Protein kinase C phosphorylation of rat liver S-adenosylmethionine synthetase: dissociation and production of an active monomer

María A. PAJARES,* Cristina DURÁN, Fernando CORRALES and José M. MATO
Instituto de Investigaciones Biomédicas, C.S.I.C. Arturo Duperier 4, 28029 Madrid, Spain

The regulation of rat liver S-adenosylmethionine synthetase (AdoMet synthetase), a key enzyme in methionine metabolism, by protein kinase C (PKC) phosphorylation has been studied. Both enzyme forms, tetramer and dimer, are phosphorylated by this kinase in the same residue, Thr-342, of the sequence. Phosphorylation of the dimer leads to its dissociation, with production of a fully-active monomer. The kinetics of the monomer have been studied, and a K_m^{Met} of 931.9 μM, a K_m^{ATP} of 708 μM and a V_{max} of 66.8 nmol/min/mg have been calculated. Alkaline phosphatase treatment of both enzyme forms (tetramer and dimer) produces a reduction in their activity with no change in the oligomeric state. On the other hand, PKC phosphorylation of the alkaline phosphatase-treated AdoMet synthetase forms leads to the dissociation of the dimer to produce a monomer. Rephosphorylation occurs again in the same residue, Thr-342, of the sequence. The significance of AdoMet synthetase regulation by PKC phosphorylation is further discussed.

INTRODUCTION

The regulatory mechanism that controls rat liver S-adenosylmethionine (AdoMet) synthetase, the enzyme that synthesizes AdoMet using methionine and ATP [1], is not well known. Only recently a modulation of the activity of this key enzyme in methionine metabolism by oxido/reduction of its sulphydryl groups has been shown. The redox state of the protein is governed by the ratio GSH/GSSG and alterations in this ratio may produce changes in the enzyme activity and in its oligomeric state [2]. AdoMet synthetase appears in the liver as a tetramer (high-M_r form) and a dimer (low-M_r form), both active, the first one being 10-fold the more active under physiological concentrations of methionine and ATP [3]. Incubation of the protein, tetramer and dimer, in the presence of GSSG results in inhibition of the enzyme activity and a displacement in the equilibrium between the different oligomeric forms. In fact, an inactive monomer of AdoMet synthetase can be detected after this incubation. [2]. The relationship between variations in the ratio GSH/GSSG and alterations in the enzyme activity have been confirmed by experiments *in vivo* using buthionine sulphoximine [4] and carbon tetrachloride treatments [5], two methods that produce a reduction in the GSH levels.

An interesting possibility of control of AdoMet synthetase activity is protein phosphorylation, a general mechanism of regulation involving the transfer of phosphate to Ser, Thr or Tyr residues of proteins [6,7]. The study of the sequences of many proteins subject to phosphorylation has led to the establishment of consensus sites for the action of different kinases. The rat-liver AdoMet synthetase sequence is known [8,9], and shows several putative phosphorylation motifs [10]. In fact, as many as six possible sites for the action of protein kinase C (PKC), 1 for cyclic AMP-dependent protein kinase, 5 for casein kinase II and 2 for Ca^{2+}-calmodulin-dependent protein kinase have been identified. An hydrophobicity profile of the enzyme reveals the presence of two of those sites in the most hydrophilic area of the molecule [10]. Interestingly, one of them, corresponding to a PKC site, contains the most exposed residue of the protein, Thr-342. These data suggest an easy access for some kinases, especially PKC, to phosphorylate AdoMet synthetase. The aim of this research has been to study the possible mechanism of regulation of AdoMet synthetase by this kinase.

EXPERIMENTAL

Materials

Male Wistar rats (150 g) were from our inbred colony. Hepes, dithiothreitol (DTT), EGTA, phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor (STI), benzamidine, leupeptin, aprotinin, ATP, histone type VS, phosphatidylserine, diolein, Trizma base, methionine, trypsin and $ZnCl_2$ were products of Sigma. Triton X-100, 2-mercaptoethanol, Me_2SO, pyridine, butan-1-ol, trifluoroacetic acid (TFA) and DC-cellulose Plastikfolien were purchased from Merck. DE-52 was obtained from Whatman. DEAE–Sephacel, phenyl Sepharose CL-4B, blue Sepharose CL-6B, 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, a protein assay kit and the electrophoresis reagents were purchased from Bio-Rad. The 10% SDS-polyacrylamide gels were obtained from Linus. [γ-^{32}P]ATP and [2-^{3}H]ATP were products of Amersham. The h.p.l.c. reverse-phase C_{18} Ultrasphere column was purchased from Beckman. Calf-intestine alkaline phosphatase was obtained from Boehringer Mannheim. Films for autoradiography were from Konica. The gel-filtration column used was a Protein Pack 300 SW from Waters. All the solvents for h.p.l.c. and the amino acid analyser were from Riedel-de-Häen and Beckman, respectively. The rest of the 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. [2]. Briefly, the rat-liver cytosol was subjected to

Abbreviations used: AdoMet, S-adenosylmethionine; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; STI, soybean trypsin inhibitor; TFA, trifluoroacetic acid; DTT, dithiothreitol.

