Site-directed mutagenesis of rat liver S-adenosylmethionine synthetase

Identification of a cysteine residue critical for the oligomeric state

Jesús MINGORANCE, Luis ALVAREZ, Estrella SÁNCHEZ-GÓNGORA, José M. MATO and María A. PAJARES*

Instituto de Investigaciones Biomédicas, CSIC. Arturo Duperier 4, 28029 Madrid, Spain

We have examined the functional importance of the cysteine residues of rat liver S-adenosylmethionine synthetase. For this purpose the ten cysteine residues of the molecule were changed to serines by site-directed mutagenesis. Ten recombinant enzyme mutants were obtained by using a bacterial expression system. The same level of expression was obtained for the wild type and mutants, but the ratio of S-adenosylmethionine synthetase between soluble and insoluble fractions differed for some of the mutant forms. The immunoreactivity against an anti-(rat liver S-adenosylmethionine synthetase) antibody was equivalent in all the cases. Effects on S-adenosylmethionine synthetase activities were also measured. Mutants C57S, C69S, C105S and C121S showed decreased relative specific activity of 68, 85, 63 and 29 %, respectively, compared with wild-type, whereas C312S resulted in an increase of 1.6-fold. Separation of tetramer and dimer forms for wild type and mutants was carried out by using phenyl-Sepharose columns. The dimer/tetramer ratio was calculated based on the activity and on the protein level estimated by immunoblotting. No monomeric forms of the enzyme were detected in any case. Comparison of dimer/tetramer ratios indicates the importance of cysteine-69 (dimer/tetramer protein ratio of 88 versus 10.2 in the wild type) in maintaining the oligomeric state of rat liver S-adenosylmethionine synthetase. Moreover, all the mutations carried out of cysteine residues between cysteine-35 and cysteine-105 altered the ratio between oligomeric forms.

the enzyme when two of the ten sulphhydryl groups per subunit were modified [16]. In addition, modification of these two groups per subunit produced the dissociation of the tetramer to an inactive dimer [16]. Moreover, one of these groups seemed to be responsible for the main activity loss produced. This sulphhydryl group was identified by amino acid analysis of the isolated peptide as the one corresponding to cysteine-150 of the sequence [17].

INTRODUCTION

S-Adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6) is the enzyme that synthesizes AdoMet using ATP and methionine as substrates [1]. The importance of this reaction is based on the fact that AdoMet is the main methyl group donor for the transmethylation reactions [2]. The rat liver enzyme has been purified [3,4], cloned [5,6] and expressed in *Escherichia coli* [7]. Gel-filtration chromatography of the liver-purified and recombinant enzymes showed two peaks corresponding to a high- (210 kDa) and a low-molecular-mass (110 kDa) forms [7]. On SDS/PAGE both forms showed a unique band of a calculated molecular-mass of 48 kDa [3]. Based on all these data it has been concluded that the high-molecular-mass form is a tetramer and the low-molecular-mass form a dimer composed of the same type of subunit.

The tetramer/dimer ratio and the activity of liver AdoMet synthetase have been shown to be altered under several circumstances, such as alcohol liver cirrhosis [8], but the mechanisms that govern these changes are not known. Protein activity and structure can be modulated by the redox state of some of the cysteine sulphhydryl groups [4]. An important role in this regulatory mechanism has been proposed for glutathione, the main cellular thiol, one of its functions being the protection of protein sulphhydryl groups against oxidation [9]. In fact, enzymes such as glycogen phosphorylase phosphatase [10], glycogen synthetase [11], phosphofructokinase [12], 3-hydroxy-3-methylglutaryl-coenzyme A reductase [13] and rat liver AdoMet synthetase [4] have been shown to be regulated *in vitro* by the redox system provided by GSH/GSSG. Moreover, decreases in glutathione levels by buthionine sulfoximine [14] or carbon tetrachloride [15] led to a decrease in the AdoMet synthetase activity in rat liver.

