Kinetic Behavior of 25-Hydroxyvitamin D-1-Hydroxylase and -24-Hydroxylase in Rat Kidney Mitochondria*

Reinhold Vieth‡ and Donald Fraser

From the Departments of Physiology and Paediatrics, University of Toronto and The Research Institute, The Hospital for Sick Children, Toronto, Canada

Although 25-hydroxyvitamin D (25-OHD)-1-hydroxylase and -24-hydroxylase have been measured extensively in fowl, no methods for assay of these enzymes have been validated for the mammal. Inhibitory activity present in all mammalian tissues is a hindrance to accurate measurement. The mechanism by which protein components from rat tissues reduce chick 25-OHD-1-hydroxylase activity was studied by an enzyme-kinetics approach, and data were analyzed using Eadie plots of enzyme activity (V) versus V/substrate concentration. Inhibitory activity affecting chick 1-hydroxylase was present in all tissues studied, including chick mitochondrial preparations themselves. This inhibition is thought to be due to binding of 25-OHD_2 to nonenzymic sites.

A method for quantitative and simultaneous assay of rat kidney mitochondrial 25-OHD-1-hydroxylase and -24-hydroxylase activities was devised. Assay conditions include incubation at 25°C instead of the conventional 37°C, and the use of 500 nM 25-OHD_2 to overcome inhibitory activity. V_{max} of rat 25-OHD-1-hydroxylase was of comparable magnitude to that of the chick enzyme under identical conditions, 2.9 and 5.5 pmol (mg x min)^{-1}, respectively. In rat mitochondria, mean K_{m} was 890 nM for 25-OHD-1-hydroxylase and 375 nM for -24-hydroxylase. It is hypothesized that the higher apparent K_{m} of rat 1-hydroxylase is due to lack of an allosteric activation factor for this enzyme in the \textit{in vitro} preparation.

Extensive studies of 25-hydroxyvitamin D-1-hydroxylase and -24-hydroxylase have been carried out in fowl (1–4). In mammalian tissues few studies of these enzymes have been made (5–7) and no method for their quantification has been validated. Inhibitory activity present in all mammalian tissues is a major hindrance to accurate measurement of 1-OHase in mammals (8–10). Botham et al. (8) reported that addition of rat kidney microsomes or rat serum to the incubation system greatly decreased chick 1-OHase activity; the inhibitor appeared to be 25-OHD_2 binding protein (10).

In our initial experiments, we studied the enzyme kinetics of chick 1-OHase with and without inhibitor from rat tissue. Based on the results of these experiments, we devised a method to overcome the inhibitory activity in mammalian samples. This method permits accurate simultaneous measurement of 1-OHase and 24-OHase activities in rat kidney mitochondrial preparations.

EXPERIMENTAL PROCEDURES

Animals and Diets—Male Wistar rats were housed in pairs in hanging wire cages in a room lit by a single inandescent light bulb. Invasive experiments were conducted under light anesthesia with ether, and the animals were killed by cervical dislocation. Plasma 25-OHD was measured by a binding assay (11).

To obtain renal tissue with high 1-OHase activity, rats weighing 120 g (approx 2 weeks after weaning) were given, \textit{ad libitum}, distilled water and a vitamin D-deficient diet (Teklad TD 77051, Madison WI) containing 0.014 g of calcium and 0.18 g of P/100 g. On Days 1 and 14 on the diet, blood was withdrawn by heart puncture and plasma 25-OHD was determined. By Day 14 plasma 25-OHD was undetectable (<1.6 ng/ml). Assay of 1-OHase was carried out between Days 21 to 30.

To obtain renal tissue with high 24-OHase activity normal rats weighing 350 g were supplied, \textit{ad libitum}, with distilled water and a regular, commercial rat diet. Assay of 24-OHase was carried out at any time.

Materials—[26,27-3H]25-Hydroxyvitamin D_3, 11.7 Ci/mmol, was purchased from Amersham Searle Corp., Arlington Heights, IL. 25-Hydroxyvitamin D_3 was a gift from the Upjohn Co., Kalamazoo, MI. The concentration of nonradioactive 25-OHD_2 was determined by UV absorbance spectrophotometry. All organic solvents were from Burdick and Jackson, Muskegon, MI.

