Expression of rat liver S-adenosylmethionine synthetase in *Escherichia coli* results in two active oligomeric forms

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A cDNA containing the complete coding sequence for rat liver S-adenosylmethionine synthetase was cloned into the prokaryotic expression vector pT7-7 and expressed in *Escherichia coli* BL21(DE3). A major additional band corresponding to a protein of 48 kDa was detected on SDS/PAGE after induction with isopropyl β -D-thiogalactopyranoside. This protein was distributed in both the soluble and insoluble fractions and accounted for approx. 30% of the total bacterial protein. The soluble enzyme was fully active, as revealed by assays *in vitro* of S-

adenosylmethionine synthetase activity. In addition, transformed bacteria exhibited highly increased levels of intracellular S-adenosylmethionine. Two active forms of the recombinant enzyme, with apparent molecular masses of 210 kDa and 110 kDa, were detected when cytosolic extracts of the transformed cells were fractionated by gel-filtration chromatography. It is concluded that the expressed S-adenosylmethionine synthetase polypeptide assemble as tetramers and dimers.

INTRODUCTION

S-Adenosylmethionine (AdoMet) synthetase (ATP:L-methionine S-adenosyltransferase; EC 2.5.1.6) catalyses the only known route for the synthesis of AdoMet. The relevance of this reaction lies in the key role that AdoMet plays in cellular metabolism, since it is the main methyl donor for transmethylation reactions and also acts as the aminopropyl donor in the biosynthesis of polyamines [1,2].

Different AdoMet synthetase isoenzymes have been found in all organisms studied. Chiang and Cantoni [3] first purified two closely related forms of AdoMet synthetase from yeast. Both isoenzymes had an apparent molecular mass of 115 kDa, by gel filtration and appeared to be composed of different subunits, of 55 and 60 kDa. Later it was reported that the two isoenzymes were encoded by different structural genes (SAM1 and SAM2) [4,5]. From *Escherichia coli*, Markham et al. [6] purified an AdoMet synthetase form of molecular mass 180 kDa, as determined by gel-filtration chromatography. However, SDS/PAGE analysis showed a single band of 43 kDa, suggesting that the *E. coli* enzyme could be a tetramer of identical subunits. These results were subsequently confirmed by the isolation of a single gene (MetK) encoding *E. coli* AdoMet synthetase [7]. Recently, an alternative form of AdoMet synthetase has been identified in conditional mutants of *E. coli* [8,9], demonstrating the occurrence of two AdoMet synthetase forms also in this organism. The presence of two AdoMet synthetase isoenzymes has also been established in *Trypanosoma brucei* [10] and *Arabidopsis thaliana* [11,12].

In mammals, the exact number of AdoMet synthetases is still a subject of controversy. Three different isoenzymes have been purified and designated α (or high- M_r AdoMet synthetase), β (or low- M_r AdoMet synthetase) and γ . The γ form is distributed in extrahepatic tissues and has been reported to consist of two or three distinct subunits, with molecular masses in the range 38–53 kDa [13–15]. The α and β forms are expressed in the liver and have apparent molecular masses of 210 kDa and 110 kDa respectively, as determined by gel-filtration chromatography

[16,17]. Various pieces of evidence have led to the proposal that the two AdoMet synthetase isoenzymes found in the mammalian liver represent different oligomeric states of the same polypeptide chain. First, both forms when analysed by SDS/PAGE show a single polypeptide band of about 48 kDa [13,16]. Second, tryptic digests of the two isoenzymes are identical [16]. Third, antibodies raised against the low- M_r form are able to recognize both isoenzymes [16]. Fourth, the high- M_r form can be converted into the low- M_r form by incubation with LiBr [17]. In addition, cDNAs coding for a single AdoMet synthetase polypeptide have been isolated from rat [18,19], mouse [20] and human [21,22] liver.

To establish whether different oligomeric forms of rat liver AdoMet synthetase exist, we have used a heterologous expression system. Bacterial cells transformed with a rat liver AdoMet synthetase cDNA construct yielded a fully active enzyme. The recombinant enzyme was contrasted with the rat liver AdoMet synthetase native forms. Evidence for the occurrence of different oligomeric forms of the enzyme is presented.