Chemical modification with *N*-ethylmaleimide (NEM) of the isolated AdoMet synthetase forms led to a total inactivation of

All these data suggested that cysteine sulphhydryl groups are important in the modulation of the activity and oligomeric state of rat liver AdoMet synthetase. In order to fully establish their role in the regulation of the enzyme, site-directed mutagenesis of these residues has been carried out.

EXPERIMENTAL

Materials

Restriction and other DNA-modification enzymes were from AGS GmbH (Heidelberg, Germany) or from Boehringer Mannheim (Mannheim, Germany). The Sequenase DNA sequencing kit was purchased from U.S.B. (Cleveland, OH, U.S.A.). The thermostable DNA polymerase Dynazyme from *Thermus brockianus* was a product of Finnzymes Oy (Espoo, Finland). [³H]ATP was obtained from Amersham (Amersham, Bucks., U.K.). ATP, methionine, mineral oil, complete and incomplete Freund's adjuvants, lysozyme, 2-mercaptoethanol, aprotinin, pepstatin A, antipain, PMSF and benzamidine were from Sigma (St. Louis, MO, U.S.A.). Sephadex G-150 and phenyl-Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden). Chemiluminescence Renaissance reagents were obtained from DuPont New England Nuclear (Boston, MA, U.S.A.). Goat anti-rabbit IgG-horseradish peroxidase and the Bio-Rad kit I were purchased from Bio-Rad (Hercules, CA,

U.S.A.). Disposable polypropylene columns were from Pierce (Rockford, IL, U.S.A.). The rest of the buffers and reagents were the best quality commercially available.

DNA manipulations

Oligodeoxynucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. DNA manipulations followed standard procedures [18].

Site-directed mutagenesis

A 1.2 kb fragment containing the rat liver AdoMet synthetase coding region was amplified by PCR from plasmid pSSRL as described previously [7] and subcloned into the *Sma*I site of pBluescript M13(+)KS (Stratagene, La Jolla, CA, U.S.A.) by 'TA cloning' [19]. This plasmid, called pSSRL-BlueT2, was used as a template for mutagenesis experiments. Site-directed mutagenesis followed the two-step PCR method of Zhao and co-workers [20], using the pUC/M13 forward and reverse primers as external primers, and the following mutagenic oligonucleotides:

5'-ATGGTCAGACAAGCCAT-3' (C9S); 5'-CTGGTCAGAGATCTTAT-3' (C35S); 5'-ACTGTCTCAGAGGCCAC-3' (C57S); 5'-TGTCTTGGACACTGTCT-3' (C61S); 5'-ATCTCTCCAGACAGGAC-3' (C69S); 5'-CACATTGGAGGTCTTGA-3' (C105S); 5'-ATGGACAGATTGGGCAA-3' (C121S); 5'-GCGGCATGGACTCCTCT-3' (C150S); 5'-TCTCCGAGAGAGCCAG-3' (C312S); 5'-ACCATAGGATGCAGTCT-3' (C377S). The substituted bases are in italic and all the oligonucleotides are complementary to the coding strand. The cysteine numbering is the same used in the revised sequence included in the GenBank/EMBL under the accession number X60822.

The first PCR reactions contained 20 ng of plasmid pSSRL-BlueT2 as template, 200 ng each of the forward primer and the mutagenic oligonucleotide, 100 μ M dNTPs, 5 μ l of 10 $\times$ Dynazyme buffer and 0.5 unit of Dynazyme in a total volume of 50 μ l, and the samples were overlaid with 50 μ l of mineral oil. The reactions were carried out in a Gene ATAQ thermal cycler (Pharmacia) and consisted of a 5 min cycle at 94 $^{\circ}$ C, followed by 30 cycles at 94 $^{\circ}$ C for 2 min, 55 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 1 min, and ending with 10 min at 72 $^{\circ}$ C.