Sample Preparation and Incubation for Assays of 1-Hydroxylase and 24-Hydroxylase in the Rat—Each rat was exsanguinated by heart puncture; the kidneys were removed, and the rat was killed. The kidneys were placed briefly in ice cold homogenization medium (250 mM sucrose, 10 mM Heps buffer, and 10 mM KCl, adjusted with NaOH to pH 7.42 at room temperature) and transferred to a stainless steel tray resting on crushed ice. Capsules, ureters, and papillae were teased away from cortical and medullary tissue with forceps. The cortical and medullary tissue was homogenized in 10 volumes (v/w) of ice cold homogenization medium using a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 4000 x g for approximately 40 s, and the supernatant fluid was centrifuged at 9000 x g and 4°C for 20 min. The pellet, which consisted mainly of mitochondria but included other subcellular particles, was suspended in 10 to 15 volumes of ice cold incubation medium (125 mM KCl, 20 mM Heps, 10 mM L-malic acid, 2 mM MgSO_4, 1 mM diithothreitol, and 0.05 mM EDTA, adjusted to pH 7.4 at room temperature); the final mitochondrial protein concentration was 2.5 to 5 mg/ml.

Each incubation sample consisted of a 1.0 ml aliquot of mitochondrial suspension.

Samples, in 25-ml Erlenmeyer flasks, were placed in a Dubnoff incubator at 37°C, shaking at 100 cycles/min. At 9 min, 500 pmol of

*This work was supported by Grant MA 5139 from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed at: The Hospital for Sick Children, Room 5128B, 555 University Ave., Toronto, Ontario, Canada M5G 1X8.

The abbreviations used are: 1-OHase, 25-hydroxyvitamin D_3-1-hydroxylase; 25-OHD_2, 25-hydroxyvitamin D_3-1,25-di(OH)_2D_3; 24,25(OH)_2D_3, 24,25-dihydroxyvitamin D_3; 24-OHase, 25-hydroxyvitamin D_3-24-hydroxylase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMS, postmitochondrial supernatant fluid; CV, coefficient of variation; HPLC, high pressure liquid chromatography; V, enzyme activity; S, substrate concentration.
$[^{3}H]25$-OHD$_3$ in 20 μl of acetone were added. $[^{3}H]25$-OHD$_3$ had been adjusted to 100 dpm/pmol, by addition of nonradioactive 25-OHD$_3$. Following substrate addition, the flask was gassed for 1 min with a direct flow of 95% O$_2$, 5% CO$_2$ at a rate of 0.5 liter/min. Immediately after gassing, the flask was sealed with a rubber stopper. Incubation with substrate lasted exactly 15 min at which time the reaction was stopped by addition of 3.75 ml of methanol:chloroform (2:1); this was also the first step of the lipid-extraction procedure.

**Lipid Extraction and Chromatography**—The contents of each Ehrlenmeyer flask were decanted into an 8-ml vial and sealed with a foil-lined screw cap. The lipids were extracted by a slightly modified version of the method of Bligh and Dyer (12). 1.25 ml of chloroform was added, the vial was capped and shaken, 1.25 ml of 4% KCl solution was added, and after shaking the vial was centrifuged at 3000 × g for 10 min. The chloroform layer was transferred to a con-bottomed 3-ml Reactivial (Pierce, Chicago, IL) and the sample was evaporated to dryness under a stream of nitrogen. The lipid was dissolved in 0.1 ml of chloroform and streaked across a strip (2 × 16 cm) of aluminium foil-backed silica gel under a stream of nitrogen. To separate 1,25-(OH)$_2$D$_3$, 24,25-(OH)$_2$D$_3$, and 25-OHD$_3$, samples were chromatographed using the continuous development, thin layer chromatography technique of Vieth et al. (13). Radioactivity was eluted from 1-cm slices of the TLC strip with 5 ml of Aquasol in a 20-ml vial and was counted directly in a Beckman LS 355 liquid scintillation counter. We have shown that for our applications, fractional recoveries of $[^{3}H]1,25$-(OH)$_2$D$_3$, $[^{3}H]24,25$-(OH)$_2$D$_3$, and $[^{3}H]25$-OHD$_3$ are equal for all three metabolites with both the lipid-extraction and the TLC procedures.²

**Protein Determinations**—Protein concentrations of the mitochondrial preparations were measured by a modified Biuret method (14) against a bovine serum albumin Fraction V protein standard.

**Calculation**—Measurement of enzyme activity was based on the radioactivity recovered as $[^{3}H]1,25$-(OH)$_2$D$_3$ or $[^{3}H]24,25$-(OH)$_2$D$_3$, relative to total radioactivity recovered. Under our standard incubation conditions, 1-OHase activity was calculated as follows: enzyme units, pmol (mg X min)$^{-1}$

\[
\frac{cpm \text{ as } 1,25-(OH)_2D_3 \times 500 \text{ pmol}}{\text{total recovered cpm mg mitochondrial protein x 15 min}}
\]

Results are the means of three incubations unless stated otherwise.

