Modulation of polyamine-biosynthetic activity by S-adenosylmethionine depletion

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The methionine-analogue inhibitor of S-adenosylmethionine (AdoMet) synthetase, L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cisAMB), was used to study the early effects of AdoMet depletion on polyamine biosynthesis. In the presence of decreased methionine (30 μM) in the medium, treatment of cultured L1210 cells with 1 mM-L-cisAMB resulted in a near-total (95%) depletion of cellular AdoMet pools by 4 h. This was accompanied by a 3-fold increase in ornithine decarboxylase (ODC) activity, a 2.5-fold increase in AdoMet decarboxylase (AdoMetDC) activity and a 20% decrease in spermidine and spermine pools. The increase in enzyme activities seemed to be partially due to prolongation of enzyme activity half-life, since that of ODC was extended from 30 to 50 min and that of AdoMetDC from 65 to 310 min. By temporal sequence characterization (0–4 h), the onset of elevations of enzyme activity (0.5–1 h) seemed to be causally related to an earlier (0–0.5 h) decline in AdoMet pools, as opposed to the 20% decrease in spermidine and spermine pools, which occurred much later (2–4 h); the latter are known to regulate decarboxylase activities negatively. Drug-induced elevations in ODC and, to a lesser extent, AdoMetDC activities were reversed by later treatment with exogenous AdoMet. However, because the latter also increased spermine pools (which could not be prevented with various enzyme inhibitors), the reversal of elevations in enzyme activities could not be directly linked to AdoMet. Although not definitive, the data raise the interesting possibility that, in addition to being negatively regulated by polyamines, ODC and AdoMetDC activities may also be subject to negative control by cellular AdoMet (or an AdoMet metabolite). The net effect of either or both of these influences would be to conserve polyamine-biosynthetic activity in the face of declining AdoMet supplies.

INTRODUCTION

In addition to its well-recognized role as a methyl donor in transmethylation reactions, S-adenosyl-L-methionine (AdoMet) is critically involved in the synthesis of the higher polyamines, spermidine and spermine. AdoMet enters the pathway via a decarboxylation reaction which is probably rate-limiting for polyamine biosynthesis, since, unlike other polyamine precursors and intermediates, decarboxylated AdoMet is not detectable in cells under steady-state conditions. The AdoMet metabolite is utilized separately by spermidine and spermine synthases as an aminopropyl donor to form spermidine from putrescine and spermine from spermidine, respectively. Given the role of AdoMet in polyamine biosynthesis and its competitive utilization by transmethylation reactions, it might be expected that the highly regulated polyamine pathway would have mechanisms to ensure AdoMet accessibility. That such mechanisms have not yet been described may be partially because selective and effective means for depleting cells of AdoMet have not been previously available.

As part of a programme to study methionine analogues as potential anti-cancer agents (Sufrin & Lombardini, 1982; Lombardini & Sufrin, 1983; Porter & Sufrin, 1986), we have identified one particular analogue, L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cisAMB). By virtue of its apparent structural resemblance to the enzyme-bound conformation of methionine, L-cisAMB is the most potent methionine-analogue inhibitor of AdoMet synthetase thus far described (Sufrin et al., 1982; Porter et al., 1984; Sufrin et al., 1986). Because L-cisAMB is capable of rapidly depleting AdoMet pools in intact cells without affecting macromolecular precursor incorporation (Kramer et al., 1987), the compound is particularly well suited for studies examining the cellular consequences of AdoMet depletion. In the first of such studies, Kramer et al. (1987) found that L-cisAMB induced a rapid AdoMet depletion and decreased methylation of total nucleic acids by 44–72% and that of RNA by 60–87%. Contrary to expectations, spermidine and spermine pools were not significantly decreased after 48 h of drug treatment (Porter et al., 1984). This appeared to be due to substantial increases in ornithine decarboxylase (ODC) and AdoMet decarboxylase (AdoMetDC) activities (Kramer et al., 1987), both of which are lead-in enzymes in the polyamine-biosynthetic pathway and are generally regarded as rate-limiting in the synthesis of spermidine and spermine. Since both ODC (Kay & Lindsay, 1973; Clark & Fuller, 1975; Kallio et al., 1977; McCann et al., 1979; Seely & Pegg, 1983) and AdoMetDC (Alhonen-Hongisto, 1980; Mamont & Danzin, 1981; Pegg et al., 1987) are known to be negatively regulated by intracellular spermidine and

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Table 1. Long-term (24 h) effects of AdoMet depletion on polyamine biosynthesis

Treatment was in media containing 30 μM-methionine. This concentration will support L1210-cell growth at control values (those obtained with 100 μM-methionine) for 48 h (Sufrin et al., 1986). Abbreviations: PUT, putrescine; SPD, spermidine; SPM, spermine.