For the second PCR, 0.5–1 μ l of the first reaction was diluted in 50 μ l of a reaction mixture containing 5 ng of plasmid pSSRL-BlueT2, 100 μ M dNTPs, 5 μ l of 10 $\times$ Dynazyme buffer and 0.5 unit of Dynazyme. After elongation of the products of the first PCR with seven cycles at 94 $^{\circ}$ C for 1 min, 65 $^{\circ}$ C for 2 min and 72 $^{\circ}$ C for 1 min, 200 ng of each of the external primers, forward and reverse, were added and the reactions continued for another 30 cycles at 94 $^{\circ}$ C for 1 min, 55 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 1 min, ending with 10 min at 72 $^{\circ}$ C. The products of the second PCR were purified and digested with *Eco*RI and *Bam*HI and ligated to the *Eco*RI/*Bam*HI sites of pBluescript M13(+)KS. The products of the ligation reactions were used to transform *E. coli* DH5 α , and 4–6 clones were sequenced using suitable primers to screen for mutations; typically the efficiency of mutagenesis was around 75%. The selected mutants were sequenced to confirm that no additional mutations had been introduced, and then subcloned into the *Nde*I/*Sal*I sites of the expression vector pT7-7 as described previously [7].

Rabbit anti-(AdoMet synthetase) antiserum production

Rat liver AdoMet synthetase purified as described by Pajares and co-workers [4] was used to immunize New Zealand rabbits.

Figure 1 Characterization of rat liver AdoMet synthetase antiserum

Rat liver AdoMet synthetase antiserum was obtained as described in the Experimental section. In order to analyse the immune response, immunoblotting of *E. coli*/pT7-7 (lane 1), *E. coli*/pSSRL-T7N (lane 2) and rat liver (lane 3) cytosols were performed. (A) Results obtained using preimmune antiserum; and (B) results using the anti-(rat liver AdoMet synthetase) antiserum.

Preimmune serum was obtained before injection of the antigen. Enzyme (250 μ g) mixed with complete Freund's adjuvant was injected in the first immunization. One month later a serum sample was obtained to test the immune response. At that time, 125 μ g of enzyme mixed with incomplete Freund's adjuvant was injected, and after 15 days a serum sample was obtained. This last procedure was repeated twice. The serum obtained after the last three injections gave a specific response as tested by immunoblotting (Figure 1), and the best results were obtained by using a 1:10000 dilution of the antiserum.

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

Wild-type and mutant proteins were expressed in *E. coli* BL21(DE3) as described previously [7], except that cells were disrupted by incubation in lysis buffer [50 mM Tris/HCl (pH 8)]/0.5 M NaCl containing 0.1% 2-mercaptoethanol, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 0.5 μ g/ml leupeptin, 2.5 μ g/ml anti-pain, 0.1 mM benzamidine and 0.1 mM PMSF] with 1 mg/ml lysozyme for 30 min on ice, followed by three cycles of freeze–thawing in liquid nitrogen.

AdoMet synthetase activity measurements

AdoMet synthetase activity was determined as described previously [3], using saturating concentrations of each of the substrates, 5 mM methionine and 5 mM ATP.

Phenyl-Sepharose CL-4B chromatography

For hydrophobic chromatography, 2 ml samples of the cytosolic fractions obtained from 30 ml cultures were loaded on 5 ml

disposable polypropylene columns containing 1 ml bed volume of phenyl-Sepharose CL-4B equilibrated in buffer A [10 mM Hepes/Na (pH 7.5) containing 1 mM EDTA and 10 mM MgSO₄]. The sample was eluted with 25 ml of buffer A, followed by 25 ml of buffer A containing 50 % DMSO, and 5 ml fractions were collected. All the AdoMet synthetase activity appeared in the first flowthrough fraction and the first DMSO fraction.

Determination of the protein levels by Western-blot analysis

For immunoblot analysis of the soluble protein fractions 5 µg samples were used. However, for the analysis of the phenyl-Sepharose eluents 100 µl aliquots (of a total of 5 ml) of the first flowthrough fraction were lyophilized and dissolved in 20 µl of SDS/PAGE loading buffer, and 10 µl aliquots (of a total of 5 ml) of the first DMSO fraction were diluted with 10 µl of the 2 × loading buffer. These aliquots were loaded on SDS/10 % polyacrylamide gels and the electrophoresis developed as described by Laemmli [21]. Proteins were electrotransferred to nitrocellulose membranes using 20 mM Tris/HCl, pH 7.5, containing 20 % methanol and the immunoblotting developed using a rabbit anti-(AdoMet synthetase) antiserum and goat anti-rabbit IgG-horseradish peroxidase. Protein bands were revealed by chemiluminescence using DuPont New England Nuclear Renaissance reagents. The films were subjected to densitometric scanning and the band intensities quantified using the NIH Image v 1.52f software.

