Stimulation *in vitro* of vitamin B₁₂-dependent methionine synthase by polyamines

Susan H. KENYON, Anna NICOLAOU, Tamara AST and William A. GIBBONS*

University–Industry Centre for Pharmaceutical Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, U.K.

Vitamin B₁₂-dependent methionine synthase is an important enzyme for sulphur amino acid, folate polyamine metabolism, *S*-adenosylmethionine metabolism and also in the methylation pathway of DNA, RNA, proteins and lipids. Consequently, studies aiming at exploring the control and regulation of methionine synthase are of particular interest. Here we report the modulation of enzyme activity *in vitro* by polyamines. Although putrescine, cadaverine, spermine and spermidine all stimulated enzyme activity, the last two were the most potent, causing

increases in enzyme activity up to 400 %. The EC₅₀ for spermine was determined as 8 μ M and for spermidine 40 μ M. The physiological concentration for spermine has been reported to be 15–19 μ M. Spermine was found to increase both the *K_m* and the *V_{max}* with respect to methyltetrahydrofolate for the enzyme. These data support the hypothesis that spermine and spermidine are feedback regulators of methionine synthase both *in vivo* and *in vitro* and are consistent with the polyamines' regulating cell signalling pathways.

INTRODUCTION

Vitamin B₁₂-dependent methionine synthase (5-methyltetrahydrofolate–homocysteine methyltransferase, EC 2.1.1.13) catalyses the formation of methionine and tetrahydrofolate (THF) from homocysteine and methyltetrahydrofolate (MTHF) [1]. The mammalian forms of methionine synthase require cobalamin as a prosthetic factor, *S*-adenosylmethionine (SAM) as a cofactor and a reducing system for activity [2,3]. The *Escherichia coli* cobalamin-dependent methionine synthase has been extensively studied [4], and is considered to provide a model for the mammalian form, which is less well characterized although it has been purified from pig liver [5], rat liver [6], human placenta [7] and pig kidney [8].

Methionine synthase is involved in many metabolic pathways (Scheme 1). The enzyme's involvement in the folate cycle means that ultimately DNA and RNA production are dependent on the correct functioning of methionine synthase. Sulphur amino acid recycling, and hence the methylation pathway and the production of polyamines, are also dependent on the enzyme's function [9]. Administration of nitrous oxide, a well known inhibitor of methionine synthase, produces symptoms similar to vitamin B₁₂ deficiency, namely macrocytic anaemia [10], neurological degeneration and psychiatric symptoms [11]. Methionine synthase has also been associated with atherosclerosis via an increase in homocysteine [12], tumour formation [13] and cell growth and differentiation [14].

A number of substances have been reported to interact with methionine synthase and change its activity. Direct inactivators include nitric oxide [15,16], nitrous oxide [17] and halocarbons such as chloroform [18]. Indirect inhibitors are ethanol [19], cobalamin analogues [20], methylmercury [21], methotrexate and antiepileptic drugs [22]. Penicillamine increases the enzyme's activity [22].

The polyamines spermidine and spermine are formed from putrescine and decarboxylated SAM (dc-SAM) [23]. They are found ubiquitously in mammalian systems and have been shown

to be involved in many different biological processes such as regulation of gene expression [24] and cell growth and differentiation [25,26]. Normal intracellular levels are of the order of 0.1–1.0 nmol per 10⁶ cells for putrescine, 0.5–3.5 nmol per 10⁶ cells for spermidine and 0.75–1.5 nmol per 10⁶ cells for spermine [27]. Polyamines have been reported to inhibit the following: cAMP-dependent kinase [27], protein kinase C [28], calcium/calmodulin-dependent protein kinase [27], Ca²⁺-ATPase [29], nitric oxide synthase [30] and the *N*-methyl-D-aspartate (NMDA) receptor channel [31,32]. They stimulate casein kinases I and II [33],

Scheme 1 Biochemical pathway showing the relationship between methionine synthase and polyamine production

Abbreviations used: dc-SAM, decarboxylated *S*-adenosyl-L-methionine; Hcy, homocysteine; SAH, *S*-adenosylhomocysteine.