Treatment (24 h)	AdoMet (nmol/10^7 cells)	Decarboxylase activity (nmol of $^{14}CO_2$/h per mg)		Polyamine pools (nmol/10^7 cells)		
		ODC	AdoMetDC	PUT	SPD	SPM
None	1.03	6.30	3.17	5.2	27.7	9.2
1 mM-L-cisAMB	0.26	13.77	11.42	37.7	27.2	6.0

spermine, it was decided to determine whether the observed increases in decarboxylase activities were indirectly due to perturbations in polyamine pools resulting from AdoMet depletion or were due directly to decreases in AdoMet itself. Data presented here indicate that, in addition to being regulated by intracellular polyamine concentrations, ODC and AdoMetDC activities may also be subject to negative control by AdoMet.

MATERIALS AND METHODS

Materials

L-cisAMB was synthesized by procedures published previously (Sufrin et al., 1982). Methylglyoxal bis-(guanylhydrazone) (MGBG) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

Murine L1210 leukaemia cells were grown as a suspension culture in RPMI-1640 medium containing 16 mM-Hepes and 8 mM-Mops as a buffer system, and 10% (v/v) Nu Serum IV (Collaborative Research, Lexington, MA, U.S.A.) as a semi-defined serum substitute. Methionine concentrations in media were either 100 μM (standard) or 30 μM (excluding the 2 μM contributed by Nu Serum). Cells were grown in 25 cm^2 or 75 cm^2 tissue-culture flasks in a total volume of 15 or 50 ml, respectively, under a humidified atmosphere containing 5% CO_2 at 37 °C. Cell number was determined by electronic particle counting (model ZF Particle Coulter Counter; Coulter Electronics, Hialeah, FL, U.S.A.). Cells previously grown in 100 μM-methionine were seeded into media containing 30 μM-methionine at 2.5 × 10^5/ml, 12 h before being treated with 1 mM-L-cisAMB for periods ranging from 0 to 4 or 24 h. Reversal studies were performed by adding 500 μM-AdoMet with or without 10 μM-MGBG to cell cultures pretreated for 4 h with 1 mM-cisAMB without removing the latter, and incubating the cells for an additional 2-4 h.

AdoMet pools

The intracellular concentrations of AdoMet were measured in portions of the same HClO$_4$ extracts prepared for polyamine-pool determinations. Analysis of the extract was performed by a slight modification of the h.p.l.c. method of Zappia et al. (1980), as described elsewhere (Porter et al., 1985).

Decarboxylase activities

Extracts for measurements of ODC activity were prepared by sonicating control or treated cells in 10 mM-

Tris/HCl (pH 7.2) containing 0.5 mM-Na$_2$EDTA, 5 mM-dithiothreitol and 50 μM-pyridoxal 5'-phosphate, followed by centrifugation at 20000 g for 20 min. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine (New England Nuclear Corp.) in the presence of saturating concentrations of pyridoxal 5'-phosphate (0.1 mM) in accordance with the methods of Pegg & Seely (1983). AdoMetDC activity was similarly assayed with S-[carboxy-^{14}C]adenosyl-methionine (New England Nuclear Corp.) as a substrate in the presence of saturating (3 mM) putrescine as described by Pegg & Pösö (1983). Results were expressed as nmol of $^{14}CO_2$ released/h per mg of protein.

Intracellular polyamines

A sample of 10^7 cells was taken for polyamine determinations after extraction with 0.6 M-HClO$_4$. The extract was analysed by h.p.l.c. by using a system based on cation exchange and post-column derivative formation with o-phthaldehyde as described by Porter et al. (1985).

