A comparison of ornithine decarboxylases from normal and SV40-transformed 3T3 mouse fibroblasts

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Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) has been purified from 3T3- and SV40-transformed 3T3 mouse fibroblasts by affinity chromatography, and the physicochemical properties of the two enzymes compared. Measured properties include molecular weight of the active species, subunit molecular weight and specific activity of the purified enzymes, kinetic parameters, thermostability, degradation rate *in vivo* and immunological cross-reactivity. Although crude extracts of the transformant possess more ornithine decarboxylase activity per mg of protein than the parent strain, there is no evidence for the appearance of an altered form of the enzyme in these cells. The results reported in the present paper indicate that the increased ornithine decarboxylase activity in the transformed cells is the result of higher enzyme biosynthesis *de novo*.

L-Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) has recently generated much interest among researchers investigating regulation of cell growth. This pyridoxal phosphate-requiring enzyme controls the rate-limiting step in the biosynthesis of polyamines (Jänne, 1967; Pegg & Williams-Ashman, 1968), which play an incompletely-characterized but nonetheless important role in cell growth and division (Calderera & Moruzzi, 1970; Heby *et al.*, 1975a; Mamont *et al.*, 1976; Tabor & Tabor, 1976; Jänne *et al.*, 1978). Not only does ornithine decarboxylase activity rise rapidly and markedly after a wide variety of growth stimuli (Russell & Snyder, 1968, 1969; Jänne & Raina, 1968a,b; Pegg *et al.*, 1970; Stastny & Cohen, 1970; Cohen *et al.*, 1970; for review see Tabor & Tabor, 1976), but a close correlation can be established between ornithine decarboxylase activity and growth rate in a number of systems (Williams-Ashman *et al.*, 1972; Morris & Fillingame, 1974; Heby *et al.*, 1975b).

A previous report from this laboratory showed that ornithine decarboxylase activity in crude extracts of the virally-transformed SV101 cell line was significantly higher than in the parent 3T3 cells

(Lembach, 1974). Elegant studies with a temperature-sensitive Rous sarcoma mutant in chick embryo fibroblasts (Don & Bachrach, 1975) also showed an increase in ornithine decarboxylase activity on transformation. This phenomenon only occurred at the permissive temperature, indicating that the effect was specific for the transformation process. Several mechanisms could account for the observed differences: (1) presence of an activator/inhibitor; (2) structural and/or kinetic differences between the two enzymes; (3) increased enzyme stability in the transformant; (4) increased enzyme biosynthesis *de novo*.

Extensive testing has thus far failed to uncover low-molecular-weight physiological effectors of the eukaryotic ornithine decarboxylase (Jänne & Williams-Ashman, 1971; Friedman *et al.*, 1972; Ono *et al.*, 1972; Morris & Fillingame, 1974; Raina & Jänne, 1975; Williams-Ashman & Canellakis, 1979); therefore the first mechanism was considered unlikely. Polyamines inhibit the enzyme *in vitro*, but at concentrations that are unphysiologically high and toxic to the cells (Clark & Fuller, 1975; Fong *et al.*, 1976). Although a so-called 'ornithine decarboxylase antizyme' has been reported (Heller *et al.*, 1976; Fong *et al.*, 1976; McCann *et al.*, 1977), attempts to detect such a protein inhibitor in 3T3 cells have failed (Clark & Fuller, 1976b).

To examine possible physicochemical differences between the enzymes, ornithine decarboxylase was purified from both 3T3 and SV101 mouse fibroblasts via affinity chromatography (Boucek &

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Lembach, 1977). The specific activities of the purified enzymes were compared, as well as molecular sizes, kinetic properties and thermostabilities. Degradation rates *in vivo* were measured to determine whether changes in enzyme stability could account for the difference in activity observed in crude extracts. The data strongly suggest that the increase in enzyme activity in the transformant can best be accounted for on the basis of biosynthesis *de novo*.

Materials and methods

Materials

All tissue culture supplies were obtained from Grand Island Biological. NCS tissue solubilizer and L-[1-¹⁴C]ornithine were purchased from Amer-sham/Searle. Econofluor was from New England Nuclear. L-Ornithine, pyridoxal 5'-phosphate, 3-isobutyl-1-methylxanthine and cycloheximide were supplied by Calbiochem. Trizma base and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Coomassie G-250 and R-250 dyes, *o*-phthalaldehyde and all molecular-weight standards were from Sigma. Acrylamide and bisacrylamide were from Aldrich. Sodium dodecyl sulphate was from Pierce. Triton X-100 was from Research Products International. DE-52 DEAE-cellulose was obtained from Whatman. Sephacryl S-200 resin was from Pharmacia. Agarose A0.5 M resin was from Bio-Gel. All salts were the highest grade commercially available.