Abbreviations used: dc-SAM, decarboxylated *S*-adenosyl-L-methionine; MTHF, methyltetrahydrofolate; NMDA, *N*-methyl-D-aspartate; SAM, *S*-adenosyl-L-methionine.

* To whom correspondence should be addressed.

transglutaminase [34] and the NMDA receptor channel [31,32], all of which are common cell signalling systems. Because of their diversity of function, polyamines have been proposed as cellular regulators [27].

Methionine synthase has been shown to be another target for polyamines [35]. Here we report a detailed study of the spermine and spermidine interaction *in vitro* with purified rat liver methionine synthase.

EXPERIMENTAL

Materials

D,L-Homocysteine, SAM (iodide salt), 2-mercaptoethanol, MTHF (barium salt), spermine, spermidine, putrescine, cadaverine, PMSF, tosyl-lysylchloromethane, trypsin inhibitor and aprotinin were all purchased from Sigma. Radiolabelled [*methy*-¹⁴C]methyltetrahydrofolate (barium salt, 56 mCi/mmol) was obtained from Amersham. Bio-Rad provided the AG1-X8 resin (200–400 mesh, chloride form), hydroxyapatite and Bradford's reagent. Q-Sepharose fast flow, Mono-Q, Superose 12 and G25 columns were from Pharmacia. Ultrafiltration membranes, XM-50 (cut-off 50 kDa), were purchased from Amicon.

Enzyme assay

Methionine synthase activity was assayed with a modification of the procedure of Weissbach et al. [2]. Assay mixtures (total volume 300 μ l) contained 50 mM potassium phosphate buffer, pH 7.2, 400 μ M D,L-homocysteine, 300 μ M SAM, 125 mM 2-mercaptoethanol, 236 μ M MTHF (5211 d.p.m./nmol) and the enzyme source. Incubations were performed in light-protected stoppered serum vials under nitrogen. Reaction mixtures were preincubated for 5 min at 37 °C. The reaction was initiated by the addition of homocysteine through a syringe and incubated at 37 °C for up to 30 min. The reaction was terminated by the addition of 400 μ l of ice-cold water and immediately passed through a 0.5 cm $\times$ 5.0 cm column of Bio-Rad AG1-X8 resin. [¹⁴C]Methionine was eluted with 2 ml of water, collected and quantified by scintillation spectrometry.

Protein assay

Protein concentrations were determined with the Bio-Rad protein assay based on the method of Bradford [31] with BSA as the standard.

Purification of methionine synthase from rat liver

Step 1: preparation of rat liver cytosol

Adult Wistar rats were killed by CO₂ intoxication and their livers immediately removed and stored at –80 °C. Rat livers (150 g) were placed in 500 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 1.5 mg of tosyl-lysylchloromethane, 12.5 mg of PMSF, 6.25 mg of trypsin inhibitor and 1.31 mg of aprotinin. The livers were blended three times for 1 min. All the following purification steps were performed at 4 °C. The homogenate was centrifuged at 1000 *g* for 15 min, the supernatant was collected and re-centrifuged at 27000 *g* for 30 min, and finally the supernatant was centrifuged at 100000 *g* for 60 min.

Step 2: batch chromatography on DEAE cellulose

The resulting cytosol was added to 200 g of DEAE-cellulose equilibrated with 20 mM sodium phosphate buffer, pH 7.2,

stirred for 60 min and then filtered. Unbound protein was removed with the equilibration buffer. Methionine synthase was eluted with a 20 mM sodium phosphate buffer, pH 7.2, containing 500 mM NaCl.

Step 3: Q-Sepharose chromatography

The active fraction was diluted 2-fold with water and loaded on to a 1.6 cm $\times$ 20 cm column equilibrated with 20 mM sodium phosphate buffer, pH 7.2. A linear gradient containing 0–1 M NaCl was applied with flow rate of 4 ml/min. The active fraction was eluted between 270 and 350 mM NaCl.

Step 4: hydroxyapatite chromatography

Enzyme from the previous step was concentrated with an Amicon ultrafiltrator fitted with a 50 kDa membrane, diluted 2-fold with water and loaded on to a 1.5 cm $\times$ 15 cm hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer, pH 7.2.

The column was eluted with a linear gradient up to 500 mM potassium phosphate (total volume 110 ml). Methionine synthase was eluted on the gradient between 10 and 25 %.

