Study of the rat liver *S*-adenosylmethionine synthetase active site with 8-azido ATP

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The active site of rat liver *S*-adenosylmethionine synthetase was studied using 8-azido ATP, a photolabile analogue of ATP. Both forms of the enzyme, tetramer and dimer, could be labelled by using concentrations of the analogue similar to the K_m^{ATP} values for each form, 350 μ M and 1 mM respectively. Labelling of both *S*-adenosylmethionine synthetase forms with 8-azido [α - 32 P]ATP, followed by tryptic digestion and purification by HPLC, afforded

one specifically labelled peptide in each case. Identification of the labelled peptide by amino acid analysis and peptide sequencing, and comparison with the enzyme sequence, indicated that the same peptide (267–286) was modified in both enzyme forms. The results are discussed on the basis of the high degree of similarity that this peptide shows in all the known *S*-adenosylmethionine synthetase sequences.

INTRODUCTION

S-Adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6) uses methionine and ATP to synthesize AdoMet [1], the main methyl donor for the transmethylation reactions [2]. The rat liver enzyme was purified and found to be eluted on gel-filtration chromatography in two forms, a high- and a low- M_r AdoMet synthetase, with molecular masses of 210000 and 110000 Da respectively. Both forms appeared on SDS/PAGE as bands of approx. 48 kDa [3]. This calculated M_r varied upon purification of both enzyme forms by using thiopropyl-Sepharose chromatography, 43 and 48 kDa for the high- and low- M_r forms respectively [4]. Both AdoMet synthetase forms showed identical peptide maps on HPLC, and were recognized by antibodies raised against the low- M_r AdoMet synthetase. These data made Cabrero et al. [3] suggest that the high- M_r form of the enzyme is a tetramer and the low- M_r form is a dimer composed of the same type of subunit.

The tetramer is about 15-fold more active than the dimer at physiological concentrations of methionine, and shows lower values for K_m^{Met} and K_m^{ATP} [3,4]. The tetramer can be converted into the dimer, the less active form, by incubation in the presence of LiBr [5] and by modification with *N*-ethylmaleimide [6]. In addition, both tetramer and dimer can be converted into inactive monomers upon oxidation in the presence of GSSG [4]. Finally, we have observed that the dimer can be dissociated to an active monomer by phosphorylation with protein kinase C [7]. All these changes in the oligomeric state of the protein lead to the formation of different species that vary in their kinetic parameters, possibly due to alterations affecting the active site. It is therefore important to establish which residues contribute to this active site, and how they are affected by changes in the oligomeric state of the enzyme. All these data may help us to understand the variations in affinity for the substrates in the different aggregation states of AdoMet synthetase.

The only data about the active site available at the present come from the analysis of the enzyme sequence [8,9]. These studies have revealed the presence of the motif that was identified by Wierenga et al. [10] as the putative ATP-binding site for all the ATP-using enzymes studied until now. This possible ATP-

binding site consists of the sequence Gly-Ala-Gly-Asp-Gln-Gly, which lies 16–28 residues from the N-terminal of a Lys residue. For AdoMet synthetase this Gly motif is located at residues 132–137, and the Lys is number 160 of the enzyme sequence [8]. In an effort to gain more insight into the AdoMet synthetase active site, we have used a photoactivable analogue of the substrate, 8-azido ATP (8- N_3 ATP). The results shown here lead to the identification of another peptide that participates in the binding site of the ATP adenine ring in AdoMet synthetase.

