*-450 preparation obtained from a pig fed a low-calcium diet.

A, Dithionite-reduced minus oxidized difference spectrum; B, dithionite-reduced-CO minus oxidized difference spectrum; C, reduced-CO minus reduced difference spectrum. The *P*-450 concentration of this preparation was 0.139 nmol/ml and the specific content was 0.27 nmol/mg of protein.

isolate the product (a) or on a reverse-phase system (b). Both of these systems can separate 10-oxo-19-nor-25-OH- D_3 from 1,25-(OH) $_2$ - D_3 , and it can also be seen that the product co-migrates with authentic 1,25-(OH) $_2$ - D_3 in both systems. In addition, the product also displaced [3H]1,25-(OH) $_2$ - D_3 from the calf thymus receptor in a manner parallel with authentic standard (results not shown), providing additional evidence for the identity of the product. In a typical reconstitution experiment containing an NADPH-generating system, adrenodoxin, adrenodoxin reductase and 12 pmol of cytochrome *P*-450, 9.67 pmol of 1,25-(OH) $_2$ - D_3 was produced during a 1 h incubation. Similar incubations containing the same amount of cytochrome *P*-450, but with either no NADPH-generating system or no adrenodoxin reductase, produced no detectable product. Likewise, omission of cytochrome *P*-450 from the incubation mixture also resulted in no production of 1,25-(OH) $_2$ - D_3 .

Catalytic properties

Utilizing affinity-enriched cytochrome *P*-450 preparations from pigs fed a low-calcium diet, production of 1,25-(OH) $_2$ - D_3 by the reconstituted system was linear with time up to 2 h and was also dependent upon the concentration of ox adrenal ferredoxin in the range of 10–40 μ g/ml (23–184 units/ml). In the presence of saturating amounts of ferredoxin, product formation was dependent upon cytochrome *P*-450 concentrations in the range of 5–30 nM (Fig. 3a). Estimation of the catalytic-centre activity from the linear portion of this relationship yields a value of approx. 1 h $^{-1}$. Production of 1,25-(OH) $_2$ - D_3 by the reconstituted system was also dependent upon substrate concentration, as seen by the reciprocal

Fig. 2. H.p.l.c. profiles the putative 1,25-(OH) $_2$ - D_3 product generated in reconstitution assays

(a) Normal-phase h.p.l.c. using a four-solvent system consisting of hexane/methylene chloride/propan-2-ol/methanol 161:30:7:2 (by vol.) A du Pont Zorbax 'Golden Series' column was used at a flow rate of 1.3 ml/min, and 1 min fractions were collected for measurement of 1,25-(OH) $_2$ - D_3 content by radioreceptor assay. Arrows indicate elution positions of authentic 10-oxo-19-nor-25-OH- D_3 and 1,25-(OH) $_2$ - D_3 respectively, chromatographed under identical conditions. (b) Reverse-phase h.p.l.c. of putative 1,25-(OH) $_2$ - D_3 product utilizing a du Pont 'Golden Series' Zorbax ODS column. The solvent system was water/methanol (1:3, v/v), and the flow rate was 1.8 ml/min.

plot of the data in Fig. 4. As previously observed by Vieth & Fraser [24], 1,25-(OH) $_2$ - D_3 production was not linear at the lowest substrate concentration (0.1 μ M), but was linear at higher substrate concentrations (0.25–2.5 μ M), yielding a significant inverse relationship:

$$y = 10.4 \text{ pmol/h} - 0.966(v/s), r = 0.92$$

The K_m of 0.97 μ M is similar to that reported in previous studies with a reconstituted 1 α -hydroxylase preparation from vitamin D-deficient pigs [5]. The V_{max} of 10 pmol/h agrees well with the catalytic-centre activity estimated from Fig. 3, since there were 10 pmol of cytochrome *P*-450 in the incubations used to generate these data.

Reconstitution of affinity-enriched cytochrome *P*-450 preparations from pigs fed a normal diet also resulted in 1,25-(OH) $_2$ - D_3 production. Utilizing a 1 h incubation and saturating amounts of substrate and ferredoxin, production of 1,25-(OH) $_2$ - D_3 was also dependent upon cytochrome *P*-450 concentrations in the range 5–40 nM (Fig. 3b). This was similar to the observation in Fig. 3(a), where a preparation from a pig fed a low-calcium diet

Fig. 3. Production of 1,25-(OH)₂-D₃ as a function of cytochrome *P*-450 concentration utilizing an affinity-enriched preparation obtained from a pig fed (a) a low-calcium diet and (b) a normal diet

Incubation conditions were the same as described in the Experimental section, and each point represents a single incubation.