EXPERIMENTAL

Materials

The pT7-7 expression vector and the bacterial host strain BL21(DE3) were kindly given by Dr. Stan Tabor (Harvard Medical School, Boston, MA, U.S.A.) and Dr. F. William Studier (Brookhaven National Laboratories, NY, U.S.A.) respectively. Enzymes used in DNA manipulation were obtained either from Biotech S.L. (Madrid, Spain) or Boehringer Mannheim (Mannheim, Germany). Taq DNA polymerase and reagents for PCR were supplied by Promega Inc. (Madison, WI, U.S.A.). The Sequenase DNA-sequencing kit was from U.S. Biochemicals (Cleveland, OH, U.S.A.). Methionine, ATP, benzamidine, phenylmethanesulphonyl fluoride (PMSF), pepstatin A, aprotinin, leupeptin, antipain and the molecular-mass standards for gel filtration were products from Sigma Chemical Co (St. Louis, MO, U.S.A.). [2-³H]ATP (20 Ci/mmol) was supplied by Amersham International (Little Chalfont, Bucks., U.K.).

Sephacryl S-300 and Optiphase HiSafe 3 scintillation fluid were purchased from Pharmacia LKB (Uppsala, Sweden). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.). Other reagents used were of the best quality commercially available. The h.p.l.c. column Ultrasil-CX (particle size 10 μ m; 4.6 mm $\times$ 250 mm) was obtained from Beckman Instruments (Palo Alto, CA, U.S.A.). Oligodeoxynucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. The thermal cycler was a model Gene ATAQ controller, from Pharmacia LKB.

Construction of the AdoMet synthetase expression vector

For constructing the AdoMet synthetase expression vector, a 2.3 kb *Eco*RI fragment of plasmid pSSRL [19] was used. This insert was adapted for cloning into the *Nde*I/*Sal*I sites of the prokaryotic expression vector pT7-7 [23] by PCR. The 5' PCR primer (5'CGGAATTCATATGAATGGACCTGTGG3') consisted of an un-annealed sequence containing *Eco*RI and *Nde*I restriction sites at the 5'-end (underlined), and a 16-nucleotide sequence identical with bases 1–16 of the rat liver AdoMet synthetase clone pSSRL. The 3' PCR primer (5'GCACTCGAGGCTCTAAACACAAG3') had a *Xho*I site at the 5'-end (underlined) and a 15-nucleotide sequence complementary to the bases 1183–1197 of the pSSRL clone. The PCR was carried out in a reaction mixture containing 150 ng of pSSRL plasmid, 25 pmol of each of the two oligonucleotides, 0.04 mM of each of the four deoxyribonucleotides, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100 and 2.5 units of *Taq* DNA polymerase in a total volume of 50 μ l. Amplification was performed for 30 cycles at 94 °C for 2 min, 55 °C for 1 min and 72 °C for 3 min. The PCR product was purified, digested with *Nde*I and *Xho*I and ligated to the *Nde*I/*Sal*I sites of the expression vector pT7-7. The ligation mixture was used to transform *E. coli* DH5 α , and recombinants were identified after digestion with *Nde*I and *Xho*I. The resulting construct, designated pSSRL-T7N, was sequenced to confirm the correct insertion and to verify that no mismatch had been generated by the *Taq* DNA polymerase.

Expression in *E. coli* and preparation of cell extract

Competent *E. coli* BL21(DE3) cells [24] were transformed with either pSSRL-T7N or the vector alone. Overnight cultures prepared from single colonies were used to inoculate 100 ml of LB medium plus ampicillin (100 μ g/ml). The cells were grown to A_{595} 0.3–0.4, and isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The incubations were continued for 6 h. For monitoring AdoMet synthetase expression, 1 ml samples were removed every 1 h during the induction period; for SDS/PAGE, samples were collected 3 h after the initiation of induction. The cells were harvested by centrifugation, and the pellets obtained were washed with water and resuspended in 100 μ l of 50 mM Tris/HCl (pH 8.0)/0.5 M NaCl containing 0.1% 2-mercaptoethanol and protease inhibitors (2 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 0.5 μ g/ml leupeptin, 2.5 μ g/ml antipain, 0.1 mM benzamidine, 0.1 M PMSF). The cells were disrupted by sonication at 4 °C (four 30 s pulses at 30 s intervals, output power level 6), and soluble (cytosolic fraction) and insoluble materials were separated by centrifugation for 15 min at 13 000 rev./min in a microfuge. The supernatants were used for measuring AdoMet synthetase activity.