EXPERIMENTAL

Materials

Male Wistar rats (150 g) were from our inbred colony. Hepes, dithiothreitol, phenylmethanesulphonyl fluoride, soybean trypsin inhibitor, benzamidine, ATP, 8- N_3 ATP, methionine, trypsin, GSH and EDTA were products of Sigma Chemical Co. 2-Mercaptoethanol, DMSO and trifluoroacetic acid (TFA) were purchased from Merck. DEAE-Sepharcel, phenyl-Sepharose CL-4B, Blue Sepharose CL-6B, Sephadex G-10, Sephacryl S-300, thiopropyl-Sepharose 6B and OptiPhase HiSafe 3 scintillation fluid were products of Pharmacia LKB Biotechnology. Bio-Gel HTP, cation-exchanger AG-50W-X4 and a protein assay kit were purchased from Bio-Rad. 8- N_3 [α - 32 P]ATP and [2- 3 H]ATP were products of ICN Radiochemicals and Amersham Corp., respectively. The HPLC reverse-phase C_{18} Ultrasphere and the cation-exchange Ultrasil CX columns were purchased from Beckman. Ultrafree MC 10000 NMWL filters were obtained from Millipore. All the solvents for HPLC were from Riedel de Hen. Other buffers and reagents were of the best quality commercially available.

Methods

AdoMet synthetase purification

High- and low- M_r AdoMet synthetases were purified as described by Pajares et al. [4]. Briefly, the rat liver cytosol was subjected to DEAE-Sepharcel chromatography, followed by phenyl-Sepharose

CL-4B column chromatography, where both AdoMet synthetase forms were separated. The isolation procedure for the high- M_r form continued by purification on Bio-Gel HTP, Blue Sepharose CL-6B, Sephacryl S-300 and thiopropyl-Sepharose 6B columns. Similarly, the low- M_r form was obtained by using the following chromatography steps: Blue Sepharose CL-6B, Sephacryl S-300, and finally thiopropyl-Sepharose 6B. Elution from this last chromatography step was performed in the presence of 50 mM GSH. Since reducing agents are known as scavengers of the active species generated by photolysis of the azido derivatives, it was necessary to lower its concentration in the protein samples. Therefore all the modifications of the protein with 8- N_3 ATP were made after dialysis against 10 mM Hepes/NaOH, pH 7.5, containing 50 μ M GSH, or after filtration using Ultrafree MC Eppendorfs.

Kinetics of 8- N_3 ATP as a substrate and an inhibitor for AdoMet synthetases.

The ability of 8- N_3 ATP to serve as substrate was tested with purified AdoMet synthetase forms. Kinetics were performed as described by Pajares et al. [4], except that ATP was replaced by 8- N_3 ATP, and therefore the reaction was carried out in the dark.

Inhibition by 8- N_3 ATP was studied in a reaction mixture containing 5 mM methionine and 5 mM ATP in the presence of concentrations of 8- N_3 ATP ranging from 0.5 to 3 mM.

The kinetic parameters were calculated by using Eadie-Hofstee and Lineweaver-Burk transformations.

Modification with 8- N_3 ATP of the high- and low- M_r AdoMet synthetases

The reaction was carried out in the dark in 10 mM Hepes/NaOH (pH 7.5)/10 mM $MgSO_4$ /1 mM EDTA and flushed with nitrogen; either 100 μ g of low- M_r AdoMet synthetase or 50 μ g of high- M_r AdoMet synthetase was used. Different concentrations of 8- N_3 ATP were added to the enzyme (final concentrations 0.5–3 mM) in the dark. The mixtures (1 ml) were preincubated for 5–20 min on ice under mild shaking, and then transferred to 1 ml quartz cuvettes. Samples were placed on ice, and irradiation was carried out in a UV-Stratalinker 2400 instrument (Stratagene; 254 nm, 120000 J/cm²) for 0.5–12 min. The UV source was located 10 cm above the samples. Controls were treated identically, except that 8- N_3 ATP was omitted from the reaction mixture. Immediately after photolysis, enzyme activity was assayed as described previously [3,4].

