Separation of Two Forms of Chick 1,25-Dihydroxyvitamin D_3 and 25-Hydroxyvitamin D_3 24-Hydroxylase (43676)

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Abstract. **We previously reported the development of monoclonal antibodies against chick kidney mitochondrial 1,25-dihydroxyvitamin D_3 24-hydroxylase. However, these monoclonal antibodies were able to immunoprecipitate only approximately 25% of total 24-hydroxylase activity present in solubilized preparations, suggesting the existence of different forms of the enzyme. Chromatography of the solubilized preparations on a mono-Q column resolved the 24-hydroxylase activity into two peaks. The protein found in the first peak of enzyme activity (peak 1) did not immunoblot with the monoclonal antibody (IVC2F10) that was raised against the partially purified 24-hydroxylase. However, the protein in the second peak (peak 2) reacted on immunoblot with the same antibody. The enzyme for both peaks had similar kinetics, required adrenodoxin and adrenodoxin reductase, and acted on both 1,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3. It is not yet clear whether these are two distinct enzymes or whether it is the same enzyme complexed with different proteins.**

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It is generally accepted that 24-hydroxylation is the initial step in the degradation of 25-hydroxyvitamin (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) which is known as a hormonally active form of vitamin D_3 (1). These reactions are mediated by 1,25-$(OH)_2D_3$ and 25-OH-D_3 24-hydroxylase. The renal 24-hydroxylase is a mitochondrial cytochrome P-450 mixed-function oxidase, which requires reduced NADP and molecular oxygen for its activity (2–5). The renal 24-hydroxylase has been solubilized and reconstituted with NADPH, beef adrenodoxin, and beef adrenodoxin reductase in chick (2), bovine (5), pig (6), and rat (7).

Recently, monoclonal antibodies against 24-hydroxylase were also generated by using bovine (5) and chicken (8) kidney preparations. In our laboratory, monoclonal antibodies to chick kidney enzyme were developed (9), but these antibodies immunoprecipitated only about 25% of the total enzyme activity.

The present study was undertaken to determine if differing forms of the 24-hydroxylase could be detected, one that is recognized by the antibodies and another that is not.

Materials and Methods

Chemicals. Sodium periodate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium cholate was obtained from Sigma (St. Louis, MO) and recrystallized prior to use. 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) and 25-OH-[26,27-^{3}H]D_3 (175 Ci/mmol) were obtained from Dupont/New England Nuclear (Boston, MA) and purified by straight-phase HPLC prior to use.

Animals. One-day-old White Leghorn chicks, purchased from a commercial source (Sunnyside, Inc., Beaver Dam, WI), were fed a 3% calcium and 0.7% phosphorus diet, supplemented with 400 IU of vitamin

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D_3/kg for two weeks (9). Induction of 24-hydroxylase was achieved by the injection of 1 μg each of 25-OH-D_3 and 1,25-$(OH)_2D_3$, which were given intramuscularly 48 and 24 hr prior to sacrifice.

Isolation and Solubilization of Mitochondria. Chick kidney mitochondria was isolated and then solubilized according to the method of Burgos-Trinidad *et al.* (1). Immediately after sacrifice, chick kidneys were removed and homogenized in 15 m*M* Tris-acetate buffer (0.25 *M* sucrose, pH 7.4). Centrifugation at 500 g for 10 min sedimented the nuclear fraction. The supernatant was centrifuged at 10,000 g for 10 min to yield a mitochondrial pellet. This was washed twice with solubilization buffer (160 m*M* potassium phosphate, pH 7.4, 20% glycerol and 1 m*M* EDTA) and resuspended at a final concentration of 5–15 mg/ml protein. For mitochondria solubilization, the pellet was resuspended in solubilization buffer containing 1 m*M* dithiothreitol (DTT), 0.25 m*M* phenylmethylsulfonyl fluoride (PMSF), and 0.6% sodium cholate. The mixture was stirred gently for 60 min. Supernatant containing 24-hydroxylase activity was obtained by centrifugation at 100,000 g for 60 min (solubilized P-450).

