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Solubilization and Reconstitution of Chick Renal Mitochondrial 25-Hydroxyvitamin D_3 24-Hydroxylase†

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ABSTRACT: Chick kidney mitochondrial 25-hydroxyvitamin D_3 24-hydroxylase has been solubilized with sodium cholate and reconstituted with NADPH, beef adrenal ferredoxin, and beef adrenal ferredoxin reductase, each component being essential for maximal 24-hydroxylase activity. The product 24(*R*),25-dihydroxyvitamin D_3 was identified by cochromatography with synthetic compound on straight-phase and reversed-phase high-performance liquid chromatography and by periodate oxidation. The enzyme has an apparent K_m for 25-hydroxyvitamin D_3 of 0.67 μM. At 1 μM 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3 production is linear with time for up to 15 min and with protein concentrations of up to 2 mg/mL. The antioxidant diphenyl-*p*-phenylenediamine (1.3×10^{-4} M) has no effect on this reaction. Reconstituted 24-hydroxylase activity is enhanced by the addition of NaCl and KCl up to 100 mM, with higher concentrations having an inhibitory effect. 1α-Hydroxylase is not present in this preparation from vitamin D replete chicks. The similarities of this reconstituted system to the 25-hydroxyvitamin D_3 1α-hydroxylase and the adrenal systems suggest that the 25-hydroxyvitamin D_3 24-hydroxylase is also a cytochrome P-450 type mixed-function oxidase.

Vitamin D is converted in the liver to 25-hydroxyvitamin D_3 (25-OH-D_3) (Blunt et al., 1968), which is further hydroxylated in the kidney to either 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] (Holick et al., 1972), a relatively inactive metabolite (DeLuca, 1984), or 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the hormonal form of the vitamin active in intestinal calcium transport and bone mineral mobilization (Fraser & Kodicek, 1970). Both 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ have been shown in vivo and in vitro to be further metabolized to 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] (Holick et al., 1973; Kumar et al., 1978; Tanaka et al., 1977), a less active analogue of 1,25-$(OH)_2D_3$ (DeLuca, 1984). Therefore, 24-hydroxylation is presumably an inactivation route in the metabolism of vitamin D_3.

Whether renal hydroxylation of 25-hydroxyvitamin D_3 occurs at the C-1 or C-24 positions appears to be regulated in a reciprocal manner by serum levels of calcium (Boyle et al., 1971), phosphorus (Tanaka & DeLuca, 1973), and 1,25-

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$(OH)_2d_3$ (Tanaka & DeLuca, 1974; Tanaka et al., 1975). In vitamin D replete animals under normal or high calcium dietary intakes, 25-hydroxyvitamin D_3 24-hydroxylase activity is maximal and 1α-hydroxylase activity is suppressed (Boyle et al., 1971). 1,25-$(OH)_2D_3$ itself is a regulator of the 24-hydroxylase activity. When given in vivo, 1,25-$(OH)_2D_3$ stimulates 24,25-$(OH)_2D_3$ production and inhibits 1,25-$(OH)_2D_3$ production (Tanaka & DeLuca, 1974; Tanaka et al., 1975). However, the molecular mechanism of this regulation is not completely understood.