Gel-filtration chromatography

The cytosolic fractions (2 ml each) prepared as described above were loaded on a Sephadex G-150 column (96 cm × 1.5 cm) equilibrated in buffer A containing 50 mM KCl, 0.1 % 2-mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine. Samples were eluted using a flow rate of 12 ml/h and 2 ml fractions were collected.

Determination of protein concentrations

Protein concentration was measured by the method of Bradford using the Bio-Rad kit I [22].

RESULTS AND DISCUSSION

Much effort has been directed towards the identification of amino acid residues essential for enzymic activities. Among these, cysteines seem to be required for the biological activity of several proteins as deduced from the results obtained by chemical modification experiments [23–25]. This type of result has been also reported for rat liver and *E. coli* AdoMet synthetases [17,26], where the NEM modification of two cysteine residues per subunit led to a total inactivation of the protein.

In order to examine in more detail the role of cysteines in rat liver AdoMet synthetase, ten enzyme mutants have been produced. For this purpose the ten cysteine residues present in its sequence have been substituted individually by serines, using site-directed mutagenesis. Replacement by serine was decided as being the most appropriate, since this amino acid is considered to be isosterical to cysteine and is found to replace cysteine in corresponding proteins of related organisms with no change in hydrophobicity [27]. Wild-type and mutant enzymes were expressed in *E. coli* in order to analyse the effect of these changes on the level of expression, activity and oligomeric state of AdoMet

Figure 2 Soluble wild-type and mutant AdoMet synthetase levels

The cytosolic fractions for each mutant and wild-type (WT) AdoMet synthetase were obtained. Protein (35 µg) was loaded on SDS/polyacrylamide gels, and the electrophoresis was performed as described in the Experimental section. The upper panel shows the Coomassie Brilliant Blue staining of the gel, and the lower panel presents the densitometric scanning of the band corresponding to AdoMet synthetase. The results shown in the Figure were obtained from a typical independent experiment out of three.

synthetase. SDS/PAGE of the homogenates showed in all the cases a new protein band of 48 kDa. In all the cases the expression level was similar. However, when soluble and insoluble fractions were separated it was possible to observe that most of the protein was present in the insoluble pellet (results not shown). The soluble fraction showed this 48 kDa band in all the cases (Figure 2, upper panel), but its level differed from one mutant to another. Densitometric scanning of the gel revealed a decrease in the amount of soluble protein produced in several cases. This decrease was especially important in two mutants, C57S and C312S, located relatively close to the N- and C-termini of the protein respectively. In fact, the amount of soluble protein of C57S varied from one batch to another. The amounts of corresponding recombinant proteins present in the soluble fraction were in these cases only 5–10 % of that detected in the wild type (Figure 2, lower panel). A decrease in the amount of soluble enzyme produced by mutation of certain residues can be due to

Figure 3 Immunoblotting of the wild-type and mutant AdoMet synthetases

A sample of the soluble fractions of *E. coli* transformed with wild-type (WT) and mutant AdoMet synthetases was loaded on SDS/polyacrylamide gels, and the immunoblotting was performed as described in the Experimental section. The upper panel shows the immunoreactive band and the lower panel the densitometric scanning of these results. The results shown in the Figure were obtained from a typical independent experiment out of three.

effects on folding or stability of these proteins, but the contribution to these processes of cysteine residues not involved in disulphide bonds is not clear [28–31].

