S-Adenosylmethionine Synthetase Deficient Mutants of Escherichia coli K-12 with Impaired Control of Methionine Biosynthesis

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Summary: Two mutants of E. coli K-12 with low levels of S-adenosylmethionine synthetase have been isolated. These strains have larger intracellular methionine pools than the wild type and also have elevated levels of cystathionine synthetase and cystathionase. Although the activities of these enzymes are partially repressible in the mutant strains they are affected to a lesser extent than those of the wild type by growth on methionine. The mutant with the lower SAM synthetase activity has the higher enzyme activities and methionine pool. These results suggest that S-adenosylmethionine is an effector in the regulation of methionine biosynthesis.

The regulation of the biosynthesis of the aspartic family of amino acids has been the subject of many studies which have been thoroughly covered in recent reviews (1,2,3). In Escherichia coli the enzymes of this branched pathway, which are unique to the biosynthesis of methionine, are repressed by addition of methionine to the growth medium (4,5,6,7,8). Recent work in our laboratory has shown that a group of mutants, which are constitutive for the methionine biosynthetic enzymes, also have elevated, nonrepressible levels of S-adenosylmethionine (SAM) synthetase; suggesting that SAM synthetase is controlled by the methionine regulatory system (9). Gross and Rowbury (10) have reported that met G mutants of Salmonella typhimurium have a reduced capacity for synthesis of methionyl-tRNA but retain the ability to control the enzymes of methionine biosynthesis. Thus, methionyl-tRNA does not appear to be an effector in repression of the methionine biosynthetic enzymes. S-adenosylmethionine has been shown to be an inhibitor of cystathionine-γ-synthetase in Neurospora crassa (11), an inhibitor of homoserine-O-transacetylase in Saccharomyces cerevisiae (12), to act

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synergistically with methionine to inhibit homoserine-O-transsuccinylase in E. coli (13), and has been postulated to be an effector for repression of methionine synthesis in S. cerevisiae (14). We have isolated two mutant strains of E. coli K-12 with greatly reduced levels of SAM synthetase which overproduce methionine and have elevated levels of cystathionine synthetase and cystationase.

Experimental

E. coli K-12 (λ) F^+ was obtained from Dr. Samson Gross of the Department of Biochemistry at Duke University. Strains D7 and E4 were isolated as spontaneous ethionine resistant variants of the wild type. Cells were generally grown on a modified Davis Mingioli medium (15) containing 7g K_2HPO_4, 3g KH_2PO_4, 1g $(NH_4)_2SO_4$, .01g $MgSO_4 \cdot 7H_2O$ and 5g dextrose per liter of distilled water with supplementation as required. For enzyme assays cells were harvested by centrifugation, washed with 0.02M Tris·HCl pH 7.6 (3°C) and resuspended in a sufficient volume of the same buffer to give an absorbancy of 20 at 550 mμ (approximately 30 mg/ml of wet cells). For routine enzyme assays cells toluenized by incubation at 37°C for 10 min in the presence of 0.05 volume of toluene were used. In separate experiments toluenized cells and sonicated extracts had comparable SAM synthetase and cystathionine synthetase activities. Cystathionase activities of toluenized cells were usually only 60-80% as great as those of sonicated extracts, but, although the measurements are not precise, assays with toluenized cells are sufficiently accurate to reveal large differences in activity.

Cystathionase was assayed by measuring reduction of Ellman's reagent (5,5' dithio-bis-(2-nitrobenzoic acid)) (16) in the presence of L-cysthionine using a modification of the procedure of Flavin (17). A control experiment using cells of a met C (cystathionaseless) mutant showed negligible activity. Cystathionine synthetase was assayed by a modification of the method of Kaplan and Flavin (18) measuring the α ketobutyric acid resulting from the elimination reaction of O-succinyl homoserine. Cells of a met B (cystathionine synthetaseless) mutant had no activity in the assay procedure. SAM synthetase activity

was determined by measurement of the conversion of ATP-8-^{14}C to labelled SAM using Dowex 50X-2 to separate the SAM from other radioactive compounds. Blank reaction mixtures without added methionine gave values less than 1 mμmole of SAM per mg cells which is negligible when compared to the amount synthesized in the presence of methionine by wild type cells but is not insignificant compared to that synthesized by the mutant strains.