The chick kidney 25-OH-D_3 24-hydroxylase, like the 1α-hydroxylase, is believed to be a cytochrome P-450 mixed-function oxidase (Knutson & DeLuca, 1974; Kulkowski et al., 1979) and in many respects is very similar to the monooxygenases of beef adrenal cortex mitochondria. The enzyme activity is primarily located in the mitochondria of kidney, although extrarenal 24-hydroxylase activity has been reported in intestine and cartilage (DeLuca, 1984). In addition to the substrate and molecular oxygen, the enzyme requires NADPH as the primary electron donor for maximal enzyme activity (Knutson & DeLuca, 1974). Although the involvement of cytochrome P-450 in the 24-hydroxylase activity has not been fully demonstrated, Madhok et al. (1977) showed that the oxygen enzymatically introduced into the 24-position of 25-OH-D_3 is derived from $^{18}O_2$, providing strong evidence that the 24-hydroxylase is a monooxygenase. Furthermore, the inhibition of the reaction by metyrapone and carbon monoxide (Bikle, 1980; Kulkowski et al., 1979) adds to the evidence for the involvement of a cytochrome P-450. Similarly, the rat kidney mitochondrial 25-OH-D_3 24-hydroxylase has been shown to require NADPH and molecular oxygen and is completely inhibited by metyrapone and carbon monoxide (Pedersen et al., 1983). Recently, a solubilized and partially purified rat kidney mitochondria P-450 catalyzing both the 1α- and the 24-hydroxylation of 25-OH-D_3 has been reported (Warner, 1982, 1983). In this solubilized system, 24-hydroxylase appears to have properties very different from those found in chicken or intact rat kidney mitochondria (Knutson & DeLuca, 1974; Pedersen et al., 1983). The enzyme does not require NADPH and is not inhibited by carbon monoxide (Warner, 1983). Both 25-OH-D_3 hydroxylases were inhibited by antioxidants and ethylenediaminetetraacetic acid (EDTA) (Warner, 1983). Furthermore, the 24-hydroxylase activity was higher in vitamin D deficient rats than in vitamin D replete rats (Warner, 1982), in contrast to previous results in whole rat (Tanaka & DeLuca, 1974) or in chicken kidney mitochondria (Knutson & DeLuca, 1974). On the basis of these data, the author proposed that the cytochrome P-450 is acting as a peroxygenase as opposed to a mixed-function oxidase.

The 25-OH-D_3 1α-hydroxylases from chicken (Ghazarian et al., 1974; Pedersen et al., 1976), pig (Saaren et al., 1978), and beef kidney (Hiwatashi et al., 1982) have been solubilized and reconstituted. In the three species, the enzyme has an absolute requirement for NADPH, a flavoprotein, and an iron–sulfur protein. The similarities between the 24-hydroxylase and the 1α-hydroxylase systems of intact mitochondria prompted us to determine whether the chick kidney 24-hydroxylase could be solubilized and reconstituted under conditions similar to those of other mitochondrial mixed-function oxidases.

This paper describes the properties of a functional 25-OH-D_3 24-hydroxylase in a soluble form from kidney mitochondria of vitamin D replete chicks. The soluble preparation possesses in vitro 24-hydroxylase activity only when reconstituted with NADPH, beef adrenal ferredoxin, and beef adrenal ferredoxin reductase.

Materials and Methods

Chemicals. 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) was obtained from Du Pont/New England Nuclear (Boston, MA). Nonradioactive 25-OH-D_3 was a gift from Dr. J. Allan Campbell and Dr. John Babcock of the Upjohn Co. (Kalamazoo, MI). 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were gifts of the Hoffmann-La Roche Co. (Nutley, NJ). Diphenyl-*p*-phenylenediamine was purchased from the Eastman Kodak Co. (Rochester, NY). Sodium cholate and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. One-day-old white Leghorn chickens were obtained from Northern Hatcheries (Beaver Dam, WI) and maintained on a 1.2% calcium, 0.7% phosphorus vitamin D deficient diet consisting of (by percent) sucrose (54.0), soy isolate protein (22.0), Solka Floc (7.0), soybean oil (Wesson) (4.0), $CaCO_3$ (2.4), KH_2PO_4 (1.7)8 $CaHPO_4 \cdot 2H_2O$ (1.0), NaCl (ionized) (0.8), L-cysteine, (0.19), L–tryptophan (0.08), trace salts (0.7), and premix (5.5) as previously reported (Hart et al., 1984). One-week-old chickens were given 5 μg each 25-OH-D_3 and 1,25-$(OH)_2D_3$ (intramuscularly) daily for 2 days. The dosing solution was 5% ethanol/propylene glycol. The chickens were killed by decapitation 24 h after the last injection.

