Decarboxylation of Ornithine and Lysine by Ornithine Decarboxylase from Kidneys of Testosterone Treated Mice

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Induction of lysine decarboxylase activity in the kidneys of testosterone treated mice was shown to be linearly correlated with the induction of ornithine decarboxylase activity. A 2500-fold purification of ornithine decarboxylase, resulting in a single protein band on polyacrylamide gel electrophoresis, did not bring about a separation of lysine and ornithine decarboxylase activities. The purified enzyme exhibited considerably higher specific activity than previously reported for ornithine decarboxylase purified from rat liver. Ornithine and lysine decarboxylase activities were inhibited to the same extent by $\alpha$-difluoromethylornithine (DFMO*), an enzyme-activated irreversible inhibitor of ornithine decarboxylase. Administration of DFMO to mice suppressed the testosterone stimulated increase in renal ornithine and lysine decarboxylase activities. Furthermore, the elevated renal and urinary levels of the diamines putrescine and cadaverine, observed after testosterone treatment, were prevented by the administration of DFMO. These observations are consistent with the view that ornithine decarboxylase from mouse kidney is not specific for $l$-ornithine but is capable of decarboxylating $l$-lysine as well.

The biological significance of the diamine putrescine and the polyamines spermidine and spermine has been thoroughly investigated in a variety of mammalian systems (for a review see Ref. 1). On the other hand, the knowledge of the diamine cadaverine is limited to a few reports. Caldarera et al. showed that the concentration of cadaverine was increased in the chick embryo during development. Cadaverine has also been reported to occur in the brain of axenic mice and in the urine from pregnant rats. A mammalian lysine decarboxylating activity, catalyzing the biosynthesis of cadaverine, was first found in the mouse kidney stimulated to growth by an anabolic steroid. It was later shown that the brain of mice and chickens during development, the placenta and ovary from pregnant rats and the ovary from rats treated with human chorionic gonadotrophin also contained lysine decarboxylating activity.

Since ornithine and lysine are structurally similar and since the kidney of mice treated with anabolic steroids is extremely rich in ornithine decarboxylase activity (EC 4.1.1.17) the question arose whether decarboxylation of lysine is due to the action of ornithine decarboxylase. Investigation of the kinetic properties of the lysine decarboxylating activity indicated that cadaverine production in these kidneys was mediated via the decarboxylation of lysine by ornithine decarboxylase. Recently Pegg and McGill confirmed and extended this hypothesis to the rat liver. Purification of liver ornithine decarboxylase did not bring about a separation of ornithine and lysine decarboxylase activities. Furthermore, kinetic studies of the highly purified ornithine decarboxylase preparation provided convincing evidence that these activities were derived from the same enzyme.

However, Alhonen-Hongisto and Jänne showed that exposure of cultured Ehrlich ascites carcinoma cells to the enzyme-activated irreversible inhibitor of ornithine decarboxylase, $\alpha$-difluoromethylornithine, resulted in increased cellular content of cadaverine. Even with inhibited ornithine decarboxylase a synthesis of cadaverine from lysine occurred in these cells. Although the lysine de-

* Abbreviations: DFMO, $\alpha$-difluoromethylornithine; ODC, ornithine decarboxylase; LDC, lysine decarboxylase.
carboxylating activity was detected only in washed whole cells but not in cell free extracts, the finding indicates that there could be other enzymes, than ornithine decarboxylase, involved in the biosynthesis of mammalian cadaverine.

Administration of testosterone propionate to mice results in a very high activity of renal ornithine decarboxylase. In the present study, the hypothesis of decarboxylation of lysine catalyzed by ornithine decarboxylase was investigated in the kidney of mice treated with testosterone propionate. Ornithine and lysine decarboxylating activities were followed during the purification of ornithine decarboxylase. The effects of DL-α-difluoromethylornithine on the two decarboxylating activities in fractions of highly purified ornithine decarboxylase were also studied. Further, the effect of the inhibitor on the in vivo synthesis of cadaverine was examined.

EXPERIMENTAL

Materials. L-[1-14C]Ornithine (2.11 TBq/mol) and DL-[1-14C]lysine (925 GBq/mol) were purchased from the Radiochemical Centre, Amersham, U.K. Testosterone propionate, dithiothreitol and pyridoxamine 5'-phosphate were obtained from Sigma and Sephadex G-150 Superfine and DEAE-Sephadex A-50 were bought from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Affi-Gel 10 was obtained from Bio-Rad. DL-α-Difluoromethylornithine was a generous gift from Centre de Recherche Merrell International, Strasbourg (France).

