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Investigation of Structure and Rate of Synthesis of Ornithine Decarboxylase Protein in Mouse Kidney†

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ABSTRACT: An immunoblotting technique was used to study the forms of ornithine decarboxylase present in androgen-induced mouse kidney. Two forms were detected which differed slightly in isoelectric point but not in subunit molecular weight (~55 000). Both forms were enzymatically active and could be labeled by reaction with radioactive α-(difluoromethyl)-ornithine, an enzyme-activated irreversible inhibitor. On storage of crude kidney homogenates or partially purified preparations of ornithine decarboxylase, the enzyme protein was degraded to a smaller size (M_r ~53 000) without substantial loss of enzyme activity. The synthesis and degradation of ornithine decarboxylase protein were studied by labeling the protein by intraperitoneal injection of [^{35}S]methionine and immunoprecipitation using both monoclonal and polyclonal antibodies. The fraction of total protein synthesis represented by renal ornithine decarboxylase was increased at least 25-fold by testosterone treatment of female mice and was found to be about 1.1% in the fully induced androgen-treated female. Both forms of the enzyme were rapidly labeled in vivo, and the immunoprecipitable ornithine decarboxylase protein was almost completely lost after 4-h exposure to cycloheximide, confirming directly the very rapid turnover of this enzyme. Treatment with 1,3-diaminopropane which is known to cause a great reduction in ornithine decarboxylase activity did not greatly selectively inhibit the synthesis of the enzyme. However, 1,3-diaminopropane did produce an increase in the rate of degradation of ornithine decarboxylase and a general reduction in protein synthesis. These two factors, therefore, appear to be responsible for the loss of ornithine decarboxylase activity and protein in response to 1,3-diaminopropane.

There have been many studies of ornithine decarboxylase in mammalian cells because of the remarkable inducibility of the activity of this enzyme which catalyzes the first step in the polyamine biosynthetic pathway (Jänne et al., 1978; Russell, 1980; McCann, 1980; Pegg & McCann, 1982). Until recently, most of these investigations were limited to measurements of enzyme activity because of the small amount of ornithine decarboxylase protein present in mammalian cells even after maximal induction and the insensitivity or unavailability of methods for studying the protein itself. Therefore, although a number of groups have proposed that there are multiple forms of this enzyme or that it may be a substrate for regulatory posttranslational modifications (Richards et al., 1981; Kuehn & Atmar, 1982; Mitchell & Mitchell, 1982; Russell, 1983; Bullock et al., 1983), definitive experiments to test these possibilities have not been carried out. Also, there is solid evidence based on the use of immunoaffinity chromatography that yeast ornithine decarboxylase is present in the cell as a larger protein than that obtained after purification owing to a rapid proteolytic cleavage which occurs in crude cell extracts (Tyagi et al., 1982). In the present experiments, monospecific antibodies to mouse ornithine decarboxylase have been used to examine the size and possible existence of multiple forms of this protein in the kidney. Androgen treatment was used to induce a high level of the enzyme in this organ (Henningsson et al., 1978; Seely et al., 1982a,b; Isomaa et al., 1983).

A variety of approaches have been used to demonstrate that ornithine decarboxylase has a rapid rate of turnover in most mammalian cells (Russell, 1980; McCann, 1980; Seely et al., 1982c), but the identification of a rapidly labeled polypeptide corresponding to ornithine decarboxylase has so far been accomplished only with variant mouse cell lines (McConlogue & Coffino, 1983a,b). These cells were selected for resistance to an ornithine decarboxylase inhibitor and greatly overproduce the enzyme, but it is not known how similar the overproduced enzyme is to that found in normal cells. We have, therefore, studied the labeling of mouse kidney ornithine decarboxylase by administration of [^{35}S]methionine and determined the synthesis rate of this enzyme and demonstrated its rapid degradation.

It is well-known that ornithine decarboxylase activity is lost rapidly after treatment with exogenous diamines including 1,3-diaminopropane (Pösö et al., 1978; Pegg et al., 1978; Canellakis et al., 1979; McCann, 1980). The mechanism by which this decrease is brought about is not clear. A protein which binds to ornithine decarboxylase and inhibits its activity appears to be induced or released in response to 1,3-diaminopropane (Canellakis et al., 1979), but radioimmunoassay techniques which can detect the complex between this protein and the decarboxylase failed to reveal substantial quantities of such an inactive complex (Seely & Pegg, 1983a,b). In these experiments, there was a substantial loss of total immunoreactive ornithine decarboxylase protein in response to 1,3-diaminopropane, suggesting that the regulation of enzyme

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activity is exerted at the level of synthesis and degradation. We have, therefore, measured the rates of synthesis and degradation of the enzyme in order to test these possibilities.