Preparation of Mitochondria and Solubilization. Immediately following sacrifice, the kidneys were removed and rinsed in 0.25 M sucrose and 15 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, pH 7.4. A 20% (w/v) homogenate was prepared with a Teflon pestle–homogenizer. The homogenate was centrifuged at 500*g* for 10 min to remove nuclei and cell debris; the pellet was washed once. The combined supernatants were centrifuged at 10000*g* for 10 min. The mitochondrial pellet was washed twice and resuspended in 20% glycerol and 15 mM Tris–acetate, pH 7.4, containing 0.6% sodium cholate at a final concentration of 15 mg of protein/mL. After 1 h on ice, the mixture was centrifuged at 100000*g* for 60 min. The supernatant containing soluble 24-hydroxylase was used in these experiments. The solubilized material was immediately assayed for protein content by the method of Bradford (1976) with bovine serum albumin as standard and was either used immediately or stored at −80 °C. The preparation is stable for as long as 6 months. the protein content of the solubilized preparation in two series of experiments was 4–6 mg/mL.

Adrenodoxin was purified from beef adrenal postmitochondrial supernatant as described by Orme-Johnson and Beinert (]969) with the omission of the electrophoresis step. The protein was further purified by DEAE-cellulose (Whatman DE-52) (Suhara et al., 1972) followed by a Sephadex G-50 chromatography. The adrenodoxin obtained by this procedure had an A_{414nm}/A_{276nm} of 0.86. Adrenodoxin reductase was purified from beef adrenal mitochondria by the affinity chromatography method described by Sugiyama and Yamano (1975).

Enzyme Assays. Each reaction mixture contained 200 μg of 24-hydroxylase fraction, 1.6 nmol of adrenodoxin, 0.08 nmol of adrenodoxin reductase, 100 nmol of NADPH, 1.0 μmol of glucose 6-phosphate, 0.4 unit of glucose-6-phosphate dehydrogenase, 15 mM Tris–acetate, pH 7.4 and 200 pmol of 25-OH-[26,27-^{3}H]D_3 (sp act. = 3000–10 000 dpm/pmol) dissolved in 10 μL of 95% ethanol in a final volume of 200 μL. Unless otherwise indicated, the reactions were kept at 0 °C until initiated by addition of the substrate and were stopped after 15 min at 37 °C with the addition of 4.5 mL of meth-

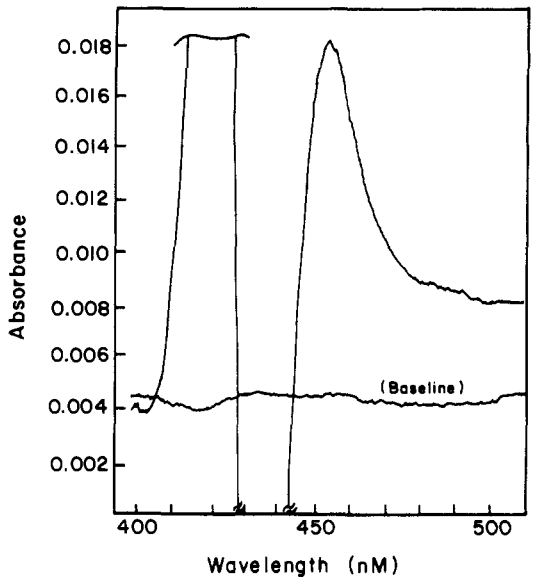

FIGURE 1: Reduced CO minus reduced difference spectrum of the cytochrome P-450 solubilized from chick kidney mitochondria. A cytochrome P-450 preparation containing 5.2 mg of protein/mL in 15 mM Tris–acetate, pH 7.4, 20% glycerol, and 0.6% sodium cholate was divided equally between a sample and a reference cuvette, and the base line was recorded. The difference spectrum was recorded by adding a few crystals of solid sodium dithionite to both cuvettes followed by bubbling CO monoxide into the sample cuvette only. The cytochrome P-450 concentration is 0.108 nmol/mL.

anol/chloroform/water (6:3:2 v/v) to give a single phase.

Metabolite Isolation and High-Performance Liquid Chromatography. An internal standard of 24(*R*),25-$(OH)_2D_3$ (150 ng) was added, and the reaction mixture was separated into two phases with the addition of 1.1 mL of water and 1.2 mL of chloroform (Bligh & Dryer, 1969). The chloroform phase was removed, and the aqueous phase was extracted twice with 2.2 mL of chloroform. The chloroform extracts were pooled, evaporated to a dryness under a stream of nitrogen, and dissolved in 200 μL of 6% 2-propanol in hexane and subjected to HPLC analysis.