Step 5: Mono-Q chromatography

The active fractions from the previous stage were loaded on to a 1 cm $\times$ 5 cm Mono-Q column equilibrated with 20 mM sodium phosphate buffer, pH 7.2. The column was eluted with a linear gradient of NaCl up to 1 M (total volume 20 ml). The enzyme was eluted between 300 and 400 mM NaCl.

During the successive steps of the purification the enzyme was stored at 4 °C.

Purified enzyme samples were stored for up to three weeks at 4 °C without loss of activity.

Determination of the effect of polyamines on methionine synthase activity

The enzyme was buffer-exchanged with a 3.5 ml Superose G25 column, in accordance with the manufacturer's instructions, immediately before use. Buffers used for the assessment of the effect of polyamines were 50 mM Hepes, pH 7.2. Incubations were performed for 30 min for the concentration dependence assay, and for times up to 10 min for the time dependence assay. In all other respects assays were performed as described above in the Enzyme assay section.

Steady-state kinetics

These were conducted with the radiolabelling assay, and determined with respect to MTHF. Samples were preincubated for 5 min, and reactions were initiated by the addition of homocysteine and incubated at 37 °C for 10 min before ending the reaction by the addition of 400 μ l of ice-cold water.

RESULTS

Highly purified rat liver methionine synthase was purified 3000-fold from rat liver by methods modified from the literature [5,7,32]. The final active preparation contained no more than three major bands on a Coomassie Blue-stained denatured SDS/PAGE gel (results not shown). The specific activity exhibited by this preparation was 90 nmol of methionine per mg of protein.

Table 1 Polyamine-induced stimulation (percentages) of rat liver methionine synthase

Concentration (μ M)	Spermine	Spermidine	Putrescine	Cadaverine
10	48 $\pm$ 6	23 $\pm$ 21	2 $\pm$ 2	—4 $\pm$ 39
100	223 $\pm$ 4	66 $\pm$ 23	9 $\pm$ 3	17 $\pm$ 11
1000	402 $\pm$ 6	272 $\pm$ 16	50 $\pm$ 11	42 $\pm$ 8

Figure 1 Dose-dependent stimulation of methionine synthase by spermine (A) and spermidine (B)

The fits to the data were generated from the complete data set. The EC_{50} was estimated from the equation for the line of best fit.

Stimulation of methionine synthase activity by polyamines

After purification the enzyme sample was buffer-exchanged to Hepes, pH 7.2; marked stimulation of the enzyme activity by polyamines was noted (Table 1). Spermine increased enzyme activity to a maximum of around 400% at 5 mM and spermidine to a maximum of 270% at 7 mM. Putrescine and cadaverine were also tested and found to give much weaker stimulation even at high concentrations (1 mM). This experiment was also performed on partly purified (three steps) enzyme [29] and a similar effect was observed (results not shown).

Figure 2 Time-dependent stimulation of methionine synthase by spermine and spermidine

Δ , Spermine (785 μ M); $\blacksquare$, spermidine (1.25 mM); $\square$, control. The fits to the data were generated from the complete data set.

Table 2 Kinetic parameters reported for isoforms of vitamin B₁₂-dependent methionine synthase

Enzyme and conditions	K_m (μ M)	V_{max} (nmol/h)
Hepes buffer, rat liver	5	8.7
Phosphate buffer, rat liver	28	78
Phosphate buffer, pig liver [5]	12.8/16.8	125
Phosphate buffer, <i>E. coli</i> [37]	27.8	18.8
Phosphate buffer, pig kidney [5]	25	

Concentration-dependent effect of spermine and spermidine on methionine synthase

The effect of spermine and spermidine was further evaluated and the EC_{50} values determined by a dose-dependence study (Figure 1). Spermine was again found to be more potent than spermidine. The EC_{50} for spermine in Hepes buffer was found to be 8 μ M. Spermidine was found to have an EC_{50} of 40 μ M.

Time-dependent effect of spermine and spermidine on methionine synthase

Both polyamines were found to increase the activity of methionine synthase after 30 s, and at 1 min the stimulation was 100% (Figure 2). The reaction was seen to be linear for 10 min, both for control and stimulated enzyme.

