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Purification and Properties of Chick Renal Mitochondrial Ferredoxin†

Poksyn S. Yoon and Hector F. DeLuca*

ABSTRACT: Chick renal mitochondrial ferredoxin, as detected by its adrenal flavoprotein dependent NADPH–cytochrome *c* reductase activity, has been isolated from vitamin D_3 replete chicks. This ferredoxin, active in the reconstituted 1α-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3), was purified about 4000-fold to homogeneity, in 2% yield, using ion exchange chromatography on DEAE-cellulose and discontinuous preparative, as well as polyacrylamide disc gel, electrophoresis. The purified protein has an estimated molecular weight of 11 900 based on migration on gel electrophoresis in the presence of sodium dodecyl sulfate and chromatography on Sephadex G-100. The oxidized form exhibits optical absorbances with λ_{max} at 412 and 454 nm, which are diminished upon reduction. Enzymatically active renal ferredoxin from mitochondrial extracts of chicks raised on vitamin D_3 deficient and vitamin D_3 replete diets showed no significant difference in total units recovered from crude mitochondrial sonic extracts. The renal ferredoxin from vitamin D_3 supplemented animals was fully functional in the reconstituted 25-hydroxyvitamin D_3–1-hydroxylase system. A soluble *b*-type cytochrome, a major contaminant in previous preparations, was separated from the renal ferredoxin, and was found to be inactive in the reconstituted 1α-hydroxylase system. Since this soluble cytochrome, which was isolated from the same mitochondrial extracts, does not support 1α-hydroxylation, nor does it affect the rate of the ferredoxin-catalyzed reaction, the iron–sulfur component, and not the *b*-type hemoprotein, is the specific electron carrier between NADPH-reduced flavoprotein and cytochrome P-450 during 1α-hydroxylation of 25-hydroxyvitamin D_3.

The metabolic activation of vitamin D_3 to the dihydroxylated hormone, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] is known to involve oxidation at the C-25 position in the liver (Blunt et al., 1968; Horsting & DeLuca, 1969) and C-1 hydroxylation in the kidney (Fraser & Kodicek, 1970; Holick et al., 1971). Whether renal hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) occurs at the C-1 or C-24 positions appears to be regulated by serum calcium concentration (Boyle et al., 1972; DeLuca, 1974), parathyroid hormone (Garabedian et al., 1972; Fraser & Kodicek, 1973), serum phosphorus concentration (Tanaka & DeLuca, 1973), sex hormones (Tanaka et al., 1976; Castillo et al., 1977), and 1,25-$(OH)_2D_3$ itself (Larkins et al., 1974; Tanaka & DeLuca, 1974). Though the mechanisms of regulation are still unknown, the chick renal

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received July 20, 1979*; *revised manuscript received February 8, 1980*. This work was supported by a grant from the National Institutes of Health (AM-14881) and the Harry Steenbock Research Fund. Part of this work was performed at EPR facilities supported by U.S. Public Health Service Research Grant GM-17170 from the National Institutes of Health.

[1] Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; KP_i, potassium phosphate buffer; DTT, 1,4-dithiothreitol; high-pressure LC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; Mops, 4-morpholinepropanesulfonic acid.

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mitochondrial 25-OH-D_3-1α-hydroxylase system is known to be a cytochrome P-450 dependent mixed-function monooxygenase based upon the observations that the reaction is supported by oxidizable substrates, molecular oxygen, and magnesium ions (Gray et al., 1971) or NADPH in the presence of calcium (Ghazarian & DeLuca, 1974), and contains a carbon monoxide sensitive P-450 hemoprotein (Ghazarian et al., 1974; Henry & Norman, 1974). The recent demonstration of a requirement for an iron–sulfur protein component strongly supports the hypothesis that the chick renal 1α-hydroxylase is a three-component system similar to monooxygenase systems found in other steroidogenic tissues (Sih, 1969).

The adrenocortical mitochondrial ferredoxin was first reported by Omura et al. (1965) and Kimura & Suzuki (1965), who proposed that this iron–sulfur protein component and an FAD-containing flavoprotein constituted an NADPH-dependent cytochrome P-450 reductase complex active in the 11β-hydroxylation of deoxycorticosterone. Both adrenodoxin[2] and adrenodoxin reductase have since been well characterized (Kimura & Suzuki, 1967; Suhara et al., 1972; Nakamura & Otsuka, 1966; Sweat et al., 1969).

In the present paper we have examined the effect of vitamin D_3 status on the enzymatic properties and specific content of renal ferredoxin in isolated chick mitochondria. We have also isolated a soluble *b*-type cytochrome estimated to be present in concentrations of up to 90.0 pmol per mg of mitochondrial protein. Utilizing the purification scheme outlined below, reconstitution studies were performed to examine possible electron transport properties of the isolated cytochrome and to determine if pure renal ferredoxin from normal animals functions as the specific electron donor for the 25-OH-D_3–1α-hydroxylase system.