* To whom correspondence should be addressed.

DEAE–Sephacel chromatography, followed by loading on a phenyl Sepharose CL-4B column, 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: a blue-Sepharose CL-6B, a Sephacryl S-300 and finally a thiopropyl Sepharose 6B column. The last mentioned purification step rendered both AdoMet synthetase forms in the presence of a high concentration of GSH. Therefore, all the protein samples were extensively dialysed before their use in any phosphorylation or alkaline phosphatase reactions.

PKC purification

PKC was partially purified as described in ref. [11]. Briefly, the rat was decapitated and the brain (1.5–2 g) was extracted. The tissue was homogenized in 8 ml of 20 mM Tris/HCl, pH 7.5, containing 0.3 M sucrose/2 mM EDTA/10 mM EGTA/2 mM DDT/2 mM PMSF/10 μg/ml benzamidine/10 μg/ml leupeptin/10 μg/ml aprotinin. The homogenate was centrifuged for 1 h at 4 °C at 105000 *g*. The supernatant obtained was loaded on a DE-52 cellulose column (10 ml) equilibrated in 20 mM Tris/HCl, pH 7.5, 2 mM DTT/2 mM EDTA/2 mM EGTA (buffer A). The column was washed with two bed-volumes of buffer A, followed by two bed-volumes of buffer A containing 0.1% Triton X-100 and one bed-volume of buffer A until $A_{280} = 0$. Elution was achieved by a 100 ml gradient from 0 to 0.3 M KCl in buffer A. The flow rate was 10 ml/h and 1 ml fractions were collected.

PKC activity assay

The assay was based on the method for measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone type VS [12,13]. The typical reaction mixture (50 μl) contained 20 mM Hepes/Na, pH 7.5/5 mM $MgCl_2$/5 mM DTT/20 μM ATP/1 μCi [γ-^{32}P]ATP/1 mg/ml histone type VS/60 μg phosphatidylserine/6 μg diolein/0.5 mM $CaCl_2$. The reaction was started by the addition of ATP and was carried out for 5 min at 30 °C. The phosphorylation reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid and 100 μg BSA, followed by collection of the acid-precipitable material by centrifugation on a microfuge at 12000 *g* for 3 min. The pellets were washed twice with 1 ml 10% trichloroacetic acid and Cerenkov counted. The specific activity of our PKC preparations was normally 6–7 nmol/min/mg.

PKC phosphorylation of AdoMet synthetase high- and low-M_r forms

The assay was carried out as described previously for the determination of PKC activity, except that the histone was substituted by 50–100 μg of either high- or low-M_r AdoMet synthetase. The reaction was carried out for 0, 2, 5, 7, 10 and 15 min at 30 °C and stopped by adding 1 ml of 10% trichloroacetic acid. After 10 min at 4 °C the samples were centrifuged for 3 min at 12000 *g* on a microfuge and the pellets were prepared for electrophoresis. Samples were boiled for 2 min in the presence of 2% SDS and 1 M 2-mercaptoethanol and subjected to 10% SDS/PAGE. The electrophoresis was developed as described by Laemmli [14]. Gels were dried and subjected to autoradiography. The staining of the gels was later performed using Coomasie Blue R-250. Incorporation of ^{32}P into AdoMet synthetase forms was determined by cutting the protein band of the electrophoresis gel and Cerenkov counting.

