Ornithine decarboxylase modification and polyamine-stimulated enzyme inactivation in HTC cells

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Ornithine decarboxylase isolated from HTC cells was separated into two distinct charged states by salt-gradient elution from DEAE-Sepharose columns. This charge difference between the enzyme forms was maintained in partially purified preparations, but enzyme form II was observed to change to form I in a time-dependent polyamine-stimulated fashion in crude cell homogenates. The enzyme modification that produces this charge diversity between the alternative enzyme states was further investigated for its role in enzyme activity induction, protein stability and rapid turnover. Inhibition of new protein synthesis by cycloheximide resulted in a much more rapid loss of form I enzyme than of form II, suggesting that during normal enzyme turnover the latter enzyme state may be derived from the former. Culture conditions that favour the stabilization of this usually labile enzyme generally induced an increased proportion of the enzyme in the form II charge state. In particular, inhibitors of synthesis of spermidine and spermine induced the stabilization of cellular ornithine decarboxylase and promoted a marked accumulation in form II. Conversely, polyamines added to the cells in culture induced a very rapid loss in both forms of the enzyme, an effect that could not be attributed merely to an inhibition of new enzyme synthesis. It appears that the polyamines, but not putrescine, may be an essential part of the rapid ornithine decarboxylase inactivation process and that they may function in part by stimulating the conversion of the more stable enzyme form II into the less stable enzyme state, form I.

Polyamine biosynthesis in eukaryotic cells is sensitively manipulated through rapid fluctuations in the activity of the enzyme ornithine decarboxylase (EC 4.1.1.17). The control of this enzyme's activity is quite complex, for, in addition to regulation at the level of transcription and translation, post-translational modifications have been indicated (Clark & Fuller, 1975; Kallio et al., 1977; McCann et al., 1979; Mitchell, 1981; Russell, 1981; Atmar & Kuehn, 1981), activity-modifying factors (e.g. antizyme) have been isolated from several tissues (Heller & Canellakis, 1981; Heller et al., 1976), and a controlled, aggressive, enzyme-inactivation mechanism has been suggested.

Of particular interest is the abnormally short half-life of this enzyme in mammalian tissues, which has been reported to be as little as 10–15 min (Russell & Snyder, 1968; Hogan et al., 1974; Prouty, 1976). This has been repeatedly observed by monitoring the loss of enzyme activity after the addition of cycloheximide. That this loss of activity is a reasonable indication of enzyme protein instability is supported by studies using [3H]DFMO (Seely et al., 1982), monospecific-antibody precipitation (Erwin et al., 1983; Seely & Pegg, 1983), and the disappearance of [35S]methionine-pulse-labelled enzyme separated on two-dimensional electrophoresis gels (McConlogue & Coffino, 1983). Since inducing conditions have been reported to increase the stability of this enzyme (Hogan et al., 1974), control of the inactivation mechanism is thought to be at least partially responsible for the noted rapid activity changes. Unfortunately, the mechanism of this inactivation and its control are unknown.

Cellular ornithine decarboxylase activity also decreases extremely rapidly on addition of the
product of this enzyme, putrescine, or the putrescine derivatives, the polyamines spermidine and spermine. The mechanism of this inactivation is also not well understood. Some investigators believe that this control may involve an inhibition of enzyme synthesis (Clark & Fuller, 1975; Kallio et al., 1977; McCann et al., 1979). Several laboratories suggest that putrescine and the polyamines inhibit ornithine decarboxylase activity by stimulating the production of a labile protein, antizyme, that binds to the ornithine decarboxylase molecule and inactivates it (Heller et al., 1976; Heller & Canellakis, 1981). Since this association and inactivation is reversible in vitro, it is not clear whether this represents a normal pathway for irreversible inactivation in vivo.

More recently, ornithine decarboxylase isolated from mammalian tissues was shown to exist in at least two different charge forms, with the relative proportion of these forms changing with time after induction by various mechanisms (Richards et al., 1981; Mitchell & Mitchell, 1982). Mitchell & Mitchell (1982) suggested that, since these enzyme forms had the same apparent Mr and assay kinetics, they might reflect modifications made in the enzyme protein in association with cellular changes in enzyme stability or activity. On the other hand, Pereira et al. (1983) observed a difference in the rate of turnover between two ornithine decarboxylase forms induced in rat liver by chloroform and suggested that these reflect two separate species of the enzyme, one being much more stable than the other. In the present paper we give evidence that these two enzyme forms may really be alternative states of a single protein, and further that they result from a modification in the enzyme that is an integral part of the modulation of this enzyme's stability and induction. These experiments also help to define the involvement of the polyamines in the normal inactivation of this enzyme.