O-succinylhomoserine was synthesized by the procedure of Flavin and Slaughter (19), ATP-8-^{14}C was purchased from Schwarz Bioresearch, Inc., L-cystathionine was purchased from Calbiochem and other reagents were the purest available grades from standard commercial sources. Radioactivity measurements were made with a Packard Tricarb Model 3375 Liquid Scintillation Spectrometer.

Results and Discussion

Overproduction of an amino acid is a frequently encountered mechanism for resistance to analogs of that amino acid. Since D7 and E4 were isolated as ethionine resistant mutants they were tested for methionine excretion using microbiological procedures. Strain D7 clearly excreted a substance which could feed *E. coli* strain AB 280 (a met B mutant), strain E4 gave inconclusive results indicating less excretion than D7, but not ruling it out altogether. Chromatography of the supernatants from log phase cultures (absorbancy of 1 at 550 mμ) of D7 and wild type K12 on a Beckman Model 120 Amino Acid Analyzer showed the D7 medium to contain 10^{-5}M methionine while the medium from the wild type cells had barely detectible methionine ($< 10^{-6}$M). The methionine pools of log phase D7, E4 and wild type cells were measured by growth of cells on media with a low (but non-limiting) concentration of sulfate containing $^{35}SO_4^{=}$, paper chromatography of perchloric acid extracts and counting of the methionine spots. Before the chromatograms were cut into strips for counting in the liquid scintillation counter they were passed through a strip counter to observe the shapes of the peaks. While D7 and E4 showed well resolved peaks of radioactivity in the methionine area, the wild type showed a rather small peak which appeared to have a significant amount of contamination from radioactive compounds smeared

Table I

Methionine Pools of Wild Type and Mutant Cells

Strain	Methionine Pool (μmoles/g) uncorrected	corrected
wild type	0.24	0.09
D7	1.52	1.35
E4	.84	.70

Low sulfur culture medium contained 8.8 g K_2HPO_4 3.8 g KH_2PO_4, 0.3 g $MgCl_2 \cdot 6H_2O$, 2.2 g NH_4Cl, 0.06 g NH_4SO_4 and 10 g dextrose per liter of water. Cells were pregrown in low fulfur medium to reduce sulfur stores and a few drops of the cultures were used to inoculate 40 ml volumes of low sulfur medium containing 35 μc of $H_2{}^{35}SO_4$ (carrier free). Cells were grown till each culture had an absorbancy of approximately one at 550 mμ. The cells were recovered by centrifugation and each pellet was extracted overnight with 6% $HClO_4$. The perchloric acid extracts and washes were neutralized with $KHCO_3$, $KClO_4$ removed and lyophilized to dryness. The residues were made up to known volumes and duplicate aliquots corresponding to 1/20 of each extract were chromatographed on Whatman #1 paper using butanol 80: acetic acid 20: water 20 for development. The strips were scanned on a Packard strip scanner to find the radioactive peaks and the area corresponding to the methionine standard (which also appeared as a radioactive peak on each strip) were cut into 0.5 cm strips and counted in 5 ml of toluene PPO, POPOP scintillation fluid. Ten μl of radioactive culture medium, dried on the same sized paper strip, was counted at the same time for calculation of the specific activity of the sulfur. The sum of the total counts across each methionine spot was used for calculation of the uncorrected values while the estimated non methionine radioactivity was subtracted before computation of the corrected values (see text).

along the chromatogram. The baseline counting rates were estimated from the counting rates of strips near, but not a part of the methionine peaks. Two values for the methionine pool of each cell type is given in Table I, the lower value has been corrected for the estimated contamination of each methionine peak, while the higher value was calculated from the total radioactivity in each peak. Regardless of the validity of the correction it can be seen that the methionine pools of both D7 and E4 are markedly larger than that of the wild type. Table II shows the SAM synthetase levels of the three strains. While the exact levels of enzyme in strains D7 or E4 are subject to some uncertainty, because they are near the blank values, they are both much lower than that of the wild type and the activities of E4 cells are consistently higher than those of D7 cells. Incubation of mixtures of wild type and D7 or

Table II

SAM Synthetase Activities of
Wild Type and Mutant Strains

Strain	SAM Synthetase Activity mμmoles/mg cells/30 min
wild type	21
D7	0.3
E4	1.4