Treatment of animals. Male mice of the NMRI strain were used. They were fed a standard pellet diet and had water ad libitum, except when urine was collected, in which case a partly synthetic diet was used. Gonadectomy was performed at the age of 8 weeks. The mice were used for experiments four weeks later. Testosterone propionate, suspended in 50 μl of arachis oil, was administered subcutaneously in a dose of 200 μg per day. DL-α-Difluoromethylornithine was administered intraperitoneally in a dose of 10 mg/mouse every 8 h. Controls received vehicles only.

Preparation of tissue extracts. Mice were stunned and exsanguinated. The kidneys were rapidly removed and dissected free of capsules. For preparation of enzyme extracts, kidneys were homogenized in 7 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA and 5×10^{-4} M dithiothreitol. The homogenate was centrifuged at 20,000×g for 20 min at 4°C. The supernatant was used as the source of enzyme.

Extracts for quantitative determination of di-amines were prepared by homogenizing the kidneys in 14 volumes of a solution of 4% sulfosalicylic acid and 0.04% EDTA. The pH of the sample was adjusted to 2.0–2.5 with NaOH and the extract was filtered (0.22 μm pore size, Millipore, Bedford, MA). Urine was collected and prepared for analysis as described earlier.

Assay for ornithine and lysine decarboxylating activities were made by determining the release of 14CO2 from carbonyl-labeled ornithine and lysine. The enzyme extracts were incubated with 1×10^{-5} M pyridoxal-5-phosphate, and 5×10^{-4} M 1-14C-ornithine (specific activity 3.7 MBq/mmol) or 1×10^{-2} M 1-14C-lysine (specific activity 925 kBq/mmol) in a total volume of 1.0 ml of the phosphate buffer used for the preparation of the enzyme extracts (see above). When purified enzyme was used 0.1 mg of bovine serum albumin was added to stabilize the enzyme. After incubation for 30 or 60 min the reactions were terminated by adding 0.5 ml of 2 M HClO4. The expelled 14CO2 was trapped into 100 μl of hydroxide of Hyamine 10-X on a 10×25 mm piece of No. 005 Munktell filter paper. Maximal absorption of 14CO2 was achieved by continued shaking for additional 45 min. The filter paper was then placed in a vial containing 8 ml Lipoluma liquid scintillation mixture and the radioactivity was measured in a Contron liquid scintillation spectrometer. All values obtained were corrected against a reaction mixture without enzyme.

Purification of ornithine decarboxylase. Normal male mice were injected with testosterone propionate for 3–7 days and the kidneys were used for purification of ornithine decarboxylase. The kidneys were homogenized in 3 volumes of cold 0.01 M phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA, 5×10^{-4} M dithiothreitol and 0.25 M sucrose. The homogenate was centrifuged as described above. The supernatant (crude extract) was adjusted to pH 4.6 by addition of chilled 2 M acetic acid as described by Ono et al. The mixture was then centrifuged at 20,000×g for 10 min whereupon the sediment was suspended and homogenized in a small volume of 0.1 M sodium phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA and 5×10^{-4} M dithiothreitol (buffer A). The suspension was dialyzed at 1°C overnight against 2×2.5 l of buffer A containing 1×10^{-4} M ornithine (to stabilize the enzyme). The suspension was then centrifuged at 30,000×g for 20 min. The sediment was washed with a small volume of buffer A containing 1×10^{-4} M ornithine and centrifuged. The supernatants were combined and applied to a Sephadex G-150 Superfine column (2.5×90 cm) equilibrated with the same buffer as used for dialysis. The enzyme was eluted with this buffer at a flow rate.

of 2.5–3.0 ml/h and fractions of 5–6 ml were collected for enzyme assay. Fractions with high ornithine and lysine decarboxylating activities were pooled and dialyzed at 1 °C overnight against 2 × 2.5 l of 25 mM Tris-HCl (pH 7.2), 1 × 10⁻⁴ M EDTA, 5 × 10⁻⁴ M dithiothreitol, 1 × 10⁻⁴ M ornithine and 0.1 M NaCl (buffer B). The enzyme solution was then applied to a DEAE-Sephadex column (1.5 × 20 cm) equilibrated with buffer B. The column was washed with 10 ml of buffer B and then eluted with a linear gradient (350 ml) of 0.1 M to 0.5 M NaCl in this buffer. The eluate was collected in 7.5 ml fractions at a flow rate of 15 ml/h. The fractions with high enzyme activities were pooled and dialyzed at 1 °C overnight against 2 × 2.5 l of buffer B without NaCl and ornithine (buffer C). The last step in the purification procedure consisted of an affinity chromatography step, essentially as described by Boucek et al. Pyridoxamine 5-phosphate was coupled with activated agarose matrix (Affi-Gel 10) according to the directions provided by the supplier. The column (1.5 × 16 cm) was then equilibrated with several volumes of buffer C. The dialyzed enzyme solution was applied to the column at a flow rate of 8 ml/h and then the column was washed with 65 ml of buffer C containing 15 mM NaCl. The enzyme was eluted and collected in 8 ml fractions with the same buffer containing 1 × 10⁻³ M pyridoxal-5-phosphate.