To determine if wild-type and mutant AdoMet synthetases had a similar antigenic character, all the samples were subjected to immunoblotting using the polyclonal antibody raised against the purified rat liver enzyme. This antiserum was able to recognize all the species, wild-type and mutant forms, but failed to react with *E. coli* AdoMet synthetase (Figure 1). The migration pattern of the immunoreactive bands was similar to that of purified rat liver AdoMet synthetase (Figure 1B). When densitometric scans from the Coomassie-Brilliant-Blue-stained gels (Figure 2) and immunoblots (Figure 3) were compared, it was observed that the antibody recognized all the recombinant AdoMet synthetase mutants to the same extent. Therefore none of the mutations appeared to alter dramatically the tertiary structure that the protein epitopes show on immunoblotting [32]. The intensity of the C57S band varied between samples of different batches, since the amount of soluble protein changed, but a correlation remained between the Coomassie Brilliant Blue staining and the intensity of the band on immunoblotting.

The effect that mutation of cysteine residues could produce on the AdoMet synthetase activity was tested using soluble fractions, and the specific activities obtained are shown in Table 1. However, since the level of mutant enzymes present in this fraction varied from one mutant to another, the activity was corrected by the densitometric scanning of the corresponding AdoMet synthetase band on Coomassie-Brilliant-Blue-stained gels (Table 1). Changes of cysteine-57, -69 and -105 decreased the enzyme's relative specific activity. This decrease was especially important for C69S, where only 15 % of the wild-type activity was detected.

Table 1 Specific activity of wild-type and mutant AdoMet synthetases

Samples of cytosolic fractions of *E. coli* transformed with AdoMet synthetase cDNA constructs corresponding to wild-type and mutant forms were used to measure the enzyme activity as described in the Experimental section. These cytosolic fractions (35 µg each) were loaded on SDS/polyacrylamide gels, and the amount of AdoMet synthetase present was determined by densitometric scanning of the Coomassie-Brilliant-Blue-stained gel. The specific activity was calculated as pmol/min per mg of protein, and a correction was also made for the amount of AdoMet synthetase detected and evaluated by densitometry. These corrected values are expressed as pmol/min per unit of densitometry. The data shown in the table represent the means $\pm$ S.E.M. of three independent experiments performed in triplicate.

Enzyme	Specific activity	
	(pmol/min per mg of protein)	(pmol/min per unit)
Wild type	13937 $\pm$ 1495	2.63 $\pm$ 0.09
C9S	13422 $\pm$ 720	2.38 $\pm$ 0.09
C35S	7896 $\pm$ 613	2.09 $\pm$ 0.09
C57S	771 $\pm$ 300	0.85 $\pm$ 0.09
C61S	10139 $\pm$ 748	2.09 $\pm$ 0.08
C69S	2871 $\pm$ 645	0.39 $\pm$ 0.09
C105S	4827 $\pm$ 981	0.96 $\pm$ 0.12
C121S	9530 $\pm$ 686	1.87 $\pm$ 0.09
C150S	9050 $\pm$ 400	2.91 $\pm$ 0.09
C312S	4281 $\pm$ 336	4.22 $\pm$ 0.11
C377S	8948 $\pm$ 668	2.07 $\pm$ 0.09

On the other hand, substitution of cysteine-312 increased the relative specific activity 1.6-fold. Replacement of key residues for protein function by other amino acids is expected to produce changes in the activity and/or affinity of these mutants [28,33,34]. In our case several mutants showed decreased relative specific activity, but none of these changes completely abolished the activity, as would be expected for a residue directly implicated in catalysis. The most surprising result concerned C150S, the residue implicated in the inactivation of rat liver AdoMet synthetase by NEM modification [17]. This mutant showed no change in the relative specific activity compared with the wild-type enzyme.

Table 2 Dimer/tetramer activity ratio in wild-type and mutant AdoMet synthetases

Dimer and tetramer forms of wild-type and mutant AdoMet synthetases were separated by phenyl-Sepharose chromatography as described in the Experimental section. Fractions corresponding to each enzyme form were assayed to determine the dimer/tetramer activity ratio. The data shown in the table represent the means $\pm$ S.E.M. of three independent experiments performed in triplicate.

