S-Adenosyl-L-methionine decarboxylase of *Acanthamoeba castellanii* (Neff): purification and properties

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S-Adenosyl-L-methionine decarboxylase (AdoMetDC) has been purified to near homogeneity from the Neff strain of *Acanthamoeba castellanii*. The holoenzyme molecular mass is 88.8 kDa, including two copies each of a 32.8 kDa α -subunit and a 10–15 kDa β -subunit. The α -subunit contains the active site. It has an N-terminal pyruvoyl group, and the first 19 amino acids are 63 and 74% identical with comparable sequences from yeast and mammals, respectively. The apparent K_m for S-adenosylmethionine (AdoMet) in the presence of 2 mM

putrescine was 30.0 μ M. The enzyme was stimulated 2-fold by putrescine, but was unaffected by spermidine. It was inhibited by the following anti-metabolites, listed with their K_i values: Berenil (0.17 μ M), pentamidine (19.4 μ M), propamidine (334 μ M), hydroxystilbamidine (357 μ M), methylglyoxal bis(guanylhydrazine) (604 μ M) and ethidium bromide (1.3 mM). Activity of the enzyme fell to undetectable levels during cell differentiation (encystment).

INTRODUCTION

Acanthamoeba is a genus of amoebae that are ubiquitous in Nature. They are opportunistic pathogens responsible for the eye disease *Acanthamoeba* keratitis and the rarer central-nervous-system disease granulomatous amoebic encephalitis ('GAE') [1,2]. The eye disease can cause blindness. It is difficult to treat medically, and better chemotherapeutic agents are needed. Inhibition of polyamine biosynthesis is one effective treatment for other protistan diseases [3], and several agents currently used to treat *Acanthamoeba* keratitis are likely to be inhibitors of polyamine synthesis. Thus we have been studying polyamine metabolism in *Acanthamoeba* as a possible basis for development of a more effective therapy [4–6].

The diamidine propamidine has been used frequently to treat *Acanthamoeba* infections [1]. Diamidines arrest multiplication. Several also induce cell differentiation (encystment) [6]. Studies on other organisms, including *Trypanosoma brucei*, rats, yeast and *Escherichia coli*, indicate that diamidines can be potent inhibitors of S-adenosyl-L-methionine decarboxylase (AdoMetDC) [7,8]. Inhibition of this enzyme could affect a variety of cell methylation reactions by causing accumulation of its substrate S-adenosylmethionine (AdoMet) [9] and could arrest polyamine synthesis by blocking production of decarboxylated S-adenosylmethionine (dcAdoMet) needed for spermidine synthesis [10]. In our studies, addition of polyamines to growing cultures of *Acanthamoeba* blocked the induction of encystment by diamidines, but could not prevent the arrest of cell multiplication by these inhibitors [6]. Thus diamidine-induced encystment could be associated with inhibition of polyamine metabolism, possibly by inhibition of AdoMetDC, whereas diamidine-induced arrest of cell multiplication might be associated with effects on other targets.

We have begun a study of relationships between AdoMetDC, diamidines, cell multiplication and cyst formation in an effort to understand the role of polyamine metabolism in amoebic differentiation and to determine whether specific inhibition of

AdoMetDC could be a viable strategy for chemotherapy of *Acanthamoeba* diseases. In this study, we describe purification of AdoMetDC from *Acanthamoeba castellanii* (Neff), physical and kinetic properties of the enzyme, its sensitivity to inhibitors, and developmental changes in activity.

MATERIALS AND METHODS

Chemicals and cultures

Propamidine, hydroxystilbamidine and pentamidine isethionates were provided by N. H. Kimberley, May and Baker, Dagenham, Essex, U.K. Other inhibitors and general chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Radioisotopes were from Amersham Corp., Arlington Heights, IL, U.S.A.

Acanthamoeba castellanii (Neff) was used in all experiments. Subclone NIH-BB, obtained from Dr. Blair Bowers, National Heart, Lung and Blood Institute, Bethesda, MD, U.S.A., was used because it grows to high densities in suspension cultures. Subclone OS4-7B, derived from a clone provided by Dr. Robert J. Neff, was used because it encysts well. Subclone NIH-BB was grown in 1.5–3 litre suspension cultures in semi-defined NIH medium [11]. Subclone OS4-7B was grown in 3 ml monolayer cultures in chemically defined medium (DGM-11) or in semi-defined OGMA medium (= OGM [12] with half the yeast extract). Population doubling times were ~ 14 h in suspension cultures (26 ± 4 °C) and 12 h in monolayers (30 °C).