RESULTS

Before studying the early drug effects of L-cisAMB in L1210 cells, the more long-term effects were documented (Table 1). After treatment for 24 h with 1 mM-L-cisAMB in the presence of lower methionine concentrations (30 μM) in media, polyamine biosynthesis was only marginally affected. Spermidine pools were unchanged and spermine pools were decreased by only 31%. A 2.2-fold increase in ODC activity, together with a 3.6-fold increase in AdoMetDC activity, were apparently partially responsible for sustaining polyamine bio-synthesis. In order to determine how these increases in enzyme activity relate to decreases in AdoMet pools, a characterization of the temporal sequence of drug effects was undertaken. Since it was previously determined (Kramer et al., 1987) that the relevant drug effects were well established after only 4 h treatment with 1 mM-L-cisAMB, these same conditions were adapted as an endpoint for characterization. The results of these experiments are presented in Fig. 1.

Treatment with 1 mM-L-cisAMB in the presence of 30 μM-methionine leads to a very rapid depletion of AdoMet pools. During the first 30 min, AdoMet pools decreased by about 75%, and then much more slowly to about 5% of control by 4 h. In control cells, AdoMet pools decreased only slightly from 1.4 nmol/10^7 cells during the first 1 h, and then stabilized at about 1.2 nmol/10^7 cells for the remainder of the incubations.
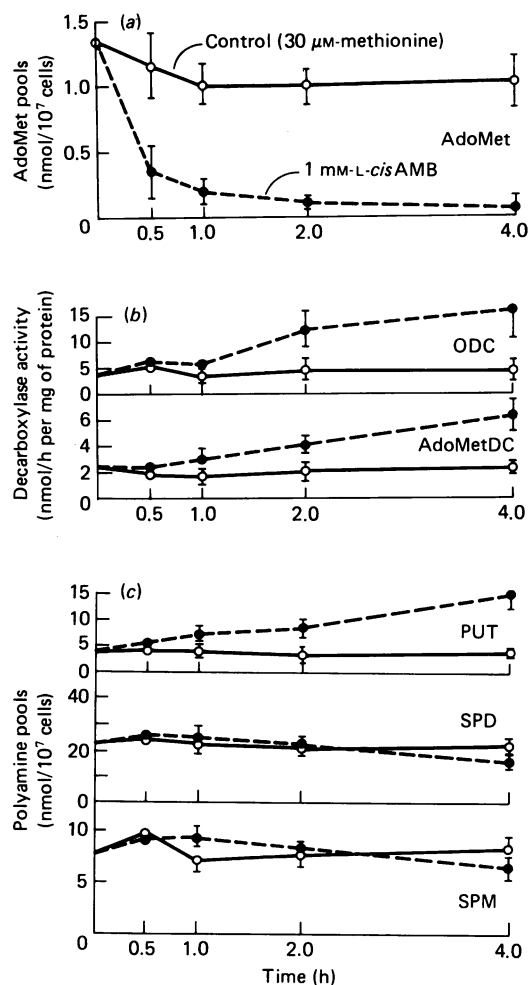
Fig. 1. Time-dependence of L-cisAMB-induced effects on AdoMet pools (a), ODC and AdoMetDC activities (b) and polyamine pools (c)

Cells were grown in media containing 30 μM-methionine for 12 h before being incubated for 0–4 h in the presence (●) or the absence (○) of 1 mM-L-cisAMB. Data represent mean values ($\pm$S.D.) for at least three experiments performed in duplicate. Abbreviations: PUT, putrescine; SPD, spermidine; SPM, spermine.

The time-dependence of L-cisAMB effects on ODC and AdoMetDC activities was very similar (Fig. 1). Increases in these enzyme activities began at 60 min (just after the greatest decrease in AdoMet pools) and continued to rise steadily thereafter. By 4 h, ODC was 3-fold greater than control enzyme activity, and AdoMetDC was 2.5-fold more. Of the three polyamine pools, only putrescine seemed to show a correlation with L-cisAMB effects on AdoMet pools. Beginning at 30 min, intracellular putrescine started to accumulate, and increased to 3.5-fold more than control by 4 h. Presumably this is related to the inability of putrescine to proceed in the pathway to spermidine and spermine as a result of AdoMet depletion. Contrary to this reasoning, spermidine and spermine pools did not decrease until after 2 h, and by 4 h were down by about 20%.