---

² R. Vieth and D. Fraser, unpublished observations.

---

**Fig. 1.** Eadie plots of V versus V/S for chick mitochondrial 1-OHase activity with control mitochondria (x—x), in the presence of rat serum (•---•), and in the presence of rat kidney postmitochondrial supernatant fluid (PMS) (△—△). Slopes emanating from the origin indicate substrate concentrations used. Inset shows V versus S plot of the data. Kidney mitochondria were prepared from chicks which had been fed for 3 weeks since hatching, with diet deficient in calcium and vitamin D. Mitochondria were prepared as described for rat mitochondria. Incubation medium for the samples in this figure consisted of 240 mM sucrose, 50 mM Heps, 10 mM L-malic acid, 2 mM MgSO$_4$ and 0.1 mM EDTA; pH was 7.4. The mitochondrial suspension was divided into three aliquots: one was a control; to the second was added 25 μl of rat serum/ml of chick mitochondrial suspension; the third aliquot was recentrifuged and the mitochondria were suspended in their original volume in PMS of a 10:1 homogenate prepared in incubation medium from kidneys of a normal rat. Each mitochondrial preparation was incubated at 37°C with eight different concentrations of 25-OHD$_3$. Substrate for each sample consisted of 50,000 dpm of $[^{3}H]25$-OHD$_3$ which had been diluted with increasing amounts of 25-OHD$_3$. 

---

Downloaded from [http://www.jbc.org/](http://www.jbc.org/) by guest on November 12, 2017
Enzyme kinetics of chick kidney mitochondria incubated with and without rat serum or rat kidney postmitochondrial supernatant fluid (containing microsomes and cytoplasmic protein) are shown in Fig. 1. \( V_{\text{max}} \) of chick 1-OHase, calculated by linear regression analysis of the data, was 5.5 pmol (mg X min)\(^{-1} \). This value was unaffected by the inhibitor. The apparent \( K_m \) of chick 1-OHase was 360 nM for both the control and the rat-serum-inhibited 1-OHase and the value was 650 nM in the portion inhibited by PMS. The linear correlations between 1-OHase and \( V/S \) in the Eadie plots at high substrate concentrations and the hooked shape of the plots at the lowest substrate concentrations suggest that the inhibitory activity disappeared at high 25-OHD\(_3 \) concentrations. The results were as expected had the mechanism of inhibition been by substrate depletion (15) and indicate that the mechanism was neither competitive nor noncompetitive inhibition of the enzyme since neither \( K_m \) nor \( V_{\text{max}} \) were affected by the inhibitor. The higher apparent \( K_m \) value with incubations inhibited by PMS was likely caused by a large decrease in the effective 25-OHD\(_3 \) concentration due to binding to PMS proteins. These findings are consistent with the interpretation of Ghazarian et al. (10) that 25-OHD binding protein is the inhibitor of 1-OHase.

Having characterized the mechanism by which mammalian tissue inhibits 1-OHase in the chick, we attempted to measure 1-OHase in rat kidney mitochondria using a 25-OHD\(_3 \) concentration of 500 nM, a substrate concentration at which we had shown inhibitory activity to be negligible in the chick 1-OHase.
preparation. However, by using the incubation conditions we had used with the chick preparation, yields of 1,25-(OH)$_2$D$_3$ were not proportional to rat mitochondrial protein concentrations or incubation time.

After several modifications of the chick method, it was found that incubation at 25°C gave higher rat 1-OHase activity and far less variability than at 37°C, the temperature previously used for chick studies. A temperature of 25°C was adopted for the mammalian assay.

To show that the inhibitory activity inherent in rat mitochondria did not affect the accuracy of the assay under the conditions finally adopted, we incubated rat mitochondria that had low or undetectable 1-OHase activity with chick kidney mitochondria. The results indicate that inhibitory activity due to the rat mitochondria was negligible at 25-OHD$_3$ concentrations > 50 nM (Fig. 2). $V_{max}$ and $K_m$ of chick 1-OHase were unaffected by the presence of rat kidney mitochondria under our incubation conditions.

It has previously been held that 1-OHase assays in the chick provide a true estimate of enzyme activity. However, when chick mitochondria were incubated alone (Figs. 1 and 2) $V$ versus $V/S$ deviated from the linear at low substrate concentrations. This suggests that even in chick preparations there exist high affinity, nonenzymic sites which bind 25-OHD$_3$ to a degree sufficient to result in a spuriously low value for 1-OHase activity.