Enzyme assays

Quantitative microassay

Poso and Pegg's [13] assay for AdoMetDC was scaled down for use with small reaction volumes. The reaction vessel was a vertical assembly of two plastic 0.5 ml microfuge tubes connected head-to-tail with a short piece of latex tubing. The cap was cut off the bottom tube, which held the reaction mixture, and the bottom tip was cut off the top tube, which held a Ba(OH)₂-saturated filter paper to trap radioactive CO₂ released in

Abbreviations used: AdoMet, S-adenosyl-L-methionine; dcAdoMet, decarboxylated AdoMet; AdoMetDC, AdoMet decarboxylase; MGBG, methylglyoxal bis(guanylhydrazine); IEF, isoelectric focusing.

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reactions. Solutions were injected through the latex tubing. The basic reaction mixture included 50 mM Tris, pH 7.2, 2 mM dithiothreitol, 2 mM putrescine and 20 μ M [*carboxy*- 14 C]AdoMet at 2.3 Ci/mol. Reaction vessels were preloaded with 90 μ l of 1.1-times-concentrated basic reaction mixture and equilibrated to 37 °C. Reactions were initiated by injecting 10 μ l (~1.5–3 m-units) of AdoMetDC into the basic reaction mixture (1 unit = amount of enzyme releasing 1 μ mol of CO₂/min). They were stopped after 30 min by injecting 50 μ l of 3 M H₂SO₄ and then were incubated for 15 min longer to ensure that all 14 CO₂ released was trapped by the filters. Radioactivity on the filters was quantified by liquid scintillation counting. The minimum detectable amount of enzyme was 1 μ -unit.

Quantitative microtitre-plate assay

A microtitre-plate assay developed by Tabor et al. [14] was adapted to screen large numbers of liquid-chromatography fractions for AdoMetDC activity. Microtitre plates (96-well) were placed on ice, and 10 μ l of sample plus 40 μ l of 1.25-times-concentrated basic reaction mixture were added to each well. Final concentrations of reactants were the same as for the quantitative microassay. The plate was quickly covered with a piece of Whatman 3 MM chromatography paper that had been soaked in saturated Ba(OH)₂, vacuum-dried over KOH pellets, and then cut to the exact dimensions of the plate. The lid to the microtitre plate was replaced and sealed with Parafilm. Total set-up time was about 5 min. The plate was incubated for 2 h at 37 °C, then disassembled, and the filter paper was dried. The dry paper was sprayed with an autofluorographic enhancing agent (En³Hance; New England Nuclear, Boston, MA, U.S.A.), dried again and exposed to Kodak XAR-5 X-ray film for 24–48 h at –80 °C. Development of the film produced an image of those wells containing AdoMetDC. As little as 300 d.p.m. of 14 CO₂ released could be detected.

Determination of K_m , K_i , pH optimum and pI

The quantitative microassay was used for all kinetic experiments. Assays were repeated at least twice, with three replicates per experimental point. Inhibitors were dissolved in water and added in a maximum of 10 μ l to make a total reaction volume of 100 μ l. The pH optimum was determined at 37 °C with 50 mM sodium phosphate in place of Tris buffer in the basic reaction mixture. The pI determination used h.p.l.c.-purified AdoMetDC thoroughly desalted by dialysis against distilled water or by ultrafiltration. We used precast isoelectric focusing (IEF) gels with pH ranges of 3–7 and 3–10 (FMC Bioproducts, Rockland, ME, U.S.A.). The anodolyte was 0.1 M acetic acid for both types of gels. The cathodolyte was 0.1 M histidine (free base) for pH 3–7 gels and 0.1 M NaOH for pH 3–10 gels. Samples (1–5 μ g of protein) were loaded and focused at a constant 1000 V (pH 3–7 gels) or 1500 V (pH 3–10 gels) for 1 h in a Pharmacia flat-bed IEF apparatus.

Purification of AdoMetDC

Preparation of cell extract

Purification procedures were at 4 °C unless indicated otherwise. NIH-BB amoebae at (4–6) $\times 10^6$ /ml were harvested by centrifugation with typical yields of 130–210 g wet wt. from ~9 litres of culture. Amoebae were resuspended in 5 ml of cold 0.15 M KCl per litre of original culture and washed twice by centrifugation. The cell pellet was resuspended in an equal

volume of lysis buffer (50 mM Tris/HCl, pH 7.2, 2 mM dithiothreitol, 2 mM putrescine, 0.5 mM phenylmethanesulphonyl fluoride and 0.1 μ M pepstatin A) and then either stored frozen at –80 °C as a thin layer in a Pyrex glass dish or placed on ice and processed immediately. Two cycles of freezing at –80 °C and thawing at room temperature lysed >90% of the amoeboid trophozoites. Cyst lysis required a single pass through a French pressure cell at 97 MPa (14000 lb/in²). The two disruption methods gave the same recovery of enzyme activity from trophozoites.