Experimental Procedures

Materials

NADPH, cytochrome *c* (type III), glucose 6-phosphate, yeast glucose-6-phosphate dehydrogenase (type VII), rotenone, Coomassie Brilliant Blue R and G-250, and cholic acid were obtained from Sigma Chemical Co. (St. Louis, MO) (cholic acid was twice recrystallized from 50% ethanol); polyacrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were from Bio-Rad Laboratories (Richmond, CA); DEAE-cellulose microgranular No. 52 was from Whatman (Clifton, NJ); sodium dithionite was from BDH Laboratories, Gallard-Schlesinger Chemical Mfg. (Carle Place, NY). Crystalline 25-OH-D_3 was a gift from the Philips-Duphar Company of Weesp, The Netherlands; 25,26-dihydroxyvitamin D_3 (25,26-$(OH)_2D_3$) was synthesized in this laboratory acording to the method of Lam et al. (1973); 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) and 1,25-$(OH)_2D_3$ were generously provided by the Hoffmann-La Roche Company of Nutley, NJ; 25-OH-[3α-^{3}H]D_3, with a specific activity of 28 Ci/mmol, was synthesized in this laboratory (S. Yamada, H. K. Schnoes, and H. F. DeLuca, unpublished experiments).

Adrenodoxin was purified from bovine adrenocortical scrapings as described by Orme-Johnson & Beinert (1969) with the omission of the electrophoretic step. Further chromatography on a Sephadex G-50 column equilibrated with 0.5 M NaCl and 0.01 M Tris-Cl, pH 7.5, gave a final preparation of adrenodoxin with an OD_{415nm}/OD_{280nm} ratio of 0.81. Adrenodoxin reductase was prepared by the procedure described by Sugiyama & Yamano (1975), and flavoprotein preparations eluted from the adrenodoxin–Sepharose affinity column were further chromatographed on a Sephadex G-50 column equilibrated with 0.3 M KCl and 0.05 M Tris-Cl, pH 7.5. The ratio of OD_{272nm}/OD_{450nm} of the final preparations was 7.8:6.9.

Methods

Assay for Renal Ferredoxin. Adrenodoxin reductase dependent NADPH–cytochrome *c* reductase activity was monitored spectrophotometrically at 25 °C by measuring the increase in absorbance of cytochrome *c* at 550 nm upon reduction. The standard reaction mixture contained 10 μmol of potassium phosphate buffer (KP_i), pH 7.4, 30.5 nmol of cytochrome *c*, 100 nmol of NADPH, and renal ferredoxin in a total reaction volume of 1.0 mL. Addition of 92 pmol of adrenodoxin reductase gave the rate of cytochrome *c* reduction, which was dependent upon the presence of the flavoprotein, with initial velocities linear with respect to renal ferredoxin concentration. Samples containing mitochondrial membrane fragments were assayed by incubation of approximately 0.4 mg of mitochondrial protein with 10^{-5} M rotenone and 5×10^{-3} M sodium cyanide in 1.9 mL of 0.01 M KP_i, pH 7.4, for 5 min at 25 °C. After the addition of 200 pmol of NADPH, equal amounts were divided between reference and sample curvettes. Adrenal reductase (92 pmol) was then added to the sample compartment, and the initial velocity was recorded. A molar extinction coefficient of 2.05×10^4 mM^{-1} cm^{-1} at 550 nm was used to determine the amount of cytochrome *c* reduced (Margoliash & Walasek, 1967).

Purification of Chick Mitochondrial Renal Ferredoxin. The purification procedure summarized below was used to purify the chick renal ferredoxin from vitamin D_3 replete animals. All steps were carried out at 5 °C unless otherwise indicated.

(*1*) *Preparation of Mitochondria.* One-day-old white Leghorn cockerel chicks were obtained from Northern Hatcheries (Beaver Dam, WI) and were maintained on a 1.1% calcium, vitamin D_3 deficient purified soy protein diet (Omdahl et al., 1971) for 6 to 8 weeks prior to sacrifice. Animals were decapitated and exsanguinated, and their kidneys were removed and placed in isolation media consisting of 0.25 M sucrose, 15 mM Tris-acetate, pH 7.4, 1 mM 1,4-dithiotreitol (DTT), and 10^{-4} M EDTA. Kidney tissue was then homogenized and subfractionated after removal of connective tissue and adhering fat. From each group of 35 animals, approximately 1.9 g of mitochondrial protein was recovered.

For studies on vitamin D_3 replete chick mitochondria 6 to 8-week-old cockerel chicks raised on a vitamin D_3 replete, 1.0% calcium diet were obtained from Dairyland Poultry, Inc. (Endeavor, WI). Renal tissue was collected and prepared as described above, with an average yield from the same number of animals of 2.4 g of mitochondrial protein.

Mitochondria from vitamin D_3 deficient chick kidneys were prepared according to a procedure described earlier (Knutson & DeLuca, 1974), which was modified for large-scale preparative isolation of mitochondria from vitamin D_3 replete chicks as follows: 250-g portions were homogenized in isolation media to give a 20% (w/v) homogenate using a Brinkman Polytron for two 15-s bursts. Nuclear pellets, cellular debris, and erythrocytes were sedimented at 400*g* for 15 min using a swinging bucket rotor. Supernatants were decanted and pellets were washed once. The combined supernatants were then centrifuged at 7000*g* for 15 min to sediment mitochon-

[2] Adrenodoxin and adrenodoxin reductase will be used synonymously with adrenocortical mitochondrial ferredoxin and adrenal ferredoxin reductase, respectively.

drial pellets, which were washed twice to minimize microsomal contamination. Final mitochondrial pellets were resuspended in 0.05 M KCl and 0.01 M Tris-Cl, pH 7.5, for a final protein concentration of 20 mg/mL.