Experimental

Chemicals

Pyridoxal 5'-phosphate, L-ornithine, Hepps, DEAE-Sepharose CL-6B, dithiothreitol, cycloheximide, EDTA, putrescine, spermidine, spermine, Sephacryl S-300 and dicyclohexylamine were purchased from Sigma. Ultragel AcA 44 was purchased from LKB. MGBG and iminobispropylamine were from Aldrich. DFM0 and α-methylornithine were gifts from Merrell/Dow Research Centre, Cincinnati, OH, U.S.A. L-[1-14C]Ornithine (50 Ci/mol) was purchased from Amersham/Searle Corp., and α-[5-3H]DFMO (11.1 Ci/mmol) from New England Nuclear.

Cell culture

HTC and HMOA cells were grown in suspension culture in Swim's 77 medium (Gibco) containing 10% (v/v) calf serum (Biolabs Inc.) as previously described by Mitchell & Mitchell (1982). Maximal induction of enzyme activity was achieved when cultures were grown to at least 1.2 x 10^6 cells/ml and maintained in this dense, slowly growing, state for 24 h before dilution in fresh media with serum.

Assay of ornithine decarboxylase activity in HTC cultures

Cell samples (0.5 x 10^7–1.5 x 10^7 cells) were washed twice with phosphate-buffered iso-osmotic saline (137 mM-NaCl/2.7 mM-KCl/65.5 mM-Na_2HPO_4/1.47 mM-KH_2PO_4, pH 7.45) (4°C) and the pellets were immediately frozen and stored until use at -20°C. Cell pellets were resuspended in 1.5 ml of 0.02 M-Hepps (pH 7.2) assay buffer (0.5 mM-EDTA/5.0 mM-EDTA/5.0 mM-dithiothreitol/50 μM-pyridoxal 5'-phosphate) and sonicated for 30 s. Duplicate 200 μl portions of these crude suspensions were added to 100 μl of the same buffer containing 0.2 mM-L-ornithine (final concn., with 0.08 μCi of L-[1-14C]ornithine). Reactions proceeded for 60 min at 37°C and were stopped by the addition of 0.5 ml of 2M-citric acid. Released 14CO_2 was captured in 0.1 ml of 1M-Hyamine hydroxide in methanol contained in plastic cups suspended from the stopper of the reaction flask. These were counted for radioactivity at 89–91% efficiency in a toluene-based scintillation fluid. Proteins were determined by the method of Bradford (1976). One unit of ornithine decarboxylase is defined as that releasing 1 nmol of CO_2/min.

Ion-exchange chromatography

Cell pellets containing 1 x 10^7–6 x 10^7 cells were sonicated in 1.5 ml of column buffer [0.02 M-Hepps (pH 7.2)/0.5 mM-dithiothreitol/1.0 μM-pyridoxal 5'-phosphate/0.02% Triton X-100/0.125 M-NaCl]. This was applied to a 1.5 ml column of DEAE-Sepharose CL-6B pre-equilibrated with this buffer. After this column was washed with 5 ml more of this buffer, a 50 ml linear gradient of 0.125–0.250 M-NaCl in the buffer was applied and 0.95 ml fractions were collected at 2.5 ml/h. After the complete gradient was run, all fractions were assayed by adding 20 μl of assay mixture to 180 μl of each fraction such that the buffer concentration was the same as in the assays above except that the L-ornithine concentration was only 0.04 mM. As reported previously (Mitchell & Mitchell, 1982), two major peaks of activity, forms I and II, were easily separated, but a minor (5% of the total activity) peak, form III, was frequently difficult to
separate from form II. For the purposes of this study, peak III was considered as part of form-II ornithine decarboxylase.