Materials and Methods

Materials. DL-[5-^{3}H]-α-(Difluoromethyl)ornithine (15.5 Ci/mmol), L-[1-^{14}C]ornithine (57 mCi/mmol), and L-[^{35}S]-methionine (1050 Ci/mmol) were purchased from New England Nuclear, Boston, MA. DL-[5-^{14}C]-α-(Difluoromethyl)ornithine (60 mCi/mmol) was synthesized by Amersham Searle, Arlington Heights, IL. Unlabeled α-(difluoromethyl)ornithine (DFMO)[1] was a generous gift from Merrell-Dow, Cincinnati, OH. Protein A bacterial adsorbent was from Miles Laboratories, Elkhart, IN. Goat anti-mouse IgM and goat anti-rabbit IgG were purchased from Cappel, Cochranville, PA, and reconstituted according to the manufacturer's instructions. Nitrocellulose paper, peroxidase-coupled goat anti-rabbit IgG, and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories, Richmond, CA. All other biochemical reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Animals. Female BALB/c mice (8–12 weeks old) were obtained from Charles River Breeding Laboratories, Wilmington, MA, and were housed under 12-h light/12-h dark lighting conditions. Testosterone propionate was given at a dose of 100 mg/kg by subcutaneous injection of a solution of 4 mg/mL in sesame oil at 4 days and again at 3 days prior to sacrifice. L-[^{35}S]Methionine (1050 Ci/mmol) was injected intraperitoneally at a dosage of 500 μCi per animal. DL-[5-^{14}C]DFMO was injected at a dose of 1 mg/kg (approximately 7 μCi per mouse) by intraperitoneal injection. Cycloheximide was administered at a dose of 20 mg/kg by intraperitoneal injection. 1,3-Diaminopropane was administered at a dose of 2 mmol/kg. All compounds were injected in a phosphate-buffered saline vehicle except where indicated.

Preparation of Tissue Extracts. Mice were killed by cervical dislocation and kidneys removed and homogenized in 4 volumes of 25 mM Tris-HCl, pH 7.5, containing 2.5 mM dithiothreitol and 0.1 mM EDTA (ODC buffer). Homogenates were centrifuged at 100000g for 45 min and the supernatants saved.

Immunoprecipitation of ^{35}S-Labeled Extracts. Animals were treated with [^{35}S]methionine, and kidney extracts were prepared as described above. The extracts were then incubated with either one of two different polyclonal antibodies: "A" prepared as described by Persson (1982) or "B" prepared as described by Seely & Pegg (1983a); or a monoclonal antibody "C" (Pegg et al., 1984) against ornithine decarboxylase. The samples were then processed according to the antiserum used as follows. In experiment A, 50 μL of the extracts (containing 0.3–0.5 mg of protein) was incubated with 1 μL of the antiserum in a total volume of 200 μL of ODC buffer for 90 min at room temperature. Thereafter, 10 μL of goat anti-rabbit IgG was added and incubated for an additional 90 min at room temperature. Then, 0.5 mL of ODC buffer was added, and the samples were centrifuged at 15000g for 5 min. The precipitate was washed in 2× 0.5 mL of ODC buffer. The immunoprecipitates were then solubilized in 0.5 mL of NCS (Amersham/Searle, Arlington Heights, IL) and counted in 10 mL of Formula 949 liquid scintillation solution (New England Nuclear, Boston, MA). In experiment B, 50 μL of the extract was incubated with 10 μL of the antiserum and 150 μL of 150 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.02% Brij 35 (RIA buffer) for 8 h at 4 °C. One hundred microliters of bacterial protein A adsorbent was then added for 2 h at room temperature, the immunocomplex was precipitated by centrifugation at 15000g for 30 s, and the immunoprecipitate was washed and recentrifuged 7 times in ODC buffer, resuspended in 200 μL of water, and counted in 10 mL of ACS II liquid scintillation fluid (New England Nuclear, Boston, MA). Alternatively, the washed immunoprecipitates from experiments A and B were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis. In experiment C, aliquots of the extract were also immunoprecipitated with a monoclonal antibody (Pegg et al., 1984) and run on $NaDodSO_4$–polyacrylamide gel electrophoresis. Fifty microliters of the labeled extract was incubated with a 1:50 dilution of the monoclonal antibody in RIA buffer for 3 h at 4 °C. Thirty microliters of a solution of goat anti-mouse IgM was then added and incubation continued overnight at 4 °C. After 16 h, bacterial protein A adsorbent was added and the sample incubated with shaking at room temperature for 2 h. The mixture was diluted by the addition of 0.75 mL of RIA buffer and centrifuged at 15000g for 30 s. The pellet was washed 4 times in the RIA buffer and prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis as described below. In experiments A and B, controls were set up in which a normal rabbit serum was used. In experiment C, the control was set up by using another monoclonal antibody not directed against ornithine decarboxylase.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out on 10% gels by using the discontinuous buffer system of Laemmli (1970). Samples were heated to 100 °C for 3 min in 2% $NaDodSO_4$, 5% β-mercaptoethanol, and 10% glycerol in 62.5 mM Tris-HCl, pH 6.8. Phosphorylase b (M_r 97 000), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000), and cytochrome c (M_r 12 500) were used for molecular weight determination. Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell (1975). The second dimension consisted of a separation on a 10% $NaDodSO_4$–polyacrylamide gel. After electrophoresis, gels were processed for either autoradiography or immunoblotting. For autoradiography, the gels were fixed overnight in 10% trichloroacetic acid, 10% acetic acid, and 30% methanol, then impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed to Kodak XAR-5 X-ray film at −70 °C.

