Studies of mammalian ornithine decarboxylase using a monoclonal antibody

Anthony E. PEGG,* James E. SEELY,* Lo PERSSON,* Meenhard HERLYN,† Karen PONSELL† and Thomas G. O'BRIEN†

*Department of Physiology, The Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033, U.S.A., and †Wistar Institute, Philadelphia, PA 19104, U.S.A.

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A monoclonal antibody of the immunoglobulin M class was produced against mouse kidney ornithine decarboxylase. Screening for the antibody was carried out using α -difluoromethyl[5-³H]ornithine-labelled ornithine decarboxylase. The antibody reacted with this antigen and with native ornithine decarboxylase. The antibody attached to Sepharose could be used to form an immunoaffinity column that retained mammalian ornithine decarboxylase. The active enzyme could then be eluted in a highly purified form by 1.0M-sodium thiocyanate. The monoclonal antibody could also be used to precipitate labelled ornithine decarboxylase from homogenates of kidneys from androgen-treated mice given [³⁵S]methionine. Only one band, corresponding to M_r of about 55000, was observed. The extensive labelling of this band is consistent with the rapid turnover of ornithine decarboxylase protein, since this enzyme represents only about 1 part in 10000 of the cytosolic protein.

Ornithine decarboxylase is the first enzyme in the mammalian polyamine biosynthetic pathway (Pegg & Williams-Ashman, 1981; Pegg & McCann, 1982). The activity of this enzyme exhibits rapid and manyfold changes in activity in response to a wide variety of stimuli and there is evidence that stimulation may be linked to cell growth and tumour promotion (Boutwell *et al.*, 1979; Russell, 1980; Pegg & McCann, 1982). Detailed studies of the underlying biochemical mechanism of the regulation of ornithine decarboxylase have been hampered by the small amounts of this protein present in mammalian tissues (Pritchard *et al.*, 1981; Seely *et al.*, 1982a) and the consequent difficulty in obtaining the purified protein. Recently, several groups have described the purification of ornithine decarboxylase to homogeneity from rat liver (Kameji *et al.*, 1982; Kitani & Fujisawa, 1983) or mouse kidney (Persson, 1981; Seely *et al.*, 1982b) and specific antisera have been raised to the purified enzyme in rabbits (Persson, 1982; Seely & Pegg, 1983). However, even from these relatively rich sources of the enzyme, only small amounts of it were obtained, and improved methods of isolating the enzyme from crude extracts will be needed to study the enzyme in other cell extracts. In the present paper, we describe the

production of a monoclonal antibody to ornithine decarboxylase and its use in the purification of the enzyme and its identification in crude tissue extracts.

Materials and methods

Materials

L-[1-¹⁴C]Ornithine (sp. radioactivity 57 Ci/mol), DL- α -difluoromethyl[5-³H]ornithine (sp. radioactivity 15 Ci/mmol) and L-[³⁵S]methionine (sp. radioactivity 1284 Ci/mmol) and EN³HANCE were purchased from NEN, Boston, MA, U.S.A. Bacterial protein A adsorbent was obtained from Miles Laboratories, Elkhart, IN, U.S.A. CNBr-activated Sepharose was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Other reagents came from Sigma Chemical Co., St. Louis, MO, U.S.A.

Production of monoclonal antibody

Ornithine decarboxylase was purified from kidneys of androgen-treated mice by the pyridoxamine-Sepharose affinity chromatography as described by Seely *et al.* (1982b). This material was about 10–20% pure, based on the comparison of its specific activity with that of the homogeneous enzyme (Seely *et al.*, 1982b). This protein was used to immunize a Balb/c female mouse by subcutaneous administration of about 5 μ g emulsified

Abbreviations used: DFMO, α -difluoromethylornithine; PBS, phosphate-buffered saline (0.12M-NaCl/0.012M-Na₂HPO₄/0.0015M-KH₂PO₄).