**Gel-filtration chromatography**

Large (26ml) DEAE-Sepharose columns were used to isolate ornithine decarboxylase forms I and II as previously reported (Mitchell & Mitchell, 1982). Samples (0.3ml) of the isolated enzyme forms were applied to 25ml columns of AcA-34 that had been pre-equilibrated with the same column buffer as in the ion-exchange chromatography above, but containing 0.2M-NaCl. The sample was eluted with this same buffer at 2ml/h and the eluate was collected in 0.5ml fractions. This column was routinely calibrated with Blue Dextran, carbonic anhydrase, bovine serum albumin and ovalbumin. All manipulations were at 4°C.

S-300 gel-filtration chromatography was performed on induced HTC-cell samples (about $1 \times 10^8$ cells) that had been sonicated in 5ml of column buffer containing either 0.125M- or 0.2M-NaCl as indicated. These 100cm columns contained 180ml of the pre-equilibrated and pre-calibrated S-300 matrix. The enzyme was eluted with the same buffer at 10ml/h and collected in 3.0ml fractions that were eventually assayed for ornithine decarboxylase as described above.

**Results**

**Physical similarity of the alternative ornithine decarboxylase forms**

The two major peaks of ornithine decarboxylase activity that are separated from crude HTC homogenates by DEAE-Sepharose column chromatography, forms I and II, appear to be very similar. Previously, these were shown to have the same assay kinetics (Mitchell & Mitchell, 1982), and here we report that they have the same pH optima (pH7.2) and sensitivity to DFMO. When the forms were isolated and then individually rechromatographed on DEAE-Sepharose columns, they again demonstrated distinct charge properties (Mitchell & Mitchell, 1982); however, when these forms were individually applied to identical calibrated gel-filtration columns (AcA-44; 26ml bed volume) and eluted with column buffer containing 0.2M-NaCl, they appeared to be the same approximate size. The peak of activity in each case was eluted shortly after the bovine serum albumin size marker, at a calculated $M_r$ of 55000 (results not shown). Under these conditions about 18% of form II, and less than 2% of form I, of ornithine decarboxylase repeatedly was eluted in earlier peaks, indicating a slight tendency for dimerization (5% of form II) and formation of higher-order polymers (13% of form II).

In complementary experiments, HTC-cell homogenates were suspended in column buffer containing 0.2M-NaCl and chromatographed on a calibrated S-300 gel-filtration column. At this ionic strength the ornithine decarboxylase activity was consistently eluted as a single peak of $M_r$ about 55000. The peak tube of activity from this column was analysed by DEAE-Sepharose column chromatography and found to contain both forms of the enzyme. When crude cell homogenates were applied at lower ionic strengths (column buffer with 0.125M-NaCl), the enzyme was again eluted as a single peak, but this time in the size range of the dimeric form of the enzyme ($M_r$,110000; Fig. 1). When individual fractions from this peak were subsequently chromatographed with DEAE-Sepharose, they each were found to again contain both of the major forms of the enzyme. There was, however, a slight increase in the proportion of activity of peak I in the later fractions eluted from

![Graph](image_url)

**Fig. 1. Elution of the two ornithine decarboxylase forms from S-300 gel-filtration columns**

A pellet of HTC cells ($1 \times 10^8$) was sonicated in column buffer containing 0.125M-NaCl and chromatographed on S-300 gel as described in the text. Eluted fractions (3ml each) were assayed for enzyme activity ($\bullet$), and several of the active fractions were subsequently diluted with an equal volume of buffer without NaCl, to lower their osmotic strength, and applied to identical DEAE-Sepharose columns. By this method the proportion of the activity corresponding to each form of the enzyme was calculated at the centre and both sides of the single S-300 peak. The relative locations of form-I ($\square$) and form-II ($\blacksquare$) activities were then plotted within this single peak. Arrows: A, exclusion volume; B, elution position of bovine serum albumin; C, peak position of ornithine decarboxylase when eluted with 0.2M-NaCl.
this column, indicating that peak I may be slightly smaller than peak II by this analysis. Since a crude homogenate was applied to this S-300 column, we could not rule out the possibility that some form-specific inhibitory factor partially overlapped with the single peak of activity, or that form conversion, as described below, was not more prevalent in the later-eluted fractions. It is clear from these experiments that the noted charge distinction is not merely due to the difference between the enzyme in the monomeric and the dimeric state, and it does not appear that there is a large size difference between the active configurations of forms I and II. The possibilities remain that either these forms are of the same $M_r$, or else form I may be slightly smaller than form II. This point may not be satisfactorily resolved without complete purification of the individual forms with subsequent amino acid analysis or peptide mapping.