Photoaffinity labelling with 8- N_3 [α -³²P]ATP

Four sample groups of the high- M_r (0.3 mg) and low- M_r (0.6 mg) AdoMet synthetases were flushed with nitrogen before use. Groups 1 and 2 received 1 mM 8- N_3 [α -³²P]ATP (30 Ci/mol), and to group 2 1 mM ATP was also added. Two more control groups were run in parallel: group 3 received the same concentration of 8- N_3 ATP, but no addition was made to group 4. All additions were made in the dark to give a final reaction volume of 0.8 ml. Preincubation was carried out on ice for 20 min, also in the dark, and photolysis of the samples was performed in the UV-Stratalinker 2400 apparatus at 254 nm for 5 min as described above. All the samples were subjected to filtration with Ultrafree MC units to a final volume of 100 μ l, in order to eliminate excess of the reagents. Controls (groups 3 and 4) were used for testing the remaining AdoMet synthetase activity.

The ³²P-labelled samples were used for calculating stoichiometry and identification of the modified positions in the active site. For that purpose, the concentrates were precipitated with 4

vol. of acetone (−20 °C). Mixtures were kept at −20 °C for 1 h and then centrifuged at 1500 *g* for 10 min. The supernatants were discarded, and the pellets dried under nitrogen and prepared for SDS/PAGE as described by Laemmli [11]. Gels were dried and subjected to autoradiography. The bands were then excised and the proteins eluted by the method of Boyle et al. [12] for trypsin treatment. The rest of the lanes containing purified AdoMet synthetase forms were stained with Coomassie Blue.

Incorporation of ³²P label was evaluated in the acetone precipitates and by counting the gel bands for radioactivity.

Trypsin treatment of the labelled AdoMet synthetases

Samples in 1 ml of 50 mM NaHCO₃, pH 8.2, were proteolysed with trypsin. Two additions of the protease were carried out: first 1:20 (w/w) of trypsin (1 mg/ml) for 1 h at 37 °C, and the reaction continued overnight after a second addition of trypsin (1:50, w/w). The reaction was stopped by freezing, and the proteolytic digest was freeze-dried.

Isolation of the azido-labelled peptides

For the purification of the azido-labelled peptides the procedure described by King et al. [13], involving cation-exchange chromatography, Sephadex G-10 and reverse-phase columns, was followed. Briefly, the freeze-dried peptides were resuspended in 100 μ l of 10 mM ammonium formate, pH 3.5, and loaded on an Ultrasil CX column (250 mm × 4.6 mm; 5 μ m particle diam.). Elution was carried out with a 50 min gradient to 0.4 M ammonium formate, pH 3.5, at a flow rate of 1 ml/min. The exclusion volume contained all the radioactivity and was collected for further purification. Next the samples were loaded on a Sephadex G-10 column (30 cm × 1 cm) equilibrated in NH₄HCO₃, pH 8.2, and eluted with the same buffer. Again the radioactive peptides were eluted in the exclusion volume, which was collected and freeze-dried. Finally, the samples were dissolved in 100 μ l of 0.1 % TFA in water and loaded on a HPLC reverse-phase C₁₈ column (250 mm × 4 mm; 10 μ m particle diam.) equilibrated in 0.1 % TFA in water. The chromatography was carried out at a flow rate of 1 ml/min, as described in [14], with a three-step gradient involving 0.1 % TFA in water (solvent A) and 0.1 % TFA in 50 % acetonitrile (solvent B). The absorbance at 214 nm was monitored, and 1 ml fractions were collected and subjected to Čerenkov counting for detection of the ³²P labelling. Those containing the labelled peptides were frozen and freeze-dried.

Amino acid analysis and sequencing of the ³²P-labelled peptides

The 8- N_3 ATP-modified peptides were hydrolysed with 6 M HCl at 110 °C for 2 h, and amino acid analysis of the samples was carried out on a Beckman System 6300 Autoanalyzer. Calculations of the amino acid content were made by considering the amount of lysine as being due to the presence of one residue, and referring the rest of the values to that.

The 8- N_3 ATP-modified peptides were also submitted to automated Edman degradation and phenylthiohydantoin-derivative analysis in an Applied Biosystems 477A unit.

Determination of protein concentration

The protein content of the samples was evaluated by the method of Bradford [15], with BSA as a standard and the Bio-Rad protein assay kit.