(*2*) *Solubilization by Osmotic Shock and Sonication.* Aliquots (100 mL) of mitochondrial suspension were sonicated with a Branson Sonifier for nine 30-s intervals at 6-A output. Temperatures were maintained below 8 °C with an ice–salt bath. The suspension was then centrifuged at 105 000*g* for 3 h, and fatty layers were aspirated off the surface of the supernatants, which were decanted and combined to give the soluble renal ferredoxin containing extract.

(*3*) *DEAE-cellulose Column.* The soluble extract was then loaded onto a DEAE-cellulose (3.5 × 17 cm) column equilibrated with 50 mM Tris-Cl, pH 8.0, at a flow rate of 1.0 mL/min. The column was washed with 250 mL of 0.18 M KCl and 10 mM Tris-Cl, pH 7.5, and renal ferredoxin was eluted from the column using a 0.18 to 0.45 M KCl gradient in 10 mM Tris-Cl, pH 7.5. Peak fractions containing renal ferredoxin, as detected by adrenal flavoprotein dependent NADPH–cytochrome *c* reductase activity, were pooled, lyophilized, and dissolved in 28 to 30 mL of distilled water. Following dialysis against 4 L of 50 mM Tris-Cl, pH 7.8, overnight, the sample was made 30% (w/v) in sucrose.

(*4*) *Discontinuous Preparative Electrophoresis.* A preparative electrophoresis system similar to one described previously (Ludden, 1977) was prepared at room temperature in a cylindrical glass column (2.9 × 13.5 cm) fitted with stopcocks 3.0 cm from the base to allow continuous flow of elution buffer containing 50 mM Tris-Cl, pH 7.8. The system, consisting of a 3.8% stacking gel layered above 9.7% separating and supporting gels, was prerun at 100 V for 2.5 h in 15 mM Tris-Cl, pH 8.9. Upper and lower chamber buffers were then changed to 84 mM Tris-glycine, pH 8.3, and 45 mM Tris-glycine, pH 8.5, respectively. After we loaded the sample at 50 V, electrophoretic separation was carried out at 100 V. Fractions (14 mL) were collected at 120 mL/h, and peak fractions of reconstituted cytochrome *c* reductase activity were combined and concentrated on a small DEAE-cellulose column (1.4 × 6.5 cm) equilibrated with 0.2 M KCl and 50 mM Tris-Cl, pH 8.0. The renal ferredoxin was eluted with 0.45 M KCl and 50 mM Tris-Cl, pH 7.8, and peak fractions were combined and concentrated by lyophilization.

(*5*) *Disc Polyacrylamide Electrophoresis.* Ten 14.6% gels (0.6 × 8 cm) were prepared and equilibrated at 5 °C, after which 8–10 μg of sample was applied to each gel and electrophoresed at 3 mA/tube for 2 h in 0.5 M Tris-glycine, pH 8.3. One gel was stained for protein, and the others were fractionated into 1-mm sections using a Gilson gel crusher. Proteins were eluted for 12 h at 5 °C from gel fractions and then assayed enzymatically for adrenal flavoprotein dependent NADPH–cytochrome *c* reductase activity, and spectrally for the presence of a *b*-type hemoprotein contaminant. The leading band was found to contain the soluble cytochrome, the second contained all the detectable amounts of renal ferredoxin, and a third was an inactive contaminant. Corresponding fractions were combined from each gel and concentrated by lyophilization.

Reconstitution of 25-OH-D_3-1α-Hydroxylase. Incubations were performed in 25-mL flasks containing components described in the legend to Table II. The reaction was initiated by the addition of 12.5 pmol of 25-OH-[3α-^{3}H]D_3 in 95% ethanol. After 60 min at 37 °C, the reaction was terminated by the addition of 10 mL of 2:1 CH_3OH–$CHCl_3$ and extracted according to the procedure of Bligh & Dyer (1959). The two-phase system formed by addition of 2.5 mL of water and 5.0 mL of $CHCl_3$ was extracted at 5 °C. The lower $CHCl_3$ phase was removed, and the upper layer was reextracted with 5.0 mL of $CHCl_3$, after which the combined $CHCl_3$ phases were evaporated to dryness and dissolved in 10% 2-propanol in *n*-hexane prior to separation of substrate and product using high-pressure liquid chromatography (LC). The reaction product was identified by comigration with nonradioactive synthetic vitamin D_3 metabolites, which were monitored spectrophotometrically by the amount of absorbance at 254 nm (Jones & DeLuca, 1975). During elution of the high-pressure LC column, 20 2.4-mL fractions were collected, evaporated to dryness, dissolved in 5.0 mL of counting solution (Knutson & DeLuca, 1974), and counted using a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiencies averaged 42% with 94–96% recovery of radioactivity from the high-pressure LC columns.