Ornithine decarboxylase form changes in vitro

In spite of the physical similarities noted above, it is still possible that these two charge forms of ornithine decarboxylase are produced by separate genes and therefore are true isoenzymes. This view is supported by the observation by Berger et al. (1984) that mouse kidney produces at least two distinct mRNA species coding for immunologically recognizable ornithine decarboxylase. The following experiments strongly suggest that these alternative enzyme charge forms actually are not entirely distinct protein species. In the study depicted in Fig. 2, identical pellets of induced HTC cells were washed, frozen and stored at $-20^\circ$C. One pellet was immediately homogenized in buffer and chromatographed on a DEAE-Sepharose column, whereas the second pellet was first thawed and then left at $4^\circ$C for 30 min before homogenization in buffer and chromatography. Although this pretreatment did not cause any measurable loss of ornithine decarboxylase activity, there was a large decrease in the second peak of enzyme activity to come off the column (form II), with a compensatory increase in form I (Fig. 2). This apparent shift in activity from the more to the negatively charged site (form II to I) was also observed when cell pellets were homogenized in buffer, at concentrations in excess of $2 \times 10^7 - 3 \times 10^7$ cells/ml, and incubated at $37^\circ$C for 60 min. More dilute cell suspensions exhibit a much less rapid shift of form II to I. In order to rule out the remote possibility that this shift in activity was due to inhibition of one enzyme species, with a precisely co-ordinated stimulation of the other, we followed this form shift by using enzyme that was labelled by $[3^H]$DFMO. This ornithine analogue is known to inhibit this enzyme specifically by binding covalently near the active site (Pritchard et al., 1981).

![Fig. 2. Changes in the pattern of ornithine decarboxylase eluted from DEAE-Sepharose with time in concentrated cell lysates](image)

Identical HTC-cell pellets containing $1.5 \times 10^7$ cells were extracted from a suspension culture 4 h after addition of fresh media and serum containing 2.5 $\mu$M-MGBG. After washing twice with ice-cold phosphate-buffered iso-osmotic saline, these were frozen and maintained at $-20^\circ$C. One pellet was quickly thawed and then placed in a $4^\circ$C water bath. After 30 min 1.5 ml of column buffer was added to both the frozen and the previously thawed samples, and they were quickly sonicated, immediately placed on identical DEAE-Sepharose columns and eluted as described in the Experimental section. Each of the 54 fractions eluted by this 0.125–0.25 $\mu$M-NaCl gradient was assayed for ornithine decarboxylase activity as described in the text. For comparison, results for the sample that was left as a concentrated cell lysate (○) were plotted on the same axis as those for the control that was immediately diluted with buffer on thawing (●).

As shown in Table 1, conditions that promoted a decrease in the proportion of active enzyme in peak II also induced a change in some of the $3^H$-labelled form-II enzyme molecules such that they emerged along with the active form-I peak. It is therefore apparent that the observed shift in activity between these peaks is actually indicative of a shift in enzyme protein molecules. A rather
Table 1. Conversion of [%H]DFMO-labelled ornithine decarboxylase form II into form I in crude homogenates