in Freund's complete adjuvant. The mouse was re-immunized 4 weeks later with a further 5 µg of protein in incomplete adjuvant given intraperitoneally. After a further 4 weeks, 5 µg of protein was given intravenously, and 4 days later splenic lymphocytes were isolated and fused to mouse myeloma P3x63Ag8 (variant 653) cells (Kearney *et al.*, 1979) with PEG4000 (Koprowski *et al.*, 1977; Herlyn *et al.*, 1979). The resulting hybridoma cells were cloned in HAT medium by limiting dilution (Galfré & Milstein, 1981). The clones were tested for the production of antibodies to ornithine decarboxylase as follows: polyvinylchloride microtitre 96-well plates (Dynatech, Alexandria, VA, U.S.A.) were coated with 50–100 µg of a 'second antibody' (goat anti-mouse immunoglobulin G) by additions of a solution in 0.05M-borate buffer, pH8, which was allowed to dry overnight at room temperature. After washing the wells three times with RIA buffer (PBS containing 10% γ-globulin-free horse serum and 0.02% NaN₃), 0.05 ml of the culture supernatants was added and incubated at 4°C overnight. The supernatant was removed and, after washing three times with RIA buffer, 0.05 ml of a solution containing about 10000 c.p.m. of [5-³H]DFMO-labelled ornithine decarboxylase was added. [This labelled protein was prepared by allowing the partially purified mouse kidney enzyme to react with [5-³H]DFMO (sp. radioactivity 15 Ci/mmol) as described previously (Seely & Pegg, 1983).] Incubation was continued for at least 1 h at room temperature or overnight at 4°C. The solution was then removed, the wells were washed three times with RIA buffer and their contents solubilized in 0.5M-NaOH and counted for radioactivity. Controls for non-specific binding were included by omitting either the second antibody or the culture supernatant. Less than 20–30 c.p.m. was bound under these conditions. One clone that gave a strong positive response, binding about 500 c.p.m. of the labelled antigen, was re-cloned several times and grown up as described above. The immunoglobulin (designated MK-1) produced by this clone was found to be of the immunoglobulin M type by Ouchterlony immunodiffusion analysis using monospecific antisera purchased from Bionetics, Bethesda, MD, U.S.A. (The commercial goat anti-mouse immunoglobulin G preparation used in the initial screening has significant ability to bind mouse immunoglobulin M molecules also, and this accounts for the initial detection of this antibody.) The hybridoma cells were grown as an ascites form by intraperitoneal injection into pristane-treated mice (Galfré & Milstein, 1981) and the resulting ascites fluid was used as a source of monoclonal antibody after removal of cells by centrifugation at 1000g for 10 min.

Precipitation of ornithine decarboxylase or [5-³H]-DFMO-labelled ornithine decarboxylase

These experiments were carried out with crude (150 units/mg of protein) or partially purified (approx. 20% pure; 360000 units/mg of protein) mouse kidney ornithine decarboxylase. These enzyme preparations were isolated as described by Seely *et al.* (1982b). Protein was determined by the method of Bradford (1976). One unit of enzyme activity is defined as the amount releasing 1 nmol of CO₂ in 30 min at 37°C under the assay conditions described by Seely *et al.* (1982a,b). Mouse kidney ornithine decarboxylase (about 30 units in 0.1 ml of buffer A, which consists of 25 mM-Tris/HCl, pH7.5, 0.1 mM-EDTA and 2.5 mM-dithiothreitol) was incubated with 0.06 ml of various dilutions of ascites fluid containing monoclonal antibody MK-1 (diluted into 150 mM-Tris/HCl, pH7.5, 1 mM-EDTA, 0.02% Brij 35; buffer A) for 2 h at 0–4°C. A further 0.06 ml of a solution (3 mg/ml) of goat anti-mouse immunoglobulin M was then added and the samples were shaken gently overnight at 0–6°C. Protein A bacterial adsorbent (0.08 ml of a 10% suspension) was then added and the samples were shaken for 2 h at 0–6°C. After centrifugation at 15000g for 1 min, 0.1 ml portions of the supernatant were assayed for ornithine decarboxylase activity (Seely *et al.*, 1982b). The precipitation of [5-³H]DFMO-labelled ornithine decarboxylase was carried out in the same way, starting with a solution containing 2592 c.p.m. in 0.1 ml. The immunoprecipitate was washed twice in 0.5 ml of buffer A, resuspended in 0.2 ml of water and counted for radioactivity as previously described (Seely & Pegg, 1983). Control experiments using another ascites fluid containing monoclonal immunoglobulin M (designated 1116NS10) that was prepared against a human adenocarcinoma cell line (Brockhaus *et al.*, 1981) indicated no loss of ornithine decarboxylase activity or precipitation of the [5-³H]DFMO-labelled ornithine decarboxylase under these conditions. A positive control was provided by the use of rabbit antiserum against ornithine decarboxylase. This was used in the same way, but the goat anti-mouse immunoglobulin M was omitted.