Cell culture

Mouse 3T3 cells (Todaro & Green, 1963) and their SV40-transformed counterparts, the SV101 line (Todaro *et al.*, 1965), were originally supplied by Dr. Howard Green, Department of Biology, MIT, Cambridge, MA, U.S.A. Cells were grown at 37°C in 490cm² Corning roller bottles as previously described (Boucek & Lembach, 1977).

Ornithine decarboxylase induction

3T3 cells were grown to a density of approx. 50 000 cells/cm² in 100 ml of Dulbecco's medium (Smith *et al.*, 1960) supplemented with 10% (v/v) calf serum. SV101 cells were grown to approx. 200 000 cells/cm² in medium with 5% (v/v) serum. The medium was aspirated and the cells were washed with 25 ml of Earle's Balanced Salts Solution. After 10 min the cells were re-fed with 50 ml of fresh serum-containing medium, supplemented with 3-isobutyl-1-methylxanthine to maximize the ornithine decarboxylase induction (3T3 and SV101 cells received 1.0 mM- and 0.2 mM-3-isobutyl-1-methylxanthine respectively).

Ornithine decarboxylase extraction

Rather than harvest the cells and release the

cytoplasmic constituents by freeze-thawing, advantage was taken of a discovery that low concentrations of non-ionic detergents will lyse the plasma membrane of cultured cells without releasing the nuclei from the basal layer that the cells grow on (Osborn & Weber, 1977). At approx. 5 h after ornithine decarboxylase induction by fresh medium, the monolayers were washed with 25 ml of ice-cold phosphate-buffered saline solution, and the ornithine decarboxylase was extracted with 3 ml of ice-cold 0.5% (v/v) Triton X-100 in TED buffer [25 mM-Tris/HCl (pH 7.5 at 22°C)/0.1 mM-EDTA/1 mM-dithiothreitol] for 2 min. This was sufficient to release 100% of the ornithine decarboxylase activity from the cells, compared with the previous freeze-thaw protocol. The enzyme was even more stable under these conditions at -70°C than in the form of cell pellets (Boucek & Lembach, 1977).

Ornithine decarboxylase purification

The following steps were performed at 4°C, unless otherwise noted.

DEAE-cellulose chromatography. The Triton extract was centrifuged at 100 000g for 30 min in a Beckman L2-65B ultracentrifuge with a 60Ti rotor, and the high-speed supernatant was chromatographed on DEAE-cellulose. For a typical purification run, approx. 100 ml of supernatant was applied to a DEAE-cellulose column (2 cm × 10 cm) pre-equilibrated with TED buffer at a flow rate of 25 ml/h. The column was washed with 200 ml of TED buffer to remove the detergent, and a linear gradient of 0–0.3 M-NaCl in TED buffer was started. Fractions (2.0 ml) were collected and assayed for ornithine decarboxylase activity, and the peak fractions (eluting at approx. 0.15 M-NaCl) containing 90% of the recovered activity were pooled.

(NH₄)₂SO₄ precipitation. The enzyme was precipitated from the pooled DEAE-cellulose fractions by the addition of ultra-pure (NH₄)₂SO₄ to 55% saturation. (NH₄)₂SO₄ was added slowly, with rapid stirring; the rate of stirring was then decreased and the enzyme was allowed to precipitate for 1 h at 4°C. The pellet was recovered by centrifugation at 20 000g for 30 min in a Sorvall RC2-B centrifuge with an SS-34 rotor, resuspended in TED buffer to a calculated concentration of about 0.12 nmol of CO₂ released/ml and dialysed overnight against 100 vol. of TED buffer to remove residual (NH₄)₂SO₄. The diffusate was referred to as fraction II ornithine decarboxylase, in accord with previous terminology (Boucek & Lembach, 1977). This preparation of enzyme was used in characterization studies, or applied to an affinity column for further purification.

Affinity chromatography. The affinity chromatography was performed as described by Boucek &

Lembach (1977), except that the final buffer was changed from 25 mM-Tris to 10 mM-Hepes, in order not to interfere with the *o*-phthalaldehyde protein assay (Butcher & Lowry, 1976). Fraction II ornithine decarboxylase was loaded on to a column (1.5 cm $\times$ 11 cm) of pyridoxamine phosphate affinity resin at 1 ml/h and washed with 20 ml of TED buffer. A 500 ml linear gradient, from 0.1 mM-ATP/1 mM-CaCl₂/15 mM-NaCl in TED buffer to 15 mM-NaCl in TED buffer, was then applied. The column was washed with 200 ml of 10 mM-Hepes (pH 7.2 at 22°C)/0.1 mM-EDTA/1 mM-dithiothreitol/15 mM-NaCl, and a 100 ml linear gradient of pyridoxal phosphate (0–6 μ M) in the same buffer was utilized to elute the bound ornithine decarboxylase. The enzyme typically eluted near the midpoint of the gradient. Fractions (2.5 ml) were collected and 0.1 ml portions were removed for ornithine decarboxylase assay. [These aliquots were incubated overnight in 0.2 mg of bovine serum albumin/ml before assay. As outlined in previous work (Boucek & Lembach, 1977), bovine serum albumin appears to stabilize the pure ornithine decarboxylase eluting from the affinity column at very dilute protein concentrations.] The remaining material was made 0.1% (w/v) in sodium dodecyl sulphate to minimize non-specific adsorption on the tubes, and frozen at –70°C for SDS/polyacrylamide-gel electrophoresis and the *o*-phthalaldehyde protein assay.