Immunoblotting. After separation by $NaDodSO_4$–polyacrylamide gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper essentially by the method of Towbin et al. (1979). The transfer was performed in 25 mM Tris-HCl, 0.19 M glycine, 0.1% $NaDodSO_4$, and 20% methanol at pH 8.3 for 3 h at 60 V. Remaining unbound sites on the nitrocellulose paper were blocked by incubating the paper in 3% gelatin in TTBS (20 mM Tris-HCl, 0.5 M NaCl, and 0.05% Tween-20, pH 7.5) for 60 min. All incubations were performed at room temperature. The paper was then incubated overnight with a specific antiserum against ornithine decarboxylase (usually antiserum A, but B also revealed the same bands) or with a control rabbit serum diluted 1:2000 with TTBS containing 1% gelatin. After 3× 10-min washes in TTBS, the paper was incubated for 1 h with a 1:2000 dilution of peroxidase-coupled goat anti-rabbit IgG in TTBS containing 1% gelatin. After the paper was washed in TTBS for 3 × 10 min, the bound antibodies were visualized by in-

[1] Abbreviations: DFMO, α-(difluoromethyl)ornithine; $NaDodSO_4$, sodium dodecyl sulfate; Ig, immunoglobulin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Immunoprecipitation of Ornithine Decarboxylase[a]

antiserum added	ornithine decarboxylase act. (nmol of CO_2/30 min)
none	82.6 ● 3.0
control serum	79.3 ● 4.1
anti-ornithine decarboxylase serum	<0.1

[a] Extracts from mouse kidneys were prepared and incubated with 1 μL of serum either from control rabbits or from a rabbit immunized with ornithine decarboxylase. After incubation at room temperature for 90 min, 10 μL of goat anti-rabbit IgG was added, and a further 90-min incubation at room temperature was carried out. The tubes were then centrifuged at 15000*g* for 5 min, and the supernatant was assayed for ornithine decarboxylase activity. The mean of three separate estimations is given.

cubation for 20 min with 4-chloro-1-naphthol (60 mg/120 mL) and 0.015% hydrogen peroxide in 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. Blots containing molecular weight markers were stained with 0.1% amido black in 45% methanol–10% acetic acid and destained with 90% methanol–2% acetic acid.

Determination of the Absolute Rate of Protein Synthesis. Methionine incorporation into renal protein was determined as described by Hutson & Mortimore (1982) by measuring the incorporation of [^{35}S]methionine into protein after several different doses of methionine and extrapolating to an infinite dose to allow for pool sizes. Androgen-treated mice were injected with 25, 50, or 125 μCi of L-[^{35}S]methionine (1.25 Ci/mol) and sacrificed after 20 min.

Miscellaneous Methods. The rate of degradation of the in vivo [^{14}C]DFMO-labeled enzyme was determined essentially as described by Seely et al. (1982a) with minor modifications. Androgen-treated mice were injected with [^{14}C]DFMO as described above and 30 min later given a dose of 100 mg/kg of unlabeled DFMO to prevent further binding of the labeled drug. Animals were sacrificed at various times later (see Table III) and the kidney extracts prepared as described above. The labeled enzyme was precipitated and washed 3 times in 1 N perchloric acid and the precipitate solubilized in 0.5 mL of NCS solubilizer and counted in 10 mL of Formula 949 scintillation fluid. In experiments where the rate of ornithine decarboxylase degradation was determined in the presence of 1,3-diaminopropane, 2 mmol/kg of 1,3-diaminopropane was administered at the same time as the unlabeled DFMO chase. In vitro labeling of ornithine decarboxylase with either [^{14}C]DFMO or [^{3}H]DFMO was carried out as described by Seely et al. (1982b).

The amount of ornithine decarboxylase protein was determined by radioimmunoassay (Seely & Pegg, 1983a). Ornithine decarboxylase activity was determined by measuring $^{14}CO_2$ production from L-[1-^{14}C]ornithine (Seely et al., 1983a). One unit of activity corresponds to the release of 1 nmol of CO_2 in 30 min. Protein was determined by the method of Bradford (1970) using bovine serum albumin as standard.