Renal mitochondria from rats conditioned to have high 1-OHase or 24-OHase activities were incubated separately at 25°C with [3H]25-OHD$_3$ at 500 nM. The products of the incubations were identified by HPLC as 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$, respectively (Fig. 3). In addition, the products co-migrated with appropriate standards using continuous development TLC.

Further studies of both rat 1-OHase and rat 24-OHase validated our method for quantification of these enzymes (Fig. 4). The pH optimum was 7.4 for both rat 1-OHase and rat 24-OHase. The time course of product formation for both enzymes was linear over a 20-min incubation interval. Production of 1,25-(OH)$_2$D$_3$ or 24,25-(OH)$_2$D$_3$ was proportional to the mitochondrial protein concentration to at least 5 mg of protein/ml.

Five sets of $K_m$ determinations of rat 1-OHase were performed. For each determination, kidney mitochondria from two rats were pooled and all incubations were carried out in triplicate, data from one set of incubations are shown in Fig. 5. The mean apparent $K_m$ was 890 ± 170 nM ($X$ ± S.E.). Three sets of $K_m$ determinations of rat 24-OHase were performed; the mean apparent $K_m$ was 375 ± 16 nM (Fig. 6).

CV (S.D./$X$) for eight replicate determinations of 1-OHase activity of one mitochondrial preparation was 19% at a mean activity of 0.33 pmol (mg x min)$^{-1}$. CV for nine replicate determinations of 24-OHase in rat mitochondria was 17% at a mean activity of 0.42 pmol (mg x min)$^{-1}$. Between-animal CV for 1-OHase was 33%, calculated from means of duplicate determinations on each of 10 rats which had an overall mean activity of 0.81 pmol (mg x min)$^{-1}$.

The limit of detectability for both enzymes was approximately 0.01 pmol (mg x min)$^{-1}$; this was represented by approximately 20 cpm recovered as product from the TLC.

**DISCUSSION**

With these studies, we have overcome problems which have previously prevented quantitative measurement of 1-OHase and 24-OHase activities in mammals and we have defined certain basic characteristics of these enzymes.

The first problem was what appeared to be inhibition of 1-OHase activity by protein present in mammalian tissues (9, 10). We have shown this "inhibition" was most profound at very low substrate concentrations and that, unlike classical competitive and noncompetitive inhibitors, almost all the "inhibition" could be overcome at substrate concentrations approaching $K_m$ of chick 1-OHase. We have used Eadie plots, $V$ versus $V/S$, to analyze the data because the more conventional Lineweaver-Burke plot, $1/V$ versus $1/S$, tends to hide deviations from Michaelis-Menten kinetics (15). The Lineweaver-Burke plot places much weight on low substrate determinations so that points at high 1/$S$ and hence high 1/$V$ can introduce marked error to the slope of the plot without significantly affecting the $r$ value. The use of Eadie plots allowed us to reject points which did not fit Michaelis-Menten kinetics; thus, our $K_m$ and $V_{max}$ determinations were based on observations made at the higher substrate concentrations.

The evidence that 25-OHD binding protein is the inhibitor (10) justifies our procedure for rejecting points which curve away from the linear $V$ versus $V/S$ relationship because the binding of substrate by a non-enzyme site introduces an error which is overcome as substrate concentration increases (15). We believe that the downward curvature of the Eadie plots in the presence of rat tissues reflected lower effective 25-OHD$_3$ concentrations and, therefore, did not reflect the kinetics of 1-OHase. Consequently, points on portions of all Eadie plots that curved away from the linear were omitted from calcula-
tions. The downward curvature was present to a small extent in control incubations of chick mitochondria, indicating that high affinity, nonenzymatic binding of 25-OH D to occurs even in the chick system, a fact that has not been considered in previous studies.

In our rat incubation system, the mitochondrial protein concentration was maintained at or below 5 mg/ml and the 25-OH D concentration was maintained at 500 nM to minimize error due to nonenzymatic binding of substrate.