HMOA cells (2x10^6 cells) were harvested 24h after dilution in fresh media containing 0.25 μM-MGBG. After homogenization in 6ml of column buffer (4°C), a 2ml sample was immediately applied to a DEAE-Sepharose column and chromatographed. The remaining sample was incubated for 2h with 10μCi of [%H]DFMO (0.9nmol), which inactivated approx. 50% of the ornithine decarboxylase. Half of this sample was then passed through a 10ml Sephadex G-25 column to remove the unbound [%H]DFMO. The other half was dialysed against column buffer overnight at 4°C to remove excess label while also permitting some enzyme conversion from form II into I. The eluted fractions from the DEAE-Sepharose columns were assayed for enzyme activity and their content of [%H]-labelled enzyme. The enzyme activity remaining and the labelled enzyme (inactive) in each peak are presented, along with their respective ratios of enzyme forms.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Form I</th>
<th>Form II</th>
<th>Ratio of forms II/I</th>
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</thead>
<tbody>
<tr>
<td>Enzyme sample before labelling</td>
<td>0.211</td>
<td>0.601</td>
<td>2.85</td>
</tr>
<tr>
<td>After [%H]DFMO labelling for 2h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rapid G-25 column separation (before extensive form conversion)</td>
<td>0.130</td>
<td>0.248</td>
<td>1.91</td>
</tr>
<tr>
<td>Sample dialysed overnight (partial II-to-I conversion)</td>
<td>0.250</td>
<td>0.148</td>
<td>0.59</td>
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These results illustrate the instability of the cellular ornithine decarboxylase enzyme preparation. In addition to the decay of activity and the conversion of form II into form I, the labelled enzyme was found to be labile to dilution and a change in ionic strength. The simplified procedure shown in Fig. 4, the activity in form I was more stable and constant in the initial column fraction and at the end of the column run. The activity in form II was less stable and more labile. The ratio of enzyme activities indicated that the activity in form I was more stable than form II. The results were consistent with the enzyme activity assay data as well as with the enzyme activity data in Table 1. Thus, the conversion of form II into form I was not due to the decay of activity but rather to the decay of enzyme activity. The results indicated that the enzyme activity was not affected by dilution or changes in ionic strength. The enzyme activity was effectively restored by the addition of spermidine to the homogenate. The results indicated that the conversion of form II into form I was not due to the decay of activity but rather to the decay of enzyme activity. The results indicated that the enzyme activity was not affected by dilution or changes in ionic strength. The enzyme activity was effectively restored by the addition of spermidine to the homogenate.
Fig. 3. Time dependence of the spermidine-induced shift of ornithine decarboxylase peak II to I
Identical HTC-cell pellets containing $1.5 \times 10^7$ cells were produced as described in Fig. 2. Each frozen cell pellet was sonicated in 1.5 ml of ice-cold buffer and either immediately chromatographed on a DEAE-Sepharose column, as in the Experimental section, or incubated at 37°C for 20, 40 or 60 min before the chromatography. Some samples were sonicated and incubated in column buffer alone (O) and others in buffer containing 0.25 mM-spermidine (●). The eluted column fractions were assayed as in Fig. 2 and the total activity in each peak was calculated. Panel (a) shows the changes in activity with time of the first peak eluted from the column (form I), and panel (b) shows the changes in activity of the second peak (form II).

Table 2. Correlation of ornithine decarboxylase activity and stability with its distribution between the major forms
This is a compilation of the relevant data from many different experiments. Each value is an average of separate determinations made over a 2½ year period, ± S.D. for the numbers of experiments in parentheses. Abbreviation: $t_1$, half-life.

<table>
<thead>
<tr>
<th>Culture conditions</th>
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<td>Cell line</td>
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<td>HTC</td>
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<td></td>
</tr>
<tr>
<td>HMOA</td>
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<td></td>
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</tbody>
</table>

* McCann et al. (1977).† This half-life was determined 20h after the change of medium instead of 24h.

was induced by a variety of treatments. The ratio of the two forms of the enzyme was then determined and compared with the extent of induction and the total enzyme stability, as measured in the presence of cycloheximide (Table 2). The low activity and short half-life of this enzyme in cells 24h or more after the last serum addition was associated with this enzyme being predominantly in the less negatively charged peak-I form. Conversely, HTC cells induced by 4h in fresh media showed a 5-fold increase in activity, along with a moderate increase in enzyme stability,
and the enzyme was more equally distributed between the two charge states.

Even greater stimulation of enzyme activity and preferential accumulation of form II was achieved when certain inhibitors of polyamine biosynthesis were added to the fresh media with 10% serum. The inhibitor of S-adenosyl-L-methionine decarboxylase, MGBG, has previously been shown to limit spermidine and spermine biosynthesis while allowing both putrescine concentration and ornithine decarboxylase activity to increase above control values (Heby et al., 1973). As shown in Fig. 6, this depression of polyamine biosynthesis enhanced both the extent and the duration of the stimulation of this enzyme by feeding. This enhancement appeared to be associated with the lengthening of this enzyme’s half-life (Table 2) and the increased proportion of this enzyme found as form II (Fig. 7a). At 4h after stimulation, MGBG appeared to stimulate only the ornithine decarboxylase activity of the form-II pool, yet Fig. 7(b) indicates that both enzyme forms were eventually increased above those in the control culture.