Results

Rabbit antiserum produced against homogeneous mouse kidney ornithine decarboxylase was able to precipitate all of the enzyme activity present in mouse kidney homogenates (Table I). This indicates that all of the protein(s) possessing ornithine decarboxylase activity in these extracts interact(s) with the antiserum. Kidney extracts were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and the proteins transferred to nitrocellulose paper by electrophoretic transfer. The papers were then examined for the presence of immunoreactive protein by immunoblotting techniques (Figures 1 and 2). As shown in Figure 1, extracts from female mice treated with testosterone contained one major band which reacted with the antibody, and this had an apparent molecular weight of about 55 000. This band was absent in extracts from untreated female mice which contained much less ornithine decarboxylase. It was not observed when a control rabbit antiserum not directed against ornithine decarboxylase was used. The band corresponds in molecular weight to that previously found for mouse kidney ornithine decarboxylase subunits and to markers of authentic ornithine decarboxylase (see below). These observations provide convincing evidence that this band is ornithine decarboxylase and that most (90% at least) of the enzyme protein in the androgen-stimulated kidney has this molecular weight. However, it can be seen from Figure 1 that the marker preparation of partially purified ornithine decarboxylase, which had been stored frozen at −20 °C for some time, did not

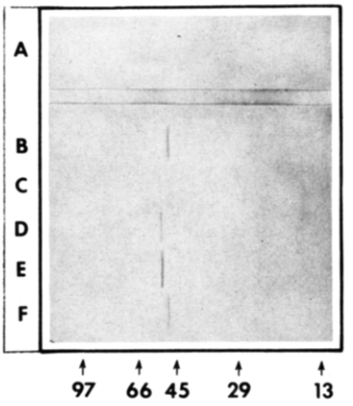

FIGURE 1: Immunoblot identification of mouse kidney ornithine decarboxylase. Kidney extracts (lanes A and C–E) and purified (~10% pure) ornithine decarboxylase (lanes B and F) were subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose paper, and the paper was exposed to antiserum to ornithine decarboxylase (lanes B–F) or control antiserum (lane A). The position of the bound antibodies was then determined by use of a peroxidase-linked anti-rabbit IgG antibody. Results are shown for samples containing 10 units (lanes A, B, E, and F), 4 units (lane D), and 1 unit (lane C) of ornithine decarboxylase activity.

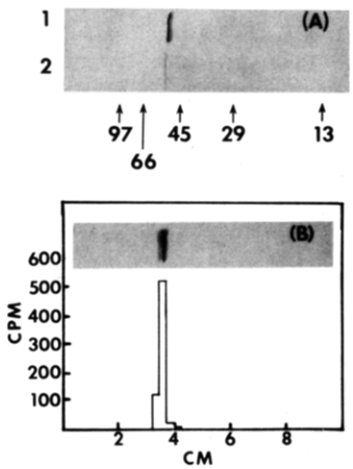

FIGURE 2: Immunoblotting of ornithine decarboxylase preparations of different ages. In panel A, lane 1 shows the results for the partially purified ornithine decarboxylase marker (stored for 2 weeks at 0 °C), and lane 2 shows fresh, unfractionated mouse kidney extract containing 7 units of ornithine decarboxylase activity. In panel B, either [^{14}C]DFMO-labeled ornithine decarboxylase (~10% pure) which had been stored for 2 months at −20 °C was separated as described in Figure 1 and developed by immunoblotting or the paper was cut into sections and assayed for radioactivity.

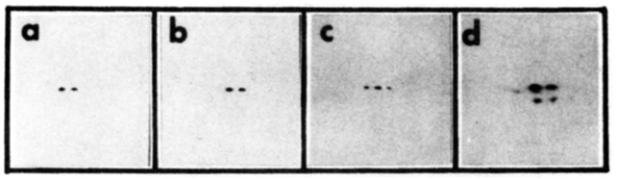

FIGURE 3: Two-dimensional electrophoretic separation of mouse kidney ornithine decarboxylase. Freshly prepared mouse kidney extracts containing about 20 units of ornithine decarboxylase activity were obtained from androgen-treated female mice. They were separated by two-dimensional gel electrophoresis, and the ornithine decarboxylase was detected by immunoblotting as described in Figure 1. Results are shown for untreated extracts (a), extracts from mice given DFMO (8 mg/kg) 1 h before death (b), and a mixture of the two extracts (c). In panel d, a stored extract (3 weeks at −20 °C) which had reacted with [^{3}H]DFMO was used, and the radioactive spots were identified by fluorography. The gels are shown with the acidic end of the isoelectric focusing run to the left and the lower molecular weight region to the bottom. The regions shown cover pH 5–6 and M_r 40 000–70 000.

coincide with the kidney extracts, but had a slightly lower molecular weight. This suggests that the marker preparation has been degraded during storage, and further evidence for this is given in Figure 2. In Figure 2B, a marker preparation of partially purified mouse kidney ornithine decarboxylase which had been labeled by reaction with [^{14}C]DFMO was subjected to the same electrophoresis and transfer procedures, and one track was cut into 2-mm sections and counted while the other was stained by immunoblotting. It can clearly be seen in the immunoblot that the marker preparation consists of two forms and both of these appear to be labeled. In this experiment, the marker preparation had been stored for a long period (2 months with several freezing and thawing steps), and most, but not all, had assumed the lower size (M_r ~53 000). In Figure 2A, a second marker preparation was used which had been stored for a shorter time, and in this case, the majority of the material corresponded to the larger form and to the ornithine decarboxylase protein present in fresh kidney extracts.