Peptide-mapping of phosphorylated AdoMet synthetases

Samples (1 mg) of each AdoMet synthetase form were phosphorylated for 7 min at 30 °C, as described above, using a reaction-mixture of 1 ml, containing 10 μCi [γ-^{32}P]ATP. The reaction was stopped by adding four volumes of acetone (−20 °C), followed by a 20 min incubation at −20 °C. The mixture was centrifugated for 10 min at 1500 *g*, the supernatant was discarded and the pellet dried under N_2. The samples were prepared for electrophoresis by boiling them in the presence of 2% SDS and 1 M 2-mercaptoethanol, and subjected to 10% SDS/PAGE. Electrophoresis was developed as described by Laemmli [14] and the gels were dried and subjected to autoradiography. The labelled AdoMet synthetase band was cut out and the protein eluted following the procedure of Boyle et al. [15]. Samples were dissolved in 300 μl of 50 mM $NaHCO_3$, pH 8.2, and 10 μg of trypsin was added. Incubation was carried out for 2 h at 37 °C before a second addition of 5 μg of trypsin, and continued overnight. The reaction was stopped by freezing and the samples were further lyophilized. The freeze-dried peptides were resuspended in 0.01% TFA in water and loaded on a reverse-phase C_{18} column for h.p.l.c. The peptide-mapping was carried out as described in ref. [16] by using two gradients, the first one using 0.01% TFA/acetonitrile and a second one where the peptides were repurified by using 10 mM ammonium acetate (pH 6.5)/acetonitrile as solvent. Furthermore, the peptides were finally loaded on a thin-layer cellulose plate after dissolving them in 10–20 μl of water. The chromatography was carried out by using butan-1-ol/pyridine/water/acetic acid (13:10:10:2 by volume) as the solvent and the ^{32}P-labelled peptides were detected by autoradiography, as described previously [16,17]. After localization, the peptides were scraped from the t.l.c. plates and eluted from the cellulose by incubation with 1 ml of the chromatographic solvent. The cellulose was then eliminated by centrifugation and the supernatant was freeze-dried.

Sequencing and amino acid analysis of the ^{32}P-labelled peptides

The sequencing procedure was performed at Biosynthesis Inc. The amino acid analysis were carried out in our own laboratory. For this last purpose, the ^{32}P-labelled peptides were hydrolysed with 6 M HCl at 110 °C overnight. The dried samples were then subjected to automatic amino acid analysis on a Beckman 6300 Autoanalyzer.

Activity assay of the phosphorylated high- and low-M_r AdoMet synthetases

High- and low-M_r AdoMet synthetases were phosphorylated with PKC as described above for 0, 2, 5, 7 and 10 min, using an assay volume of 100 μl, in the absence of [γ-^{32}P]ATP. The reaction was stopped at the indicated times by adding 20 μl of 50 mM EGTA and the AdoMet synthetase assay was immediately started by the addition of 62 μl of a reaction-mixture containing 5 mM methionine and 5 mM [2-^3H]ATP (1 Ci/mol) as described by Cabrero et al. [3]. The reaction was carried out for 30 min at 37 °C and stopped by dilution with 5 ml of water. The samples were immediately loaded on 1 ml cation-exchanger columns AG-50W-X4 and the AdoMet produced was eluted and determined as described previously [3,16].

Kinetic assays for the phosphorylated high- and low-M_r AdoMet synthetases

Activity assays to determine the kinetic parameters after phosphorylation by PKC were performed as described above, but

using different concentrations of either ATP or methionine. For the ATP kinetics, a range of 10 μM–1 mM was used at a fixed methionine concentration of 5 mM, whereas methionine kinetics were done from 1 μM up to 10 mM, while keeping a constant ATP concentration of 5 mM.

Alkaline phosphatase-treatment of AdoMet synthetase, high- and low-M_r forms

A 400 μg amount of AdoMet synthetase was used in each assay. The final volume was 500 μl, containing the AdoMet synthetase/48 units of alkaline phosphatase/10 mM Tris/HCl, pH 8/1 mM $MgCl_2$/1 mM $ZnCl_2$. The reaction was carried out for different periods of time at room temperature and stopped by the addition of Na_3PO_4 in 20 mM Hepes/Na, pH 7.5, to a final concentration of 100 mM. Aliquots of the AdoMet synthetases, high- and low-M_r, after the treatment were taken in order to test the enzyme activity as described above.

Phosphorylation of the alkaline phosphatase-treated AdoMet synthetases with PKC

Phosphorylation experiments were carried out immediately after a 3 h alkaline phosphatase treatment as described previously, but using an ATP concentration of 200 μM and 4 μCi [γ-^{32}P]ATP in a final reaction volume of 50 μl. The phosphorylation times used were 0, 2, 5, 7, 10 and 15 min and the reaction was stopped by adding 1 ml of 10% (w/v) TCA. The acid-precipitable material was prepared for electrophoresis and loaded onto 10% polyacrylamide–SDS gels, that were fixed, dried and subjected to autoradiography. For the determination of the ^{32}P-incorporation the AdoMet synthetase bands were cut and Cerenkov counted.

The isolation and identification of the labelled peptides after rephosphorylation with PKC for 7 min was carried out as described for the enzymes without alkaline phosphatase treatment, using 1 mg of each AdoMet form in a reaction mixture containing 200 μM ATP and 40 μCi [γ-^{32}P]ATP.