Acid precipitation and (NH₄)₂SO₄ fractionation

The cell lysate was placed in a pre-chilled beaker on ice and the pH lowered to 5.0–5.2 by addition of 1 M sodium acetate/acetic acid, pH 4.5. The resulting precipitate was removed by centrifugation (14000 g for 20 min). The supernatant was collected, placed in a beaker on ice, and the pH was returned to 7.2–7.3 by addition of 1 M Tris, pH 9.5. AdoMetDC was then precipitated with (NH₄)₂SO₄ in the fraction between 35% and 65% saturation. The precipitate was collected by centrifugation, re-suspended in lysis buffer at $\frac{1}{10}$ the original volume and kept overnight. The suspension was cleared by centrifugation and the AdoMetDC-containing supernatant was filtered through a 0.2 μ m-pore membrane filter.

Hydrophobic-interaction chromatography

The filtered enzyme preparation (~30 mg protein) was loaded on a 1.6 cm $\times$ 60 cm phenyl-Sepharose CL-4B column (Pharmacia, Piscataway, NJ, U.S.A.) previously equilibrated with basic column buffer (50 mM Tris, pH 7.2, 2 mM putrescine, 2 mM dithiothreitol) supplemented with 0.3 M (NH₄)₂SO₄. AdoMetDC passed through the column at this (NH₄)₂SO₄ concentration. U.v.-absorbing effluent fractions were pooled, and the (NH₄)₂SO₄ concentration of the pooled fraction was increased to 0.8 M. The column was washed with 50% ethylene glycol, re-equilibrated with basic column buffer supplemented with 0.8 M (NH₄)₂SO₄, and then loaded with the pooled fractions containing AdoMetDC. The enzyme was retained under these conditions. The column was washed with 5 column vol. (250 ml) of basic column buffer plus 0.8 M (NH₄)₂SO₄. The enzyme then was eluted with a 600 ml descending linear gradient of 0.8–0.3 M (NH₄)₂SO₄. Effluent fractions with AdoMetDC activity were pooled, concentrated by (NH₄)₂SO₄ precipitation at 65% saturation, and dialysed against basic column buffer.

DEAE-Sepharose anion-exchange chromatography

The dialysed enzyme was loaded on a 1.5 cm $\times$ 45 cm DEAE-Sepharose column (Pharmacia) previously equilibrated in basic column buffer. Enzyme was eluted with a 600 ml ascending linear gradient of 0–0.5 M KCl in basic column buffer. Effluent fractions containing AdoMetDC were pooled and enzyme was concentrated with a 30 kDa-cut-off centrifugal ultrafilter (Centricon 30; Amicon, Beverly, MA, U.S.A.) or by (NH₄)₂SO₄ precipitation, followed by dialysis against basic column buffer.

Final purification and determination of holoenzyme and enzyme-subunit molecular masses

DEAE-Sepharose-purified extract was loaded on 10 mm $\times$ 250 mm TSK-250 h.p.l.c. gel-filtration columns (Bio-Rad, Richmond, CA, U.S.A.) or 10 mm $\times$ 150 mm Superose 12 f.p.l.c. columns (Pharmacia) previously equilibrated in basic column buffer plus

Table 1 Purification of AdoMetDC from *Acanthamoeba castellanii*

Purification step	Total protein (mg)	Total activity (μmol of CO_2/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Crude homogenate	9018.0	1.14	0.126	1	100
Acid precipitate	694.0	0.53	0.764	6	46.5
$(\text{NH}_4)_2\text{SO}_4$ precipitate	284.0	0.52	1.83	15	45.6
Phenyl-Sepharose	28.8	0.40	13.9	110	35.1
DEAE-Sepharose	2.1	0.24	114	905	21.0
TSK-250	1.2	0.19	158	1254	16.7

0.2 M NaCl. Effluent fractions absorbing u.v. at 280 nm were collected and tested for AdoMetDC activity. H.p.l.c. and f.p.l.c. also were used to determine holoenzyme molecular mass. Subunit composition was determined by SDS/PAGE with 4–20% gradient acrylamide gels.