Determination of AdoMet synthetase activity

AdoMet synthetase activity was assayed as described by Cabrero et al. [16]. Samples (160 μ l) were incubated with 90 μ l of a reaction mixture containing 75 mM Tris/HCl, pH 8.0, 250 mM KCl, 9 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM methionine and 5 mM [2- 3H]ATP (4 Ci/mmol). The incubation was carried out for 30 min at 37 °C and was stopped by addition of 3 ml of distilled water. The samples were immediately loaded on cation-exchanger AG 50W-X4 columns (1 ml) equilibrated in water. The columns were washed with water (20 ml), and the [3H]AdoMet was then eluted with 4 ml of 3 M NH_4OH . The radioactivity was determined in the presence of 1 ml of acetic acid and 10 ml of scintillation liquid.

Gel-filtration chromatography

Gel-filtration chromatography was carried out using cytosolic extracts from rat liver as well as transformed and non-transformed bacteria.

A 30 ml culture of *E. coli* BL21(DE3) transformed with pSSRL-T7N was induced with IPTG. At 3 h after induction, cells were collected and lysed. Subsequent steps were carried out at 4 °C. The cytosolic fraction (2 ml) was loaded on a Sephacryl S-300 column (96 cm $\times$ 1.5 cm) equilibrated in 10 mM Hepes/NA (pH 7.5)/1 mM EDTA/10 mM $MgSO_4$ /50 mM KCl. The chromatography was carried out at 12 ml/h with the equilibration buffer containing 0.1% 2-mercaptoethanol, 0.1 mM benzamidine, 0.1 mM PMSF and 2 μ g/ml aprotinin, and 3 ml fractions were collected. Samples (160 μ l) from the fractions were assayed for AdoMet synthetase activity as described above, but by using a reaction mixture with a specific radioactivity of 1 Ci/mol. The same protocol, without IPTG induction, was used to analyse the cytosol of non-transformed bacteria. In this case, a reaction mixture with a specific radioactivity of 4 Ci/mol was used to detect significant AdoMet synthetase activity.

For gel-filtration analysis of rat liver AdoMet synthetase, the liver of a male rat was homogenized in 4 vol. of 0.3 M sucrose/10 mM Tris/HCl (pH 7.4)/0.1 mM EGTA/0.1% 2-mercaptoethanol and protease inhibitors (0.1 mM PMSF, 0.1 mM benzamidine and 10 μ g/ml soybean trypsin inhibitor). The homogenate was filtered through two layers of gauze and centrifuged for 20 min at 10 000 *g*. The supernatant was collected and centrifuged again for 60 min at 100 000 *g*. The cytosolic fraction was then loaded on to the Sephacryl S-300 column. The whole procedure was carried out at 4 °C.

Measurement of AdoMet levels

AdoMet levels were determined in either bacterial cells bearing pSSRL-T7N or non-transformed cells. At 3 h after IPTG induction, 1 ml samples were immediately deproteinized by homogenization in 10% trichloroacetic acid/0.05 M HCl at 4 °C. Trichloroacetic acid was eliminated by three successive washes with diethyl ether saturated with 0.05 M HCl, and samples were freeze-dried. Samples were then resuspended in 100 μ l of 0.01 M ammonium formate, pH 4.0, and analysed in an Ultrasil CX h.p.l.c. column (46 mm $\times$ 250 mm; particle size 10 μ m). After a 5 min washing period with 0.01 M ammonium formate, pH 4.0, the elution was carried out with a 50 min gradient from 0.01 M to 0.8 M ammonium formate, pH 4.0, at a flow rate of 1 ml/min. AdoMet was detected by measuring the A_{254} .

For measuring protein concentration, 1 ml samples of the same cultures were centrifuged, resuspended in 100 μ l of 50 mM Tris (pH 8.0)/0.5 M NaCl and sonicated.

Other methods

Gel electrophoresis was performed on 10 %-polyacrylamide/SDS gels, under reducing conditions, in the buffer system described by Laemmli [25]. The protein concentration was determined by the method of Bradford [26], with BSA as standard.

RESULTS

Construction of the expression vector

For prokaryotic expression of rat liver AdoMet synthetase, we utilized the expression vector pT7-7 [23]. The entire coding sequence for rat liver AdoMet synthetase was amplified by PCR, using the pSSRL cDNA [19] as template. Synthetic primers corresponding to the 5' and 3' ends of the coding sequence were designed to include *Nde*I and *Xho*I recognition sites, respectively, to allow cloning of the insert into the *Nde*I/*Sal*I sites of the vector pT7-7. The resulting expression plasmid was designated pSSRL-T7N.