Kinetic evaluation of the interaction between spermine and methionine synthase

The K_m and V_{max} values with respect to MTHF were evaluated in phosphate buffer and found to be of the order of 27 μ M and 78 nmol of methionine per hour respectively. These values are comparable to those found by other groups (Table 2). In Hepes

Figure 3 Effect of spermine on methionine synthase kinetics in Hepes buffer

Concentrations of spermine were: $\square$, zero; $\blacklozenge$, 14.6 μM ; $\triangle$, 2.4 mM. The fits to the data were generated from the complete data set.

buffer these values changed to K_m 5 μM and $V_{\max}$ 9 nmol/h. The addition of spermine increased both the $V_{\max}$ and the K_m : 4.6 μM spermine increased the K_m to 10 μM and the $V_{\max}$ to 13 nmol/h; 2.4 mM spermine increased the K_m to 19 μM and $V_{\max}$ to 20.5 nmol/h. The Lineweaver-Burk plot (Figure 3) at spermine concentrations of 0 μM , 14.6 μM and 2.4 mM is consistent in form with stimulation of the enzyme by spermine. The parallel nature of the three lines is consistent with a mechanism by which spermine (and spermidine) stimulates the rat liver methionine synthase in an uncompetitive manner [36].

DISCUSSION

All the polyamines tested in these experiments were found to stimulate methionine synthase. Spermine was the most active of the polyamines tested (EC_{50} 8 μM), whereas putrescine caused stimulation only in the millimolar range. The number of positive charges associated with the polyamines was correlated with their ability to stimulate enzyme activity, because spermine has four positively charged groups, spermidine three and putrescine two. To test this hypothesis further, the effect of cadaverine, also with two charged groups, was examined and it was found to be similar to that of putrescine. Thus at least part of the effect of polyamines on methionine synthase was probably due to electrostatic binding. In most of their functions polyamines are documented to interact by electrostatic binding, although polyamines have been shown to bind covalently to amino acids and proteins [38]. Polyamines have also been reported to exhibit radical scavenging

Table 4 Comparison of the concentration of spermine, spermidine and putrescine in different cell types as adapted from the literature [27]

Cell type	[Putrescine] (μM)	[Spermidine] (μM)	[Spermine] (μM)
Erythrocytes	0.6–0.9	18–28	15–19
Resting fibroblasts	29	159	635
Tumour transformed cells			
Ascites	43	430	602
sv-313	229	1835	694

properties [38]. Because the cobalt of the vitamin B_{12} prosthetic group is known to be susceptible to oxidation, polyamines acting as scavengers could offer protection against oxidizing radicals and hence stimulate enzyme activity. Alternatively it is possible that the polyamines inactivated a natural inhibitor of methionine synthase that had not been removed during purification. However, the magnitude of stimulation is the same in partly purified and highly purified enzyme samples [34], suggesting a more specific interaction.

The EC_{50} values found here for spermine and spermidine, 8 μM and 40 μM respectively, compare with the reported EC_{50} values for the interaction of polyamines with other enzymes, and the NMDA receptor channel (see Table 3). Estimates of the normal physiological levels of polyamines in nmol per 10^6 cells are quoted in the Introduction section, and these, converted into concentrations [27], are shown in Table 4. Serum and plasma concentrations of the order of 0.1–0.5 nmol/ml for putrescine, 0.1–0.3 nmol/ml for spermidine and below 0.1 nmol/ml for spermine have been reported [27]. In the cerebrospinal fluid polyamine levels are quoted as: putrescine, 184 ± 54 nM; spermidine, 150 ± 48 nM; and spermine, undetectable [38]. Looking at the polyamine concentration quoted in Table 3, the EC_{50} value for the interaction between spermine and methionine synthase is within the physiological intracellular range and the EC_{50} value for spermidine is within the range found in tumour cells. These results give credence to the hypothesis that the polyamine-induced stimulation of methionine synthase might be physiologically relevant.