Fig. 4. Eadie-Hofstee plot of the dependence of 1,25-(OH)₂-D₃ production on substrate concentration (v versus v/s)

Because of non-linearity at the lowest substrate concentration (0.1 μ M, open symbols), the regression line includes only the points at the higher substrate concentrations (0.25–2.5 μ M, closed symbols).

was used. This is somewhat surprising, since serum 1,25-(OH)₂-D₃ levels in pigs fed the low-calcium diet averaged 298 ± 39 pM ($n = 6$), whereas those in normal pigs of the same age averaged 156 ± 45 pM ($n = 5$), $P < 0.001$.

Production of other metabolites

Production of a metabolite co-migrating with authentic 24,25-(OH)₂-D₃ could not be demonstrated utilizing affinity-enriched cytochrome *P*-450 preparations from pigs fed either diet and utilizing a protein-binding assay for 24,25-(OH)₂-D₃ which could detect about 3 pmol of the product per incubation, assuming a detection limit of 0.1 ng/tube and 30% recovery through the extraction and purification procedures. Since production of this metabolite has been reported in crude solubilized preparations from avian kidney [11], reconstitution experiments were carried out utilizing the crude solubilized mitochondria obtained from pigs fed a normal diet. This crude extract was subjected to hydroxyapatite chromatography to remove detergents before reconstitution. Fig. 5 shows absorbance spectra of the treated material. The CO-reduced difference spectrum is similar to that obtained by Burgos-Trinidad & DeLuca [11] for avian kidney and shows the presence of cytochrome *P*-450 along with cytochrome oxidase [3,11]. The calculated *P*-450 content from this spectrum is 0.019 nmol/mg, which is in good agreement with the value of 0.108 nmol/5.2 mg reported previously [11]. Reconstitution of this material with adrenal ferredoxin, ferredoxin reductase and an NADPH-generating system produced a metabolite of 25-OH-D₃ co-migrating on normal-phase h.p.l.c. with authentic 24,25-(OH)₂-D₃. As Table 1 shows, production of this metabolite was absolutely dependent upon the presence of the solubilized protein and was also

Fig. 5. Absorbance spectra of a crude solubilized extract of mitochondria obtained from pigs fed a normal diet

A, Dithionite-reduced minus oxidized difference spectrum; B, dithionite-reduced-CO minus oxidized difference spectrum; C, reduced-CO minus reduced difference spectrum. The cytochrome *P*-450 concentration of this preparation was estimated to be 0.33 nmol/ml and the protein content was 1.72 mg/ml, giving a specific content of 0.019 nmol/mg.

Table 1. Reconstitution of 25-hydroxyvitamin D₃ 1 α - and 24-hydroxylases from a crude solubilized extract

The complete system contained 2.5 mg of protein (approx. 48 pmol of cytochrome *P*-450) along with the NADPH-generating system, ferredoxin and ferredoxin reductase as described in the Experimental section. Incubation was at 30 °C for 1 h. The detection limit of the assay for quantification of 1,25-(OH)₂-D₃ is estimated to be 0.1 pmol/incubation, assuming 40% recovery of the sample and 25% of the sample taken for analysis. The detection limit for quantification of 24,25-(OH)₂-D₃ is estimated to be about 3 pmol/incubation, assuming a detection limit of 0.1 ng/tube and 30% recovery through the extraction and purification procedures. All values represent the averages of duplicate incubations. The measurements were within 5–10% of each other.