RESULTS

High- and low- M_r AdoMet synthetases from rat liver, purified as described by Pajares et al. [4], were incubated in the presence of 8- N_3 ATP in order to determine its ability to serve as a substrate of the enzyme. Kinetics were performed by using reaction mixtures containing this analogue, and the AdoMet synthetase activity was measured in the dark. K_m values of 900 and 372 μ M were obtained for the low- and high- M_r forms respectively. These results are similar to the values observed with the normal substrate, ATP (Table 1). On the other hand, the calculated V_{max} values were 186.3 and 270.8 pmol/min per mg for the dimer and tetramer respectively. These values are about 50- and 15-fold lower than those observed with ATP in the preparation used (Table 1).

We have also tested the ability of 8- N_3 ATP to serve as an inhibitor of the enzyme activity. For that purpose, AdoMet synthetase activity was measured as described in the Experimental section in the presence of increasing amounts of the analogue. The synthesis of AdoMet was decreased under these conditions, and the K_i values calculated for this competitive inhibitor were not significantly different from the K_m obtained.

These data indicate that 8- N_3 ATP binds to AdoMet synthetase, high- and low- M_r forms, in a manner very similar to ATP. Since this analogue can be efficiently cross-linked to proteins by UV light, it can be used as an active-site modifying agent, providing information about this area of the enzyme. This type of photoaffinity reagent is susceptible to the presence of reducing agents in the reaction mixtures. Therefore, since AdoMet synthetase forms are isolated with 50 mM GSH, it was necessary to lower its concentration by dialysis or filtration before any photolabelling reaction was carried out.

In order to find the appropriate conditions for this modification, both AdoMet synthetase forms were preincubated with 8- N_3 ATP for different periods of time in the dark, and several UV-irradiation times were used. Preincubation times ranging from 5 to 20 min at different temperatures were studied, and the best results were obtained with 20 min at 4 °C. Therefore, the other parameters were tested under these conditions.

Concentrations of 8- N_3 ATP between 0.5 and 3 mM were used for modification, followed by 5 min irradiation at 254 nm. The effectiveness of the reaction was measured by testing the remaining AdoMet synthetase activity. A decrease in the enzyme activity was observed that appeared to reach its maximum at concentrations close to the calculated K_m^{ATP} values, 1 mM for the dimer and 350 μ M for the tetramer. 8- N_3 ATP concentrations higher than 1.5 mM did not result in significant enhancement of

Figure 1 Effect of different concentrations of 8- N_3 ATP on low- M_r AdoMet synthetase activity

Low- M_r AdoMet synthetase was preincubated with 8- N_3 ATP (0.5–3 mM) for 20 min at 4 °C in the dark and subsequently irradiated with UV light (254 nm, 5 min); the enzymic activity was then assayed as described in the Experimental section. The Figure shows the results of a typical experiment done in triplicate, as means $\pm$ S.D.

the inhibition obtained after photolysis of the low- M_r AdoMet synthetase (Figure 1). Similar results were observed with the high- M_r form of the enzyme. Therefore, concentrations of 1 mM and 350 μ M 8- N_3 ATP were chosen to perform our studies.

Different UV-irradiation conditions ranging from 0.5 to 12 min were also tested. A decrease in the AdoMet synthetase activity of both enzyme forms that correlates with an increase from 0.5 to 4 min irradiation was observed in the presence of 8- N_3 ATP at the concentrations mentioned above. The maximal decrease obtained was 22 % relative to the control after 5 min irradiation of the dimer (Figure 2). This effect could be completely prevented when the analogue was UV-irradiated for 5 min before incubation with either of the enzyme forms. Extensive irradiation, longer than 7 min, also diminished the AdoMet synthetase activity in the absence of the photolabile analogue. Similar results were again obtained with the tetramer form of the enzyme.