Molecular weight determination of the phosphorylated AdoMet synthetases

AdoMet synthetase samples (200–250 μg), high- and low-M_r, were used in this assay. The PKC phosphorylation of the samples was carried out as described above in a final reaction volume of 100 μl for 7 min at 30 °C. The reaction was stopped on ice and the samples were immediately injected onto a Protein Pak 300 SW column (300 × 7.8 mm) connected to a Waters 600 E Advance Protein Purification System. The column was equilibrated and run in 10 mM Hepes/Na, pH 7.5/10 mM $MgSO_4$/1 mM EDTA/50 mM KCl, using a 0.5 ml/min flow rate and 200 μl fractions were collected. Blue dextran (M_r 2000000), β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), phosphorylase b (M_r 97400), bovine serum albumin (M_r 66200) and ovalbumin (M_r 45000) were used as standards for the molecular mass determinations. The elution volume of the markers was 4.7, 6.5, 7.1, 7.4, 8.25, 8.55 ml, respectively. Absorbance at 280 nm was followed on a Waters 440 u.v. Detector and the fractions were tested for the presence of AdoMet synthetase activity.

The possibility of changes in the molecular weight of the AdoMet synthetase forms following alkaline phosphatase treatment was studied. Samples (400–500 μg) of the proteins were incubated at room temperature in the presence of alkaline phosphatase for 3 h in a final reaction volume of 250 μl. The reaction was stopped by the addition of Na_3PO_4 in 20 mM Hepes/Na, pH 7.5, to a final concentration of 100 mM, and 100 μl of the samples were injected onto a Protein Pak 300 SW column as described above. The remaining alkaline phosphatase-treated AdoMet synthetases, approximately 200–250 μg (150 μl), were phosphorylated in the presence of PKC and 200 μM ATP for 7 min at 30 °C and immediately injected on the Protein Pak 300 SW column under the conditions previously described. The molecular mass of the rephosphorylated samples was determined using the same standards as above. Fractions (200 μl) were collected and assayed for AdoMet synthetase activity.

Activity assay of the phosphorylated alkaline phosphatase-treated AdoMet synthetases

AdoMet synthetases, high- and low-M_r forms, after the alkaline phosphatase treatment were phosphorylated in the presence of PKC. The reaction was carried out as described above, using a 100 μl reaction-volume containing 200 μM ATP for different periods of time. The phosphorylation was stopped by the addition of 20 μl 50 mM EGTA and the AdoMet synthetase activity was immediately measured as described for the phosphorylated enzymes.

Protein concentration determination

The protein concentration of the samples was measured by the method of Bradford [18], using the Bio-Rad protein assay kit.

RESULTS

The possible regulation by PKC phosphorylation of rat-liver AdoMet synthetase activity has been studied. Purified AdoMet synthetases, high- and low-M_r forms, were incubated in the presence of a partially-purified isozyme mixture of brain PKC and incorporation of ^{32}P into the proteins was determined by SDS/PAGE followed by autoradiography of the gels.

The low-M_r form of the enzyme showed a phosphorylation band with a R_F corresponding to the protein band with an M_r of 48000 stained on the gel (Figure 1). However, the autoradiogram of the phosphorylated AdoMet synthetase tetramer showed incorporation of ^{32}P-labelling on a band with an R_F corresponding to a protein band with an M_r of 43000 stained on the gel (Figure 1). This difference in the AdoMet synthetase M_r on SDS/PAGE has been shown to occur after chromatography on thiopropyl Sepharose [2]. In both cases, it is possible to see other phosphorylated bands that might be contaminants of either the AdoMet synthetase or the PKC preparations, or PKC autophosphorylation. Densitometric scanning of the autoradiograms and stained gels revealed that the maximum incorporation of ^{32}P-labelling occurs after 7 min phosphorylation in both cases. Correction was made for the high-M_r AdoMet synthetase autoradiogram at 15 min, since the more intense band detected correlated with the presence of a higher amount of protein as revealed by densitometric scanning of the stained gel. Stoichiometry of the incorporation was estimated to be 0.5 and 0.2 for the dimer and tetramer, respectively.

The identification of the residues that were phosphorylated by PKC in the high- and low-M_r forms of the enzyme was carried out. The proteins were incubated in the presence of PKC for 7 min, loaded on SDS–polyacrylamide gels and subjected to autoradiography. Only the AdoMet synthetase bands were cut out, the proteins eluted and tryptic peptide-maps of the ^{32}P-labelled enzymes were performed. The labelled peptides were isolated using two different h.p.l.c. gradients and a final purification step on cellulose t.l.c. Only one peptide was labelled in