Immunoaffinity chromatography

The MK-1 antibody was purified from ascites fluid by (NH₄)₂SO₄ precipitation and gel filtration on Sephacryl S-300 (Hudson & Hay, 1980). Fractions eluted from this column containing pure antibody (determined by polyacrylamide-gel electrophoresis under denaturing conditions) were used to prepare immunoaffinity columns. CNBr-activated Sepharose 4B was reconstituted and washed according to the manufacturer's instructions. It was then coupled with the MK-1 antibody by over-

night incubation with the antibody (2–5 mg of protein/ml) in PBS, pH 8.0. Determination of the A_{280} of the solution before and after coupling indicated that about 80–95% of the antibody was coupled to the Sepharose. Remaining sites on the gel were blocked by reaction with 0.5 M-ethanolamine at room temperature for several hours or overnight at 4°C. Columns containing about 2 ml of the gel was prepared, washed with buffer A containing 0.3 mM-L-ornithine and 20 μ M-pyridoxal phosphate and stored at 4°C until use.

Immunoprecipitation of labelled ornithine decarboxylase from mice treated with [35 S]methionine

Female Balb/c mice were used untreated or treated with androgens to induce ornithine decarboxylase as described previously (Seely & Pegg, 1983). The mice were then given 500 μ Ci of [35 S]-methionine (sp. radioactivity 1284 Ci/mmol) by intraperitoneal injection of a 0.2 ml solution in PBS. The mice were killed 30 min later and the kidneys removed, homogenized in 3 vol. of 25 mM-Tris/HCl (pH 7.5)/0.1 mM-EDTA/2.5 mM-dithiothreitol and centrifuged at 100 000 g for 45 min. Portions (0.05 ml) of the supernatant were incubated with 0.15 ml of a 1:50 dilution of the MK-1 ascites fluid (diluted in buffer A) for 3 h at 0–4°C. Goat anti-mouse immunoglobulin M (30 μ l; 30 mg/ml) was then added and incubation was continued overnight at 0–4°C. After 16 h, bacterial protein A adsorbent (0.3 ml of a 10% solution) was added and the sample was incubated with shaking for an additional 2 h at room temperature. The mixture was diluted by addition of 0.75 ml of buffer

A and centrifuged at 15000 g for 30 s. The pellet was washed four times in buffer A and finally resuspended in 0.1 ml of a solubilizing buffer containing 2% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 10% glycerol and 62.5 mM-Tris/HCl, pH 6.8. After heating in a boiling-water bath for 5 min the sample was centrifuged at 15000 g for 1 min and the supernatant was used for polyacrylamide-gel electrophoresis on a 10% discontinuous buffer system as described by Laemmli (1970). The gel was fixed overnight in 10% trichloroacetic acid/10% acetic acid/30% methanol, impregnated with EN 3 HANCE, dried and exposed to Kodak XAR-5 X-ray film at –70°C for 3 days. Control samples using either a monoclonal antibody not directed against ornithine decarboxylase or a polyclonal rabbit antiserum to ornithine decarboxylase were prepared in the same way.