The same immunoblotting technique was used to identify ornithine decarboxylase separated by two-dimensional gel electrophoresis. Mouse kidney extracts contained two distinct proteins which reacted with the antibody and differed in charge, but not in molecular weight (Figure 3a). When the ornithine decarboxylase was allowed to react with DFMO prior to subjection of the extracts to electrophoresis, both spots moved to a higher isoelectric point (Figure 3b). This indicates that the two forms of the enzyme are both enzymatically active since covalent binding of DFMO requires enzyme activity (Metcalf et al., 1978; Seely et al., 1982b). When extracts which had reacted with DFMO were mixed with control extracts, three spots were seen (Figure 3c), indicating that the difference in charge between the two forms is similar to the charge which can be brought about by the binding of DFMO. Although this is not known precisely, it is likely that this is a single positive charge (McConlogue & Coffine, 1983a). A similar two-dimensional separation was carried out on [^{3}H]-DFMO-labeled ornithine decarboxylase which had been stored for several weeks. An autoradiograph of the gel was then prepared (Figure 3d), and four spots were seen in two pairs corresponding to the forms of different molecular weights. Therefore, the portion which is lost does not appear to affect the isoelectric point.

In order to study the synthesis of ornithine decarboxylase, mice were treated with 500-μCi doses of [^{35}S]methionine. When the labeled proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions, a radioactive

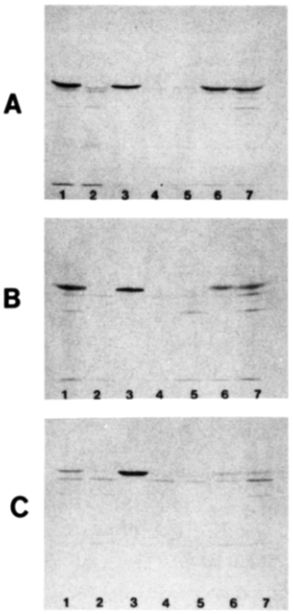

FIGURE 4: Separation of mouse kidney protein labeled with [^{35}S]-methionine and precipitated by antibodies to ornithine decarboxylase. The same extracts were used for the gels shown in panels A–C. Female mice were treated with 0.5 mCi of [^{35}S]methionine for 15 min (lane 6), 30 min (lanes 1, 2, and 5), or 4.5 h (lanes 4 and 7). Extracts were prepared and immunoprecipitates obtained by using rabbit antiserum to ornithine decarboxylase (A and B) or a monoclonal antibody (C). The immunoprecipitates were separated on polyacrylamide gels and the labeled proteins detected as described in Figure 4. Results are shown for extracts from androgen-treated female mice (lanes 1, 6, and 7), for extracts from control female mice (lane 2), and for androgen-treated female mice labeled with [^{35}S]methionine for 30 min followed by 20 mg/kg cycloheximide for 4 h before death (lane 4). Lane 3 shows an undegraded [^{3}H]DFMO-labeled ornithine decarboxylase marker which was also precipitated by the antibodies before electrophoresis. Lane 5 shows a control precipitation by preimmune rabbit serum (A and B) or another monoclonal antibody not directed against ornithine decarboxylase (C) of the same extract used in lane 1.

band of M_r ~55 000 which corresponded to an ornithine decarboxylase marker could be seen in extracts from testosterone-treated female mice, but not in those from untreated female mice. The band was removed by treatment with rabbit antiserum to ornithine decarboxylase prior to electrophoresis, and little change was produced in the other proteins present. These results demonstrated the feasibility of studying ornithine decarboxylase synthesis and degradation by immunoprecipitation techniques. In order to be certain that the labeled protein precipitated was ornithine decarboxylase, experiments were carried out with three different antibodies (Figure 4). Results are shown in Figure 4A for experiments with rabbit antiserum raised to ornithine decarboxylase as described by Persson (1982), in Figure 4B for experiments with rabbit antiserum produced by Seely & Pegg (1983a), and in Figure 4C for a monoclonal antibody against ornithine decarboxylase (Pegg et al., 1984). It can be seen that a prominent labeled band corresponding to ornithine decarboxylase is precipitated by all three of these antibody preparations from extracts from androgen-treated mice (Figure 4, lane 1). This band was not present in extracts from untreated female mice (lane 2), nor was it seen when control antisera or a control monoclonal antibody not directed against ornithine decarboxylase was used (lane 5). The ornithine decarboxylase band was intensely labeled after a 15-min exposure to [^{35}S]methionine (lane 6) as well as 30 min (lane 1) and declined in labeling after a 4.5-h exposure (lane 7). This confirms that the ornithine decarboxylase protein turns over very rapidly. The decline in

Table II: Synthesis Rate of Ornithine Decarboxylase[a]

time of exposure to [^{35}S]Met (min)	time of treatment with 1,3-diamino-propane (min)	radioact. in ornithine decarboxylase (cpm/mg of soluble protein)	total radioact. incorporated into protein (cpm/mg of soluble protein)	% of total radioact. incorporated which was in ornithine decarboxylase	ornithine decarboxylase act. (units/soluble protein)
7.5	none	491	38 110	1.3	181
15.0	none	1084	125 622	0.9	194
30.0	none	2241 ± 390	195 425 ● 24 041	1.1 ± 0.1	196 ● 13
60.0	none	1948	162 315	1.2	183
15.0	45	635	64 182	1.0	104
15.0	615	2004	125 082	1.6	220
30.0	60	182	34 041	0.5	64
30.0	600	891	156 808	0.6	11
60.0	660	2060	242 575	0.9	51