The contribution of half-life prolongation in increasing enzyme activity was assessed by studying cessation of total protein synthesis with cycloheximide

(Table 2). The half-life of ODC activity in untreated cells was 30 min, whereas that of the L-cisAMB-induced enzyme was 50 min. AdoMetDC activity had a half-life of 310 min after L-cisAMB treatment, as compared with 65 min for the untreated cells. If, after treatment, L-cisAMB was removed just before cycloheximide treatment, the half-life of enzyme activity was only 70 min, indicating that sustained presence of the drug and/or blockade of AdoMet synthesis is apparently a critical factor.

In an attempt to determine more definitively whether the increases in ODC and AdoMetDC activities were due to L-cisAMB-induced decreases in AdoMet, as suggested by temporal sequence data, or to decreases in spermidine and/or spermine, reversal studies using exogenous AdoMet were undertaken (Table 3). Cells were incubated in the absence or presence of 1 mM-L-cisAMB for 4 h to increase decarboxylase activities and then treated for an additional 2–4 h ('post-treatment') with 500 μM-AdoMet. The latter increased intracellular AdoMet pools in untreated cells from $\sim$ 1 to $\sim$ 2 nmol/10^7 cells. In cells treated with 1 mM-L-cisAMB, AdoMet pools increased from undetectable values to about 0.3 nmol/10^7 cells. Interestingly, AdoMet pools never rose to the same values as seen in untreated cells, even when the post-treatment was extended from 2 to 4 h. Apparently, L-cisAMB-treated cells utilized exogenously supplied AdoMet more rapidly than did untreated cells.

In untreated cells, exogenous AdoMet lowered ODC activity by 87% and AdoMetDC activity by only 30%. The basis for these effects was not unambiguous, since the increase in AdoMet pools was accompanied by a simultaneous increase (60%) in spermine pools (spermidine pools remained the same). In fact, reversal studies with exogenous spermine produced decreases in enzyme activities which were nearly identical with those obtained with exogenous AdoMet.

In cells treated with L-cisAMB, the effect of exogenous AdoMet on enzyme activity was more impressive (Table 3). ODC activity, which was about 3-fold greater than control as a consequence of L-cisAMB treatment, fell from 16.96 to 0.95 nmol of CO_2/h per mg (a 94% decrease), whereas AdoMet decarboxylase activity was decreased by about the same extent as in untreated cells, i.e. 25%. Extending post-treatment from 2 to 4 h had no additional effect on ODC or AdoMetDC activity. As with control cells treated with exogenous AdoMet, spermine pools were approximately doubled and spermidine pools were unchanged, so that the basis for the decrease in enzyme activity could not be directly attributed to increases in AdoMet pools. Likewise, exogenous spermine also lessened drug-induced increases in enzyme activities in a manner similar to AdoMet. After 4 h post-treatment with AdoMet, putrescine pools were finally decreased by AdoMet, as a probable result of the cumulative effects of lower ODC activity, sustained AdoMetDC activity and renewed AdoMet availability.

To separate AdoMet effects from spermine effects in the negative regulation of decarboxylase activities, inhibitors of AdoMetDC or of spermine and spermidine synthases were included during the reversal period with exogenous AdoMet in an attempt to prevent the accumulation of spermine. Since the inhibitors themselves affected enzyme activities, the data from these experiments were not readily interpretable. When the reversible AdoMet decarboxylase inhibitor MGBG

Table 2. Effect of cycloheximide (CHX) on decarboxylase activities of untreated and 1 mM-L-cisAMB-treated cells

All experiments were performed in 30 μM-methionine. Data represent means values of two experiments run in duplicate.

Treatment (4 h)	Decarboxylase activity (nmol of $^{14}CO_2$/h per mg)		Post-treatment (0.5–6 h)	Decarboxylase half-life (min)	
	ODC	AdoMetDC		ODC	AdoMetDC
None	5.86	2.46	50 μM-CHX	30	65
			50 μM-CHX plus 1 mM-L-cisAMB	30	170
1 mM-L-cisAMB	16.65	4.88	50 μM-CHX	45	70
			50 μM-CHX plus 1 mM-L-cisAMB	50	310

Table 3. Reversal of L-cisAMB-induced effects on polyamine-biosynthetic activity by exogenous AdoMet

Data represents mean values of two experiments performed in duplicate. Abbreviations: PUT, putrescine; SPD, spermidine; SPM, spermine.