AdoMet synthetase inactivation by 8- N_3 ATP could be prevented in the presence of the normal substrate, ATP. Three groups of samples were prepared for each enzyme form. Group 1, the controls, was kept at 4 °C, while the other groups were treated. Group 2 of each enzyme form received 40 mM ATP, and then both groups 2 and 3 were irradiated for 5 min at 254 nm in the presence of 1 mM or 350 μ M 8- N_3 ATP, for the dimer and tetramer samples respectively. All the groups were dialysed and assayed for AdoMet synthetase activity. In the presence of ATP, the inactivation of the enzyme activity provided by 8- N_3 ATP photolysis was prevented (results not shown).

The identification of the modified position in the active site and calculations of stoichiometry were carried out with 8- N_3 [α - 32 P]ATP. For this purpose, four types of samples of each AdoMet synthetase form were prepared. Group 1 received 1 mM 8- N_3 [α - 32 P]ATP (30 Ci/mol); group 2, 1 mM 8- N_3 [α - 32 P]ATP (30 Ci/mol) plus 1 mM ATP; group 3, 1 mM 8- N_3 ATP; and group 4, no addition. All the samples were preincubated at 4 °C for 20 min and then irradiated for 5 min at 254 nm. Excess of the reagents was eliminated by Ultrafree MC filtration. Groups 3 and 4 were used to measure AdoMet synthetase activity, and

Table 1 Kinetic parameters of high- and low- M_r AdoMet synthetases in the presence of either 8- N_3 ATP or ATP

Purified AdoMet synthetase forms were incubated with reaction mixtures containing different concentrations of either 8- N_3 ATP or ATP, and the enzyme activity was measured as described in the Experimental section.

	Low- M_r AdoMet synthetase	High- M_r AdoMet synthetase
ATP		
K_m	950 μ M	251 μ M
V_{max}	9.75 nmol/min per mg	3.9 nmol/min per mg
8- N_3 ATP		
K_m	900 μ M	372 μ M
V_{max}	0.18 nmol/min per mg	0.27 nmol/min per mg

Figure 2 Effect of the irradiation time on low- M_r AdoMet synthetase activity

The enzyme was irradiated with UV light (254 nm, 5 min) in the presence of 1 mM 8- N_3 ATP before determination of enzymic activity for different periods of time. The Figure shows the results of a typical experiment done in triplicate, as means $\pm$ S.D.

Figure 3 SDS/PAGE of the 8- N_3 [32 P]ATP-labelled high- and low- M_r AdoMet synthetases

Two types of samples of each enzyme form were prepared for electrophoresis: controls of both purified AdoMet synthetases, and samples labelled with 8- N_3 [32 P]ATP as described in the Experimental section. Electrophoresis on 10%-polyacrylamide gels was carried out, and the lanes containing the 32 P-labelled enzyme were subjected to autoradiography, while controls were stained with Coomassie Blue. Lanes (a) and (c) show the stained high- (20 μ g) and low- (50 μ g) M_r AdoMet synthetases respectively. Lanes (b) and (d) correspond to the 32 P-labelled tetramer and dimer preparations respectively.

groups 1 and 2 were precipitated with acetone and prepared for SDS/PAGE.

Samples were loaded on 10%-polyacrylamide gels, dried and subjected to autoradiography. Two major labelled bands were observed in either the low- or the high- M_r AdoMet synthetases, one of them corresponding to the main Coomassie-Blue-stained band, with molecular mass of 48 000 and 43 000 Da for the low- and high- M_r AdoMet synthetases respectively (Figure 3).

The 32 P-labelled enzyme bands were excised from the gel, hydrated, and Čerenkov-counted to determine the stoichiometry of incorporation. Calculations made from these data revealed very poor labelling, about 10 pmol/100 pmol of protein in both enzyme forms.