Results

One of the major advantages of the hybridoma technique for the production of specific antibodies is that a homogeneous antigen is not required for immunization, and in our experiments only partially purified ornithine decarboxylase protein was used. However, it is necessary to have either the purified protein of interest or a specific way to identify its binding to the antibodies in order to screen potential clones of antibody-secreting cells. In our experiments, we achieved this by using [3 H]DFMO to specifically label ornithine decarboxylase. DFMO is an enzyme-activated

Table 1 *Precipitation of ornithine decarboxylase by a monoclonal antibody*

Expt. (A): Precipitation of native enzyme

Antibody added (0.06 ml of dilution shown)	Ornithine decarboxylase activity remaining (units)	Amount precipitated (%)
None	27.1	0
Rabbit antiserum (1:50)	0.1	99
Monoclonal antibody (1:50)	8.7	68
Monoclonal antibody (1:100)	8.9	67
Monoclonal antibody (1:500)	8.8	68
Monoclonal antibody (1:2500)	22.7	16

Expt. B: Precipitation of enzyme that had been labelled and inactivated by reaction with [3 H]DFMO

Antibody added (0.06 ml of dilution shown)	[3 H]DFMO-labelled ornithine decarboxylase precipitated (c.p.m.)*	Amount precipitated (%)
None	0	0
Rabbit antiserum (1:50)	2756	93
Monoclonal antibody (1:50)	2133	72
Monoclonal antibody (1:500)	1153	39
Monoclonal antibody (1:2500)	250	8

* A total of 2952 c.p.m. was added.

irreversible inhibitor of this enzyme and forms a covalent bond with the enzyme. This interaction is extremely specific, and even in crude cell extracts ornithine decarboxylase is the only protein that becomes labelled (Seely *et al.*, 1982*b,c*). The use of [5-³H]DFMO-labelled ornithine decarboxylase to identify positive clones probably helped in the isolation of an antibody, MK-1, which recognizes the native enzyme, since the soluble antigen was used for detection rather than insolubilized protein used in many ELISA techniques. However, it is unlikely that antibodies directed at the active site of ornithine decarboxylase would be found using this screening method, since DFMO binds at the active site. When added to solutions containing either native ornithine decarboxylase from rat or mouse tissues or [5-³H]DFMO-labelled ornithine decarboxylase, the monoclonal antibody, MK-1, did not lead to rapid loss of the activity or precipitation of the radioactivity (results not shown). However, precipitation was achieved by addition of a second antibody (immunoglobulin G) specific for mouse immunoglobulin M, followed by protein A (Table 1).

Not all of the ornithine decarboxylase was precipitated even under these conditions, and the amount precipitated was somewhat variable (from 40–80%) from experiment to experiment. In a typical experiment shown in Table 1, Expt. A, with mouse kidney extracts that contained about 25 ng of ornithine decarboxylase in 0.1 ml, approx. 70% of the activity was precipitated by about 1:500 dilutions of the ascites fluid. Increasing the antibody concentration by 10-fold did not increase the extent of precipitation, but all of the enzyme could be precipitated by polyclonal rabbit antiserum under the same conditions. In these experiments, the monoclonal antibody was incubated with the enzyme for 2 h at 4°C, but increasing the time or temperature did not increase the percentage precipitated. Similarly, the monoclonal antibody was able to precipitate about 70% of the radioactivity present in [5-³H]DFMO-labelled ornithine decarboxylase in the presence of the second antibody and protein A, although rabbit antiserum could precipitate more than 90% of this material (Table 1, Expt. B). Larger amounts of the monoclonal antibody were needed to bring about precipitation of the inactivated labelled antigen than of the native enzyme (Table 1).