Chromatography was performed with a Waters Model ALC/GPC 204 (Waters Associates, Milford, MA) HPLC using a Zorbax-Sil column (0.46 × 25 cm, Du Pont Instruments, Wilmington, DE) at a flow rate of 2 mL/min with 6% 2-propanol in hexane. The 24,25-$(OH)_2D_3$ peak was collected, dried, and further purified by reversed-phase HPLC on a Zorbax-ODS (0.46 × 25 cm) column with 22% water in methanol at a flow rate of 2 mL/min. Fractions of 0.8 mL were collected and mixed with 3 mL of Scint A (United Technologies/Packard) and counted in a Packard Prias Model 400 CL/D liquid scintillation counter (Downers Grove, IL). Radioactivity in the 24,25-$(OH)_2D_3$ peak was corrected for recovery on the basis of UV absorbance of the internal standard. The percent recovery was routinely 70–90%.

Periodate Oxidation. For product identification, each 0.8-mL fraction collected from reversed-phase HPLC was dried and dissolved in 1 mL of methanol. Half of the fraction was counted, and the other half was dried and subjected to periodate cleavage by dissolving in 300 μL of methanol and treating with 200 μL of a 5% aqueous solution of $NaIO_4$. After 20 h at room temperature, the reaction mixture was evaporated to dryness, and the radioactivity remaining was determined.

Spectral Measurements. The concentration of cytochrome P-450 was determined by the method of Omura and Sato (1994). The cytochrome P-450 content was estimated with

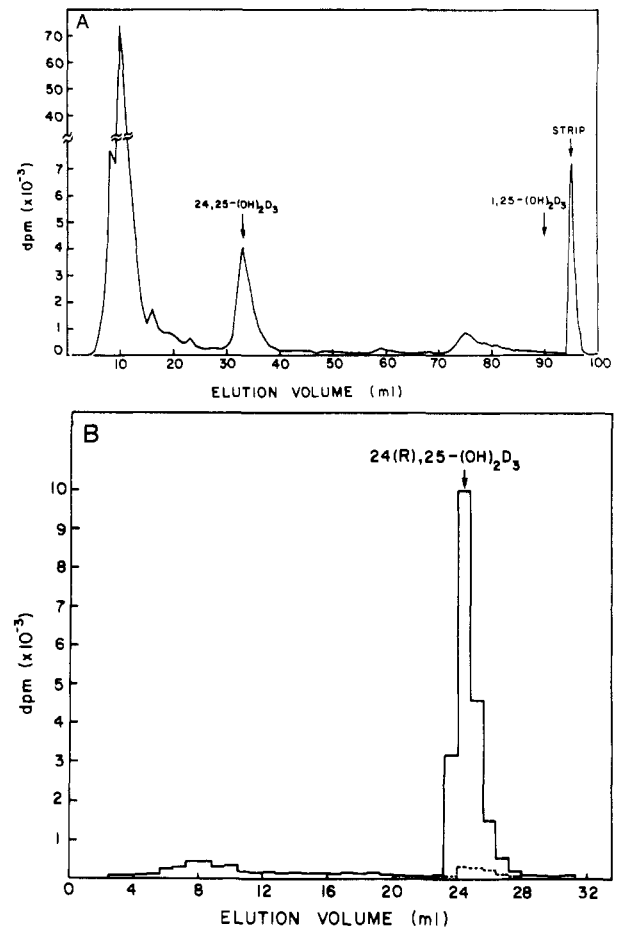

FIGURE 2: HPLC chromatography of reconstituted 24-hydroxylase reaction product. The extract of the incubation containing the reconstituted 25-OH-D_3 24-hydroxylase components was subjected to straight-phase HPLC in 6% 2-propanol in hexane (A), and the 24,25-$(OH)_2D_3$ was purified further on reversed-phase HPLC with 22% water in methanol (B). The dashed line in (B) represents the radioactivity remaining after periodate treatment of the in vitro product as described under Materials and Methods. The elution position of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ was assessed with unlabeled standards by measuring absorbance at 254 nm.

an extinction coefficient of 91 mM^{-1} cm^{-1} for the absorbance difference between 453 and 490 nm in the spectrum of the sodium dithionite reduced reduced CO complex minus dithionite reduced. The absorption spectra was recorded at room temperature on Amino DW-2 UV/vis spectrophotometer.