Disc electrophoresis on polyacrylamide gels was performed with 7.5 % separating gels (pH 9.2) and 4 % stacking gels (pH 6.1) at 3 mA/gel for 1 h at 4 °C, mainly as described by Davis. The gels were stained for protein with 0.1 % Coomassie brilliant blue.

Quantitative determination of diamines and protein. Chromatographic separation and quantitative estimation of putrescine and cadaverine in the kidneys and the urine were carried out by high performance liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL 3201. Protein was measured mainly by the method of Lowry et al. with bovine serum albumin as standard. Since dithiothreitol interfered with this method a commercially available protein assay kit obtained from Bio-Rad was also used.

RESULTS

Ornithine and lysine decarboxylating activities in the mouse kidney after treatment with testosterone propionate. If decarboxylation of lysine is carried out by ornithine decarboxylase, a linear correlation between the two activities should be expected. In order to test this assumption, castrated mice were treated with testosterone propionate for different times, whereupon ornithine and lysine decarboxylating activities in the kidneys were determined. As seen in Fig. 1 lysine decarboxylating activity was directly proportional to ornithine decarboxylase activity. In agreement with kinetic results presented earlier the ornithine decarboxylase activity was in magnitude much greater than the lysine decarboxylating activity. Hence these results support the theory of a common enzyme responsible for decarboxylation of both ornithine and lysine.

Purification of ornithine decarboxylase. The purification of ornithine decarboxylase contributed even
Table 1. Purification of ornithine decarboxylase from testosterone propionate stimulated mouse kidney.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Enzymatic activity</th>
<th>Total protein (mg)</th>
<th>Total activity (μmol/h)</th>
<th>Specific activity [nmol/(mg protein h)]</th>
<th>Purification (-fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 20,000 x g Supernatant</td>
<td>ODC</td>
<td>3861</td>
<td>1072</td>
<td>278</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LDC</td>
<td>132</td>
<td>34</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Acid treatment (pH 4.6)</td>
<td>ODC</td>
<td>960</td>
<td>695</td>
<td>724</td>
<td>2.6</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>LDC</td>
<td>86</td>
<td>90</td>
<td>2.7</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>3. Sephadex G-150 Superfine</td>
<td>ODC</td>
<td>190.3</td>
<td>574</td>
<td>3016</td>
<td>10.8</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>LDC</td>
<td>70</td>
<td>368</td>
<td>10.8</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>4. DEAE-Sephadex</td>
<td>ODC</td>
<td>5.02</td>
<td>368</td>
<td>73307</td>
<td>264</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>LDC</td>
<td>45</td>
<td>8964</td>
<td>264</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>5. Affinity chromatography</td>
<td>ODC</td>
<td>0.338</td>
<td>242</td>
<td>714296</td>
<td>2569</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>LDC</td>
<td>29</td>
<td>85478</td>
<td>2514</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

more to the evidence that ornithine decarboxylase could be capable of decarboxylating lysine. As shown in Table 1, the purification of ornithine decarboxylase was 2500-fold and yet it did not bring about a separation of ornithine and lysine decarboxylase activities. The ratio between these two enzyme activities was constant during each step in the purification procedure (Table 1). Fig. 2 shows the elution profiles of the individual chromatographic steps employed in the purification of ornithine decarboxylase. As seen in this figure the elutions of both the decarboxylating activities were very similar in all three steps.

Disc gel electrophoresis of the purified enzyme revealed a single major protein band (Fig. 3). Unfortunately the method resulted in total loss of enzymatic activities and thus the protein band could not be shown to be identical with ornithine decarboxylase. Although crude extracts from kidneys containing ornithine decarboxylase of lesser specific activity were used for purification, the final specific activity obtained after purification was comparable to that obtained with crude extracts with higher enzyme activity (Table 2).