Polyacrylamide-gel electrophoresis

Subunit molecular weight and purity were assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with the Maizel discontinuous system (Maizel, 1971). The 10% (w/v) acrylamide resolving gels (pH 8.9 at 22°C) were poured the night before, to ensure complete polymerization. The 3% (w/v) acrylamide stacking gels (pH 6.8 at 22°C) were poured 1 h before electrophoresis. After removing portions for protein determination, peak affinity-column fractions were pooled (4 ml total volume), dialysed for 4 h against 0.1% (w/v) sodium dodecyl sulphate (to remove salts), freeze-dried, and taken up in the following sample buffer: 50 mM-Tris/HCl (pH 6.8 at 22°C)/2% (v/v) mercaptoethanol/10% (v/v) glycerol/0.005% (w/v) Bromophenol Blue. Standards were taken up in the same buffer, with sodium dodecyl sulphate added to achieve the same final concentration (2%, w/v). All samples and standards were heated to 100°C for 5 min and, after cooling, were layered directly on to the stacking gel. Electrophoresis was performed at 1 mA/gel for 1 h at 20°C, then the voltage increased to 2.5 mA/gel for about 3 h, until the tracking dye had moved to within 2 cm of the bottom of the gels. Gels were fixed in 25% (v/v) propan-2-ol overnight and stained in 0.002% (w/v) Coomassie R-250/10% (v/v) acetic acid/10% (v/v) methanol for 24 h. This

modification of the methodology of Bibring & Baxandal (1978) results in well-stained bands with very little background staining. The gels were cleared for a couple of hours in water before photography.

o-Phthalaldehyde protein assay

This ultrasensitive protein assay was used to measure the purified enzyme after affinity chromatography, and was performed as described by Butcher & Lowry (1976). Bovine serum albumin standards were made up in the same buffer used to elute ornithine decarboxylase from the affinity resin, with sodium dodecyl sulphate added to achieve a final concentration of 0.1% (w/v). All standards and samples were hydrolysed by adding an equal volume of conc. HCl in acid-washed hydrolysis tubes and heating for 4 h at 120°C. After cooling, equal volumes of 6 M-NaOH were added to neutralize the solutions, and the protein assay was performed as described for samples containing 0–1 μ g of total protein. Fluorescent intensities were monitored on an Aminco Fluoro-Microphotometer at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Assay of ornithine decarboxylase activity

Enzyme activity was measured by the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine, as previously described (Boucek & Lembach, 1977). The assay was performed at 37°C in the presence of saturating concentrations of cofactor (pyridoxal 5'-phosphate) and substrate (L-ornithine). To a sample of 0.1 ml was added 10 μ l of 2.4 mM-pyridoxal 5'-phosphate, and the sample incubated on ice for 10 min. After preincubation with cofactor, 10 μ l of 12 mM-L-[1-¹⁴C]ornithine (25 μ Ci/ml) was added and the extracts incubated for 20 min at 37°C. The released CO₂ was captured in 0.2 ml of NCS tissue solubilizer contained in polypropylene wells attached to rubber stoppers that capped the incubation tubes. The reaction was halted by placing the tubes on ice and injecting 1 ml of 2 M-citric acid through the rubber stoppers. After a further incubation for 1 h at 37°C to ensure complete CO₂ capture, the wells were counted for radioactivity in 10 ml of Econofluor. All assays were performed in duplicate, and all values corrected for radioactivity in control samples containing buffer in lieu of cell extract. Under these conditions the assay is linear with time for at least 1 h.

Protein was quantified by the method of Bradford (1976), with crystalline bovine serum albumin as a standard. This protein assay was chosen since neither dithiothreitol nor low concentrations of Triton X-100 interfere. Enzyme activities are expressed as nmol of CO₂ released/h per mg of protein.