High- and low- M_r AdoMet synthetases were eluted from the gel as described in the Experimental section, and the proteins were proteolysed with trypsin. Purification of the modified peptides was carried out by three different chromatographic

Figure 4 Reverse-phase HPLC purification of the 8- N_3 [32 P]ATP-labelled tryptic peptides from the high- M_r AdoMet synthetase

High- M_r AdoMet synthetase was labelled with 8- N_3 [32 P]ATP; extraction and purification of the tryptic peptides was carried out as described in the Experimental section. Chromatography of the labelled peptides on a reverse-phase HPLC column was carried out with a TFA/acetonitrile gradient. Fractions (1 ml) were collected and counted for Čerenkov radiation. (a) Profile obtained for the modified enzyme form in the presence of 8- N_3 [32 P]ATP; (b) data obtained in the presence of 8- N_3 [32 P]ATP and ATP. ●, Radioactivity eluted; the continuous line represents the A_{214} profile.

steps, cation-exchange HPLC, gel filtration and, finally, C_{18} reverse-phase. The radioactivity and UV profiles in the presence and absence of ATP were compared for both enzyme forms. Figure 4 shows the results obtained for the tetramer, but identical data were produced when dimer samples were analysed. In both cases, there is a peak showing a retention time of 23 min that is diminished in the samples containing ATP, in both the absorption and the radioactivity profiles. This peptide derived from high- and low- M_r AdoMet synthetases was collected and freeze-dried for acid hydrolysis and amino acid analysis. The results obtained were similar for both AdoMet synthetase forms (Table 2). The azido-modified peptides obtained from tetramer and dimer AdoMet synthetases were subjected to automated sequencing, giving the initial sequence of the same peptide, Ile-Ile-Val-Asp-Thr. Identification of the labelled peptide from both enzyme forms was carried out by comparison of the partial sequence and the data of the amino acid composition with the theoretical tryptic peptides derived from the known sequence for rat liver AdoMet synthetase [8]. The labelled peptide corresponds in both cases to that between residues 267 and 286 of the enzyme

Table 2 Amino acid analysis of the 8-N₃ [α -³²P]ATP-labelled peptides

High- and low- M_r AdoMet synthetases were incubated and irradiated in the presence of 8-N₃ [α -³²P]ATP as described in the Experimental section. The enzyme forms were then subjected to proteolysis with trypsin, and the ³²P-labelled peptides were isolated by HPLC. The purified peptides were then hydrolysed in the presence of HCl, and subjected to amino acid analysis. The number for Lys was chosen arbitrarily as 1, and the content of all the other amino acids was referred to that value. The Table shows the closest integer to the average of three independent experiments. Tryptophan was not determined.

	Low- M_r AdoMet synthetase	High- M_r AdoMet synthetase	Peptide 267–286
Asx	1.1	1.34	1
Thr	0.57	0.83	1
Ser	1.12	2.03	1
Gly	8.7	5.69	7
Ala	1.7	1.4	2
Val	0.73	0.74	1
Ile + Leu	3.02	1.49	2
Tyr	1.05	0.71	1
Phe	0.51	0.74	1
Lys	1	1	1
His	0.91	0.45	1
Trp	—	—	1

I	I	V	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Rat liver
I	I	V	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Rat kidney
I	I	V	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Human liver
I	I	V	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Human kidney
I	I	V	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Mouse liver
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Ara1
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Ara2
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Ara3
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Dianthus
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Populus
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Petroselinum
I	I	I	D	T	Y	G	G	W	G	A	H	G	G	G	A	F	S	G	K	Yeast 1
I	I	V	D	A	Y	G	G	A	S	S	V	G	G	G	A	F	S	G	K	Yeast 2
I	I	V	D	T	T	G	G	M	A	R	H	G	G	G	A	F	S	G	K	E. coli
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure 5 Comparison of the sequences of several AdoMet synthetases equivalent to the peptide 267–286 of the rat liver enzyme

The sequences of known AdoMet synthetases have been compared, and aligned in order to find the percentage of similarity in different areas of the molecule. The sequences equivalent to the rat liver peptide 267–286 for several of those AdoMet synthetases are shown: * indicates those amino acids that are conserved in this family of enzymes. Abbreviation: Ara, *Arabidopsis*.

sequence, a peptide that is very well conserved in all the AdoMet synthetases whose sequences are known (Figure 5).