Dicyclohexylamine, which reportedly inhibits spermidine synthase (Hibisami et al., 1980), allowed the accumulation of the precursor, putrescine, and also paradoxically enhanced the stimulation of ornithine decarboxylase activity by fresh media with serum (J. L. A. Mitchell, D. W. Mahan, P. P. McCann & P. Qasha, unpublished work). In the presence of this inhibitor, ornithine decarboxylase activity was maximal at about 24h, and was associated with a marked stabilization of the enzyme as well as a strong preferential accumulation of form II (Table 2). α-Methylornithine, a competitive inhibitor of ornithine decarboxylase that diminishes cellular putrescine and spermidine contents (Mamont et al., 1976), is known to have a stabilizing effect on this enzyme (McCann et al., 1977). As shown in Table 2, form II accumulates preferentially in response to this inhibitor, as it did with dicyclohexylamine.

The HTC subline HMOA, which has been extensively studied because of its unusually stable
ornithine decarboxylase (Pritchard et al., 1982), was also noted to maintain most of its enzyme as the more negatively charged state, form II (Table 2). It thus appears likely that the stability of this enzyme in a cell is directly related to the proportion of it in the more stable state, form II.

**Polyamine-induced cellular ornithine decarboxylase inactivation**

Consistent with the observation that interfering with a cell's ability to produce spermidine tends to enhance ornithine decarboxylase stability, the addition of exogenous polyamines is known to induce a very rapid loss of this enzyme's activity. This inactivation is thought to be due partially to an inhibition of new enzyme synthesis (Clark & Fuller, 1975; Kallio et al., 1977; McCann et al., 1979), and partially to the stimulation of the production of a regulatory protein, antizyme, that is thought to combine with, and somehow inactivate, the enzyme (Heller et al., 1976; Heller & Canellakis, 1981). Since it was demonstrated that the polyamines can stimulate the conversion of ornithine decarboxylase form II into I, it was decided to examine possible correlations between changes in these forms and the rapid loss of activity induced by the polyamines.

To this end, polyamines were added to cultures of HTC cells that had been induced 4h earlier by re-feeding with fresh media containing 10% serum and 2.5 μM-MGBG. As shown in Fig. 8, the addition of 10 mM-spermidine decreased enzyme activity by about 40% in 15 min, a decrease that was approximately equal for each form of the enzyme. In general, the rate of enzyme-activity loss caused by polyamine addition (for 0.5 mM-spermidine, t½ = 19.3 ± 4.5 min) was much greater than that with cycloheximide (t½ = 55.4 ± 8.6 min; Table 2) in cultures that were induced with MGBG. Table 3 extends these observations by showing that, for several polyamines and at several different extents of induced activity, approximately equally rapid inactivation of both forms is ob-

![Graph](image)

**Fig. 6. Effect of MGBG on the induction of ornithine decarboxylase**

HTC cells grown in stirred flasks to a density of 1.5 × 10⁶ cells/ml were diluted to 4.0 × 10⁵ cells/ml in two identical flasks containing fresh media plus 10% calf serum. One flask (○) also contained 2.5 μM-MGBG (●, control). At each of the indicated time points samples were removed and analysed for ornithine decarboxylase activity.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Compound</th>
<th>Conc. (mM)</th>
<th>Time (min)</th>
<th>10³ × Enzyme activity (units/mg of protein)</th>
<th>Ratio of forms II/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>20</td>
<td>77.4</td>
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<td></td>
<td>Spermidine</td>
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<td>40.6</td>
<td>1.89</td>
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<tr>
<td></td>
<td>Spermine</td>
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<td>Putrescine</td>
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<td>2</td>
<td>Control</td>
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<td>15</td>
<td>120.6</td>
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<td>Spermidine</td>
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<td>Iminobispropylamine</td>
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<td>91.6</td>
<td>1.26</td>
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<tr>
<td>3</td>
<td>Control</td>
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<tr>
<td></td>
<td>Spermidine</td>
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<td>15</td>
<td>73.2</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Figure 7. Changes in the relative activity of the major ornithine decarboxylase forms after enzyme induction with and without MGBG