Gel filtration

The enzymes were chromatographed on both Sephacryl S-200 and agarose A0.5M resins. Column size for the S-200 resin was 1.5 cm × 80 cm, corresponding to a bed volume of 140 ml (56.5 ml void volume). The column was pre-equilibrated with 500 ml of TED buffer containing 50 mM-NaCl at a flow rate of 30 ml/h; this salt concentration was chosen to minimize non-specific electrostatic interactions without inhibiting ornithine decarboxylase activity. The samples were applied in 0.5 ml of the same buffer containing 10% (v/v) glycerol to aid in layering on to the resin. Blue Dextran was used to find the void volume, and 1 M-NaCl to find the salt volume (measuring conductivity). The column was calibrated with aldolase, bovine serum albumin, horse liver alcohol dehydrogenase, haemoglobin, ovalbumin and chymotrypsinogen. Fractions (1.0 ml) were collected; protein peaks were detected by absorbance at 230 nm, by Soret bands at 455 nm (haemoglobin), by enzyme activity (liver alcohol dehydrogenase; Bonnichsen & Brink, 1955), by rocket immunoelectrophoresis (bovine serum albumin; Laurell, 1972), and by the Bradford protein assay (Bradford, 1976). Separate calibrations were done every time the run conditions were altered.

Agarose-gel filtration was performed as described by Boucek & Lembach (1977).

Thermostability studies

Thermostability studies were performed at 40°C, with partially purified ornithine decarboxylase (fraction II) from each cell type. Thermostability was markedly affected by the protein concentration (results not shown), and thus care was taken to adjust all preparations of enzyme to the same protein concentration (0.7 mg/ml) for the thermal incubations, although not necessarily the same ornithine decarboxylase activity. Higher concentrations of protein increased the stability of the enzyme activity. Samples were placed in small capped vessels to minimize evaporation, and incubated in a 40°C water bath. Portions were removed at various time points for ornithine decarboxylase assay. Thermostability was monitored in the presence and absence of both cofactor and substrate, using the same (saturating) concentrations as in the ornithine decarboxylase assay: 0.2 mM-pyridoxal phosphate and 1 mM-ornithine.

Kinetics

The kinetic parameter K_m was measured with fraction II ornithine decarboxylase from both types of cells by adjusting the concentration of the substrate, L-ornithine. The concentration dependence of enzyme activity on the cofactor was compared by adjusting the amount of pyridoxal 5'-phosphate added in the assay. Enzyme assays

were otherwise performed as described above. K_m values were derived by linear regression analysis of the straight lines produced on Eadie-Hofstee plots (v versus $v/[S]$).

Immunological techniques

Antibodies to SV101 cell ornithine decarboxylase were raised by the method of Johnston (1972), which requires relatively small quantities of antigen. Roughly 50 µg of purified SV101 cell ornithine decarboxylase was freeze-dried, resuspended in sterile saline solution, and injected intraperitoneally into a starved New Zealand White rabbit. Macrophages were harvested and reinjected as described. Three booster injections were given at biweekly intervals thereafter, using 25–50 µg of purified SV101 cell ornithine decarboxylase in a Freund's complete adjuvant emulsion. At 2 weeks after the last immunization, the animal was bled and a globulin fraction was purified from the serum by DEAE-cellulose ion-exchange chromatography (Fahey, 1967). Immunoglobulin-containing fractions were pooled and dialysed overnight against 0.1 M-NaCl/0.2% (w/v) NaN₃, and stored at 4°C. Control serum was obtained from a non-immunized rabbit, and was purified in a similar fashion.

Immunotitration studies were performed with an extract of cells induced as described above. At 4 h after induction, cells were harvested by trypsinization, lysed in TED buffer by alternating freeze-thaw cycles, centrifuged at 8400 g for 10 min in a Beckman L3-50 ultracentrifuge with a 50Ti rotor, and the supernatants were diluted to approximately the same ornithine decarboxylase activity. Equal portions of enzyme were incubated overnight at 4°C in the presence of 1 mg of bovine serum albumin/ml and various amounts of anti-(ornithine decarboxylase) or control globulins. Ornithine decarboxylase assays were performed as described above.

Degradation rates

The half-life of ornithine decarboxylase *in vivo* was measured in each cell type by administration of cycloheximide after induction of the enzyme. Cells were set up in Linbro plates (tissue culture dishes containing twenty-four 2 cm² wells apiece, obtained from Linbro Scientific) at a density of 40 000 3T3 cells/well or 80 000 SV101 cells/well. After 24 h the monolayers were washed with serum-free Dulbecco's medium and 'stepped down' to 0.25% (v/v) calf serum in Dulbecco's medium overnight. The following day the cultures were re-fed with fresh serum-containing medium; this protocol results in an induction of enzyme activity that peaks in roughly 4–6 h (Lembach, 1974). At 3 h after induction cycloheximide was added to half the cultures to a final concentration of 50 µg/ml; time points were taken every 30 min thereafter for 2.5 h. The medium

was aspirated, each well rinsed briefly with 1 ml of ice-cold phosphate-buffered saline solution, and ornithine decarboxylase was extracted by overlaying for 2 min with 50 μ l of ice-cold 0.5% (v/v) Triton X-100/TED buffer/0.2 mM-pyridoxal 5'-phosphate. Extracts from duplicate wells were combined to minimize culture heterogeneity and provide an increased amount of enzyme activity. Ornithine decarboxylase was assayed as described, except that no additional pyridoxal 5'-phosphate was put in the assay.