Expression of rat liver AdoMet synthetase

E. coli BL21(DE3) [24] cells transformed with pSSRL-T7N were induced with IPTG. The cells were centrifuged and lysed as described in the Experimental section. Proteins from the total extract, soluble and insoluble fractions were resolved on SDS/PAGE under reducing conditions. As shown in Figure 1, there was no detectable expression of the rat liver AdoMet synthetase in the absence of IPTG. Induction with IPTG resulted in accumulation of a protein with a calculated molecular mass of 48 kDa. This additional protein presented electrophoretic behaviour identical with that described for the rat liver AdoMet synthetase [13,16]. The protein was present mostly in the insoluble fraction, although significant amounts were also found in cytosol (Figure 1). Densitometric scans of the Coomassie-Blue-stained polyacrylamide gels indicated that the amount of the recombinant protein produced represented up to 30% of the total bacterial protein.

To examine whether the recombinant protein was active, soluble and insoluble fractions of bacterial cells transformed with pSSRL-T7N were obtained and assayed for AdoMet synthetase activity at saturating concentrations of the substrates, methionine and ATP. Enzymic activity was determined at different times after IPTG induction. No detectable enzymic activity was found in the insoluble fraction. In the cytosolic fraction, a 12-fold increase in AdoMet synthetase activity was observed 3 h after the addition of IPTG. This value remained unchanged for up to 6 h (Figure 2). Bacterial cells harbouring the plasmid pT7-7 without the AdoMet synthetase cDNA insert did not exhibit changes in the enzymic activity, measured under the same conditions. The basal AdoMet synthetase activity detected in pSSRL-T7N transformants before IPTG induction and in cells transformed with the pT7-7 vector is attributed to the intrinsic activity of the *E. coli* enzyme.

Determination of intracellular AdoMet concentration

To investigate the effect of AdoMet synthetase over-expression on the intracellular AdoMet production, the concentration of AdoMet was measured in both pSSRL-T7N-transformed and non-transformed bacterial cells. The determinations were carried out 3 h after IPTG induction. The levels of AdoMet were 1.1 ± 0.5 and 482 ± 60 nmol/mg of protein for non-transformed and pSSRL-T7N-transformed cells respectively.

Analysis of AdoMet synthetase oligomeric forms

The product of the expression of pSSRL-T7N in bacteria was compared with AdoMet synthetase forms from rat liver and *E. coli*. For this purpose, cytosolic extracts from rat liver, *E. coli* BL21(DE3) transformed with the recombinant plasmid, and non-transformed cells were prepared as described in the Ex-

Figure 1 SDS/PAGE analysis of rat liver AdoMet synthetase expression in *E. coli*

Cultures of *E. coli* transformed with pSSRL-T7N were induced with 0.5 mM IPTG, and samples at 0 h (lanes 1, 3, 5) and 3 h (lanes 2, 4, 6) were collected. Portions of the whole cell lysate (lanes 1, 2), supernatant fraction (3, 4) and pellet fraction (5, 6) were resolved on SDS/PAGE and stained with Coomassie Blue. The calculated molecular mass of the recombinant protein is shown on the right. Molecular masses (kDa) of the standards are shown on the left: phosphorylase *b* (97 400), BSA (66 200), ovalbumin (45 000), bovine carbonic anhydrase (31 000).

Figure 2 Time course of AdoMet synthetase induction by IPTG

E. coli BL21(DE3) transformed with pSSRL-T7N (●) or the pT7-7 vector (○) were induced with 0.5 mM IPTG. Samples were collected at the indicated times, and AdoMet synthetase activity was assayed in the cytosolic fraction as described in the Experimental section.

Figure 3 Separation of AdoMet synthetase forms by gel-filtration chromatography

The cytosolic fractions from (a) rat liver, (b) pSSRL-T7N-transformed bacteria and (c) non-transformed bacterial cells were analysed by gel-filtration chromatography on Sephacryl S-300 as described in the Experimental section. Fractions (3 ml) of the eluate were collected, and AdoMet synthetase activity was determined in a reaction mixture with a specific radioactivity of 1 Ci/mol for the fractions shown in (a) and (b), and 4 Ci/mol for the fractions in (c). The elution positions of the molecular-mass standards (kDa) are indicated: A, Blue Dextran (2000); B, β -amylase (200); C, alcohol dehydrogenase (150); D, phosphorylase *b* (97.4); E, carbonic anhydrase (29).