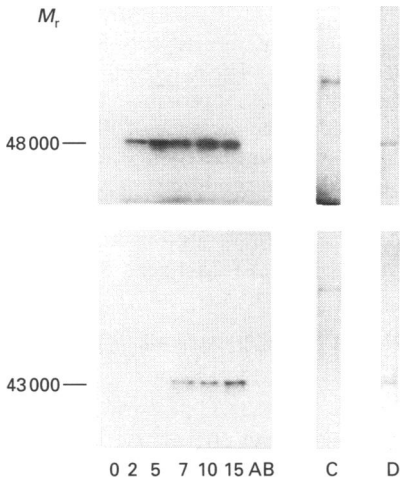

Figure 1 Phosphorylation of AdoMet synthetase by PKC

Samples of the high- (50 μg) and low-M_r AdoMet synthetase (50 μg) were incubated in the presence of a partially-purified PKC mixture at the indicated times. The time course (in min) of ^{32}P incorporation was followed by gel electrophoresis and autoradiography. The figure shows results of a typical experiment performed as described in the Experimental section. The upper and lower panels show the results obtained with the low- and high-M_r AdoMet synthetase forms, respectively. The left side of the figure shows the time course of phosphorylation by PKC. Lane A, 15 min of phosphorylation without PKC. Lane B, 15 min phosphorylation in the absence of Ca^{2+} and phospholipids. Lane C, 15 min incubation without AdoMet synthetase. Lane D, Coomasie Blue staining of the gel.

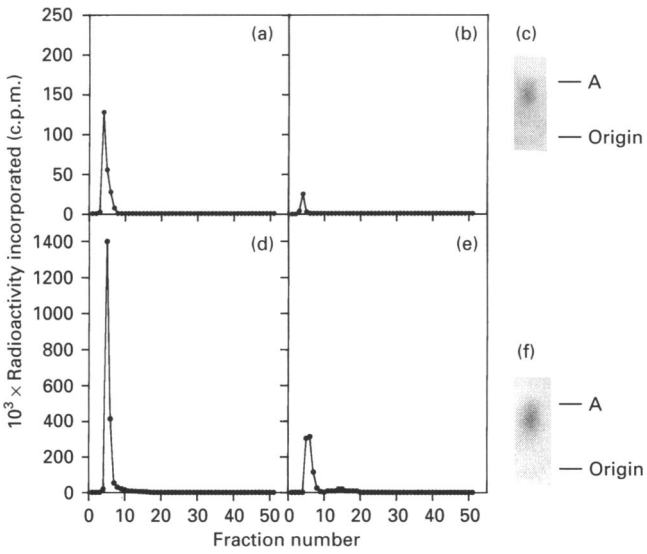

Figure 2 Tryptic peptide maps of AdoMet synthetase after phosphorylation with PKC

1 mg of either the high- or low-M_r AdoMet synthetases was phosphorylated in the presence of PKC for 7 min and prepared as described under Experimental. The samples were digested with trypsin and subjected to h.p.l.c. and 1 ml fractions were collected for counting. The upper and lower panels of the figure show the results for the high- and low-M_r AdoMet synthetase, respectively. (**a**) and (**d**) show the first h.p.l.c. gradient where TFA/acetonitrile were used as solvents. (**b**) and (**e**) show the rechromatography of the labelled peptide using an ammonium acetate/acetonitrile gradient. (**c**) and (**f**) show the t.l.c. of the peptide after its purification by h.p.l.c.

Table 1 Amino acid analysis of the phosphorylated high- and low-M_r AdoMet synthetase peptides obtained from alkaline phosphatase-treated and non-treated samples

Samples of high- and low-M_r AdoMet synthetases were phosphorylated in the presence of PKC and [γ-^{32}P]ATP, and the labelled peptides were obtained as described under Experimental. In the same experiment, samples of both enzyme forms were treated in the presence of alkaline phosphatase followed by incubation with PKC and [γ-^{32}P]ATP. The labelled peptides were obtained and purified as described. After acid-hydrolysis amino acid analysis of the labelled peptides was carried out and the results obtained were compared with the enzyme sequence. Calculations were made on the basis that the lysine content represents the presence of one residue and referring the rest of the values to that.

	High-M_r		Low-M_r		
Alkaline phosphatase-treated	−	+	−	+	341–353
Asp-Asn	1.7	1.9	2.22	1.74	2
Thr	0.8	0.85	1.16	1.07	1
Glu	3	2.82	2.77	2.98	3
Gly	5	5	9	5	–
Val	1.6	1.8	2.15	1.73	2
Ile + Leu	1.88	2	2	1.84	2
Lys	1	1	1	1	1
Arg	1	1	1	1	1

Table 2 Comparison of the K_m values for ATP and methionine of the high- and low-M_r AdoMet synthetases and the enzyme forms phosphorylated in the presence of PKC

High- and low-M_r AdoMet synthetases purified from rat liver were used to establish their K_m values for methionine and ATP. The isolated enzyme forms were phosphorylated in the presence of a partially-purified PKC mixture to obtain the K_m values for the phosphorylated AdoMet synthetases. The experiments were done in triplicate as described under Experimental.