Results

Although it would be desirable to work solely with purified material for the characterization studies, the difficulty of obtaining large amounts of pure enzyme from a tissue culture system (where the amount of starting material is small to begin with), coupled with the extremely unstable nature of highly purified ornithine decarboxylase (half-life of under 1 hour at 4°C; Boucek & Lembach, 1977), made this impossible. Thus a partially purified preparation of the enzyme (fraction II) was used for the following analyses, except as noted otherwise.

Polyacrylamide-gel electrophoresis

The 3T3 cell ornithine decarboxylase was evidently purified to homogeneity by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis criteria, running as a single band with a subunit molecular weight of 55 000. This is identical with the previously reported estimate of the subunit molecular weight of the purified SV101 cell ornithine decarboxylase (Boucek & Lembach, 1977).

Gel filtration

Owing to the relative instability of the enzyme, gel filtration was performed on Sephacryl S-200 columns. This resin has the sizing characteristics of Sephadex G-200, but far superior flow characteristics, yielding a recovery of enzyme activity of 80% or greater.

Both the 3T3 and the transformed cell enzymes chromatographed as single symmetrical bands of activity at 4°C, eluting within a fraction of one another on successive column runs. The apparent molecular weight was 105 000 [derived by plotting log (molecular weight) versus V_e/V_0], corresponding to a physiological dimer. Typical activity profiles for the two enzymes are shown in Fig. 2.

The filtration characteristics of SV101 cell ornithine decarboxylase were tested in the presence and absence of cofactor, for pyridoxal phosphate had a marked stabilizing effect on the thermal stability. This might be mediated via a change in the aggregation state of the enzyme. However, the molecular weight did not change in the presence or

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified 3T3 cell ornithine decarboxylase

The gel on the right is the result of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of approx. 17 μ g of purified ornithine decarboxylase from 3T3 cells, as described in the Materials and methods section. A single band was detected by Coomassie staining, corresponding to a subunit molecular weight of 55 000. Purified SV101 cell ornithine decarboxylase yielded similar results (not shown). The gel on the left shows molecular-weight standards: 1, β -galactosidase (130 000); 2, lactoperoxidase (80 000); 3, bovine serum albumin (69 000); 4, catalase (58 000); 5, ovalbumin (43 000); 6, aldolase (40 000); 7, chymotrypsinogen (25 700); 8, cytochrome c (11 700), running with the tracking dye.

absence of pyridoxal phosphate. The concentration of ornithine decarboxylase activity that was applied to the column was varied by a factor of ten in an attempt to change the aggregation state, also without success (results not shown).

The 3T3 and SV101 cell enzymes behaved in a

Fig. 2. Gel chromatography of 3T3 and SV101 cell ornithine decarboxylase on Sephacryl S-200 resin. Partially purified ornithine decarboxylase from 3T3 (○) and SV101 (□) cells was chromatographed on Sephacryl S-200 resin at 4°C in TED buffer containing 50 mM-NaCl, as described in the Materials and methods section. Arrows mark elution positions of molecular-weight standards: 1, Blue Dextran (void volume); 2, aldolase (158 000); 3, liver alcohol dehydrogenase (80 000); 4, haemoglobin (64 500); 5, chymotrypsinogen (35 700).

similar fashion on an agarose A0.5M column (results not shown), with an apparent molecular weight of about 105 000.

Thermostability

The thermostabilities of 3T3 and SV101 cell ornithine decarboxylase were compared at 40°C under a variety of conditions. The results are shown in Fig. 3. Several interesting facts are immediately apparent. (a) Thermostabilities of 3T3 and SV101 cell ornithine decarboxylase are very similar under all conditions tested. (b) Addition of substrate (ornithine) has no effect. (c) Addition of cofactor (pyridoxal phosphate) results in a rapid and marked stabilization of activity. (d) Addition of both substrate and cofactor results in a degree of stabilization intermediate between that of cofactor alone or substrate alone.

Crude extract kinetics

The kinetic parameter K_m was measured in

partially purified ornithine decarboxylase preparations for the substrate ornithine and the cofactor pyridoxal 5'-phosphate. Within experimental error, the values determined were identical for the enzymes from both cell types, as shown in Table 1.

The addition of isobutylmethylxanthine to the induction medium results in significantly greater amounts of ornithine decarboxylase activity in both cell types (results not shown). If this effect was due to enzyme activation or production of a more active ornithine decarboxylase isoenzyme, one might expect to see different kinetic characteristics from those found in control cultures. Therefore ornithine decarboxylase was also isolated from 3T3 and SV101 cells induced only with fresh serum-containing medium, and the K_m for ornithine was measured. The apparent K_m did not change in either cell line, arguing against the above possibilities.