Treatment (4 h)	Post-treatment (2 h)	AdoMet (nmol/10^7 cells)	Decarboxylase activity (nmol of $^{14}CO_2$/h per mg)		Polyamine pools (nmol/10^7 cells)		
			ODC	AdoMetDC	PUT	SPD	SPM
None	None	0.96	6.75	2.80	5.85	25.30	7.60
	5 μM-spermine	1.10	1.35	2.05	3.50	22.40	10.90
	500 μM-AdoMet						
	2 h	2.04	0.90	1.98	4.90	26.50	12.05
	4 h	2.10	1.60	1.58	2.95	23.35	12.30
1 mM-L-	1 mM-L-cisAMB	< 0.05	16.96	8.10	27.20	20.80	6.45
cisAMB	1 mM-L-cisAMB plus 5 μM-spermine	< 0.05	1.90	7.85	16.15	15.70	9.50
	1 mM-L-cisAMB plus 500 μM-AdoMet						
	2 h	0.28	0.95	5.96	22.05	22.15	12.55
	4 h	0.35	1.20	5.78	14.70	18.30	12.30
	1 mM-L-cisAMB plus 500 μM-AdoMet and 10 μM-MGBG	0.51	0.85	5.54	15.60	15.30	9.10

(Corti et al., 1974) was included during reversal with exogenous AdoMet, the L-cisAMB-induced increase in ODC activity was reversed, but spermine pools were still increased (Table 3). Similar difficulties were experienced with 5'-methylthioadenosine, an inhibitor of spermine and spermidine synthases (Raina et al., 1982), and 5-dimethylthioadenosine, an inhibitor of spermine synthase (Pegg & Coward, 1985).

DISCUSSION

Given the role of AdoMet as an aminopropyl donor in the synthesis of spermidine and spermine, it is reasonable to expect that near-total depletion of cellular AdoMet pools might result in a substantial decrease in these polyamine pools. As documented in Table 1 and reported previously (Porter et al., 1984; Kramer et al., 1987), L-cisAMB-induced AdoMet depletion under conditions of decreased methionine in media did not give rise to such an effect. This appears to be primarily due to several-fold increases in ODC and AdoMetDC activities, but it is also recognized that enhanced AdoMet synthetase activity, leading to a partial recovery of AdoMet pools, may also

play a contributing role (Kramer et al., 1987). These findings have given rise to the consideration that decarboxylase activities, which are generally regarded as rate-limiting for polyamine biosynthesis, might be under the regulatory control of AdoMet. It is well recognized that, in eukaryotes, both ODC (Kay & Lindsay, 1973; Clark & Fuller, 1975; Kallio et al., 1977; McCann et al., 1979; Seely & Pegg, 1983) and AdoMetDC (Alhonen-Hongisto, 1980; Mamont & Danzin, 1981; Pegg et al., 1987) activities are negatively regulated by polyamines through what probably involves translational or post-translational mechanisms (Murakami & Hayashi, 1985; Kahana & Nathans, 1985; Holtta & Pohjanpelto, 1986; Persson et al., 1986; Kameji & Pegg, 1987a; Sertich & Pegg, 1987). Typically, enzyme activities rise in response to inhibitor-induced decreases in polyamines and fall in response to increases in intracellular polyamines (and polyamine analogues) derived from exogenous sources (Porter et al., 1987a,b). Thus, in delineating the regulatory influences which mediate L-cisAMB-induced increases in decarboxylase activities, careful consideration must be given to the possibility that subtle decreases in polyamine pools or the synthetic flux

occurring secondarily to AdoMet depletion might account for rises in enzyme activity.

It would appear from an analysis of the temporal sequence of drug effects which occurred during the 4 h treatment with L-cisAMB (Fig. 1) that the increases in both ODC and AdoMetDC activities were causally related to decreases in AdoMet pools. Enzyme activity began to increase just after AdoMet pools underwent their greatest decrease. The alternative possibility, that the enzyme activities may have increased in response to a decrease in spermidine and/or spermine pools, seems unlikely, since the latter was not detected until some 1–2 h after increases in enzyme activity were initiated.

Because measurements of polyamine pools do not necessarily reflect flux through those pools, the above interpretations are made with caution. Firstly, intracellular spermidine and spermine are not necessarily static, as the pools suggest, but have the ability to exchange with one another via the interconversion pathway (Pegg et al., 1981; Mamont et al., 1981). In addition, pool-size determinations are representative of the total cellular polyamine content and fail to reflect bound versus unbound pools or the concentrations within specific cellular compartments such as those harbouring the regulatory site(s) for decarboxylase activity. Given the relative rapidity of onset of drug effects (i.e. 30 min), it is doubtful that alternative means of study, such as measuring accumulation in media of methylthioadenosine (Iiazasa & Carson, 1985), the main by-product of polyamine biosynthesis, or following radiolabel through the polyamine pathway, could be successfully applied.