Reaction system	24,25-(OH) ₂ -D ₃ (pmol·h ⁻¹)	1,25-(OH) ₂ -D ₃ (pmol·h ⁻¹)
Complete system	15.9	11.9
Minus reductase	<3.0	<0.1
Minus protein	<3.0	<0.1

Fig. 6. Reverse-phase h.p.l.c. of putative 24,25-(OH)₂-D₃ product generated in reconstitution assays

A Zorbax 'Golden Series' ODS column was utilized, with a solvent system of water/methanol (1:3, v/v) and a flow rate of 1.5 ml/min. Fractions (1 min) were collected, and one-half of each fraction was treated with aq. sodium periodate for 20 h as previously described [11]. —, [³H]24,25-(OH)₂-D₃ displacement from its binding protein by the untreated half of each fraction; ◆, identical assays of the periodate-treated half of each fraction. The arrows indicate the elution positions of authentic 24,25-(OH)₂-D₃ and 23,25-(OH)₂-D₃ chromatographed under identical conditions.

dependent upon the presence of ferredoxin and ferredoxin reductase. Isolation of 24,25-(OH)₂-D₃ product from normal-phase h.p.l.c. and re-chromatography on reverse-phase h.p.l.c. is shown in Fig. 6. When the product from reverse-phase h.p.l.c. was treated with periodate, the product could no longer be detected by the protein-binding assay, showing that the product was completely periodate-sensitive. Although the product was not well

separated from 23,25-(OH)₂-D₃ in this system (Fig. 6), the latter metabolite is not periodate-sensitive. Thus, it would appear that 23,25-(OH)₂-D₃ was not produced in these incubations. As expected, production of 1,25-(OH)₂-D₃ was also detected in this preparation (Table 1), but the activity expressed on a protein basis was much lower than that found in affinity-enriched preparations (4.75 as against 268 pmol/h per mg respectively). This would suggest that the specific activity of the 1 α -hydroxylase is increased over 50-fold as a result of purification by *p*-chloroamphetamine-Sephacryl chromatography.

Hydroxylase activities of fractionated cytochrome *P*-450

Further fractionation of the mitochondrial cytochrome *P*-450 by DEAE-Sephacryl chromatography is shown in Fig. 7. There were two areas where cytochrome *P*-450

Fig. 7. Fractionation of cytochrome *P*-450 by DEAE-Sephacryl chromatography

(a) The KCl gradient is indicated by the diagonal continuous line and the protein absorbance at 280 nm is indicated by the arrow. Open bars indicate cytochrome *P*-450 content per fraction. The material eluted from the column before starting the salt gradient was collected, concentrated and analysed for catalytic activity and cytochrome *P*-450 content as a single fraction. After starting the salt gradient, 3 min (2.8 ml) fractions were collected and analysed individually. (b) The open bar indicates reconstituted 1 α -hydroxylase activity and the hatched bar indicates 24-hydroxylase activity of the respective cytochrome *P*-450 fractions shown in (a).

Table 2. 25-OH-D₃ 1 α - and 24-hydroxylases in crude and partially purified cytochrome *P*-450 from normal pigs

Reconstitution assays were performed as described in the Experimental section after removal of detergents with hydroxyapatite. Specific contents of cytochrome *P*-450 in these preparations ranged from 0.02 nmol/mg in the crude extract to 0.35 nmol/mg in the DEAE-Sepharose purified preparation.

Preparation	Activity (pmol/h per mg)	
	1 α -Hydroxylase	24-Hydroxylase
Crude	4.8	6.3
Affinity-purified	268	< 3.0
Affinity-purified + DEAE-Sepharose	430	53

was eluted from the column. The first, containing about 35–40 % of the recovered *P*-450, was eluted in the sample wash and was not catalytically active in either 1,25-(OH)₂-D₃ or 24,25-(OH)₂-D₃ production. Another area of cytochrome *P*-450 (Fig. 7a), representing about 10–30 % of the recovered material, was eluted shortly after starting the salt gradient and appeared to contain different cytochrome *P*-450 components. The first component was eluted at about 22 % of the gradient and produced only 1,25-(OH)₂-D₃ after removal of detergents and reconstitution (Fig. 7b). The second component, which appeared as a shoulder, was eluted at about 32 % of the gradient and produced primarily 24,25-(OH)₂-D₃ (Fig. 7b). This fraction also produced a small, but detectable, amount of 1,25-(OH)₂-D₃. The remainder of the column contained very little cytochrome *P*-450 and was not catalytically active in reconstitution assays. Table 2 shows the relative enrichment of both enzyme activities upon fractionation. The activity of the 1 α -hydroxylase was increased about 90-fold over the starting material, whereas that of the 24-hydroxylase was increased only about 8-fold. These results show that DEAE-Sepharose chromatography achieves some purification and separation of the two enzymes.