Enzyme	Activity ratio
Wild type	2.80 $\pm$ 0.44
C9S	3.97 $\pm$ 0.49
C35S	4.11 $\pm$ 0.45
C57S	0.99 $\pm$ 0.43
C61S	2.99 $\pm$ 0.38
C69S	4.08 $\pm$ 0.84
C105S	4.86 $\pm$ 2.43
C121S	10.09 $\pm$ 2.57
C150S	3.27 $\pm$ 1.32
C312S	1.49 $\pm$ 0.27
C377S	2.94 $\pm$ 0.67

This type of result has been obtained in several proteins when the data derived from chemical modifications were re-examined by site-directed mutagenesis, among them *Drosophila* alcohol dehydrogenase [35] and *E. coli* AdoMet synthetase [36]. The steric constraints due to NEM modification [17] were not expected to be the same as those due to amino acid substitution. However, we cannot rule out the location of a non-essential thiol near the binding site in those cases where protection by the substrates against chemical modification has been shown [17,26,36,37].

It is well known that rat liver AdoMet synthetase and the recombinant wild-type enzyme in *E. coli* appear as tetramers and dimers [3,7]. Previous studies by NEM modification [17] and GSSG incubation [4] have indicated a possible role for cysteine residues in maintaining the oligomeric state. To study this possibility, the amount of each oligomeric form was studied for each mutant using phenyl-Sepharose CL-4B, which is a procedure that gives better resolution of tetramers and dimers [38–40]. The high-molecular-mass form does not bind to this type of gel, whereas the dimer binds tightly and can only be eluted in the presence of 50% DMSO. The soluble fraction of wild-type and mutant AdoMet synthetases was loaded on phenyl-Sepharose columns, and both oligomeric forms were separated. Once isolated, the activity of the tetramer and dimer forms was measured, and the amount of each protein was evaluated by immunoblotting. Several alterations in the ratio of dimer/tetramer activities were observed (Table 2). C57S and C312S showed a lower ratio, with approximately equal activity for both oligomeric forms. On the other hand, C121S showed a much higher ratio, where dimer activity was about 10-fold higher than that of the tetramer. Other mutants, C9S, C35S, C69S and C105S, also had an altered dimer/tetramer ratio showing higher dimer activity (about 4-fold). These data must be interpreted bearing in mind several parameters: first, elution of the dimer in the presence of DMSO leads to a slight activation of this form, even when the assay is carried out under saturating concentrations of methionine [3,39]. Secondly, *E. coli* presents its own AdoMet synthetase, which appears as a tetramer [41] and which on phenyl-Sepharose chromatography behaves like the recombinant tetramer. Therefore, it is conceivable that the total and tetramer activities measured would be a mixture of both AdoMet synthetases. Thirdly, the exact contribution of the bacterial tetramer cannot be evaluated without the separation of both. However, it is not expected to be very important since *E. coli* AdoMet synthetase expression and activity is regulated by AdoMet, which acts as a co-repressor [42] and an inhibitor

Figure 4 Dimer/tetramer ratio evaluated by immunoblotting

Dimer (D) and tetramer (T) forms of wild-type (WT) and mutant AdoMet synthetases were separated by phenyl-Sepharose chromatography as described in the Experimental section. Samples of tetramer (protein corresponding to 100 μ l) and dimer (protein present in 10 μ l) fractions were loaded on SDS/polyacrylamide gels, and the immunoblotting was performed. The upper panel shows bands obtained on immunoblotting and the lower panel the densitometric scans of the dimer/tetramer ratio. The results shown in the Figure were obtained from a typical independent experiment out of three.

respectively, even at lower concentrations than the ones detected in the wild-type transformed bacteria (millimolar range) [7].