Disc gel electrophoresis was carried out on 8.0-cm gels according to the method of Davis (1962), and gels were stained with 0.04% Coomassie Blue G-250 as described by Reisner et al. (1975).

Sodium dodecyl sulfate ($NaDodSO_4$) gels were run on 10% polyacrylamide gels using the method described by Weber & Osborn (1969) and stained by the method of Frazekas et al. (1963).

Protein concentration was determined by the method of Lowry et al. (1951) or that of Gornall et al. (1949).

Cytochrome P-450 plus P-420 was determined by the method of Omura et al. (1967) as described in the legend to Figure 5C. The concentration of the mitochondrial *b*-type hemoprotein, believed to be soluble cytochrome b_5, was estimated by assuming the extinction coefficients were identical with those used for microsomal cytochrome b_5, based upon similarities of optical absorption spectral properties. The concentration of cytochrome b_5 was determined according to the method of Klingenberg (1958). All spectrophotometric measurements were run at 25 °C using cuvettes of 1.0-cm path length, in a Cary 14 spectrophotometer equipped with a 0–0.1 slide-wire.

Results

Effect of Vitamin D_3 Status on Renal Ferredoxin Activity and Concentration. To determine if enzymatically active renal ferredoxin, as detected by cytochrome *c* reduction, could be isolated from vitamin D_3 replete as well as vitamin D_3 deficient chicks, mitochondrial sonic extracts were subjected to one-step DEAE-cellulose chromatography under identical conditions. As shown in Figure 1, similar amounts of total enzyme units were recovered from mitochondrial extracts of both normal and deficient animals. High levels of contaminating hemoproteins and low concentrations of renal ferredoxin in crude mitochondrial soluble extracts precluded use of optical absorbance or electron paramagnetic resonance (EPR) spectroscopy to determine the concentration of the renal ferredoxin. The total number of enzyme units recovered from 5.9 to 6.1 g of mitochondria from both groups of animals was: 514.2 total units recovered for vitamin D_3 deficient chicks and 534.8 total units obtained from vitamin D_3 replete animals. Recoveries of total units were 74–76% for both groups from the ion exchange column. Based on these results, the total amount of enzymatically active renal ferredoxin, as monitored by adrenal flavoprotein dependent cytochrome *c* reduction, is similar for both groups.

Purification of Chick Renal Ferredoxin. Table I summarizes the procedure utilized for the purification of renal ferredoxin from vitamin D_3 replete animals. The four-step

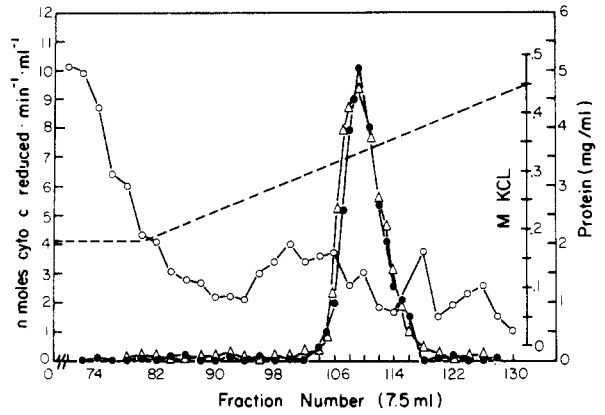

FIGURE 1: DEAE-cellulose column (2.6 × 12.4 cm) profiles of mitochondrial extracts from vitamin D_3 deficient and vitamin D_3 replete chick mitochondria. Renal ferredoxin was detected by measuring adrenocortical flavoprotein dependent NADPH–cytochrome *c* reductase activity. Mitochondria (5.8 g) were obtained from chicks raised on a vitamin D_3 deficient, 1.1% calcium diet for 5 weeks prior to sacrifice. Mitochondria (6.2 g) were isolated from 5-week-old chicks fed ad libitum vitamin D_3 supplemented, 1.0% calcium diets. Soluble submitochondrial extracts were obtained from each group and chromatographed as described under Experimental Procedures: (●) adrenodoxin reductase dependent cytochrome *c* reductase activity of extracts from vitamin D_3 replete chicks; (△) adrenal flavoprotein dependent cytochrome *c* reductase activity of vitamin D_3 deficient extracts; (○) protein concentration was determined according to the method of Gornall et al. (1949).