RESULTS

A soluble preparation containing 25-OH-D_3 24-hydroxylase activity was obtained upon treatment of kidney mitochondria from vitamin D replete chicks with 0.6% sodium cholate. This method has been described by Mitani and Horie (1969) for solubilization and purification of bovine adrenal mitochondria cytochromes P-450. This solubilized preparation could be stored at −80 °C in the dark for at least 6 months without any loss of catalytic activity. The CO difference spectrum of the solubilized preparation is shown in Figure 1. The absorption maxima is at 453 nm, illustrating the presence of a cytochrome P-450. The presence of overwhelming concentrations of cytochrome oxidase likely accounts for the large absorbance difference at 420 and 440 nm.

Incubation of the solubilized mitochondrial preparation with 25-OH-[26,27-^{3}H]D_3, adrenodoxin, adrenodoxin reductase, and a NADPH generating system resulted in the formation of 24(*R*),25-$(OH)_2D_3$ (Figure 2). The identity of the product was established by cochromatography with synthetic standard

Table I: Reconstitution of 25-Hydroxyvitamin D_3 24-Hydroxylase

reaction system	pmol of $24,25\text{-}(OH)_2D_3$/15 min[c]
complete system[a]	13.82
minus NADPH	0.33[d]
minus adrenodoxin	1.71
minus adrenodoxin reductase	6.99
minus solubilized fraction[b]	0.57[d]

[a] The complete system contained 40 μL of 24-hydroxylase fraction (200 μg), 25 μL of NADPH generating system, pH 7.4 (100 nmol of NADPH, 1.0 μmol of glucose 6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase), 12.5 μL of adrenodoxin reductase (0.08 nmol), 103.5 μL of 15 mM Tris–acetate, pH 7.4, and 200 pmol of 25-OH-[26,27-^{3}H]D_3 with a specific activity of 2000 dpm/pmol (added to the reaction tube and dried under N_2 before addition of components). Omissions from the assay system were replaced with 15 mM Tris–acetate, pH 7.4. The reaction was initiated with the addition of 19 μL of adrenodoxin (1.6 nmol). The reaction conditions, extraction, and chromatography of the samples were as described under Materials and Methods. [b] A 40-μL aliquot of 20% glycerol and 15 mM Tris-acetate, pH 7.4, containing 0.6% sodium cholate was substituted for the solubilized fraction. [c] These values represent the mean of duplicate experiments. Duplicate measurements were within 5% of each other. [d] These values are column background.

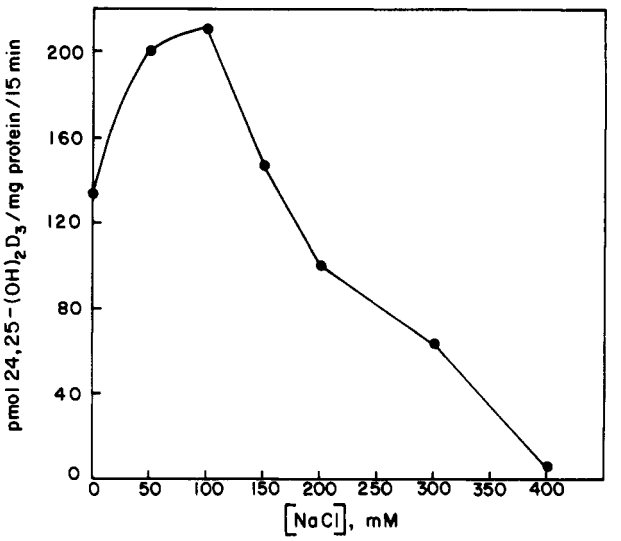

FIGURE 3: Effect of NaCl on 25-OH-D_3 24-hydroxylase activity. Assays were carried out as described under Materials and Methods. Each value represents the mean of duplicates. The duplicates were within 5% of each other.