N-terminal labelling of the catalytic subunit of AdoMetDC

Specific labelling of the catalytic subunit of AdoMetDC was possible because the N-terminal residue was a pyruvoyl group [15]. Purified AdoMetDC was incubated under standard reaction conditions, except that the radioactive label was 2–4 μCi of [methyl- ^{14}C] AdoMet and the basic reaction buffer was supplemented with 200 mM NaCNBH₃. Under these conditions, the reaction product [methyl- ^{14}C]dcAdoMet became covalently bound to the pyruvoyl group. The preparation was dialysed to remove unreacted AdoMet and product and then subjected to SDS/PAGE. The labelled subunit was identified by autoradiography of dried gels, or after electrophoretic transfer of the protein to Immobilon-P membranes (Millipore Corp., Bedford, MA, U.S.A.).

Amino acid sequence determination

The N-terminal amino acid sequence of the catalytic subunit was determined by the Ohio State University Biochemical Instrumentation Center, using a model 470 gas-phase peptide sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The N-terminal pyruvate was converted into alanine before sequencing by reductive amination [15]. The conversion reaction was for 2 h at 37 °C and included 100 μg of h.p.l.c.-purified AdoMetDC, 0.2 M $(\text{NH}_4)_2\text{CO}_3$ and 0.15 M NaCNBH₃ in 100 μl . The enzyme was desalted by dialysis against distilled water, concentrated, and the subunits were separated by SDS/PAGE. Proteins were electrophoretically transferred to Immobilon-P membrane and stained with Coomassie Blue (Millipore Corp., Immobilon-P Technical Reference). The 32–33 kDa band was excised and used for sequencing.

RESULTS

Purification of AdoMetDC

Freezing and thawing was the most useful method of disrupting large quantities of amoebae (> 100 g wet wt.), but a French press was necessary to disrupt cysts. The crude cell lysate was cleared of particulate matter and a large quantity of soluble proteins by precipitation at pH 5. This resulted in a 6-fold purification (Table 1). A 10-fold decrease in total volume and a further 2–3-fold purification was achieved by $(\text{NH}_4)_2\text{SO}_4$ precipitation. A 7–8-fold purification was obtained by two-step hydrophobic

Figure 1 Chromatographic purification of AdoMetDC

(a) Hydrophobic interaction chromatography with phenyl-Sepharose CL-4B; (b) anion-exchange chromatography with DEAE-Sepharose; (c) h.p.l.c. gel-filtration chromatography using TSK-250. —, u.v. absorbance; ·····, relative AdoMetDC activity; ----, salt concentration.

interaction chromatography using Phenyl-Sepharose CL-B4 (Figure 1a). In the first step, proteins more hydrophobic than AdoMetDC were removed from the extract. In the second step, AdoMetDC was bound and separated from less hydrophobic proteins. Anion-exchange chromatography using DEAE-Sepharose typically resulted in a 7–8-fold further purification

AdoMetDC source	N-terminal amino acid sequence	Identity (%)
Mammal	Pyr-S-S-M-F-V-S-K-R-R-F-I-L-K-T-C-G-T-L-L	
		74
<i>Acanthamoeba</i>	Pyr-S-S-M-F-V-W-N-T-K-L-I-L-K-T-C-G-T-X-L	
<i>Saccharomyces</i>	Pyr-S-S-L-F-V-F-D-H-K-L-T-M-K-T-C-G-T-T-T	63

Figure 3 Kinetic properties of AdoMetDC

Curve fitting was performed by the Levenberg–Marquart least-squares method of non-linear curve fitting incorporated into the MSDOS program Deltagraph Pro (Delta Software). The correlation coefficient for the fit was $r^2 = 0.9948$. $V_{\max} = 1.424$ nmol of CO_2/min and $K_m = 30.0$ μM .

Table 3 Inhibition of AdoMetDC by diamidines and related compounds

The quantitative microassay was used for all experiments. Inhibitors were added in a maximum of 10 μl of aqueous solution and final reaction volumes were 100 μl . Each inhibitor concentration was tested twice with three replica assays per experiment. $K_i = (K_m[I])/(K_m^{\text{app}} - K_m)$, where K_m was determined in the absence of inhibitor and K_m^{app} in the presence of varying inhibitor concentrations (I).