Polyamines have been reported to exhibit many different effects both *in vivo* and *in vitro*. Phospholipase A_2 and phospholipase C are inhibited by polyamines, which have also been shown to suppress lipolysis and stimulate *sn*-glycerol 3-phosphate acyltransferase; this has anti-inflammatory implications [39]. Platelet aggregation in response to ADP and thrombin was inhibited by polyamines, possibly by inhibition of binding or through the relevant second-messenger system [40]. Polyamines have been shown to inhibit several cell signalling processes. The cell-signalling kinase, protein kinase C, is inhibited by polyamines [28]. The movement of Ca^{2+} has been shown to be affected by polyamines [41]. The time-dependent gating of inward rectifier K^+ channels has been reported to be mediated by polyamines, which could influence cell growth and division [42]. Low concentrations of polyamines increased the frequency of opening of the NMDA receptor channel, with high concentrations decreasing the opening time and receptor sensitivity; thus polyamines could modulate brain activity [43]. Polyamines were reported to be essential for cell growth and differentiation, partly through binding to DNA [25]. As has been discussed above, the interaction between polyamines and methionine synthase could occur *in vivo*. Indeed as the reported intracellular concentration of spermine is higher than the EC_{50} for the interaction it may be that

Table 3 Comparison of the reported EC_{50} values for polyamines and various systems

System	Spermine EC_{50}	Spermidine EC_{50}
Ca^{2+} -ATPase [29]	200 μM	180 μM
Casein kinase II [33]	< 250 μM	1 mM
NMDA receptor [31]	< 10 μM , stimulation; > 10 μM , inhibition (IC_{50} 200 μM)	
Inward rectifier K^+ channels dissociation constant [42]	7.5 nM	17.9 nM
Methionine synthase	8 μM	40 μM
Yeast mitochondrial Ca^{2+} uptake [44]	12 μM	50 μM
Nitric oxide synthase [30]	56 μM	139 μM

methionine synthase is chronically stimulated by spermine. The stimulation of methionine synthase by polyamines could help prepare for cell division, as methionine synthase activity is required for protein, DNA and RNA synthesis. The concentration of polyamines in cancer cells was found to be much higher than in normal cells and of a magnitude to stimulate methionine synthase maximally. This interaction could provide a link between the observations that inhibition of methionine synthase by nitrous oxide can be used to halt tumour growth, as can inhibition of ornithine decarboxylase inhibit the formation of spermine and spermidine.

The data in Table 3 show that polyamines affect a variety of enzymes involved in cell signalling processes and are thus probably themselves involved in cell signalling, as well as in growth and differentiation. It is therefore conceivable that vitamin B₁₂-dependent methionine synthase is also involved in cell signalling processes, and could certainly be a future target for cancer chemotherapeutic agents.