The above procedure was conducted at 0 = 4°C.

FPLC Analysis. For chromatography of the 24-hydroxylase, 1.5 ml of solubilized mitochondria was applied to a 1 ml Mono Q HR5/5 column (Mono Q, Pharmacia, Uppsala, Sweden). The column was equilibrated with Mono Q buffer (20 m*M* Tris-HCl, pH 7.7, 20% glycerol, 1 m*M* EDTA, 0.1 m*M* DTT, 0.6% sodium cholate). The column was washed with the same buffer and eluted with Mono Q buffer with a continuous gradient of NaCl at a flow rate of 0.4 ml/min and 0.8 ml fractions were collected. Selected fractions were analyzed by SDS-PAGE and immunoblotted. 24-Hydroxylase activity in the indicated fractions was also measured.

24-Hydroxylase Assay. The 24-hydroxylase activity was assayed as previously described (4). In the standard hydroxylase assay, the reaction mixture contained 20 μl of sample (column eluant), 17 μl of the beef adrenal reconstitution system, 153 μl of 10 m*M* potassium phosphate, pH 7.4 in a total volume of 190 μl. The reconstitution system consisted of 0.1 nmol adrenodoxin, 0.05 nmol adrenodoxin reductase, 70 nmol NADP, 0.6 μmol glucose 6-phosphate, and 0.6 units glucose-6-phosphate dehydrogenase. Reactions were initiated by the addition of either 1,25-$(OH)_2$-[26,27-^{3}H]D_3 or 25-OH-[26,27-^{3}H]D_3 in 10 μl ethanol. The incubation proceeded for 60 min at 37°C and terminated by the addition of 30 μl of 1 *M* acetic acid. In the case of kinetic measurements, initial reaction velocity was measured. The amount of product was determined by periodate cleavage and measurement of the [^{3}H]-acetone was carried out as described (4). This assay was shown to be equal to the HPLC assay for these chick kidney preparations (4). No evidence for significant 23- or 26-hydroxylation was found.

Analytical Procedure. The protein concentrations were measured by the Bio-Rad protein microassay with bovine serum albumin as the standard (11). Cytochrome P-450 was measured by carbon monoxide difference spectral analysis using the method of Omura and Sato (12) with a Shimadzu UV 2100 Spectrophotometer. Adrenodoxin and adrenodoxin reductase were purified from beef adrenal mitochondria (13, 14).

Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis was carried out in 9.0% acrylamide gels according to Laemmli (15). For immunoblotting, polyvinylidene difluoride (PVDF) membrane (Immobilon Transfer Membrane, Millipore, Bedford, MA) and alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Madison, WI) were used. Color development was performed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega).

Monoclonal antibodies developed against chick kidney 24-hydroxylase were produced as previously described (9). One of these antibodies, IVC2F10 (IgG_1), was used for immunoblot analysis.

Results

As shown in Figure 1, 1,25-$(OH)_2D_3$ 24-hydroxylase activities eluted from the Mono Q HR 5/5 column in two peaks at 0.25 and 0.4 *M* NaCl. Additionally, when 25-OH-D_3 24-hydroxylase activity of each frac-