Inhibitor	Inhibitor concns. tested (μM)	Average K_i (μM)
Ethidium bromide	0, 200, 500	1304
MGBG	0, 250, 500	604
Hydroxystilbamidine	0, 100, 500	357
Propamidine	0, 100, 250	334
Pentamidine	0, 25	19.4
Berenil	0, 0.2, 0.5	0.17

Figure 4 Effects of Geneticin on protein synthesis and AdoMetDC activity

AdoMetDC specific activity (Sp. Act.; —) and the incorporation of ^{14}C -labelled amino acids into protein (----) were measured in exponential-phase cultures in the absence or presence of 20 μg of Geneticin (G_{418})/ml added to inhibit protein synthesis. Symbols: $\square$, $\blacksquare$, control cultures without inhibitor; $\circ$, $\bullet$, cultures supplemented with Geneticin.

agent for treatment of *Acanthamoeba* keratitis [1]; methylglyoxal bis(guanyldrazone) (MGBG), pentamidine and Berenil inhibit AdoMetDC in *A. culbertsoni* [22] and other organisms [7,8]; and

Figure 5 Developmental changes in AdoMetDC activity

(a) AdoMetDC activity during growth. Enzyme activity was determined at various cell densities in OS4–7B cultures growing in monolayers in OGMA. (b) AdoMetDC activity during encystment. Enzyme activity was determined before and after exponentially growing cultures were transferred to nutrient-free medium to induce cell differentiation [23]. Starvation was initiated when cells reached 800 amoebae/ mm^2 (zero time). The percentage of cysts, counted as previously described [21], was used to measure the extent of cell differentiation (----); —, AdoMetDC specific activity in homogenates.

Berenil, ethidium bromide, hydroxystilbamidine and pentamidine induce encystment in the Neff strain [6,21]. The average K_i values determined here were: Berenil, 0.17 μM ; pentamidine, 19.4 μM ; propamidine, 334 μM ; hydroxystilbamidine, 357 μM ; MGBG, 604 μM ; and ethidium bromide, 1304 μM (Table 3). Spermidine had no effect on AdoMetDC activity even at concentrations up to 1 mM.

Stability of activity in vivo after inhibition of protein synthesis

The stability of AdoMetDC activity was examined after treatment of cells with 20 $\mu\text{g}/\text{ml}$ Geneticin (G_{418}). This inhibitor blocked the uptake of ^{14}C -labelled amino acids after 4 h (Figure 4). After cessation of protein synthesis, AdoMetDC activity disappeared with a half-life of 2.3 h.

Developmental changes in activity

AdoMetDC activity was relatively constant during exponential growth, but was rapidly lost during starvation-induced encystment (Figure 5). Within 24 h after initiation of starvation, 20% of the amoebae had encysted and AdoMetDC activity had decreased by 20%. After 48 h of starvation, 70% of the amoebae had encysted and AdoMetDC activity had decreased by at least 99%.

DISCUSSION

Several modifications to published procedures for *A. culbertsoni* and other organisms aided purification of *A. castellanii* AdoMetDC. The initial ultracentrifugation step used in other protocols was replaced by low-pH precipitation. MGBG affinity chromatography, used to purify AdoMetDCs from *E. coli*, yeast and mammals [10], was of little use and was eliminated. Hydrophobic-interaction chromatography and h.p.l.c. gel-filtration steps were useful additions that increased purification up to 100-fold over that reported by Gupta et al. [22] for *A. culbertsoni*.

The 88.8 kDa AdoMetDC holoenzyme from *A. castellanii* is the same size as the yeast enzyme [18], but larger than the 76 kDa mammalian enzyme [16] and smaller than the 110 kDa reported for the enzyme of *A. culbertsoni* [22]. The amoeba enzyme, like the mammalian and yeast enzymes, appears to have

both α and β subunits. The molecular mass of an amoeba holoenzyme including two copies each of the 32.8 kDa and 14.6 kDa polypeptides observed by SDS/PAGE (Figure 4) would be on the high side, but within the 88 ± 8 kDa range measured for the intact enzyme. Our experience with other small proteins suggests, however, that the β -subunit size may be overestimated by the gradient-gel system used.

Divergences in α -subunit N-terminus amino acid sequences among *Acanthamoeba*, yeast and mammals (Table 2) are comparable with sequence divergences among contractile proteins and rRNAs in these organisms [24–26]. However, most of the sequence is conserved, possibly because the N-terminus of this subunit is near the active site and also may be essential for proper processing of a proenzyme to the mature α and β subunits [27].