Since the effect of the "inhibitor" was overcome by the use of a high-substrate concentration, the ideal approach to the assay would have been to measure rat 1-OHase and 24-OHase by using high 25-OH D concentrations with which activities were near Vmax. However, this would have required methods which could measure very small amounts of 1,25-(OH)2D3 and 24,25-(OH)2D3 directly. Such methods were not available to us so that we were obliged to use radiolabeled substrate, incubation for a predetermined time interval, and chromatographic separation of substrate from products. Consequently, enzyme rate determinations were based on relative conversion to product rather than on absolute conversion. These conditions necessitated use of relatively low substrate concentrations so that the fraction converted to product was large enough to be measured reproducibly. These restrictions can be reconciled by the fact that, for fixed-time enzyme measurement, assays carried out at substrate concentrations below Km (pseudo-first order reactions) are preferable, in terms of accuracy, precision, and dynamic range, to assays conducted at substrate concentrations at which activities are near Vmax (16). This is because, in first order reactions, fractional conversion to product tends to be independent of small errors in substrate concentration. The apparent Km values for 1-OHase and 24-OHase in rat renal mitochondria were 890 and 275 nM 25-OH D, respectively. Theoretical considerations favor use of 25-OH D concentrations below Km (i.e. <375 nM). However, the importance of overcoming substrate sequestration necessitates the compromise concentration we adopted, namely 500 nM.

The incubation temperature is the other factor which has hindered development of an assay of 1-OHase activity in rats. Our attempts to validate rat 1-OHase assay methods at 37°C failed. When we tested the reaction at different temperatures it became apparent that rat 1-OHase was much less active at 37°C than at 25°C. The use of 25°C, which has been recommended by Bergmeyer as the standard temperature for all enzyme assays (17), produced much more reproducible and consistent results.

Vmax of rat 1-OHase was of similar magnitude to Vmax observed under identical conditions with chick tissue, 2.9 versus 5.5 pmol (mg X min)-1 (Figs. 2 and 5). The apparent Km for control preparations of chick mitochondrial 1-OHase was approximately 1/3 that observed for the rat, 235 versus 890 nM. Use of low substrate concentrations accentuates differences in activity due to differences in affinity; this is a further reason why estimates of rat 1-OHase activity have been much lower than those observed with chick tissue.

The extent to which the enzyme activities and kinetics presented above reflect what goes on in vivo cannot be known from information which is presently available. This is because it is not possible to define fully or reproduce in vivo conditions for an enzyme assay. For 1-OHase and 24-OHase, the attainment of physiological conditions in vivo is further complicated by the limited solubility of 25-OH D in aqueous systems, a fact that is emphasized by the wide use of protein "solubilizers" in 25-OH D binding-assay systems (11, 18). The microsomal or cytoplasmic binding proteins in the postmitochondrial supernatant, which we have shown to reduce 1-OHase activity in vitro, may in fact facilitate the action of the enzyme under in vivo conditions by delivering 25-OH D to the mitochondria.

It is tempting to speculate about the 3-fold difference in affinity between 1-OHase and 24-OHase in rat kidney mitochondria as indicated by the difference in apparent Km values. Enzyme activity can be controlled either by altering the total amount of enzyme (Vmax) or by changing the affinity of existing enzyme for substrate (Km). From a teleological point of view, the latter control mechanism would be very useful for an enzyme whose product has as potent an effect and as short a plasma half-life as has 1,25-(OH)2D3. Changing the Km of the 1-OHase could surely be carried out much more readily than either synthesis or breakdown of the enzyme. The high apparent Km of rat 1-OHase may reflect the lack of an allosteric activating factor in this in vitro system. Possible candidates for such a factor include Ca2+, cAMP, and the product of their interaction with other cytoplasmic or mitochondrial constituents.

Since the activities of 1-OHase and 24-OHase appear to be inversely related, it is important to study them simultaneously. Alternative methods of measuring product could not easily provide simultaneous assays and, thus, would provide only part of the information obtainable with the method described in this paper.

The availability of rat 1-OHase and 24-OHase assays makes it possible to extend to the mammal previous studies done in the chick. We have successfully applied our method to sheep, pig, and monkey. Information about the physiological regulation of these enzymes in mammals has been inferred from in vivo concentrations of their products but must still be verified by direct assay. These assays also make possible more detailed investigations into biochemical mechanisms which control the activities of 1-OHase and 24-OHase in mammals.

Acknowledgments—We wish to thank Dr. Glenville Jones, who carried out HPLC chromatography of our samples, and who with Dr. Brian Robinson and Dr. Sang Whay Kooh, provided helpful suggestions and interpretations of these studies, and Esther Rampsers for her secretarial assistance.

REFERENCES

5 The plasma half-life of [H1]25-(OH)2D3 in lambs is 1 h and that of [H2]25,26-(OH)2D3 and [H3]25-(OH)2D3, is approximately 10 h (R. Vieth and W. S. Kooh, unpublished observations).
Kinetic behavior of 25-hydroxyvitamin D-1-hydroxylase and -24-hydroxylase in rat kidney mitochondria.
R Vieth and D Fraser