DISCUSSION

The method of Warner [7] has proven to be a convenient and efficient method for solubilization and isolation of mammalian mitochondrial cytochrome *P*-450. However, the specific content of preparations from pig kidney, which ranged from 0.058 to 0.29 nmol/mg, were somewhat lower than those reported by Warner [7], although the yields of 0.11–0.13 nmol of cytochrome *P*-450/g of kidney were similar.

Production of 1,25-(OH)₂-D₃ by the reconstituted system was absolutely dependent upon the presence of ferredoxin reductase and adrenal ferredoxin, the latter enzyme being required in fairly large amounts to achieve maximal product formation. This is in agreement with studies in the avian systems as well as with previous studies with mammalian kidney [5,6,9,15]. However, recent reports by Warner [7,8] have failed to demonstrate a requirement for ferredoxin and ferredoxin reductase in a similarly purified cytochrome *P*-450 preparation from rat kidney. These studies relied upon conversion of

radioactive 25-OH-D₃ into labelled 1,25-(OH)₂-D₃, a method which may not be as discriminating or sensitive as the radioreceptor assay used in the present studies. In addition, the use of the radioreceptor assay to quantify 1,25-(OH)₂-D₃ production has provided a more sensitive assay for 1 α -hydroxylase activity and has made it possible to study the activity of the enzyme in solubilized preparations from vitamin D-replete animals.

The catalytic properties of the 1 α -hydroxylase from pigs fed the low-calcium diet are similar to those previously reported by Saarem *et al.* [5], who used a solubilized preparation from vitamin D-deficient pigs. The *K_m* of approx. 1 μ M is similar to the value of 0.6 μ M reported previously, and the dependence of product formation on cytochrome *P*-450 concentration in the range of 5–30 nM is also similar in both studies [5]. Demonstration of saturating amounts of substrate and ferredoxin have also made it possible to estimate the catalytic-centre activity of 1 α -hydroxylase activity, which is about 1 h⁻¹. Such a low value might be expected, since these preparations are still rather crude and may contain other cytochrome *P*-450 species not related to 1-hydroxylation or even to vitamin D metabolism.

Production of 1,25-(OH)₂-D₃ utilizing an affinity-enriched preparation from pigs fed a normal diet was very similar to that observed with preparations from pigs fed the low-calcium diet in spite of a 2-fold increase in serum 1,25-(OH)₂-D₃ levels in the latter group. In addition, the yield of cytochrome *P*-450/g of kidney as well as the specific content of the cytochrome *P*-450 were similar in several affinity-enriched preparations from pigs fed either the normal or the low-calcium diets. A similar observation has also been made previously in studies using avian kidney [3]. These observations would argue against a role for increased cytochrome *P*-450 as a possible mechanism for the 18-fold increase in enzyme activity observed in isolated mitochondria from pigs fed a low-calcium diet [25]. Another possible mechanism for activation of the 1 α -hydroxylase is suggested from recent studies showing modification of a ferredoxin (iron-sulphur protein) in response to parathyroid hormone or phosphorylation [26,27]. These studies would argue for activation of existing cytochrome *P*-450_{1 α} rather than synthesis of additional cytochrome. However, since the cytochrome *P*-450_{1 α} may be only a small fraction of the total *P*-450, final resolution of this issue will require quantification of the amount of the cytochrome by e.l.i.s.a using specific antibodies and estimation of mRNA levels for the cytochrome using specific cDNA probes.

Previous studies using solubilized cytochrome *P*-450 preparations from normal mammalian kidney have failed to demonstrate 24-hydroxylase activity [5,6]. However, other studies support the involvement of cytochrome *P*-450 in 24-hydroxylase activity [28–30]. The present studies also support this concept, as demonstrated by the presence of reconstituted 24-hydroxylase activity in a cytochrome *P*-450 fraction isolated by DEAE-Sepharose chromatography. This procedure appears to resolve the 1 α - and 24-hydroxylase activities into separate fractions. These results demonstrate for the first time that it is possible to separate these two enzymes into distinct cytochrome *P*-450 species and provide a basis for their further purification and characterization. Furthermore, it would appear that the mammalian enzymes are associated with a relatively small fraction of the total mitochondrial cytochrome *P*-450, since a large portion

of the *P*-450 which did not bind to DEAE-Sepharose did not produce either metabolite in reconstitution assays. It is possible that other metabolites of 25-OH-D₃ might be produced by this cytochrome *P*-450 species, but this is beyond the scope of the present study.

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