Acanthamoeba AdoMetDC kinetic properties that are typical for eukaryotes include a K_m in the 30–115 μ M range, stimulation by putrescine (50–300% increase in activity), and competitive, yet almost completely irreversible, inactivation by Berenil [7,8,10,20]. The AdoMetDCs of *A. castellanii* and *A. culbertsoni* [22] are much less sensitive to MGBG ($K_i = 600$ and > 100 μ M, respectively) than are AdoMetDCs of rat ($K_i = 0.3$ μ M) [20], *E. coli* ($K_i = 13$ μ M) [20] or *Trypanosoma brucei* ($K_i = 32$ μ M) [8].

Four aromatic diamidines, Berenil (diminazene aceturate), propamidine, pentamidine and hydroxystilbamidine, and the aromatic polycyclic compound ethidium bromide inhibited *Acanthamoeba* AdoMetDC. Berenil was the most potent inhibitor by several orders of magnitude, and ethidium bromide was the weakest. These compounds share a single common feature, the presence of at least two amine groups at the ends of the molecule. All except ethidium bromide have terminal guanido moieties. How MGBG and these molecules act as competitive inhibitors of AdoMetDC is unclear. Jänne et al. [28] suggested that MGBG might act as a congener of AdoMet. Bacchi [29] suggested that diamidines may be analogues of polyamines, and noted that AdoMetDC activities from several organisms can be modulated by polyamines. He suggested that diamidines might affect AdoMetDC by interaction with polyamine-binding sites, rather than by directly competing for the substrate-binding site. Berenil, the most effective inhibitor, is the only aromatic inhibitor used that has internal secondary amines, and they might make the compound a closer analogue to naturally occurring polyamines. Ethidium bromide, the least effective inhibitor, is the least structurally similar to polyamines. Nevertheless, this inhibitor does have a superficial resemblance to spermidine [30] and can compete with this polyamine for binding in some instances. The possibility that the inhibitors that we tested could act as analogues of spermidine is intriguing, since this polyamine affects the activity of AdoMetDCs from all other eukaryotes. However, the amoeba enzyme did not respond to spermidine concentrations up to 1 mM in our tests.

AdoMetDC activity decreases to undetectable levels during encystment (Figure 5). This is consistent with evidence that the enzyme is associated with active growth in other organisms [10] and that the cyst is metabolically inactive [31]. The decrease in AdoMetDC activity during encystment is accompanied by and might be partially responsible for decreases in concentrations of polyamines [6]. In contrast with the observation of Zhu et al. for *A. culbertsoni* [32], we found only minor decreases in concentrations of the substrate AdoMet during encystment (E. Hugo, unpublished work). Thus the decrease in enzyme activity probably is not due to limiting substrate levels. We did find that AdoMetDC activity in trophozoites decreased rapidly after inhibition of protein synthesis (Figure 4), suggesting that AdoMetDC is rapidly degraded as in mammals [10], or otherwise inactivated.

Berenil, pentamidine, hydroxystilbamidine and ethidium bromide all can induce encystment of the Neff strain of *A. castellanii* [6], an effect that could be problematic for their use as chemotherapeutic agents, but the mechanism of induction is unknown. Berenil and ethidium bromide, at least, inhibit mitochondrial nucleic acid synthesis, and berenil stimulates release of encystment-enhancing factors from amoeba mitochondrial fractions [21]. Thus mitochondria might have a role in inhibitor-induced encystment. We now have shown that all four compounds inhibit the amoeba AdoMetDC, but there is no clear correlation between inhibition of the enzyme and the ability to induce encystment. Berenil, the most effective inducer of differentiation in our experience, had the lowest K_i (0.17 μ M), but ethidium bromide, the next best inducer, had the highest value (1.3 mM). Berenil induced encystment best at 3 μ M [6], somewhat above the K_i , but the inducing concentrations for ethidium bromide (40 μ M) [21], pentamidine (1–2 μ M) and hydroxystilbamidine (2–5 μ M) [6] were substantially below their K_i values. Whether inhibition of AdoMetDC is essential for differentiation will only be answered when the enzyme can be inactivated specifically by a genetic approach. There is increasing evidence that variations in polyamine levels resulting from inhibition of polyamine synthesis are less important than previously thought [33]. Attention now is shifting to perturbations in cell methylations resulting from accumulations of AdoMet caused by inhibition of polyamine synthesis [C. J. Bacchi et al. (1992) Abstr. 45th Ann. Meeting, Soc. Protozoologists]. The latter effect could turn out to be the most important result of inhibiting AdoMetDC by diamidines and related compounds, and might provide a link between the enzyme and cell differentiation.

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