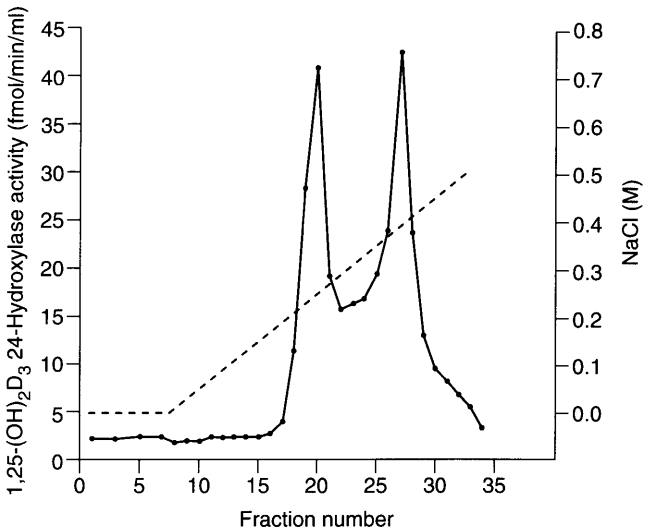

Figure 1. Chromatography of solubilized chick kidney 24-hydroxylase on a Mono Q column. Solubilized mitochondria (1.5 ml) was applied to a Mono Q HR 5/5 column and eluted with a NaCl gradient (0–0.5 *M*) at 0.4 ml/min. Fractions (0.8 ml) were collected, and 1,25-$(OH)_2D_3$ 24-hydroxylase activities were measured as described in Materials and Methods.

tion was measured, the same pattern was obtained (not shown).

As shown in Figure 2, peaks 1 and 2 were subjected to immunoblotting analysis with monoclonal antibody, IVC2F10. Peak 1 protein (lane 3) was not immunoblotted by this antibody despite the presence of 24-hydroxylase activity. On the other hand, peak 2 (lane 2) shows a band that has a similar size as the one in solubilized mitochondria (lane 4).

To evaluate the dependency of peak 1 and peak 2 24-hydroxylase activity on adrenodoxin or adrenodoxin reductase, the enzyme activity was assayed with and without adrenodoxin or adrenodoxin reductase (Table I). The 24-hydroxylase activity from peak 1 and peak 2 in the absence of adrenodoxin or adrenodoxin reductase were 7.6% and 6.6%; and 3.2% and 2.8% of control, respectively, indicating that both adrenodoxin and adrenodoxin reductase are required by both enzymes.

The K_m of the 24-hydroxylases in peak 1 and peak 2 from Mono Q column were determined from Lineweaver-Burk plots and are shown in Table II. The V_{max} for 1,25-$(OH)_2D_3$ were 0.22 (peak 1) and 0.13 (peak 2) pmol/pmol P-450/min, whereas for 25-OH-D_3, they were 0.22 (peak 1) and 0.42 (peak 2) pmol/pmol P-450/min. The K_m values of peak 1 and peak 2 for 1,25-$(OH)_2D_3$ were 0.4 μM, while the K_m values of peaks 1 and 2 for 25-OH-D_3 were 1.5 μM. The products produced by peak 1 and peak 2 enzymes were also verified by HPLC to be 1,24,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$.