perimental section. The extracts were directly fractionated by gel-filtration chromatography under conditions known to separate the different AdoMet synthetase isoenzymes present in the rat liver [16,27]. Two peaks of AdoMet synthetase activity were detected after gel-filtration chromatography of the cytosolic extract from pSSRL-T7N-transformed bacterial cells (Figure 3). These peaks of activity coincided with the elution positions for the rat liver high- M_r (210 kDa) and low- M_r (110 kDa) AdoMet synthetase (Figures 3a and 3b). By contrast, a single peak of AdoMet synthetase activity (Figure 3c) was found when a cytosolic extract from the wild-type *E. coli* BL21(DE3) strain was analysed. In this case a reaction mixture with higher specific radioactivity was used to detect significant AdoMet synthetase activity. The calculated molecular mass for the peak obtained was 180 kDa, a result that agrees with the expected molecular mass of the *E. coli* native AdoMet synthetase [6].

From these results and those obtained by SDS/PAGE, it can be deduced that by providing *E. coli* with the coding sequence for a single polypeptide, two oligomeric forms of AdoMet synthetase are produced, a tetramer and a dimer.

DISCUSSION

Rat liver AdoMet synthetase has been expressed in bacteria by using the T7 RNA polymerase/promoter system. To construct the expression vector, the AdoMet synthetase cDNA was manipulated by using the PCR technique, to confer suitable restriction sites at the desired positions, while keeping the correct reading frame.

Successful expression of a protein with an electrophoretic behaviour identical with that described for the native rat liver AdoMet synthetase [13,16] was achieved in the presence of IPTG. This protein accounted for approx. 30% of the total bacterial protein and was obtained mostly as an insoluble form. This is a common problem encountered when either cytosolic or membrane-bound proteins are expressed in *E. coli* [28], and references therein).

Assays *in vitro* of AdoMet synthetase activity in the cytosolic fraction demonstrated that the enzyme produced was fully active. Under saturating concentrations of the substrates, the specific activity measured was about 16 nmol/min per mg. This value is much higher than that described for AdoMet synthetase activity in rat liver cytosol, under the same conditions (70–100 pmol/min per mg) [29–31]. This difference is conceivable, as judged by the fact that the ratio AdoMet synthetase/total protein is higher in the transformed bacteria than in rat liver.

The functionality of the recombinant enzyme was also demonstrated *in vivo*, by the surprising finding that the intracellular AdoMet pool was dramatically increased in the transformed *E. coli* cells. It is noteworthy that in *E. coli* [32], *T. brucei* [10] and *Saccharomyces cerevisiae* [33] AdoMet synthetase is negatively regulated by its product, in order to maintain this metabolite at normal concentrations. Moreover, several studies carried out in rat and human liver have shown that marked alterations of AdoMet synthetase activity do not result in significant changes in the AdoMet levels [29–31,34]. Therefore it seems that cells try to maintain AdoMet concentration constant. From these data, it is difficult to explain the high accumulation of AdoMet observed in the transformed bacteria, although it might be due to an incapacity of the cell to use such an amount of the metabolite.

The existence of several AdoMet synthetase forms in rat liver has been evidenced. Based on kinetic studies of crude rat liver extracts and by hydrophobic chromatography, Liao et al. [35,36] and Sullivan and Hoffman [37] identified three different AdoMet synthetase isoenzymes. Later, Tsukada's group reported the existence of only two forms of the enzyme [13,38,39], a result that was subsequently confirmed upon purification to apparent homogeneity [16]. Based on their elution positions on gel-filtration chromatography, the molecular masses of the two isoenzymes were estimated as 210 kDa and 110 kDa [16]. An identical pattern was also found when human liver samples were directly fractionated on a Sephacryl S-300 column [29]. It was then postulated that liver AdoMet synthetase appears as two oligomeric forms, a tetramer and a dimer, composed of the same type of subunit [16,17]. The results obtained upon chromatography of the cytosol of *E. coli* cells transformed with pSSRL-T7N definitely confirm this hypothesis. The coding sequence for a single polypeptide chain originates two oligomeric forms of AdoMet synthetase that show molecular masses similar to those expected for a tetramer and a dimer. Since native *E. coli* enzyme appears only as a tetramer [6], the information required for rat liver AdoMet synthetase to assemble also as a dimer must be inherent to its primary structure.

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