When the ratio between AdoMet synthetase forms was studied

Figure 5 Scheme of the localization of the cysteine residues present in several AdoMet synthetase sequences

All the sequences were obtained from Genbank and aligned using programs from the GCG package.

by immunoblotting, several changes were also observed compared with the wild type (Figure 4). Higher dimer/tetramer ratios were detected in C69S, C312S and C377S, especially C69S, which showed most of the protein as a dimer. On the other hand, C35S, C57S, C61S and C105S showed a lower ratio, approximately twice dimer to tetramer. The possible presence of the mutant enzymes as monomer forms was also studied using Sephadex G-150 columns. None of the enzymes showed activity or the presence of AdoMet synthetase immunoreactive bands as judged by immunoblotting of the fractions eluting in the region expected for monomer recovery (results not shown). All these data indicate changes in the dimer/tetramer ratio in several mutant enzymes, the most important ones being those related to the area between cysteine residues 35 and 105. The most dramatic of these is C69S, whose mutated residue is located in the most hydrophobic area of the AdoMet synthetase molecule as observed in a hydrophobicity profile [43], as would be expected for a residue implicated in subunit interactions.

Changes in the oligomeric state upon mutation of key cysteine residues have been reported previously [44,45]. In fact, mutation of cysteine-90 of *E. coli* AdoMet synthetase (C90S and C90A) led to the appearance of dimers [36]. These results would support the idea that the corresponding rat liver AdoMet synthetase residue (cysteine-105) would play the same role in the subunit interaction; however, the most dramatic change is obtained by replacement of another residue, in C69S.

Finally, the replaced cysteine residues present in all the known sequences of AdoMet synthetases, C57S and C105S (Figure 5), are not the ones producing the most important changes either in activity or oligomeric state. This role is reserved for that in C69S, a residue typical of rat, mouse and human liver enzymes (Figure 5). It is also noteworthy that rat and human liver are the tissues that present AdoMet synthetase in two oligomeric forms, dimer and tetramer [3,8]. Other cysteine residues present only in hepatic AdoMet synthetases (cysteine-9 and cysteine-377) do not produce such changes. Therefore it is possible to deduce that cysteine-69 is the residue implicated in the distribution of oligomeric forms observed in liver AdoMet synthetase. Moreover, all the mutations carried out of the cysteine residues between cysteine-35 and cysteine-105 alter the ratio between oligomeric forms. Altogether, these results suggest the involvement of this area in binding between subunits. Kinetic and crystallographic studies of the purified mutant and wild-type enzymes may yield further insight into interactions involved in protein oligomerization.

J. M. and E. S.-G. are fellows of the Ministerio de Educación y Ciencia and Comunidad de Madrid respectively. This work was supported by grants from the Fondo de Investigaciones Sanitarias (94/0231), Boehringer Ingelheim España and the Science Programme of the European Community (SCI*-CT92-0780).