The purified ornithine decarboxylase was extremely labile (Fig. 4). Incubation at 37 °C of enzyme obtained after affinity chromatography, resulted in a loss of more than 65 % of the enzymatic activities within 5 min. However, addition of 100 μg of bovine serum albumin to the incubate

Table 2. Four different purifications of ornithine decarboxylase from kidneys containing different amounts of enzyme activity. Crude extracts were obtained from kidneys of mice treated with testosterone propionate for 3 (Nos. 2 and 4) or 7 (Nos. 1 and 3) days.

<table>
<thead>
<tr>
<th>Purification No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity of crude extract [nmol/(mg protein h)]</td>
<td>278</td>
<td>150</td>
<td>272</td>
<td>123</td>
</tr>
<tr>
<td>Specific activity of purified enzyme [nmol/mg protein h]</td>
<td>714 296</td>
<td>665 723</td>
<td>629 064</td>
<td>712 036</td>
</tr>
<tr>
<td>Relative purification (-fold)</td>
<td>2 569</td>
<td>4 438</td>
<td>2 313</td>
<td>5 789</td>
</tr>
</tbody>
</table>

Fig. 2. Elution profiles of ornithine decarboxylase activity (△), lysine decarboxylase activity (○) and proteins (●) after gel-filtration (A), ion exchange chromatography (B) and affinity chromatography (C). Arrow indicates the addition of $1 \times 10^{-3}$ M pyridoxal-5-phosphate to the buffer.

stabilized the enzyme. Hence, albumin was routinely added to the assay mixtures. As also shown in Fig. 4 there was an identical decrease in the two decarboxylating activities, when incubated at 37 °C without addition of external protein. This finding further supports the view of a single protein responsible for ornithine and lysine decarboxylating activities. It should be mentioned that the purified enzyme was much more stable when stored at 1 °C, losing only a few per cent of the activity during the first week.

The newly discovered enzyme-activated irreversible inhibitor of ornithine decarboxylase, N-α-difluoromethylornithine, was employed in the present study to further confirm the theory of a single enzyme catalyzing the decarboxylation of both ornithine and lysine. Purified ornithine decarboxylase obtained after affinity chromatography was preincubated with different concentrations of the inhibitor for 30 min, whereupon ornithine and lysine decarboxylase activities were measured. As seen in Fig. 5, DFMO at various concentrations was shown to inhibit ornithine decarboxylase activity and lysine decarboxylase activity to the same extent. This implies that ornithine decarboxylase is involved in the decarboxylation of lysine.

Inhibition of in vivo synthesis of putrescine and cadaverine. The results presented so far undoubtedly support the hypothesis of a single enzyme responsible for the synthesis of both putrescine and cadaverine in the mouse kidney. Putrescine and

Fig. 3. Polyacrylamide gel electrophoresis of purified enzyme (5 μg).

Fig. 4. Time course of inactivation of ornithine (●) and lysine (○) decarboxylase activities when preincubated at 37 °C without albumin. Albumin present during the preincubation (■, □). Each value represents the mean ± S.E. of the mean, n = 10.
Fig. 5. Effect of DL-α-difluoromethylornithine on ornithine (●) and lysine (○) decarboxylase activities. Enzyme aliquots were preincubated at 37 °C with various concentrations of the inhibitor. Each value represents the mean ± S.E. of the mean, n = 4.

cadaverine contents have been shown to be increased in the kidneys and urine from castrated mice after treatment with testosterone propionate. Hence it would be most interesting to investigate if the biosynthesis of cadaverine could be blocked by the administration of DFMO. Thus, castrated mice were treated with testosterone propionate for 3 days and urine was collected and determined for diamines during the last 24 h. Half of the mice receiving testosterone propionate were given 10 mg of the inhibitor 8 h before the onset of urine collection and then every 8 h onward. As seen in Table 3, the increase in urinary putrescine after testosterone propionate treatment was nearly abolished when the mice were given the inhibitor. DFMO also totally prevented the elevation in urinary excretion of cadaverine induced by androgen treatment (Table 3).