Specific activity of purified ornithine decarboxylase

The enzyme was purified via affinity chromatography from both cell types, and the peak column fractions were hydrolysed in 6 M-HCl for protein determination via the *o*-phthalaldehyde protein assay. *o*-Phthalaldehyde binding to the amino groups released by hydrolysis results in an enhancement of fluorescence and an extremely sensitive protein determination, suitable for measuring the low (approx. 10 µg/ml) concentrations of protein in the peak affinity column fractions. An alternative protein assay similar to the above, but with ninhydrin rather than *o*-phthalaldehyde to quantify released amino acids, was also developed in this laboratory. Although 100-fold less sensitive, the ninhydrin assay provided useful confirmation for the *o*-phthalaldehyde data (results not shown).

The specific activity obtained was slightly higher than reported previously for the SV101 cell ornithine decarboxylase (Boucek & Lembach, 1977), probably due to the different protein assay methodology. For both cell types, the specific activity of purified ornithine decarboxylase was approx. 125 000 nmol of CO₂ released/h per mg of protein, as shown in Table 1.

Immunology

A typical enzyme titration curve is shown in Fig. 4. The 3T3 cell enzyme appears to cross-react completely with the SV101 cell enzyme, yielding virtually identical inactivation profiles.

Enzyme half-life

The half-life of ornithine decarboxylase activity *in vivo* was measured in the two cell types in the presence of cycloheximide. Ornithine decarboxylase was induced in duplicate cultures by the addition of

Fig. 3. Thermostability of 3T3 and SV101 cell ornithine decarboxylase in vitro at 40°C
The thermostability at 40°C of partially purified ornithine decarboxylase from both 3T3 (a) and SV101 (b) cells was measured in the presence and absence of saturating concentrations of substrate and cofactor, as described in the Materials and methods section. Time points were taken every 30 min for 2 h in control samples (O), samples containing 1 mM-ornithine (Δ), 0.2 mM-pyridoxal phosphate (◇) and 1 mM-ornithine plus 0.2 mM-pyridoxal phosphate (□). Ornithine decarboxylase activity remaining is expressed as a percentage of the initial activity of the samples.

Table 1. Molecular properties of 3T3 and SV101 cell ornithine decarboxylase
Experimental details are given in the Materials and methods section.

	3T3 cells	SV101 cells
Subunit molecular weight (Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis)	55 360	56 210
Active species (S-200 gel chromatography)	104 440	109 080
Specific activity (nmol of CO ₂ released/h per mg)	125 000	120 000
Kinetic parameters		
<i>K_m</i> (ornithine) (μM)	30.1	24.7
<i>K_m</i> (pyridoxal phosphate) (μM)	0.3	0.2
Degradation rate (enzyme half-life, min)	113	52

fresh serum-containing medium to serum-starved cells. Cycloheximide was added to half the cultures at 3 h after enzyme induction, and ornithine decarboxylase activity was measured at short time intervals thereafter. As can be seen in Fig. 5, the activity decay was first-order for both cells, corresponding to a calculated half-life of about 1 h in the transformants and 2 h in the normals. The 2 h half-life value for the 3T3 cell ornithine decarboxylase is in excellent agreement with the half-life

previously reported for these cells under similar induction conditions (Clark, 1974; Clark & Fuller, 1976a). This would imply slightly decreased enzyme stability in the SV101 cells.
To determine whether the induction conditions would alter the degradation rates, half-lives were measured in the presence of 3-isobutyl-1-methylxanthine. No significant change in enzyme stability could be detected (results not shown).

These results are summarized in Table 1.

Fig. 4. Immunological cross-reactivity of ornithine decarboxylase from 3T3 and SV101 cells

Extracts containing roughly equal amounts of ornithine decarboxylase activity were prepared from 3T3 (○) and SV101 (□) cells and titrated with increasing amounts of antibody raised against purified SV101 ornithine decarboxylase, as described in the Materials and methods section. Residual ornithine decarboxylase activity is expressed as a percentage of the activity present in control samples incubated with sera obtained from a non-immunized rabbit.

Discussion

Numerous researchers feel ornithine decarboxylase plays a pivotal role in overall growth control. Not only does ornithine decarboxylase catalyse the rate-limiting step in the synthesis of polyamines, but one of the earliest detectable events after a wide variety of growth stimuli in many different systems is the rapid and substantial increase in ornithine decarboxylase activity. Thus a study of ornithine decarboxylase regulation may well provide insights into the more general phenomenon of regulation of cell growth and division.

The 3T3/SV101 mouse fibroblast system represents a model tissue culture system for the study of growth regulation. Viral transformation manifests itself in this system primarily as a loss of density-dependent inhibition of growth. This phenomenon is accompanied by a decrease in serum requirements for cell growth and division. Changes in regulation of ornithine decarboxylase may be involved in this process.