Cells were grown overnight at 37°C, harvested and toluenized as described in the text. One ml incubation mixtures containing 150 μmoles Tris·HCl pH 8.5, 100 μmoles KCl, 15 μmoles $MgCl_2$, 8 μmoles glutathione, 20 μmoles L methionine 10 μmoles ATP-8-^{14}C (0.25 μc) and 0.4 ml toluenized cells were incubated for 30 min at 37°C. The reactions were stopped by addition of 1 ml of 6% $HClO_4$ to each tube and after removal of precipitated material 1 ml of each supernatant solution was pipetted onto a 0.6 x 4 cm column of Dowex 50 x-2 (H) (200-400 mesh). Each column was washed with three 10 ml aliquots of 1NHCl followed by 5 ml of water and the SAM was removed by 9 ml 1N NH_3. One ml of concentrated HCl was added to each NH_3 eluate and 4 ml aliquots were counted using the 2:1 toluene: Triton mixture of Patterson and Greene (20). Values given above were corrected for radioactivity from incubation mixtures lacking methionine (1 mμmole/mg/30 min for wild type and 0.6 mμmole/mg/30 min for D7 and E4).

E4 sonicated extracts give within 90% of the calculated results suggesting that the low activities of these strains are not due to accumulation of an inhibitor. The SAM pools of log phase E. coli K12 cells are quite low and difficult to quantitate, but qualitative results obtained from ^{35}S labelled cell extracts show that D7 and E4 have lower intracellular pools of SAM than wild type cells. The cystathionase and cystathionine synthetase activities of the three cell types grown on minimal medium or in the presence of methionine are given in Table III. D7 has the highest enzyme activities which are the least subject to repression. E4 occupies an intermediate position with activities that are lower and more easily repressed than those of D7 but still consistently higher than those of the wild type. Results from cells grown in the presence of SAM give some indication of repression but the results are inconclusive. Separate experiments show that little, if any, SAM is taken up from the medium by E. coli cells, so intracellular concentrations of SAM may be too low to cause clear cut repression.

Table III

Cystathionase and Cystathionine Synthetase Activities of Cells Grown in Minimal and Methionine Containing Media

Strain	Addition to Growth Medium	Cystathionine Synthetase Activity mμmoles/20 min/mg cells	Cystathionase Activity mμmoles/min/mg cells
wild type	none	6.4	0.96
	10^{-3}M methionine	0.93	0.42
	$5X10^{-3}$M methionine	0.34	0.28
D7	none	78	4.7
	10^{-3}M methionine	70	4.4
	$5X10^{-3}$M methionine	39	1.5
E4	none	32	1.7
	10^{-3}M methionine	10	1.1
	$5X10^{-3}$M methionine	8.7	0.52

Cells grown overnight at 37°C on medium supplemented with methionine as indicated were harvested and toluenized as described in the text. For cystathionine synthetase assays 0.5 ml. incubation mixtures containing 100 μmoles Tris·HCl pH 8.1, 0.125 μmole pyridoxal phosphate, 5 μmoles O-succinyl homoserine and 0.1 ml or 0.2 ml of washed toluenized cell suspension (depending on the activity) are incubated at 37°C for 20 min. Reactions are stopped by the addition of 0.1 ml 6% $HClO_4$. Ketobutyrate content of suitable aliquots of the neutralized supernates are measured by lactic dehydrogenase catalyzed oxidation of NADH. Values are corrected for blanks without O-succinyl homoserine (average value approximately 2 mμmoles/20 min/mg cells). For cystathionase assays the rate of reduction of Ellman's reagent in 1 ml reaction mixtures containing 160 μmoles potassium phosphate pH 7.4, 0.125 μmole $MgSO_4$, 1 μmole Ellman's reagent, 1 μmole L cystathionine, and 0.05 or 0.1 ml of toluenized cells was measured at 410 mμ with a Gilford model 2000 spectrophotometer. Blanks in the absence of cystathionine were insignificant (less than five percent).

Although the exact nature of the genetic defect in these strains is not known there appears to be an inverse correlation between the SAM synthetase activity (and presumably the SAM pool) of a strain and its level of methionine biosynthetic enzymes. The methionine biosynthetic enzymes of these strains still appear to be at least partially repressible but higher concentrations of methionine are required and the strain with the lowest level of SAM synthetase is the least sensitive to added methionine. These results are consistent with the hypothesis that SAM or one of its metabolites is an effector for repression of the methionine biosynthetic enzymes in *E. coli*.

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