Preliminary trials with immunoaffinity columns containing bound MK-1 antibody indicated that both active ornithine decarboxylase and [5-³H]-DFMO-labelled derivative were retained on these columns. Several different strategies for eluting the material from these columns were tested. Low-ionic-strength buffer of either high (11.5) or low (2.5) pH eluted the protein, but were not compat-

ible with the recovery of enzyme activity in good yield. However, as shown in Fig. 1(*a*), a stepwise gradient of the chaotropic agent NaSCN eluted the enzyme with excellent retention of activity (measured after dialysis to remove the thiocyanate). About 82% of the enzyme activity applied was recovered, with 91.5% of this amount (75% of the total) in the NaSCN fractions. These columns could also be used to purify crude preparations of ornithine decarboxylase (Fig. 1*b*). After application of a crude mouse kidney extract, virtually all the protein was eluted in the first four fractions, whereas 93% of the recovered ornithine decar-

Fig. 1. Immunoaffinity chromatography of native ornithine decarboxylase

(*a*) Partially purified mouse kidney ornithine decarboxylase [through the DEAE-cellulose chromatography step of the procedure described by Seely *et al.* (1982*b*)] was applied to an immunoabsorbent column prepared as described in the Materials and methods section. The column was eluted with 2 ml fractions of buffer A containing 0.3 mM-L-ornithine and 20 μ M-pyridoxal phosphate (loading buffer) containing the concentration of NaSCN shown, and the ornithine decarboxylase (ODC) activity eluted was determined after overnight dialysis against 60 vol. of buffer A to remove NaSCN. (*b*) Crude mouse kidney extracts [purified about 2-fold by (NH₄)₂SO₄ fractionation and dialysis (Seely *et al.*, 1982*b*)] were applied to the column. The column was washed with 8 ml of loading buffer (1 ml per fraction) and then ornithine decarboxylase was eluted with 1 M-NaSCN in loading buffer (arrow). All fractions were assayed for ornithine decarboxylase (ODC) activity (after dialysis as described for *a*) (●) and for protein (○).

Fig. 2. Immunoaffinity chromatography of $[5\text{-}^3\text{H}]\text{DFMO}$ -labelled ornithine decarboxylase

The same procedure as used in Fig. 1 was employed, except that $[5\text{-}^3\text{H}]\text{DFMO}$ -labelled ornithine decarboxylase was chromatographed and elution was carried out with 0.4M -NaSCN (arrow). Approx. 84% of the applied radioactivity was recovered from the column with 26.5% in the peak after 0.4M -NaSCN.

boxylase activity was eluted as a sharp peak when 1M -NaSCN was applied. Even with crude extracts that had been fractionated only by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Seely *et al.*, 1982b), at least 60-fold purification was achieved by a single pass through the immunoaffinity column (Fig. 1b). When the extracts were partially purified by DEAE-cellulose chromatography (Seely *et al.*, 1982b) before immunoaffinity chromatography, at least 2000-fold purification was achieved at this step (results not shown).

The immunoaffinity columns also bound $[5\text{-}^3\text{H}]\text{DFMO}$ -labelled ornithine decarboxylase, and this could be eluted by NaSCN (Fig. 2), although a larger fraction of the labelled ornithine decarboxylase was not retained by this column. This result is consistent with the results of Table 1 and other data suggesting that the monoclonal antibody has a greater affinity for unmodified ornithine decarboxylase than for the DFMO-inactivated protein. The immunoaffinity columns could be re-used several times without significant loss of activity (results not shown). Immunoaffinity columns prepared in the same way with a mouse monoclonal immunoglobulin M (1116NS10) directed against the Le^b blood-group antigen (Brockhaus *et al.*, 1981) did not retain native or DFMO-labelled ornithine decarboxylase, indicating that the results are not due to a non-specific adherence of ornithine decarboxylase to the column adsorbent.