AdoMet synthetase forms	K_m^{ATP} (μM)	K_m^{Met} (μM)
Low-M_r form	812.5	967.17
Phosphorylated low-M_r form	708	931.9
High-M_r form	1209.78	36.1
		718.8
Phosphorylated high-M_r form	1007	37.5
		604.17

both the low- and high-M_r AdoMet synthetases (Figure 2). The retention times on h.p.l.c. were 5 min in the TFA/acetonitrile gradient and 5 min using ammonium acetate/acetonitrile as solvent, while the R_F on t.l.c. was 0.09. These data were the same for the peptide labelled in both forms of AdoMet synthetase. The amino acid analysis of the ^{32}P-peptides after acid-hydrolysis gave the composition shown in Table 1. By comparison of the amino acid analysis data and the enzyme sequence, the phosphorylated peptide was identified as that comprised of residues 341–353, the labelled amino acid being Thr-342 of the sequence. The presence of an excess of Gly might be explained by its normal existence as contaminant in this type of determination [19,20]. The identification of peptide 341–353 as the ^{32}P-labelled one was also confirmed by sequencing of a portion of the isolated peptides that gave Lys-Thr-Glu-Arg as the initial sequence of the peptide 341–353.

The effect that PKC phosphorylation could have on the activity of the high- and low-M_r AdoMet synthetases was also studied. For that purpose, the AdoMet synthetase activity of the tetramer and dimer forms was tested at different phosphorylation times. No change in the high-M_r AdoMet synthetase activity was

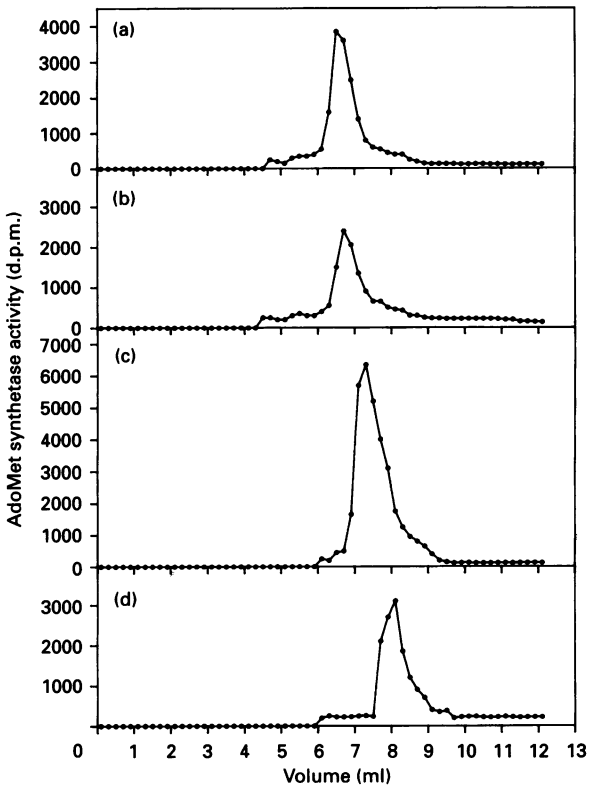

Figure 3 Gel filtration chromatography of AdoMet synthetase forms after PKC phosphorylation

Samples of either the high- or low-M_r AdoMet synthetases were injected on a Protein Pak 300 SW column and 200 μl fractions were collected to measure the enzyme activity. (**a**) The profile of a high-M_r AdoMet synthetase sample; (**b**) the profile of phosphorylated high-M_r AdoMet synthetase; (**c**) the profile of a low-M_r AdoMet synthetase sample and (**d**) its elution profile after PKC phosphorylation. The figure shows the results of a typical experiment, where the following proteins were used as standards: blue dextran (M_r 2000000), β-amylase (M_r 200000), alcohol dehydrogenase (M_r 150000), phosphorylase *b* (M_r 97400), BSA (M_r 66200) and ovalbumin (M_r 45000). The elution volume (ml) of the standards was as follows: 4.7, 6.5, 7.1, 7.4, 8.25, 8.55, respectively.