Unfortunately, reversal studies with AdoMet and spermine were found to be no more definitive than the temporal sequence data. Restoring AdoMet pools in L-cisAMB-treated cells lowered ODC activity by 80%. AdoMetDC activity was lowered to a lesser extent, probably owing to the marked increase in half-life of this enzyme in the presence of L-cisAMB. Likewise, increasing AdoMet pools in control cells also decreased both enzyme activities. Although these observations again tend to implicate AdoMet further in a regulatory function, they are complicated by the finding that spermine pools are significantly increased by exogenous AdoMet. In fact, increases in enzyme activity could be similarly reversed by either exogenous AdoMet or spermine (Table 3). Unfortunately, the two effects could not be separated by reversal studies with AdoMet and inhibitors of spermine synthesis. MGBG, a reversible inhibitor of AdoMetDC (Corti et al., 1974), was included during the reversal incubation mixture to minimize the accumulation of spermine (Hibasami et al., 1980). Under these conditions, exogenous AdoMet lowered ODC and AdoMetDC activities, but also increased spermine. Thus, although the temporal sequence data suggest a regulatory role for AdoMet in polyamine biosynthesis, our attempts to delineate such a mechanism further have been unable to confirm this possibility. On the other hand, they have not excluded it. It is interesting to recognize that, by either or both control mechanisms, the net effect of AdoMet depletion would be to preserve polyamine-biosynthetic activity in proliferating cells. Whether this is also the case in non-proliferating or differentiating cells remains to be determined.

The prospect that AdoMet might control polyamine-biosynthetic activity has not been previously raised.

Tisdale (1981) reported that methionine deprivation increased AdoMetDC activity, and proposed that the apparent induction in enzyme activity was mediated by the amino acid itself. In light of data showing that methionine deprivation profoundly lowers cellular AdoMet pools (Kramer et al., 1987), it would seem more likely that the enzyme effect was mediated by decreases in AdoMet pools (which were not measured by Tisdale, 1981).

Although it is reasonable to expect that AdoMet might affect AdoMetDC activity, the case for ODC is less obvious. The former enzyme interacts catalytically with AdoMet as a substrate, but ODC does not. There are precedents, however, of enzymes whose activities are regulated by AdoMet, but which do not utilize AdoMet as a substrate or cofactor (Rognes et al., 1980; Lombardini & Talalay, 1971), thus lending credence to the case for ODC.

When considering the consequences of the AdoMet-related effects on both decarboxylase activities in terms of overall polyamine-biosynthetic activity, certain interesting perspectives emerge. The increase in ODC activity, whether mediated by AdoMet depletion itself or by a related decrease in spermine pools, contributes to the accumulation of putrescine which, in addition to being a substrate for AdoMetD , serves as a potent activator of the enzyme (Kay & Lindsay, 1973) as well as increasing its synthesis (Kameji & Pegg, 1987b). This, together with the several-fold rise in AdoMetDC activity, would serve to potentiate the overall ability of the polyamine-biosynthetic pathway to utilize AdoMet under conditions of compromised AdoMet supplies. As we have determined elsewhere (Kramer et al., 1987), this occurs at the expense of transmethylation processes involving nucleic acids and perhaps other molecules as well.

Cantoni (1986) has proposed that regulation of AdoMet fluxes through transmethylation reactions and polyamine biosynthesis could be determined by the relative affinity of S-adenosylhomocysteine for the various methyltransferases and AdoMetDC. S-Adenosylhomocysteine is the by-product of transmethylation reactions mediated by AdoMet and a competitive inhibitor of most enzyme reactions in which AdoMet participates as a methyl donor. Although this hypothesis might obtain under steady-state conditions, our present findings indicate that, under conditions of AdoMet depletion, other regulatory controls are evoked, in particular, increases in AdoMetDC and ODC activities. Together, these latter serve to divert available AdoMet from transmethylation reactions to polyamine biosynthesis. Thus far, this has been observed to occur in proliferating cells, although the situation may be different in non-proliferating or differentiating cells.

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