Samples (about $1 \times 10^7$ cells) were removed from the cultures described in Fig. 6 at 4h (left panel) and 5h (right panel) after feeding. These were later chromatographed on DEAE-Sepharose, as described in the Experimental section, and eluted fractions assayed for ornithine decarboxylase activity. For each time point, the distribution of activity was compared between the culture induced with (○) and without (●) the addition of 2.5μM-MGBG.

The conversion of form II into I occurs readily in concentrated cell homogenates, yet not at all once the forms have been isolated and partially purified, even if spermidine is present. Isolated peak-II enzyme is modified, however, if it is added to crude homogenates and incubated with spermidine (results not shown). It therefore appears that this change in the ornithine decarboxylase charge form requires some as yet unidentified factor present in the crude homogenate.

Discussion

Several research groups have reported the presence of distinct charge forms of ornithine decarboxylase derived from mammalian tissues (Richards et al., 1981; Mitchell & Mitchell, 1982; Pereira et al., 1983). This is the first evidence, however, that these enzyme forms are actually alternative charge states of the same molecule, suggesting that one form is produced by post-translational modification of the other. Little is known of the nature of this post-translational enzyme modification, except that it results in a discrete chemical or conformational difference, since charge states intermediate between peak I and II are not observed. Also this modification does not appear to involve the addition or removal of an associated protein subunit, as the $M_r$ values of these forms are not appreciably different either before or after their separation on DEAE-Sepharose.

The conversion of form II into I occurs readily in concentrated cell homogenates, yet not at all once the forms have been isolated and partially purified, even if spermidine is present. Isolated peak-II enzyme is modified, however, if it is added to crude homogenates and incubated with spermidine (results not shown). It therefore appears that this change in the ornithine decarboxylase charge form requires some as yet unidentified factor present in the crude homogenate.

There are many modifications in ornithine decarboxylase that could explain these multiple enzyme states in vivo and the change in enzyme charge observed in crude homogenates. Unfortunately, at present there is no clear evidence to support or eliminate any possibility. The diminished rate of this conversion in enzyme that was labelled with the irreversible inhibitor DFMO does suggest that the active site of this enzyme may somehow be involved in this form-converting reaction. It is also conceivable that form I may be derived from form II by a specific proteolytic removal of a small peptide. We do not, however, consider that it is due to random proteolytic attack in the crude cell lysates, because the conversion from form II into I is discrete, without observable intermediates or other obvious charge states being produced. Furthermore, conversion in vitro was not affected by the presence of phenylmethanesulphonyl fluoride or EDTA.
Fig. 8. Acute effect of exogenous spermidine on the distribution of cellular ornithine decarboxylase between its constituent forms

A stirred culture of HTC cells was induced with fresh media containing MGBG as in Fig. 6. After 4.5 h, 10 μM spermidine was added to the culture and cell samples were taken immediately ○ and again after 15 min (●). These samples were chromatographed on DEAE-Sepharose, and the enzyme activity of the eluted fractions was assayed as described in the Experimental section.

The observation that form I is much more labile in vivo than form II is somewhat consistent with a study by Pereira et al., (1983) using chloroform-induced ornithine decarboxylase from rat liver. These authors may have oversimplified the interpretation of their cycloheximide study by assuming that these forms were entirely distinct enzyme species. Now that it appears that these ornithine decarboxylase forms are closely related and perhaps due to a post-translational modification of the enzyme, it is much more difficult to interpret precisely the instability noted in the enzyme form I.

Past work has indicated that an induced activity of ornithine decarboxylase represents a dynamic state of rapid enzyme synthesis in critical balance with rapid continuous enzyme degradation. Now it also appears that, in between this synthesis and degradation, a modification may be made in the enzyme protein that affects the charge on the molecule and perhaps is influential in controlling this enzyme's turnover. Possible relationships between these enzyme (ODC) forms and turnover processes are shown in Scheme 1.