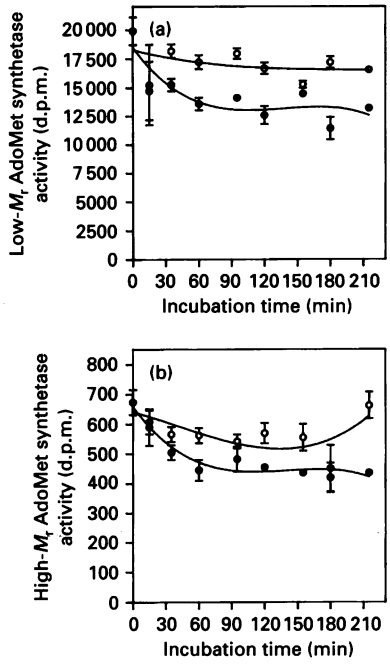

Figure 4 Effect of alkaline phosphatase treatment on the high- and low-M_r AdoMet synthetase activities

Samples of each AdoMet synthetase form were incubated in the presence of alkaline phosphatase at the indicated times. The enzyme activity was immediately measured as described under Experimental. The Figure shows results of a typical experiment done in triplicate ± standard deviation. (**a**) and (**b**) show the effect on the low- and high-M_r AdoMet synthetases, respectively. (●) Represents the activity of the alkaline phosphatase-treated samples and (○) that of the controls.

observed due to phosphorylation of this enzyme form, while a slight increase, approximately 1.4-fold, was detected after a 7 min phosphorylation of the low-M_r AdoMet synthetase (66.8 and 48.3 nmol/min/mg for the phosphorylated and non-phosphorylated forms, respectively). These results were obtained using a reaction mixture containing 5 mM methionine.

The possibility of a change in the kinetic parameters, K_m^{Met} and/or K_m^{ATP}, of the AdoMet synthetases due to their incubation in the presence of PKC was also studied. In these experiments, no changes in the K_m^{Met} and K_m^{ATP} values were observed, either in the phosphorylated tetramer or phosphorylated dimer when compared with the data for the non-phosphorylated AdoMet synthetase forms (Table 2). In fact, the double sigmoidal plot that the tetramer showed in its kinetics for methionine in the absence of GSH was retained by the phosphorylated high-M_r AdoMet synthetase. These two K_m values seem to correspond to different oxidative states of the tetramer, the lower to a reduced form of the enzyme and the higher to a partially oxidized state. The fact that the K_m^{Met} for the dimer resembles the highest value of the tetramer seems to indicate that this is also a partially-oxidized form of the enzyme.

Changes in the oligomeric state of the enzyme forms due to phosphorylation have also been tested using a Protein Pak 300 SW column. The high-M_r AdoMet synthetase, the tetramer (Figure 3a), did not change its elution position after phosphorylation with PKC (Figure 3b). The calculated M_r for this form was 190000 on this type of column.

On the other hand, the low-M_r AdoMet synthetase, the dimer (Figure 3c), changed its elution position to a higher volume after phosphorylation (Figure 3d). The M_r of the protein was calculated and the values obtained were 125000 and 60000 for the non-phosphorylated and phosphorylated forms, respectively. The calculated M_r after phosphorylation was slightly higher than that expected for the monomer form. However, when the eluted protein was subjected to SDS/PAGE it showed an M_r of 48000 (data not shown). This is the first time that a fully active monomer of AdoMet synthetase has been obtained.

The incorporation of ^{32}P on AdoMet synthetase by the action of PKC *in vitro* might be limited by the presence of the enzyme in an already phosphorylated form. Therefore, alkaline phosphatase treatment of AdoMet synthetase, high- and low-M_r forms, was carried out at room temperature for different times. In both cases a decrease in the enzyme activity was observed after this reaction (Figure 4). The inhibition obtained was around 20% after a 3 h treatment for the tetramer and about 30% for the dimer. Rephosphorylation of the alkaline phosphatase-treated AdoMet synthetases was performed using a brain PKC isoenzyme mixture in the presence of 200 μM ATP. The dephosphorylated enzymes are able to incorporate ^{32}P by the action of this kinase mixture as can be observed by autoradiography of an SDS–polyacrylamide gel of the samples (data not shown). In both cases, the main incorporation occurs on a band with an M_r of 60000, corresponding to a contaminant present in the alkaline

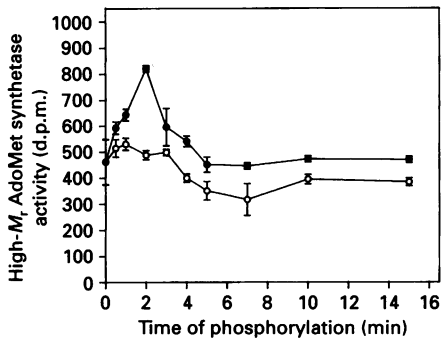

Figure 5 Activity of the alkaline phosphatase-treated high-M_r AdoMet synthetase after PKC phosphorylation

Samples of the high-M_r AdoMet synthetase were treated for 3 h in the presence of alkaline phosphatase at room temperature. These samples were immediately phosphorylated in the presence of PKC at the indicated times and the enzyme activity was measured as described under Experimental. The Figure shows results of a typical experiment done in triplicate ± standard deviation. (●) Represents the activity of the phosphorylated samples and (○) that of the controls.

phosphatase preparation, but bands of M_r 48000 and 43000 were also labelled. Densitometric-scanning of the autoradiograms showed that the maximum phosphorylation was again obtained after 7 min. The stoichiometry of ^{32}P-labelling was estimated to be 0.2 and 1 for the tetramer and dimer, respectively.