on straight-phase and reversed-phase HPLC and verified by periodate oxidation (Figure 2B). Reaction of $24,25\text{-}(OH)_2$-[26,27-^{3}H]D_3 with periodate releases the radioactivity as tritiated acetone, which is removed through evaporation. The percent radioactivity remaining in the nonvolatile fraction following treatment with periodate of standard $24,25\text{-}(OH)_2$-[26,27-^{3}H]D_3, the in vitro product, and 25-OH-[26,27-^{3}H]D_3 was 10, 7, and 97%, respectively. These results and the cochromatography on two systems leave no doubt about the identity of the product. Furthermore, the lipid peroxygenase inhibitor diphenyl-*p*-phenylenediamine (1.3×10^{-4} M) had no effect on $24,25\text{-}(OH)_2D_3$ production, supporting the involvement of a monooxygenase mechanism.

The reconstitution conditions for the 24-hydroxylation of 25-OH-D_3 were analyzed in a series of experiments, and the results are shown in Table I. As expected, NADPH, adrenodoxin, adrenodoxin reductase, and the solubilized fraction containing 24-hydroxylase activity are essential for maximal activity. The data strongly support the concept that the reduction of the 24-hydrolyase by NADPH via electron transfer to the flavoprotein (adrenodoxin reductase) and an iron–sulfur protein (adrenodoxin) is indeed an essential step for the interaction of the cytochrome with molecular oxygen leading to the production of $24,25\text{-}(OH)_2D_3$. The lower but detectable formation of $24,25\text{-}(OH)_2D_3$ in the absence of adrenodoxin reductase is due to the presence of endogenous ferredoxin reductase in the solubilized preparation as determined by the cytochrome *c* reductase assay of Sugiyama and Yamano (1975).

The 25-OH-D_3 24-hydroxylase activity was found to be linear with time up to at least 15 min and with protein concentrations up to 2 mg of protein/mL. Final sodium cholate concentration in the assay up to 0.24% had no effect on 24-hydroxylase activity. The incubation conditions selected for subsequent assays were 15 min and 1 mg of solubilized protein/mL. An apparent K_m of 0.67×10^{-6} M was determined from the Lineweaver–Burk plot of the 24-hydroxylation of 25-OH-D_3 vs. substrate concentration.

Since monovalent ions have been shown to markedly affect adrenal cytochrome P-450s (Hanokoglu et al., 1981), their effect on 24-hydroxylase activity was examined. In the complete system, we observed a bell-shaped dependence on NaCl concentration, with peak activity between 50 and 150 mM NaCl and a decline in activity at higher concentrations (Figure 3). The dependence on KCl was essentially identical with that of NaCl (data not shown). The results observed here are similar to those of Hanokoglu et al. (1981) for the adrenal system.

The solubilized preparation from vitamin D replete chicks did not produce significant amounts of $1,25\text{-}(OH)_2D_3$ (Figure 2A). Conversely, a solubilized preparation from vitamin D deficient chicks reconstituted with the same components produced $1,25\text{-}(OH)_2D_3$ but no significant amounts of $24,25\text{-}(OH)_2D_3$ (Ghazarian et al., 1974; Pedersen et al., 1976; Pedersen et al., 1976; Yoon & DeLuca, 1980).

Discussion

This study reports the solubilization and reconstitution of the 25-OH-D_3 24-hydroxylase from vitamin D replete chicks and the optimization of the in vitro production of $24,25\text{-}(OH)_2D_3$ by the reconstituted system. The soluble preparation catalyzes the 24-hydroxylation of 25-OH-D_3 when supplemented with NADPH, adrenodoxin, and addrenodoxin reductase. The product of this enzymatic reaction is $24,25\text{-}(OH)_2D_3$.