Complementary models in which form II is synthesized and then modified to form I are not illustrated, as they are not consistent with the results obtained when new enzyme synthesis was blocked with cycloheximide. Since it is unlikely that this inhibitor has an immediate effect on the rate of enzyme modification or degradation, the expected initial effect is a rapid depletion of the newly synthesized enzyme pool. Thus the observation that cycloheximide produces a more rapid decline in enzyme form I refutes the possibility that enzyme form I is derived from newly synthesized form II by a post-translational modification.

The above models are both consistent with our observation of the preferential increase in form-II enzyme associated with a diminished rate of ornithine decarboxylase degradation. In the first scheme, slowing enzyme degradation relative to the constant rates of synthesis and form I-to-II modification would promote selective accumulation of enzyme form II. In the second model degradation could be decreased, and enzyme form II would accumulate, by the controlled favouring of enzyme form I-to-II conversion over the reverse reaction.

How do the polyamines induce the very rapid loss of ornithine decarboxylase activity? Although the polyamines may block new enzyme synthesis, their effect is clearly not limited to this step, as (a) they produce a much more rapid loss of enzyme activity than cycloheximide does in MGBG-induced cells, and (b) they promote the rapid loss of both charge forms and not form I selectively, as obtained when new protein synthesis is blocked with cycloheximide. Both of the models in Scheme 1 are consistent with the possibility that the polyamines stimulate ornithine decarboxylase degradation, perhaps through a mechanism involving antizyme. To obtain equally rapid loss of both forms of this enzyme in the second model, it would in addition be necessary for the polyamines to stimulate the enzyme form II-to-I conversion, and this of course was observed in crude homogenates.

Putrescine has generally been considered as the
important intermediate in the feedback control of ornithine decarboxylase activity. Thus it is noteworthy that the polyamines, and not putrescine, were found to stimulate form II-to-I conversion in crude homogenates. This specificity of the polyamine-dependent post-translational modification is similar to that of the ornithine decarboxylase A-to-B (active-to-inactive) modification previously reported in Physarum (Mitchell et al., 1982). Furthermore, it appears that the polyamines, and not putrescine, are also responsible for stimulating the degradation of cellular ornithine decarboxylase. The inhibitors MGBG and dicyclohexylamine both decrease the production of spermidine from putrescine, allowing putrescine concentrations to rise; yet, in spite of these higher putrescine concentrations, both inhibitors stimulate an increase in the half-life of this enzyme, with preferential accumulation of form II. Even exogenous putrescine was much less effective than the polyamines and their analogues in decreasing ornithine decarboxylase activity in HTC cultures induced with MGBG. These results strongly suggest that the polyamines spermidine and spermine are the critical elements in at least these two components of the product feedback control of this enzyme and are essential for the normally rapid enzyme degradation observed.

In spite of the above evidence that these ornithine decarboxylase forms are physically and kinetically quite similar, and that their charge difference can be eliminated in vitro, it is still possible that these forms are two distinct, but very closely related, gene products in which the obvious charge difference is readily removed in vitro (e.g. the proteolytic nick removal of a signal peptide). This possibility is somewhat consistent with the observation (Berger et al., 1984) that there are two distinct mRNA species for ornithine decarboxylase in a mammalian tissue. Experiments are required to determine the extent of the isolation of these two forms of enzyme and possible conversions between them within the intact cell.

In this study we have presented evidence that form-II enzyme can be modified to produce form I in cell homogenates, suggesting these forms may represent alternative charge states of a single basic enzyme protein. It was also shown that forms I and II exhibit different half-lives in vitro, with the relative cellular pool sizes of these forms correlating closely with changing enzyme stability. Finally, it was suggested that the polyamines, and not putrescine, are an essential part of the rapid enzyme-inactivation process, and that one of their functions may be to stimulate the conversion of the more stable form II into the less stable form I. Although these results expand our understanding of this complex problem of rapid modulation of cellular ornithine decarboxylase activity, several questions have been left unanswered, and even more have been raised. In particular we need to determine: (a) which (if not both) form of the enzyme is the precursor to inactivation; (b) what is the function of antizyme in this enzyme turnover; (c) what is the role of the minor form, III, reported previously by Mitchell & Mitchell (1982); and (d) what is the chemical nature and physiological control of the observed post-translational modification of this enzyme.

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