[a] Androgen-treated female mice were given [^{35}S]methionine and 1,3-diaminopropane as described under Materials and Methods. The radioactivity in ornithine decarboxylase was determined by measuring the amount of immunoprecipitable radioactivity using both antiserum A and antiserum B. These results agreed closely, and the mean values are shown. Total radioactivity incorporated into soluble protein was determined by precipitation with hot trichloroacetic acid (Hutson & Mortimore, 1982). Results at 30 min are the mean ± SD of six estimations.

labeling between 30 min and 4.5 h was not as great as it should be because no chase of unlabeled methionine was given to prevent continued incorporation of the radioactive amino acid into the protein. However, the remarkably rapid turnover of ornithine decarboxylase protein was seen more clearly in mice given cycloheximide to block protein synthesis for 4 h after the 30-min labeling period (lane 4). Such treatment produced an almost complete disappearance of the labeled ornithine decarboxylase.

Two-dimensional gel electrophoresis of the labeled proteins present in the kidney extracts showed that both of the forms of the enzyme identified in Figure 2 were rapidly labeled (Figure 5). The spots described by arrows (Figure 5) were identified as ornithine decarboxylase by their correspondence to the spots detected by immunoblotting, by their removal by the antisera to ornithine decarboxylase, and by their ability to react with DFMO (results not shown). Both spots disappeared in the samples from mice treated with cycloheximide for 4 h after the labeling period.

The labeling of ornithine decarboxylase protein was quantitated by counting the immunoprecipitate directly and subtracting the radioactivity present in immunoprecipitates obtained with the control antiserum. (This was carried out for both rabbit antisera with similar results. The monoclonal antibody could not be used for this purpose because as shown in Figure 4C only a fraction of the ornithine decarboxylase was precipitated and retained during washing when the monoclonal antibody was used. The fraction of total protein synthesis accounted for by ornithine decarboxylase protein obtained from labeling periods of 1 h or less was about 1.1% in the androgen-treated female mice (Table II). There was too little radioactivity incorporated into this protein in the untreated female mice for an accurate estimation of the fraction of synthesis in these animals, but this value was less than 0.04%. Androgens had little effect on the total protein synthesis rate. Therefore, androgen treatment enhances the absolute rate of synthesis of this protein by at least 25-fold.

The absolute amount of ornithine decarboxylase protein synthesis can also be obtained from these data, providing that the rate of methionine incorporation into protein is known and it is assumed that the methionine content of ornithine decarboxylase is similar to that of the kidney-soluble protein as a whole. The methionine incorporation into renal protein was determined by measuring the incorporation of [^{35}S]methionine into protein after several different doses of methionine and extrapolating to an infinite dose to allow for pool sizes (Hutson & Mortimore, 1982). Assuming that methionine makes up

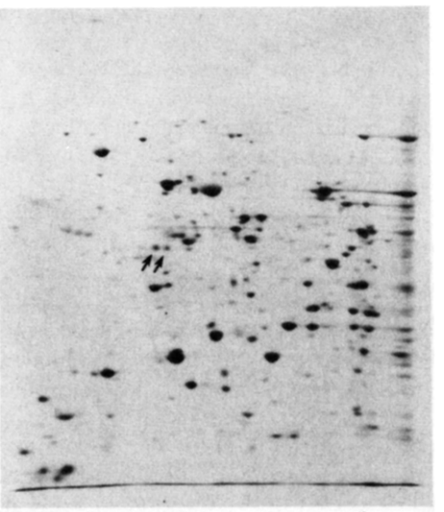

FIGURE 5: Two-dimensional electrophoretic separation of mouse kidney proteins labeled with [^{35}S]methionine. Freshly prepared kidney extract (containing about 20 units of ornithine decarboxylase) from a testosterone-treated mouse given 0.5 mCi of [^{35}S]methionine 30 min before death was separated by two-dimensional gel electrophoresis. The arrows indicate the position of ornithine decarboxylase. The entire gel is shown with the acidic end of the isoelectric focusing end to the left and the lower molecular weight region to the bottom.

2% of the protein, these experiments indicated that the protein synthesis rate in the kidney was 17 ± 6 μg of protein (30 min)$^{-1}$ (mg of soluble protein)$^{-1}$. This corresponds to the synthesis of 187 ± 66 ng of ornithine decarboxylase (30 min)$^{-1}$ (mg of soluble protein)$^{-1}$ in the kidneys of mice which contain about 189 units of ornithine decarboxylase activity/mg of soluble protein (Table II). Homogeneous preparations of mouse kidney ornithine decarboxylase have a specific activity of 1.4 units/ng (Seely et al., 1982a), so 189 units corresponds to 135 ng of protein, and the half-life of ornithine decarboxylase calculated by this method is 10 ± 3 min.