Table I: Purification of Mitochondrial Renal Ferredoxin from Vitamin D_3 Replete Chicks[a]

purifn step	total protein (mg)	total act.[b]	yield (%)	sp act. (units × mg^{-1})	*x*-fold purifn
sonicated mitochondria[b]	18667	31680	100	1.7	1.0
submitochondrial soluble extract (105000*g* supernatant)	5463	16679	52.6	3.1	1.8
DEAE-cellulose[c] (2.6 × 12.4 cm) column	190	12628	39.9	66.4	39.3
lyophilized, dialyzed concentrate	92.9	10602	33.4	114	67.5
discontinuous preparative electrophoresis[d]	15.9	5648	17.8	355	210
disc polyacrylamide	0.10	676	1.8	6758	3999

[a] Chick renal ferredoxin from vitamin D_3 replete mitochondrial extracts was purified to homogeneity from crude sonic extracts as described under Experimental Procedures. The amount of adrenal flavoprotein dependent NADPH-cytochrome *c* reductase activity was determined in the presence of excess NADPH and adrenodoxin reductase, where the rate of cytochrome *c* reduction was dependent upon the concentration of renal ferredoxin present. One enzyme unit is defined as the amount of ferredoxin catalyzing the reduction of 1.0 nmol of oxidized cytochrome *c* min^{-1} mL^{-1}. [b] Crude mitochondrial sonic extracts containing respiratory chain electron transport particles were assayed in the presence of 10^{-5} M rotenone in 95% ethanol and 5×10^{-3} M NaCN in 10 mM KP_i, pH 7.8. Both inhibitors were added in 5 μL of the appropriate solvent. [c] Peak fractions of renal ferredoxin, as detected by highest adrenodoxin reductase dependent NADPH-cytochrome *c* reduction. [d] Values were determined for a total of 12 gels; each gel sample contained 9–10 μg of protein.

procedure resulted in 4000-fold purification over crude sonic extracts, in 1.8 to 2.2% yield. Further sonication of pellets sedimented during the first high-speed centrifugation failed to increase the amount of activity recovered in the soluble fraction. The most effective step during purification was

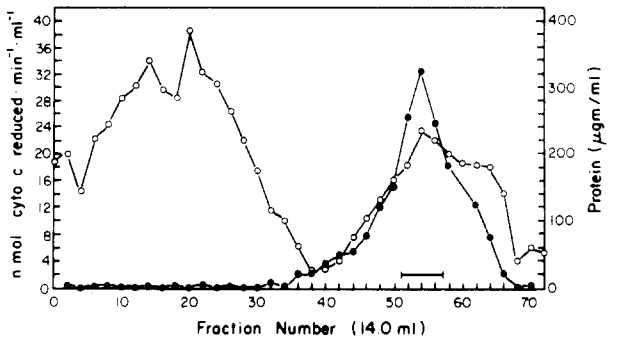

FIGURE 2: Discontinuous preparative electrophoresis of concentrated, dialyzed pooled fractions from DEAE-cellulose column. The concentration of the sample was 3.38 mg/mL in a total volume of 27.5 mL. The collection of fractions with continuous flow of elution buffer containing 50 mM Tris-Cl, pH 7.8, was initiated after tracking dye had migrated 3 cm into the 9.7% separating gel toward the anode: (●) amount of NADPH–cytochrome *c* reduction upon addition of 92 pmol of adrenodoxin reductase; (○) protein concentration according to the method of Lowry et al. (1951).

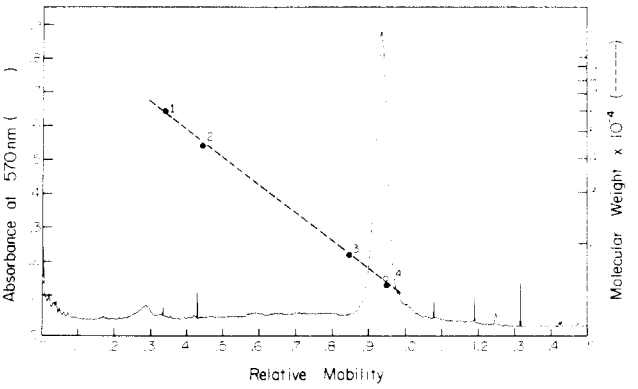

FIGURE 3: Polyacrylamide electrophoresis in the presence of 0.1% $NaDodSO_4$ on 10% gels of purified renal mitochondrial ferredoxin and the marker proteins: (1) catalase (60000); (2) ovalbumin (43000); (3) myoglobin (17200); (4) cytochrome *c* (11700). Each gel was loaded with 8–10 μg of protein following denaturation by incubation at 40 °C for 2 h in the presence of 1% $NaDodSO_4$ and 1% β-mercaptoethanol. Samples were loaded onto the gels and run at 8 mA/tube for 9 h at 5 °C. The densitometer scan of 9.7 μg of renal ferredoxin containing gel was run on a Gilson gel scanner at 570 nm. Relative mobilities are expressed relative to the bromophenol blue dye front, and have been corrected for changes in gel length after staining.

preparative electrophoresis, which gave up to a 96-fold increase in specific activity over the previous step (Figure 2). The soluble cytochrome *b* type was successfully separated from the renal ferredoxin during the final step, which yielded homogeneous iron–sulfur protein.

Purity and Molecular Weight. Renal ferredoxin migrated as a single band on 10% polyacrylamide gels run in the presence of 0.1% $NaDodSO_4$ over a range of protein concentration from 6 to 20 μg. As illustrated in Figure 3, the densitometer trace shows one major symmetrical peak with a relative mobility close to that of cytochrome *c*. Mobilities of marker proteins of known molecular weight are determined as described in the figure legend. The elution profile of chick renal ferredoxin, as detected by its characteristic adrenal flavoprotein dependent cytochrome *c* reductase activity on Sephadex G-100, is shown in Figure 4. The peak of enzymatic activity did not contain any detectable cytochrome b_5, based upon optical absorption spectra. Both activity and protein peaks eluted at 206–208 mL.