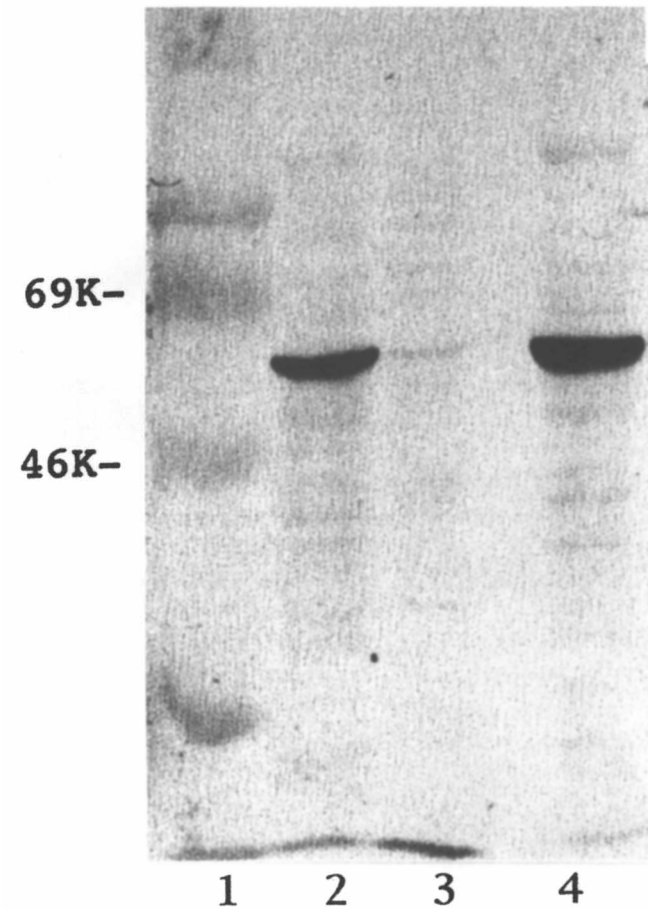

Figure 2. Immunoblot of peak 1 and peak 2 from Figure 1. Eluates to Mono Q column were subjected to electrophoresis in 9% SDS-polyacrylamide gel under denaturing conditions and blotted on PVDF membrane. Transferred protein was detected with IVC2F10 monoclonal antibody. Lane 1, molecular weight standard; lane 2, peak 2 (2 μg); lane 3, peak 1 (2 μg); lane 4, solubilized mitochondria (10 μg).

Table I. Dependence of the Hydroxylase Fractions on Adrenodoxin and Adrenodoxin Reductase for Activity[a]

	No adrenodoxin % of control activity	No adrenodoxin reductase % of control activity
Peak 1	7.6	3.2
Peak 2	6.6	2.8

[a] Effect of beef adrenodoxin and adrenodoxin reductase on 24-hydroxylase activities of peak 1 and peak 2. 1,25-$(OH)_2D_3$ 24-Hydroxylase activity was measured with or without either adrenodoxin or adrenodoxin reductase as described in Materials and Methods.

Discussion

The present report demonstrates that the solubilized 1,25-$(OH)_2D_3$ 24-hydroxylase from chick kidney mitochondria can be resolved into two distinct fractions by FPLC using a strong anion exchange column. The major fraction (peak 1) does not immunoblot by Western analysis using our monoclonal antibody directed against the chick kidney 24-hydroxylase, while the minor fraction (peak 2) clearly immunoblots with this antibody. This, therefore, explains our previous experience that this antibody can only immunoprecipitate 25% of the solubilized 24-hydroxylase from chick kidney. This might suggest two different 24-hydroxylases. However, both fractions have similar kinetic characteristics (V_{max}, K_m) and both act on 25-OH-D_3 as well as 1,25-$(OH)_2D_3$. Additionally, both hydroxylase fractions require adrenodoxin and adrenodoxin reductase for activity. It is, therefore, premature to conclude that two antigenically distinct forms of the 24-hydroxylase exist. It is equally possible that the antibody may be directed against a protein that binds the 24-hydroxylase. If that is the case, the binding protein must have a molecular weight similar to the 24-hydroxylase (ca. 59,000) and it must significantly alter the charge of the complex to allow its separation from noncomplexed 24-hydroxylase (peak 1). This question requires further examination especially since at least one of the 24-hydroxylases from rat (16) and human HL-60 cells (17) have been cloned and sequenced.

Since the periodate assay could also detect 26-hydroxylation, the possibility that one of the peaks

Table II. Kinetic Values for the Hydroxylase Fractions[a]

	V_{max}		K_m	
	$1,25\text{-}(OH)_2D_3$ (pmol/pmol P-450/min)	25-OH-D_3	$1,25\text{-}(OH)_2D_3$ (μmol)	25-OH-D_3
Solubilized P-450	0.13	0.28	0.20	1.50
Peak 1	0.22	0.22	0.40	1.50
Peak 2	0.13	0.42	0.40	1.50

[a] K_m and V_{max} values were calculated from Lineweaver-Burk plots. $1,25\text{-}(OH)_2D_3$ and 25-OH-D_3 24-hydroxylase activity was carried out under conditions of initial reaction velocity.

could be a 26-hydroxylase was examined. By direct HPLC assay only, $1,24,25\text{-}(OH)_3D_3$ and not $1,25,26\text{-}(OH)_3D_3$ could be detected with the solubilized chick kidney preparations used here. Either the 26-hydroxylase is not present in chick kidney mitochondria or may not be active under the conditions used here. In any case, the two fractions studied have only 24-hydroxylase activity.

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Note Added in Proof

After this paper was submitted, we determined that the antibody that immunoprecipitates 25% of the 24-hydroxylast is actually directed against a protein that binds the 24-hydroxylase and not the hydroxylase itself (see [18]).