DISCUSSION

This work shows that 8-N₃ ATP is an effective affinity label for the active site of rat liver AdoMet synthetase, high- and low- M_r forms. The use of 8-azidopurine analogues has proved to be very useful in answering a number of biochemical and biological questions concerning nucleotide-binding sites and nucleotide-binding proteins. Their structural properties are very similar to those of the naturally occurring substrates, and moreover they present a reactive group which has the potential to form an irreversible covalent bond upon irradiation [16]. This type of compounds has been used in several opportunities to identify the ATP-binding sites of proteins, among them, 2-5A synthetase [17], the catalytic subunit of protein kinase A [18], the β -subunit of phosphorylase kinase [19], (Na + K) ATPase [20], (Ca + Mg) ATPase [21] and F₁-ATPase [22].

The conditions for modification of the enzyme forms, concen-

tration of 8-N₃ ATP, preincubation and UV-irradiation times have been studied in each case. The maximum effect on AdoMet synthetase activity, about a 22% decrease in the synthesis of AdoMet, was produced by 8-N₃ ATP concentrations in the range of the K_m^{ATP} of each enzyme form, 1 mM for the dimer and 350 μ M for the tetramer [4]. The results were influenced by the length of UV treatment, 5 min being the preferred time. Longer irradiation periods caused enzyme inhibition even in the absence of the photolabile analogue, an effect that was observed by Glazer et al. [23] and Galarzy et al. [24] as being due to protein degradation. Moreover, 5 min irradiation of 8-N₃ ATP results in complete destruction of the compound, making it likely to be a sufficient period of time to photolyse protein-bound 8-N₃ ATP.

The specificity of the labelling was proved by several experiments: (1) 8-N₃-ATP can serve as a substrate for rat liver AdoMet synthetase in the dark, as occurs with other enzymes, such as the *Escherichia coli* AdoMet synthetase [25]; (2) incubation in the presence of the analogue inhibits the synthesis of AdoMet; (3) no labelling was observed when a pre-irradiated analogue was used; and (4) modification with 8-N₃ ATP can be protected in the presence of the natural substrate, ATP. The maximum labelling achieved was 10 pmol of ³²P/100 pmol of protein, and the inactivation observed was a 22% decrease in the enzyme activity in both AdoMet synthetase forms. Both values are low, however; results in this range have been observed in other studies [26–36]. Several authors have also indicated that photoaffinity labelling is not a stoichiometric reaction ([13], and references therein), but this problem has been overcome by using larger amounts of protein to obtain information about the active site.

To identify which part of the enzyme has been labelled by 8-N₃ ATP, two types of samples of both AdoMet synthetase forms were incubated in the presence of 8-N₃ [α -³²P]ATP either alone or plus ATP. Comparison of the results obtained with both types of samples allows us to identify the specific labelling. The α -labelled analogue has been chosen, since it is known that AdoMet synthetase shows pyrophosphatase activity [3]. Therefore, to increase the possibility of labelling, the use of 8-N₃ [γ -³²P]ATP was excluded. At the same time, to overcome this problem partially, a high specific radioactivity (30 Ci/mol) was used in order to include a minimal amount of labelling into the protein, which allowed identification of the modified peptide(s). The use of a high excess of the photoproduct has been recommended by several authors [37], even when values in the millimolar range produce higher unspecific labelling [16].

An additional problem is due to the fact that purified high- and low- M_r AdoMet synthetases are obtained in the presence of 50 mM GSH [4]. This represents the presence of a high concentration of reducing agent in the assay, as well as an increased amount of contaminant for the amino acid analysis of the samples. The effect of reducing agents, such as 2-mercaptoethanol [38] or dithiothreitol [37], as nitrene scavengers is well known. However, in our case, and since the concentrations of 8-N₃ ATP used are high, the presence of a small amount of GSH, which acts as nitrene scavenger, may be an advantage for decreasing the unspecific labelling. Therefore, the samples were either dialysed against a buffer containing 50 μ M GSH, or filtered through Ultrafree MC Eppendorfs to decrease the amount of this compound from the protein preparation to limits that improve the assay.