It is well established (Kimura & Suzuki, 1967; Omura et al., 1966) that adrenal steroidogenic cytochromes P-450 require both a flavoprotein (adrenodoxin reductase) and a ferredoxin-type iron–sulfur protein (adrenodoxin), which function in the transport of electrons from NADPH to the cytochrome P-450. In a reconstituted system for the chick kidney mitochondria 25-OH-D_3 1α-hydroxylase, adrenodoxin and adrenodoxin reductase were shown to reduce the chick kidney cytochrome P-450 and were required for $1,25\text{-}(OH)_2D_3$ production (Ghazarian et al., 1974), suggesting that this enzyme was similar to the adrenal mitochondrial hydroxylase system. The isolation (Pedersen et al., 1976) and subsequent purification (Yoon & DeLuca, 1980) of a chick renal mitochondrial ferredoxin fully active in a reconstituted 1α-hydroxylase system strongly substantiated this hypothesis. The similarities between the chick 24-hydroxylase and the 1α-hydroxylase, that is, the requirement for NADPH and molecular oxygen (Ghazarian & DeLuca, 1974; Gray et al., 1971; Knutson & DeLuca, 1974) and inhibition of the enzymes by metyrapone and carbon monoxide (Bikle, 1980; Gray et al., 1971; Kulkowski et al., 1979), together with these reconstitution data and the lack of

inhibition by the antioxidant diphenyl-*p*-phenylenediamine strongly support the idea that the chick kidney 25-OH-D_3 24-hydroxylase is also a three-component mixed-function oxidase.

The simulation of the 24-hydroxylase activity by NaCl and KCl resembles the effect obtained on the activity of purified adrenal mitochondrial cytochrome P-450 specific for cholesterol side-chain cleavage (Hanukoglu et al., 1981). In this system, metal ions strongly modulate adrenodoxin binding to adrenodoxin reductase and to the cytochrome P-450 and can activate adrenodoxin reduction by adrenodoxin reductase, as well as cholesterol side-chain cleavage. The nature of the system, when compared to the reconstituted 24-hydroxylase system, suggests that a similar mechanism might be involved.

Previous evidence from in vivo rat studies (Tanaka & DeLuca, 1974) and in vitro studies in chick kidney mitochondria (Bikle, 1980; Tanaka et al., 1975) has clearly demonstrated that the 24-hydrolyxase and the 1α-hydroxylase are reciprocally regulated, with the 24-hydroxylase predominating in vitamin D replete animals and the 1α-hydroxylase in vitamin D deficient animals. Similar results have been found with purified cytochrome P-450 from bovine kidney mitochondria (Hiwatashi et al., 1982) and pig kidney mitochondria (Saaren et al., 1978). In agreement with these results, our solubilized fraction from vitamin D sufficient chicks was active in the conversion of 25-OH-D_3 to 24,25-$(OH)_2D_3$ but was unable to support the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$. In this context, a solublized preparation from vitamin D deficient chicks, reconsituted with the same components used for the 24-hydroxylase studies, produced 1,25-$(OH)_2D_3$ but no detectable amounts of 24,25-$(OH)_2D_3$ (Ghazarian et al., 1974; Pedersen et al., 1976; Yoon & DeLuca, 1980). Furthermore, when soluble preparations of 24-hydroxylase from vitamin D sufficient chicks and 1α-hydroxylase from vitamin D deficient chicks were incubated together, the 24-hydroxylase activity is not affected by the vitamin D deficient preparation and vice versa (A. J. Brown, M. Burgos-Trinidad, and H. F. DeLuca, unpublished results). These results together with the finding that purified renal ferredoxin from normal chicks is active in the reconstitution of 25-OH-D_3 1α-hydroxylase when incubated with cytochrome P-450 from rachitic chicks (Yoon & DeLuca, 1980) are consistent with the hypothesis (Pedersen et al., 1976) that the regulation of both 25-OH-D_3 hydroxylases by 1,25-$(OH)_2D_3$ is at the level of the cytochrome P-450 and provide evidence that it is not due to the presence of soluble inhibitory factors. This solubilized preparation from chick renal mitochondria makes possible the purification and the study of the regulation of this enzyme at the molecular level.

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Registry No. P-450, 9035-51-2; 25-OH-D_3, 19356-17-3; 25-OH-D_3 24-hydroxylase, 53112-53-1.

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