REFERENCES

- Banerjee, R. V. and Matthews, R. G. (1990) *FASEB J.* **4**, 1450–1459
- Weissbach, H., Peterkofsky, A., Redfield, B. G. and Dickerman, H. (1963) *J. Biol. Chem.* **238**, 3318–3324
- Banerjee, R. V., Harder, S. R., Ragsdale, S. W. and Matthews, R. G. (1990) *Biochemistry* **29**, 1129–1135
- Banerjee, R. V., Frasca, V., Ballou, D. P. and Matthews, R. G. (1990) *Biochemistry* **29**, 11101–11109
- Chen, Z., Crippen, K., Gulati, S. and Banerjee, R. (1994) *J. Biol. Chem.* **269**, 27193–27197
- Ast, T., Nicolaou, A., Anderson, M. M., James, C. H. and Gibbons, W. A. (1994) *Biochem. Soc. Trans.* **22**, 217S
- Utley, C. S., Marcell, P. D., Allen, R. H., Anthony, A. C. and Kolhouse, F. J. (1985) *J. Biol. Chem.* **260**, 13656–13665
- Burke, G. T., Magnum, J. H. and Brodie, J. D. (1971) *Biochemistry* **10**, 3079–3085
- Allen, R. H., Stabler, S. P., Savage, D. G. and Lindenbaum, J. (1993) *FASEB J.* **7**, 1344–1353
- Chanarin, I., Deacon, R., Lumb, M. and Perry, J. (1989) *Blood Rev.* **3**, 211–215
- Green, R. and Jacobsen, D. W. (1990) in *Biomedicine and Physiology of Vitamin B12: the Role of Cobalamins in the Nervous System* (Linnell, J. C. and Bhatt, H. R., eds.), pp. 107–119, London Children's Medical Charity, London
- Mohammed, R. and Lammond, M. (1986) *Ann. Rech. Vet.* **17**, 447–450
- Wainfan, E. and Poirier, L. A. (1992) *Cancer Res. Suppl.* **52**, 2071s–2077s
- Refsum, H. and Ueland, P. M. (1990) *Trends Pharmacol. Sci.* **11**, 411–416
- Nicolaou, A., Ast, T., Garcia, C. V., Anderson, M. M., Gibbons, J. M. and Gibbons, W. A. (1994) *Biochem. Soc. Trans.* **22**, 296S
- Nicolaou, A., Kenyon, S. H., Gibbons, J. M., Ast, T. and Gibbons, W. A. (1996) *Eur. J. Clin. Invest.* **26**, 167–170
- Yagiela, J. A. (1991) *Anesth. Prog.* **38**, 1–11
- Alston, T. A. (1991) *Biochem. Pharmacol.* **42**, R25–R28
- Barak, A. J., Beckenhauer, H. C. and Tuma, D. J. (1991) *Alcohol Alcohol.* **26**, 125–128
- Stabler, S. P., Brass, E. P., Marcell, P. D. and Allen, R. H. (1991) *J. Clin. Invest.* **87**, 1422–1430
- Smith, J. R. and Smith, J. G. (1990) *Bull. Environ. Contam. Toxicol.* **45**, 649–654
- Ueland, P. M. and Refsum, H. (1989) *J. Lab. Clin. Med.* **114**, 473–501
- Seiler, N. and Atanassov, C. L. (1994) *Prog. Drug Res.* **43**, 87–141
- Koza, R. A. and Herbst, E. J. (1992) *Biochem. J.* **281**, 87–93
- Milam, K. M., Deen, D. F. and Morton, L. J. (1989) *Cell Tissue Kinet.* **22**, 269–277
- Fredlund, J. O., Johansson, M. C., Dahlberg, E. and Oredsson, S. M. (1995) *Exp. Cell Res.* **216**, 86–92
- Morgan, D. M. L. (1990) *Biochem. Soc. Trans.* **18**, 1089–1094
- Morrucci, M., Borbiroli, B., Monti, M. G., Tadolini, B., Hakim, G. and Mezzetti, G. (1987) *Biochem. J.* **247**, 175–180
- Hughes, G., Starling, A. P., East, J. M. and Lee, A. G. (1994) *Biochemistry* **33**, 4745–4754
- Hu, J., Mohmoud, M. I. and El-Fakahony, E. E. (1994) *Neurosci. Lett.* **175**, 41–45
- Rock, D. M. and MacDonald, R. L. (1992) *Mol. Pharmacol.* **42**, 157–164
- Wei, T. and Tao, M. (1993) *Arch. Biochem. Biophys.* **307**, 206–216
- Munir, M., Subramaniam, S. and McGanigie, P. (1993) *Brain Res.* **616**, 163–170
- Wang, J. Y., Viar, M. D. and Johnson, L. R. (1994) *Proc. Soc. Exp. Biol. Med.* **205**, 20–28
- Kenyon, S. H., Ast, T., Nicolaou, A. and Gibbons, W. A. (1995) *Biochem. Soc. Trans.* **23**, 444S
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Palmer, T. (1995) *Understanding Enzymes*, 4th Edn., Ellis Horwood/Prentice Hall, London
- Seiler, N. (1991) *Prog. Drug Res.* **37**, 107–159
- Villanueva, V. R. (1983) *Adv. Polyamine Res.* **4**, 297–306
- Lee, K.-Y., Chun, S. H., Choi, C.-S. and Lee, M. W. (1991) *Korean J. Biochem.* **23**, 167–174
- Scott, R. H., Sutton, K. G. and Dolphin, A. C. (1993) *Trends Neurosci.* **16**, 153–160
- Ficker, E., Tagliatela, M., Wible, B. A., Henley, C. M. and Brown, A. M. (1994) *Science* **266**, 1068–1073
- Seiler, N. (1981) *Neurochem. Int.* **3**, 95–110
- Votyakova, T. V., Bazhenova, E. N. and Zvjagilskaya, R. A. (1993) *J. Bioenerg. Biomembr.* **25**, 569–574