High- and low- M_r AdoMet synthetases after photoaffinity labelling with 8-N₃ [α -³²P]ATP in the absence or presence of ATP were subjected to SDS/PAGE, where two major bands were identified on autoradiograms. One of them showed the expected molecular mass for the thiopropyl-Sepharose-purified enzyme

A	H	G	G	A	F	S	G	K	D	Y	T	K	
X	X	X	X	P	G	X	G	K	G	T	X	X	
X	X	X	X	G	X	X	X	G	K	X	X	L	
G	L	F	G	G	A	G	V	G	K	T	X	X	
X	X	X	G	E	S	G	A	G	K	T	X	X	
X	X	X	G	X	K	V	X	X	K	X	X	X	

Rat liver AdoMet synthetase
Adenylate kinases
ras proteins
ATP synthase β -subunits
Myosin heavy chains
Phosphoglycerate kinases

Figure 6 P-loop consensus motif for different enzyme groups

The sequences of the phosphoryl group, or P-loop, of several enzyme families are compared with the peptide residues 267–286 of rat liver AdoMet synthetase. The sequences have been aligned in order to find the equivalent residues that are shown in boxes.

forms, 43 and 48 kDa, for the tetramer and dimer respectively [4]. The bands of interest to us were extracted by a procedure developed for phosphorylated proteins [12], proteolysed with trypsin, and peptide maps were performed.

Isolation of the labelled peptides was done by three chromatographic steps, as recommended for photoaffinity-labelled peptides, since it is known that the analysis of this type of samples by reverse-phase HPLC presents several difficulties [13]. Several peaks of ^{32}P radioactivity were observed by this procedure, but only one, with a retention time of 23 min, was decreased in the presence of ATP, indicating the specificity of the labelling. Comparison with the theoretical composition of the tryptic peptides derived from the rat liver AdoMet synthetase sequence [8,9] was carried out. The results obtained for both enzyme forms were very similar. Our data correlate very well with the calculated amino acid composition for the peptide comprising residues 267–286. Minor differences in the data correspond to Trp, which was not evaluated after acid hydrolysis [39], and to Gly and Ser, which appear as normal contaminants in these type of determinations [40,41]. The results obtained by sequencing of the modified peptides from both AdoMet synthetases gave the initial sequence of the labelled peptides, which coincides with that of the peptide comprising residues 267–286. The peptide identified should be present in the active site of the enzyme; therefore, since all AdoMet synthetases use the same substrates, this should be a common recognition sequence for all of them. Comparison of the sequences of the known AdoMet synthetases showed that this peptide (267–286) corresponds to a very well conserved area of the molecule, and contains a motif that seems to be characteristic of this type of enzyme [8,9,42–54]. Moreover, this peptide contains the sequence GXXXXGK, which matches a common motif present in several families of proteins that use ATP [55] (Figure 6). This sequence is proposed to interact with the phosphoryl groups of ATP and is named the phosphate-binding or P-loop [56].

Since the analogue which we have used has the reactive azido group at the adenine ring, we can assume that peptide 267–286 contains the area where the adenine ring and the phosphoryl groups of ATP are attached at the active site of AdoMet synthetase. Computer modelling of 8- N_3 ATP has shown the presence of both areas of the molecule quite close, explaining the proximity of their binding sites [57].

Other data about the rat liver AdoMet synthetase active site correspond to the localization of the motif identified by Wierenga et al. [10]. Those authors established a consensus sequence for the ATP-binding site, Gly-Ala-Gly-Asp-Gln-Gly, which has been located at residues 132–137 of AdoMet synthetase [8]. Another requirement was the presence of a Lys 16–28 residues upstream in the former sequence [10]. This Lys residue has been identified in the rat liver AdoMet synthetase sequence as Lys-160 [8]. All these data together give a better idea about the enzyme active site.

In summary, we have identified a new peptide that, with the Gly motif and Lys-160, is located at the ATP-binding pocket of rat liver high- and low- M_r AdoMet synthetases. It remains obscure which are the areas of the enzyme responsible for methionine binding.

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