Spectral Properties. The optical absorption spectrum of the oxidized renal ferredoxin exhibited λ_{max} at 412 and 454

Table II: Reconstitution of 25-Hydroxyvitamin D_3-1α-Hydroxylase[a]

components added to incubation mixture	incubation numbers													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cytochrome P-450[b] (nmol)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.26	0.26	0.26	0.26	0.25	0.26
chick renal ferredoxin[c] (nmol)		2.20			1.11	2.20	4.43		1.11	2.20	4.43			
chick renal cytochrome b_5[d] (nmol)			2.31						1.20	2.31	4.50	1.20	2.31	4.50
bovine adrenodoxin (nmol)				3.11					3.11					
bovine adrenodoxin reductase (nmol)					0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
1α-hydroxylase act. (pmol of 1,25-$(OH)_2D_3$ formed h^{-1} mL^{-1})[e]	0.00	0.00	0.00	0.01	1.04	2.53	3.84	2.20	1.03	2.30	3.86	0.01	0.00	0.01
% conversion[f]	0.0	0.0	0.0	0.1	7.9	19.2	29.2	16.7	7.8	17.5	29.3	0.1	0.0	0.1

[a] Incubations were performed in 25-mL stoppered Erlenmeyer flasks containing 3.3 μmol of glucose 6-phosphate, 100 nmol of NADPH, 1.0 μmol of magnesium acetate, 1.5 units of yeast glucose-6-phosphate dehydrogenase, and isolated protein components as indicated. All cofactor and salt solutions were freshly prepared in 25 mM Mops, pH 7.4, for a total reaction volume of 1.1 mL. The mixture was gassed for 1 min with 100% O_2 and equilibrated at 37 °C for 5 min prior to addition of 12.5 pmol of 25-OH-[3α-^{3}H] D_3 in 95% ethanol to initiate the reaction. After 60 min, the reaction was terminated with 10.0 mL of CH_3OH–$CHCl_3$ and extracted according to Bligh & Dyer (1959) as described under Experimental Procedures. [b] Added in 150 μL containing soluble cytochrome P-450 in 0.1 M KP_i, pH 7.4, 1 mM DTT, 10^{-4} M EDTA, and 25% glycerol. The amount of cytochrome P-450 was determined from the optical absorbance properties of the reduced carbon monoxide vs. the reduced difference spectrum according to Omura et al. (1965). [c] Concentration of renal ferredoxin was calculated from comparison of the value of the double integral of the reduced EPR spectrum with the value of the double integral of a Cu^{2+} EDTA standard of known concentration. [d] The amount of the soluble renal *b*-type cytochrome was determined from the optical absorption properties of the reduced vs. oxidized spectrum (Klingenberg, 1958). [e] Calculated values based upon total amount of radioactivity recovered from high-pressure LC column which cochromatographed with authentic 1,25-$(OH)_2D_3$. [f] Represent average values of duplicate incubations for each group.

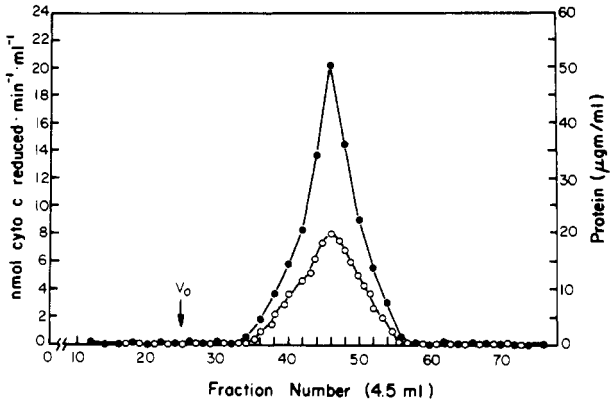

FIGURE 4: Sephadex G-100 chromatography of chick renal ferredoxin. A sample containing 130 μg of purified chick renal ferredoxin was applied to a Sephadex G-100 (2.2 × 70 cm) column equilibrated with 0.2 M KCl and 10 mM Tris-Cl, pH 7.8, and eluted in the same buffer at a flow rate of 4.0 mL/min into 4.5-mL fractions: (●) denotes adrenal flavoprotein dependent NADPH–cytochrome *c* reductase activity, indicating the presence of chick renal ferredoxin; (○) protein concentration as determined according to the method of Lowry et al. (1951).

nm, which were bleached upon reduction with sodium dithionite as shown in Figure 5A. Also shown are spectra of chick renal mitochondrial cytochrome b_5 (Figure 5B) (this soluble cytochrome b_5 preparation exhibited spectra that were insensitive to the addition of carbon monoxide) and solubilized cytochrome P-450 (Figure 5C) utilized in the reconstitution of 25-OH-D_3-1α-hydroxylase activity.

Characterization of the adrenal flavoprotein dependent cytochrome *c* reductase activity as chick renal ferredoxin was confirmed by low-temperature EPR spectral characteristics of the reduced forms, which are very similar in *g* values and line shape to those observed for reduced bovine adrenal mitochondrial ferredoxin.