Fig. 5. Half-life of ornithine decarboxylase activity in 3T3 and SV101 cells in culture

Ornithine decarboxylase was induced in 3T3 (○) and SV101 (□) cells as described in the Materials and methods section, and 50 μg of cycloheximide/ml was added 3 h later. Time points were taken every 30 min for 2.5 h. Activity is expressed as a percentage of ornithine decarboxylase activity in control cultures that did not receive cycloheximide.

Qualitatively the induction of ornithine decarboxylase in the two cells is quite similar. In each strain the enzyme is induced by typical growth stimuli, such as the refeeding of quiescent cultures with serum-containing medium, the addition of growth factors etc. Quantitatively they show great differences. As previously described, the transformant has far greater amounts of ornithine decarboxylase activity than the parent strain at any given concentration of serum (the effect is greatest at low concentrations of serum, but still quite significant at higher concentrations, e.g. about 6-fold greater at 5% serum). This holds true for steady-state ornithine decarboxylase activities as well as under typical induction conditions (Lembach, 1974).

One obvious explanation for the difference in specific activities found in crude extracts is the appearance of an enzyme molecule in the transformant with different physicochemical properties, either via a post-translational modification or through induction of a high-specific-activity isoenzyme. These studies provide the first direct comparison of highly-purified ornithine decarboxylases from a transformed cell and its parent line. No significant differences could be found between the

purified enzymes, with regard to either specific activity or subunit molecular weight on sodium dodecyl sulphate/polyacrylamide-gels. The specific activities agree well with a previous estimate for the specific activity of SV101 cell ornithine decarboxylase, also purified via a pyridoxamine phosphate affinity procedure (Boucek & Lembach, 1977). They are significantly higher than specific activities reported in all other systems with alternative (non-affinity) purification schemes (Friedman *et al.*, 1972; Ono *et al.*, 1972; Obenrader & Prouty, 1977a). The subunit molecular weight of the purified enzyme from 3T3 cells is virtually identical with that found previously for both SV101 cell (Boucek & Lembach, 1977) and rat liver (Obenrader & Prouty, 1977a) enzymes.

The results of molecular sizing via gel filtration show the enzymes to be identical as far as state of subunit aggregation is concerned. The enzyme runs as an apparent dimer on gel-filtration chromatography, in agreement with previous work with SV101 cell (Boucek & Lembach, 1977) and rat liver (Obenrader & Prouty, 1977a) ornithine decarboxylases. If this result represented a rapid equilibrium between a monomer and a polymer, one might expect the equilibrium to shift towards the monomer as the enzyme concentration decreased, and towards the polymer as it increased. However, no change in molecular weight was observed when the ornithine decarboxylase concentration was varied by a factor of ten, arguing strongly against such a rapid equilibrium system. No activity could be detected at the monomer molecular-weight elution volume, suggesting that this species is either inactive or not present under these conditions.

The thermostability of ornithine decarboxylase activity *in vitro* at 40°C was investigated in detail. In the absence of cofactor, the enzyme is quite unstable at elevated temperatures. The addition of substrate has little or no effect. The addition of cofactor results in a marked stabilization of activity, whereas substrate and cofactor in combination yield a degree of stabilization intermediate between either one alone.

This can be explained in the following manner. Owing to the nature of pyridoxal phosphate-mediated decarboxylation (Mandel *et al.*, 1954), the covalently-bound cofactor must be released from the ϵ -amino group of a lysine residue peripheral to the active site in order to interact with substrate. Since the affinity of ornithine decarboxylase is relatively low for pyridoxal phosphate, during catalysis the cofactor may diffuse out of the active-site cleft, allowing the enzyme to regain its former unstable conformational state. Thus the substrate may contribute to the rate of thermal degradation by promoting release of pyridoxal phosphate from the active site. As substrate is

gradually removed from the system by catalysis, one would expect the activity to stabilize at some intermediate value, which indeed it does (Fig. 3).

To investigate the nature of this protection by the cofactor, an attempt was made to monitor a change in the aggregation state of the enzyme in the presence of pyridoxal phosphate on the S-200 column without success. Any conformational change must therefore be taking place at a more subtle level, perhaps involving a slight folding of the enzyme about the cofactor to a more thermally resistant form.

The mechanism *in vitro* and significance *in vivo* of these studies are unclear. Although evidence for ornithine decarboxylase isoenzymes that differ in cofactor affinity has been found in *Physarum* (Sedory & Mitchell, 1977), the results reported herein argue against such a system in mouse fibroblast cells. Litwack & Rosenfield (1973) believe that cofactor dissociation may be the rate-limiting step for enzyme degradation *in vivo*, but kinetic studies by Clark & Fuller (1975) suggest this may not be the case. Whether thermal degradation involves the action of an endogenous proteinase is not known; however, a group-specific proteinase capable of selectively degrading the apoenzyme form of pyridoxal phosphate-requiring enzymes has been reported (Kominami *et al.*, 1972).