Administration of 1,3-diaminopropane produced a rapid decline in renal ornithine decarboxylase activity and a subsequent increase back to control levels. A typical experiment is shown in Figure 6 where activity reached a minimum of 10% of the starting level by 3 h after treatment and increased back to control levels by 12–16 h. In some other experiments (see Table II), the time of return to control levels was rather variable although the return always took place by 16 h. The reason for this variation is unclear but could relate to diurnal changes in the metabolism or clearance of 1,3-diaminopropane

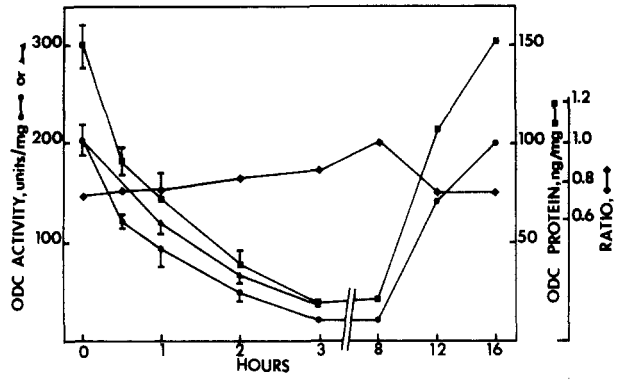

FIGURE 6: Effect of 1,3-diaminopropane on ornithine decarboxylase activity in androgen-treated mouse kidneys. Androgen-treated female mice were treated with 1,3-diaminopropane (2 mmol/kg) or cycloheximide (20 mg/kg), and the decline in ornithine decarboxylase (ODC) activity and protein content was measured. Results are shown for protein (■) and activity (●) after 1,3-diaminopropane addition and for activity (▲) after cycloheximide addition. The ratio of activity to protein content after 1,3-diaminopropane (♦) addition is also shown.

since the experiments were not always started at the same time of the day. The changes in ornithine decarboxylase activity brought about by 1,3-diaminopropane were due to changes in the total amount of enzyme protein as indicated by radioimmunoassay (Figure 6). For investigation of the mechanism by which the amount of protein was changed, the synthesis rate and the rate of degradation of the protein were measured in mice treated with 1,3-diaminopropane. The results for the synthesis rate are shown in Table II and indicate that at 45 and 60 min after exposure to the diamine when the amount of protein is declining rapidly there is little change in the fraction of total methionine incorporation going into ornithine decarboxylase. At 45 min, there was no change in this rate, and at 60 min, although the rate was apparently halved to 0.5%, the absolute value of ornithine decarboxylase protein was also reduced by 60–70%. Therefore, 1,3-diaminopropane does not greatly selectively inhibit the synthesis of ornithine decarboxylase. However, the dose of the diamine used, which is similar to that employed by earlier investigations, did substantially reduce the overall rate of protein synthesis (by almost 80% at 60 min), and this profound, but transient, inhibition is likely to play a significant role in reduction of ornithine decarboxylase activity.

It is also apparent from Figure 6 that treatment with 1,3-diaminopropane may increase the rate of degradation of ornithine decarboxylase because the decline in ornithine decarboxylase activity brought about by 1,3-diaminopropane is actually faster than that seen when protein synthesis is completely inhibited by cycloheximide. More direct proof that this is the case was obtained by labeling ornithine decarboxylase in vivo by injection of [^{14}C]DFMO and following the loss of the labeled protein in control mice and those receiving 1,3-diaminopropane (Table III). The diamine significantly increased the rate of degradation of the protein.

Table III: Effect of 1,3-Diaminopropane on Degradation of Ornithine Decarboxylase[a]

	radioact. bound to protein (cpm/mg)	
time (h)	control mice	1,3-diaminopropane-treated mice
0	477 ± 102 (100)[b]	477 ± 102 (100)
1	251 ± 54 (53)	175 ± 15 (37)
2	172 ± 15 (36)	85 ± 8 (18)

[a] Ornithine decarboxylase was labeled by administration of 7 μCi (1 mg/kg) of [^{14}C]DFMO 35 min prior to treatment with 1,3-diaminopropane (2 mmol/kg). Unlabeled DFMO (100 mg/kg) was given 5 min before the injection of 1,3-diaminopropane to prevent additional labeling of the ornithine decarboxylase. Results are shown ± SD for four to six animals at each time point. [b] Values in parentheses are percentages.