Reconstitution of 25-OH-D_3-1α-Hydroxylase Activity. Summarized in Table II are results from reconstitution experiments designed to test purified renal ferredoxin as an intermediate electron carrier between NADPH-reduced flavoprotein and oxidized cytochrome P-450 solubilized from vitamin D_3 deficient chicks. Under the conditions given, the rate of product formation was dependent upon the concentration of renal ferredoxin present. The *b*-type hemoprotein believed to be a mitochondrial cytochrome b_5, when substituted at equimolar concentrations, was totally inactive (incubation no.'s 12–14). When the isolated cytochrome was added to incubation mixtures containing renal ferredoxin (incubation no.'s 10–12), the rate of product formation was identical with that observed when only the iron–sulfur component served as electron donor to the P-450 hemoprotein (incubation no.'s 5–7).

High-Pressure LC of Reaction Product from Reconstitution Experiments. The high-pressure LC profiles of chloroform extracts from incubation no. 7 of Table II are shown in Figure 6. Based upon cochromatography with nonradioactive internal standards, only the 1α-hydroxylated reaction product was observed. Radioactivity appeared only in the region corresponding to 1,25-$(OH)_2D_3$ and the substrate, 25-OH-D_3.

Discussion

Utilizing the purification scheme in Table I, chick renal mitochondrial ferredoxin has been purified to apparent homogeneity based upon electrophoresis on 10% polyacrylamide gels run in the presence of $NaDodSO_4$ and chromatography of the purified protein on Sephadex G-100. Overall fold purification ranged from 3300- to 4000-fold, in 1.8–2.2% yield, from crude mitochondrial extracts. The final electrophoretic step was found to be essential for the separation of a soluble *b*-type cytochrome contaminant. Reconstitution studies, as shown in Table II, clearly demonstrate that the hemoprotein neither substitutes for the ferredoxin component, nor does it affect the ferredoxin-catalyzed rate of product formation. The former observation indicates that cytochrome b_5 will not serve as an intermediate electron donor for the P-450 hemoprotein, while the latter results demonstrate that cytochrome b_5 will not compete with the P-450 terminal oxidase for reduction by renal ferredoxin. Hence, the iron–sulfur component, and not