The kidneys of the mice, from which urine was collected, were also examined for ornithine and lysine decarboxylase activities as well as for content of diamines (Table 3). The administration of DFMO resulted in a nearly completely restriction of the testosterone propionate stimulated increase in renal ornithine decarboxylase activity. The inhibitor was equally effective in suppressing the elevated renal

Table 3. Effect of DL-α-difluoromethylornithine (DFMO) on renal ornithine and lysine decarboxylase activities, renal concentrations of putrescine and cadaverine, and on urinary excretion of putrescine and cadaverine. Each value represents the mean ± S.E. of the mean, n = 5. N.D., not detectable.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC [nmol/(mg h)]</th>
<th>LDC [nmol/(mg h)]</th>
<th>Kidneys</th>
<th>Putrescine (nmol/g)</th>
<th>Cadaverine (nmol/g)</th>
<th>Urine</th>
<th>Putrescine (nmol/24 h)</th>
<th>Cadaverine (nmol/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>137 ± 39.5</td>
<td>13.9 ± 4.13</td>
<td></td>
<td>17.3 ± 1.59</td>
<td>N.D.</td>
<td></td>
<td>295 ± 14.9</td>
<td>13.4 ± 1.70</td>
</tr>
<tr>
<td>Testosterone + DFMO</td>
<td>2.4 ± 0.44</td>
<td>N.D.</td>
<td></td>
<td>32.8 ± 3.91</td>
<td>N.D.</td>
<td></td>
<td>600 ± 112.9</td>
<td>13.0 ± 2.73</td>
</tr>
</tbody>
</table>

lysin decarboxylating activity observed after androgen treatment. As to the contents of putrescine and cadaverine in the kidneys after steroid treatment, DFM0 was shown to inhibit the expected increase in the concentrations of both these diamines.

The above results strongly indicate that the in vivo synthesis of cadaverine seen in mice after treatment with testosterone propionate is solely due to the activity of the enzyme ornithine decarboxylase.

DISCUSSION

The present results strongly confirm the view of a mammalian ornithine decarboxylase as not specific for L-ornithine, but for decarboxylating L-lysine as well. However, as shown in previous reports, the affinity of the enzyme for L-lysine is much lower than for L-ornithine. This would explain the contradictory results in earlier reports, in which the addition of 10 mM lysine had no effect on the decarboxylation of ornithine. The concentrations of ornithine in the assays for ornithine decarboxylase was, in these studies, well above saturating levels. No effect is to be expected by the addition of 10 mM lysine, on the decarboxylation of ornithine, if ornithine is present in such high concentrations, since the Km for lysine is as great as 10 mM.

Thus, ornithine decarboxylase will in vivo favour ornithine as a substrate as long as this amino acid is available in significant amounts. However, if lysine is present in considerably higher concentrations than ornithine, the decarboxylation of lysine would be significant and cadaverine could thus be detected. The finding that treatment with the irreversible specific inhibitor of ornithine decarboxylase, D,L-α-difluoromethylornithine, resulted in a total suppression of urinary cadaverine, indicates that all cadaverine excreted in the urine of testosterone propionate treated mice is synthesized by the action of ornithine decarboxylase. During the preparation of the present work, Pegg and McGill reported that ornithine decarboxylase from rat liver was also capable of decarboxylating lysine to cadaverine. None the less, it remains to be established whether ornithine decarboxylases from any mammalian tissue are capable of decarboxylating lysine, or if enzyme forms exist that are specific for ornithine.

The purification of ornithine decarboxylase in the present study was 2500-fold with a recovery of more than 20%. Polyacrylamide gel electrophoresis of the purified enzyme revealed a single major protein band. Together with the finding that the specific activity of the purified enzyme was the same although crude extracts of much lower activity was used for purification, it indicates that the purification resulted in a highly purified ornithine decarboxylase. The extreme lability of the purified enzyme is the same as that reported for ornithine decarboxylase purified from simian virus 40-transformed 3T3 mouse fibroblasts, which likewise was stabilized by the addition of albumin. Hitherto, this lability has not been reported for purified ornithine decarboxylase from other mammalian tissues. The purification of mouse kidney ornithine decarboxylase did not reveal any multiple forms of the enzyme as described for ornithine decarboxylase purified from rat liver.

The specific activity of the purified enzyme is the highest ever reported for purified ornithine decarboxylase. Purification of ornithine decarboxylase from simian virus 40-transformed 3T3 mouse fibroblasts and from rat liver resulted in enzymes with specific activity 7-fold and 50-fold, respectively, lower than that reported in the present study. These variations in specific activity are most probably not due to differences in the degree of purification, since analytical gel electrophoresis, in these studies, revealed only a single major protein band. More likely there are intrinsic variations in the ornithine decarboxylase protein, which give rise to enzyme units with great differences in the ability to decarboxylate ornithine. To clarify the significance of the finding, further work appears relevant.

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