Discussion

Ornithine decarboxylase in the mouse kidney after stimulation by androgens consists of two closely related forms which have the same molecular weight and differ slightly in isoelectric point. Both of these forms are enzymatically active and turn over very rapidly. Any other form of the enzyme must amount to less than 5% of the total, which represents the sensitivity of the immunoblotting technique. Bullock and colleagues (Bullock et al., 1983) have reported preliminary evidence that the unstimulated female mouse kidney contains two forms of ornithine decarboxylase in equal proportions which differ in their half-life and heat sensitivity. It is not possible to use the present technique to study the situation in the uninduced female mouse which has a very low content of ornithine decarboxylase, and it remains possible that an isoenzyme of ornithine decarboxylase which is only slightly or not at all induced by androgens is present. The two forms which we have identified and separated are so similar that they may represent a posttranslational modification or a minor difference in sequence. Our results are in agreement with the findings of Isomaa et al. (1982) that their purified ornithine decarboxylase preparations were heterogeneous with respect to isoelectric point. However, the rapid loss of a small portion of the ornithine decarboxylase protein during storage of extracts which we have observed necessitates the use of fresh homogenates for definitive studies of the forms of ornithine decarboxylase present in the original tissue. Although the size of the peptide lost during this degradation is considerably smaller with the mouse kidney enzyme than with yeast, our finding that such degradation can take place without loss of enzymatic activity is similar to that made with the yeast ornithine decarboxylase by Tyagi et al. (1982). It is an interesting possibility that the degradation may be the first step in the very rapid turnover of the mouse kidney protein, but we have never observed the smaller, partially degraded protein in fresh extracts, although it appears within a few days of storage (unpublished observations). Therefore, if this cleavage is involved in the degradation of ornithine decarboxylase in vivo, it must be a rate-limiting step, and the smaller form does not accumulate in significant quantities. Although the degradation to the smaller form appears not to affect the enzyme activity or isoelectric point, our results emphasize that caution should be exercised in interpreting results indicating heterogeneity of ornithine decarboxylase activity during purification procedures as indicating the presence of multiple forms of the enzyme.

Androgen treatment of female mice results in a 200–400-fold increase in the amount of ornithine decarboxylase protein present in the kidney (Seely & Pegg, 1983a; Isomaa et al., 1983). This increase is partly due to a reduction in the rate of degradation of the protein, but this reduction is only 4–10-fold (Seely et al., 1982c; Isomaa et al., 1983). The present experiments provide direct proof that the rate of synthesis of the protein is also increased at least 25-fold, and such an increase is consistent with the overall change in the amount of protein. It is probable that the increased synthesis is due to androgen induction of the mRNA for ornithine decarboxylase. Two different mRNA species which are highly androgen inducible have been isolated by plasmid recapture using plasmids containing cDNA sequences of androgen-inducible mRNAs and shown to code for the synthesis of a

protein which is precipitable by antiserum to ornithine decarboxylase.[2]

The results shown in Figure 4 indicate that the metabolically labeled ornithine decarboxylase turns over very rapidly as predicted on the basis of experiments using less direct techniques. It also should be pointed out that the experiments in which the mice received labeled methionine 4.5 h before death showed no indication of a second form of the enzyme which turns over slowly and, therefore, did not become labeled in the short pulse experiments. Because of the rapid turnover of the protein, the fraction of total protein synthesis accounted for by ornithine decarboxylase is quite high, amounting to slightly more than 1%, and therefore, the mRNA for this protein should be relatively abundant. This is consistent with the levels of mRNA which have recently been tentatively identified as coding for ornithine decarboxylase.[2,3]

Direct calculations of the synthesis of ornithine decarboxylase protein based on methionine incorporation indicate a half-life for the protein of 10 min. This method for measuring the turnover is laborious and may be subject to significant experimental errors (see below), but it has the advantage that no extraneous agents such as cycloheximide or binding of DFMO which could influence the turnover rate are involved. Therefore, these results provide conclusive proof that this protein in a normal mammalian organ does turn over extremely rapidly. Our indirect measurement of ornithine decarboxylase turnover in mouse kidney using cycloheximide or DFMO suggests that the half-life is 80 min (Seely et al., 1982c; Seely & Pegg, 1983a). Although both 10 and 80 min indicate a very rapid turnover, this is a substantial discrepancy, and the reasons for it are not clear. Since the methionine content of ornithine decarboxylase is not known, one possibility is that this is quite different from that of the average kidney protein. A second factor may be that the kidney ornithine decarboxylase induced by androgens is predominantly located in the proximal tubule cells of the kidney (Pegg et al., 1982) and the overall protein synthesis rate is determined for the kidney as a whole.

The regulation of mammalian ornithine decarboxylase protein by diamines is not well understood, although in virtually all experiments reported, the activity of the enzyme declines rapidly after exposure to diamines [reviewed by Canellakis et al. (1979) and McCann (1980)]. The most commonly used nonphysiological diamine in such experiments is 1,3-diaminopropane, but the results shown in Table II confirm previous reports that this compound is a strong inhibitor of protein synthesis (Kay & Benzie, 1980; Tuomi et al., 1980). Therefore, part of the transient decline in ornithine decarboxylase activity is due to the transient but general inhibition of protein synthesis which affects short-lived proteins like ornithine decarboxylase much more dramatically than the average protein. 1,3-Diaminopropane produced little, if any, selective inhibition of the synthesis of ornithine decarboxylase, but did significantly enhance the rate of degradation. This increase in degradation could be due to the induction of the antizyme protein which may play a role in this degradation (McCann, 1980; Seely & Pegg, 1983b).

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[2] F. Berger, personal communication.
[3] O. Jänne, personal communication.

Registry No. Ornithine decarboxylase, 9024-60-6; testosterone, 58-22-0.

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