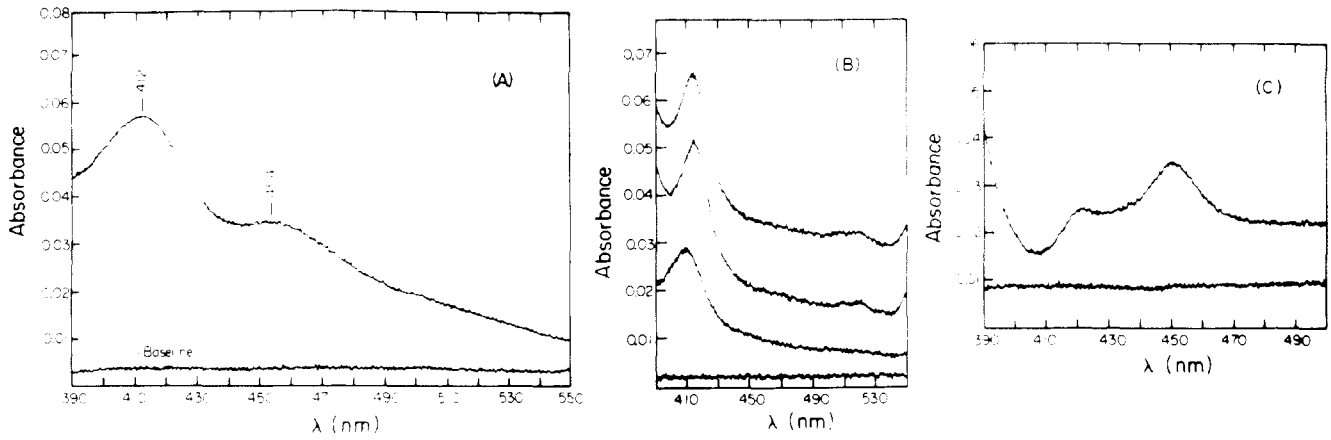

FIGURE 5: Optical absorption spectra of chick renal ferredoxin, renal cytochrome b_5, and solubilized cytochrome P-450. (A) Absolute oxidized spectrum of purified chick renal ferredoxin. After recording of the buffer blank, oxidized protein was placed in the sample compartment and the spectrum was recorded, while the reduced spectrum was obtained after addition of solid sodium dithionite. Protein concentration of the sample was 45 μg/mL as determined by the method of Lowry et al. (1951). (B) Optical absorption spectra of renal mitochondrial cytochrome b_5 from the same preparation. The absolute oxidized and reduced spectra were obtained by recording the spectra of oxidized and dithionite-reduced samples as shown in A and B, respectively. The reduced vs. oxidized difference spectrum was obtained by placing an equivalent amount of oxidized sample in the reference compartment, after recording the reduced spectrum (C). Protein concentration was 8.4 μg/mL, and the concentration of cytochrome b_5 was determined from the reduced vs. oxidized difference spectrum using an extinction coefficient of 185 cm^{-1} mm^{-1} for the amount of absorbance between 424 and 409 nm (Klingenberg, 1958). (C) Reduced carbon monoxide vs. reduced difference spectra of cytochrome P-450 solubilized from rachitic chick mitochondria. The P-450 hemoprotein was solubilized as indicated under Experimental Procedures. The sample containing 2.7 ng of protein per mL was equally divided between reference and sample cuvettes and the base line was recorded. Carbon monoxide was then bubbled through the sample compartment, and the oxidized carbon monoxide vs. oxidized spectrum was recorded to test for hemoglobin contamination. The cytochrome P-450 and P-420 spectra were obtained by the addition of solid sodium dithionite to both reference and sample compartments. The concentration of cytochrome P-450 was determined from the reduced carbon monoxide vs. reduced difference spectrum using a value of 91 cm^{-1} mm^{-1} for the extinction increment between 424 and 409 nm as described by Omura et al. (1967). All spectra were recorded at 25 °C using cuvettes of 1.0-cm path length as described under Experimental Procedures.

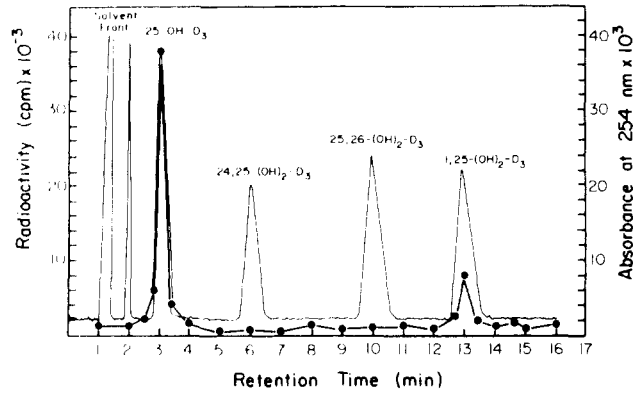

FIGURE 6: High-pressure LC of reconstituted 1α-hydroxylase reaction product. The reaction mixture contained 4.4 nmol of purified renal ferredoxin, 0.25 nmol of cytochrome P-450, 0.16 nmol of adrenodoxin reductase, and an NADPH-generating system is given under Experimental Procedures. Combined $CHCl_3$ extracts were evaporated to dryness under N_2, and the residue was dissolved in 10% 2-propanol in *n*-hexane. Twenty-five microliters was removed to determine column recoveries, and 30 μL of sample was co-injected with synthetic nonradioactive vitamin D_3 standards into a high-pressure LC Waters U6K injector fitted with a Zorbax-SIL (0.46 × 26 cm) column. Straight-phase elution under 900 psi of N_2 using the same solvent system was carried out at a flow rate of 2.0 mL/min into 2.4-mL fractions. Recovery of radioactive counts averaged 94–96%: (—) absorption at 254 nm of nonradioactive vitamin D_3 metabolites; (●) radioactivity extracted into the organic phase of reconstituted 25-OH-D_3–1α-hydroxylase above incubation mixture and cochromatography in 10% 2-propanol in *n*-hexane.

the b_5 hemoprotein, is the specific electron donor to cytochrome P-450 in the reconstituted system, in contrast to previous observations in microsomal mixed-function oxidase systems where a synergistic role for the b_5 cytochrome has been proposed (Lu et al., 1974; Hrycay & Prough, 1974). Other b_5-type hemoproteins have been reported for mitochondria from rat liver (Davis & Kreil, 1968), rabbit liver and kidney, and pig heart (Fukushima et al., 1972; Fukushima & Sato, 1973).

Whether chicks are raised on vitamin D_3 deficient diets, which result in elevated 1α-hydroxylase activity in vivo (Fraser & Kodicek, 1970), or are maintained on vitamin D_3 replete diets resulting in suppressed C-1 hydroxylation (Kuntson & DeLuca, 1974), results shown in Figure 1 demonstrate that the amount of total enzyme units recovered following ion-exchange chromatography is identical for both groups of animals. Hence, the amount of enzymatically active renal ferredoxin appears to be independent of vitamin D status. Chick kidney iron–sulfur proteins from either vitamin D deficient or vitamin D replete animals have similar enzymatic and physical properties, as well as optical absorption spectral characteristics of the active site of electron transfer. Other mitochondrial ferredoxins have recently been reported from rat liver (Atsuta & Okuda, 1978) and bovine liver and kidney (Ohashi & Omura, 1978).

In the present report, our finding that purified renal ferredoxin from normal animals is fully active in the reconstituted 1α-hydroxylation of 25-OH-D_3 when incubated with cytochrome P-450 from rachitic chick mitochondria strongly suggests that specificity for C-1 or C-24 oxidation is determined at the level of the P-450 terminal oxidase. In addition, results from reconstitution studies using isolated components clearly indicate that the renal ferredoxin, and not the *b*-type cytochrome, is the specific electron carrier between reduced flavoprotein and cytochrome P-450 of the renal 25-OH-D_3–1α-hydroxylase.

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