The monoclonal antibody in conjunction with protein A could be used to demonstrate the rapid synthesis of ornithine decarboxylase in the kidneys

Fig. 3. Polyacrylamide-gel electrophoresis of labelled ornithine decarboxylase precipitated by monoclonal antibody

Samples from mice given $[^3\text{S}]\text{methionine}$ were analysed as described in the Materials and methods section. The lanes shown are: (1) and (4), 0.05ml of androgen-treated mouse extract precipitated with monoclonal 1116NS10; (2) and (7), 0.05ml of androgen-treated mouse extract precipitated with monoclonal MK-1; (6), 0.05ml of androgen-treated mouse extract precipitated with rabbit antiserum against ornithine decarboxylase; (3), 0.05ml of female mouse extract precipitated with monoclonal MK-1; (5), $[5\text{-}^3\text{H}]\text{DFMO}$ -labelled ornithine decarboxylase marker precipitated with MK-1; (8), 0.017ml of extract from androgen-treated mouse. Note that a vacant lane was left between (7) and (8) to allow for some contamination by the very heavily labelled total extract sample placed in lane (8).

of androgen-treated female mice. As shown in Fig. 3, a band of labelled protein having a M_r of about 55000 could be precipitated from extracts of kidneys of such mice given $[^3\text{S}]\text{methionine}$ 30 min before death (lanes 2 and 7). As shown in lane 3, this band was not present in similarly treated extracts from female mice not treated with androgens, which have a 400-fold lower content of ornithine decarboxylase (Seely & Pegg, 1983). The band corresponded exactly to a marker of $[5\text{-}^3\text{H}]\text{DFMO}$ -labelled ornithine decarboxylase (lane 5) and to a band precipitated by polyclonal rabbit antiserum to ornithine decarboxylase (lane 6). This band was not found when another monoclonal antibody not directed against ornithine decarboxylase was used as a control (lanes 1 and 4). The band precipitated by the rabbit antiserum (lane 6) was more intense than that precipitated by MK-1 (2 and 7), which is consistent with the more complete precipitation of ornithine decarboxylase by the rabbit antiserum. However, the bands had identical mobility.

Discussion

The immunoaffinity purification described in the present paper should prove useful in the isolation of ornithine decarboxylase from mammalian cell extracts, in which this enzyme represents only a very small percentage of the total protein. Although the species specificity of the interaction of the monoclonal antibody has not been investigated in detail, it does interact with the enzyme from mouse, rat and hamster tissues, and cells from these species could be used as sources of enzyme. Although the degree of precipitation of the enzyme by the monoclonal antibody was rather variable from experiment to experiment, the experiments provided no evidence in favour of multiple forms of the enzyme. The labelled band of ornithine decarboxylase precipitated by the monoclonal antibody from kidney extracts of mice given [35 S]-methionine coincided exactly with the band precipitated by the polyclonal rabbit antiserum and with the [$5\text{-}^3\text{H}$]DFMO-labelled marker of ornithine decarboxylase. The substantial incorporation of [35 S]methionine into this band provides direct experimental evidence showing that ornithine decarboxylase has a rapid rate of synthesis and degradation. Ornithine decarboxylase represents only 1 part in 5000–10000 of the soluble protein in the mouse kidney even after androgen stimulation (Seely *et al.*, 1982a,b), but the radioactivity incorporated into the ornithine decarboxylase band amounts to about 1% of the total incorporation into protein. This indicates that the enzyme protein must turn over much more rapidly than the average kidney protein. These results therefore confirm previous reports based on indirect evidence that ornithine decarboxylase protein has a very short half-life (Russell, 1980; Seely *et al.*, 1982c). Studies *in vitro* of the mechanism by which the rapid degradation of ornithine decarboxylase is brought about should be aided by the availability of the radioactive labelled protein. This substrate can now be prepared by administration of [35 S]-methionine to androgen-treated mice, followed by immunoaffinity chromatography of the kidney extracts.

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