REFERENCES

- Cantoni, G. L. (1965) in *Transmethylation and Methionine Biosynthesis* (Shapiro, S. K. and Schlenk, R., eds.), pp. 21–32, University of Chicago Press, Chicago.
- Cantoni, G. L. (1975) *Annu. Rev. Biochem.* **44**, 435–451.
- Cabrero, C., Puerta, J. L. and Alemany, S. (1987) *Eur. J. Biochem.* **170**, 299–304.
- Pajares, M. A., Durán, C., Corrales, F., Pliego, M. M. and Mato, J. M. (1992) *J. Biol. Chem.* **267**, 17598–17605.
- Horikawa, S., Ishikawa, M., Ozasa, H. and Tsukada, K. (1989) *Eur. J. Biochem.* **184**, 497–501.
- Alvarez, L., Asunción, M., Corrales, F., Pajares, M. A. and Mato, J. M. (1991) *FEBS Lett.* **290**, 142–146.
- Alvarez, L., Mingorance, J., Pajares, M. A. and Mato, J. M. (1994) *Biochem. J.* **301**, 557–561.
- Martín-Duce, A., Ortíz, P., Cabrero, C. and Mato, J. M. (1988) *Hepatology (Baltimore)* **8**, 65–68.
- Meister, A. and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760.
- Shimazu, T., Tokutake, S. and Usami, M. (1978) *J. Biol. Chem.* **253**, 7376–7382.
- Ernest, M. J. and Kim, K.-H. (1974) *J. Biol. Chem.* **249**, 5011–5018.
- Gilbert, H. F. (1982) *J. Biol. Chem.* **257**, 12086–12091.
- Dotan, I. and Schechter, I. (1982) *Biochim. Biophys. Acta* **713**, 427–434.
- Corrales, F., Ochoa, P., Rivas, C., Martín-Lomas, M., Mato, J. M. and Pajares, M. A. (1991) *Hepatology (Baltimore)* **14**, 528–533.
- Corrales, F., Giménez, A., Alvarez, L., Caballería, J., Pajares, M. A., Andreu, H., Parés, A., Mato, J. M. and Rodés, J. (1992) *Hepatology (Baltimore)* **16**, 1022–1027.
- Corrales, F., Cabrero, C., Pajares, M. A., Ortíz, P., Martín-Duce, A. and Mato, J. M. (1990) *Hepatology (Baltimore)* **11**, 216–222.
- Pajares, M. A., Corrales, F., Ochoa, P. and Mato, J. M. (1991) *Biochem. J.* **274**, 225–229.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York.
- Zhao, L.-J., Zhang, Q. X. and Padmanabhan, R. (1993) *Methods Enzymol.* **217**, 218–227.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Cappel, R. E. and Gilbert, H. F. (1988) *J. Biol. Chem.* **263**, 12204–12212.
- Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E. and Curran, T. (1992) *EMBO J.* **11**, 3323–3335.
- Toledano, M. B. and Leonard, W. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4328–4332.
- Markham, G. D. and Satishchandran, C. (1988) *J. Biol. Chem.* **263**, 8666–8670.
- Schultz, G. E. and Shirmer, R. H. (1979) in *Protein Engineering* (Cantor, C. R., ed.), pp. 14–15, Springer Verlag, New York.
- Miller-Martini, D. M., Chirgwin, J. M. and Horowitz, P. M. (1994) *J. Biol. Chem.* **269**, 3423–3428.
- Jambou, R. C., Snouwaert, G., Abendroth Bishop, G., Stebbins, J. R., Freling, J. A. and Fowlkes, D. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9426–9430.
- Taniyama, Y., Yamamoto, Y., Kuroki, R. and Kikuchi, M. (1990) *J. Biol. Chem.* **265**, 7570–7575.
- Liu, J., Albers, M. W., Chen, C.-M., Schreiber, S. L. and Walsh, C. T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2304–2308.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor.
- Fertala, A., Westerhausen, A., Morris, G., Rooney, J. E. and Prockop, D. J. (1993) *Biochem. J.* **289**, 195–199.
- Dixon, R. A. F., Sigal, I. S., Candelore, M. R., Register, R. B., Scattergood, W., Rands, E. and Strader, C. D. (1987) *EMBO J.* **6**, 3269–3275.
- Chen, Z., Lu, L., Shirley, M., Lee, W. R. and Chang, S. (1990) *Biochemistry* **29**, 1112–1118.
- Reczkowski, R. S. and Markham, G. D. (1995) *J. Biol. Chem.* **270**, 18484–18490.
- Zapata, G., Roller, P. P., Crowley, J. and Vann, W. F. (1993) *Biochem. J.* **295**, 485–491.
- Hoffman, J. L. and Sullivan, D. M. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T. and Creveling, C. R., eds.), pp. 683–686, Macmillan Press, London.
- Hoffman, J. L. and Kunz, G. L. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1231–1236.
- Sullivan, D. M., and Hoffman, J. L. (1983) *Biochemistry* **22**, 1636–1641.
- Markham, G. D., Hafner, E. W., White Tabor, C. and Tabor, H. (1983) *Methods Enzymol.* **94**, 219–222.
- Markham, G. D., De Parasis, J. and Gatmaitan, J. (1984) *J. Biol. Chem.* **259**, 14505–14507.
- Pajares, M. A., Corrales, F., Durán, C., Mato, J. M. and Alvarez, L. (1992) *FEBS Lett.* **309**, 1–4.
- Östman, A., Andersson, M., Bäckström, G. and Heldin, C. H. (1993) *J. Biol. Chem.* **268**, 13372–13377.
- Andersson, M., Östman, A., Bäckström, G., Hellman, U., George-Nacimento, C., Westermark, B. and Heldin, C. H. (1992) *J. Biol. Chem.* **267**, 11260–11266.