No significant differences between 3T3 and SV101 cell ornithine decarboxylase could be detected by the kinetic analyses (Table 1). The apparent K_m for ornithine is in excellent agreement with the value of 28 μM reported for rat liver ornithine decarboxylase by Murphy & Brosnan (1976), but is considerably lower than the value of 130 μM reported by Obenrader & Prouty (1977a), also for rat liver ornithine decarboxylase. The reason for this discrepancy is not clear. The latter group, however, reported a K_m for cofactor of 0.25 μM , which is virtually identical with the values reported in the present paper; the value of 0.4 μM obtained by Clark & Fuller (1976a) for 3T3 cells is also very close.

The half-life of ornithine decarboxylase activity induced in cell cultures by refeeding yields the perhaps surprising result that the enzyme is approximately twice as stable in the parent strain, under the conditions tested. These data are in agreement with half-life studies on 3T3 cells, wherein the enzyme half-life was monitored at different stages of the induction period (Clark, 1974). At least during the first 8 h of growth stimulation, enzyme stability varied inversely with total ornithine decarboxylase activity. Since the transformant has much more ornithine decarboxylase activity one might expect a shortened enzyme half-life in these cells.

There is indirect evidence that enzyme synthesis *de novo* is involved in ornithine decarboxylase induction. Not only is the induction dependent on

transcription and translation (Lembach, 1974), but immunological studies have demonstrated the emergence of immunoreactive material concurrent with the appearance of ornithine decarboxylase activity (Hölttä, 1975; Obenrader & Prouty, 1977b).

Although studies that utilize the disappearance of enzyme activity as a means of quantifying immunoreactive protein must be viewed with caution, they are valuable as supporting evidence. By using such methodology to compare ornithine decarboxylases from 3T3 and SV101 cells, we find that the two enzymes appear to have approximately the same immunological cross-reactivity to antibodies raised against purified SV101 cell ornithine decarboxylase.

An additional argument against structural and/or kinetic differences between the two enzymes is their similar behaviour throughout the purification procedure. Both elute from the ion-exchange resin at about 0.15 M-NaCl, indicating a similar distribution of net charge; both precipitate out of solution at 55% saturation with $(\text{NH}_4)_2\text{SO}_4$; and both bind the affinity resin to the same extent, eluting at roughly $3\text{ }\mu\text{M}$ -pyridoxal phosphate, evidence for identical cofactor affinity.

On the basis of the studies described in the present paper we conclude that the two enzymes are similar if not identical, and that activity differences between 3T3 and SV101 cells are due solely to a change in the amount of active enzyme present in the cell. The difference in the specific activities of the purified enzymes is negligible, and thus cannot explain the 6-fold higher activities found in the transformant. The half-life data presented above argue strongly against an increase in stability of ornithine decarboxylase in SV101 cells; the enzyme actually appears to be more stable in the parent cells. No low-molecular-weight physiological effectors of the enzyme have been found, but the possibility remains that an activator or inhibitor could play an important role in regulation of ornithine decarboxylase activity. Considering the very rapid turnover of this enzyme, such a control mechanism would appear to be superfluous. Our conclusion is that the ornithine decarboxylase activity in these cells is controlled largely by the rate of synthesis *de novo*.

However, studies by Heller *et al.* (1978) indicate that high concentrations of putrescine can induce a protein inhibitor of ornithine decarboxylase in 3T3 cells, in direct contradiction to the previous work of Clark & Fuller (1976b). If indeed 3T3 cells contain ornithine decarboxylase 'antizyme' that can be detected without recourse to unphysiologically high concentrations of polyamines, it would be of interest to compare antizyme concentrations with those found in the transformant.

Why, then, does the transformant synthesize ornithine decarboxylase at such a tremendously elevated rate, an order of magnitude faster than the

parent cell line (taking degradation rates into account)? There is excellent evidence that the polyamines exert direct feedback inhibition on to the induction of ornithine decarboxylase activity in 3T3 cells, probably at the translational level (Clark & Fuller, 1975). Bethell & Pegg (1979) have shown that SV101 cells are not as sensitive to polyamine feedback inhibition as the parent line. This relative lack of feedback inhibition undoubtedly accounts for part of the difference in ornithine decarboxylase activity seen between the cells, but another part of the answer may lie in the realm of cyclic AMP metabolism. The addition of 3-isobutyl-1-methylxanthine, a potent phosphodiesterase inhibitor, to the induction medium causes a 'superinduction' of ornithine decarboxylase (J. M. Weiss, unpublished work) that is much greater in the parent cells. By adding optimal concentrations of 3-isobutyl-1-methylxanthine to both cell types, the ornithine decarboxylase activity in 3T3 cells can be increased to such an extent that it actually matches the transformant. Further investigations into the regulation of ornithine decarboxylase in these two cell types must take into account the interactions between two complex systems: polyamine metabolism and cyclic AMP metabolism. Only a complete understanding of this interaction will elucidate the mechanisms by which this key enzyme is controlled.

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