The phosphorylated residues after alkaline phosphatase treatment were identified. For that purpose, the rephosphorylated proteins were loaded on SDS–polyacrylamide gels and the AdoMet synthetase bands extracted for the obtention of their tryptic peptide-maps. The labelled peptides were isolated as described previously, and again only one peptide was phosphorylated in either the low- and high-M_r AdoMet synthetases. This peptide has the same retention time on h.p.l.c. (5 min in both gradients) and the same R_F on t.l.c. (0.09) in both enzyme forms, and these values are the same as for the non-treated enzyme forms. After acid hydrolysis, the amino acid analysis of the ^{32}P-peptides was performed and localization of the residues was made by comparison with the enzyme sequence (Table 1). Labelling by PKC rephosphorylation occurs in the peptide comprised of residues 341–353 of the sequence, and the phosphorylated amino acid is Thr-342. Again, as for the phosphorylated enzymes, the amino acid analysis shows an excess of Gly.

The activity of the rephosphorylated enzyme forms was measured in order to test if a reactivation of the enzymes was produced. No recovery of the initial enzyme activity was obtained in the dimer form, while a transient reactivation was observed for the tetramer (Figure 5). These results could be due to the fact that alkaline phosphatase is a non-specific phosphatase and therefore it eliminates phosphate groups included by several types of kinases. Since AdoMet synthetase presents multiple putative phosphorylation motifs, it is possible that reactivation of the dimer needs an additional phosphate, included by a kinase different from PKC.

The alkaline phosphatase-treated enzymes were tested for possible changes in their oligomeric state due to this treatment. No change in the calculated M_r for either tetramer or dimer was observed after dephosphorylation. However, when the alkaline phosphatase-treated enzymes were rephosphorylated by PKC, changes in the elution position of the timer were observed. The calculated M_r for the rephosphorylated dimer was 60000, slightly higher than the value expected for a monomer of the protein (48000). Again SDS/PAGE of the eluate, showed the presence of a protein band with M_r = 48000 and no apparition of a lower M_r band that could explain the difference (data not shown).

DISCUSSION

Rat-liver AdoMet synthetase appears in two oligomeric forms, a tetramer and a dimer, composed of the same type of subunit [3]: the high-M_r form being around 10-fold more active at physiological concentrations of methionine [3]. The equilibrium between the tetramer and dimer forms must be controlled in order to maintain AdoMet levels in the cell. Therefore, the enzyme activity and/or its oligomeric state must be regulated by a mechanism(s) that for the moment remains unknown. Previous studies have revealed the existence of a regulation of the enzyme activity depending on its oxido/reduction state due to the ratios GSH/GSSG [2,4,5]. A detailed study of the enzyme sequence has shown the presence of several putative sites of phosphorylation by different protein kinases [10]. This fact raised the interesting possibility that a general mechanism of control such as phosphorylation might direct the AdoMet synthetase equilibrium and, therefore, its activity.

Tetramer and dimer forms of the enzyme can be phosphorylated *in vitro* by a partially-purified brain PKC mixture, showing its maximum labelling after 7 min. This ^{32}P incorporation is higher in the dimer than in the tetramer (0.5 versus 0.2), maybe due to easier access for the kinase in the former enzyme form. The labelling is not observed in the absence of calcium and phospholipids, or without PKC, excluding the possibility of either phosphorylation by another kinase present in the preparation or an unspecific binding of [γ-^{32}P]ATP in the AdoMet synthetase forms. Under these conditions only one peptide of the protein was labelled, the same one in tetramer and dimer. The peptide that corresponds to residues 341–353 of the sequence was deduced from the amino acid analysis of the samples and by a partial sequence. The phosphorylated amino acid is Thr-342, the most exposed residue of the protein, as shown in an hydrophobicity profile of the enzyme [10]. This residue is located in one of the six consensus sequences for PKC present in the protein that fit the conditions XRXXS/TXRX [21,22].

One way by which phosphorylation regulates enzyme activities is dissociation of complexes [23]. This is the case for myristoylated alanine-rich C-kinase substrate whose phosphorylation by PKC leads to a reduction in its affinity for calmodulin, and therefore, a dissociation of the complex [24,25]. The same result is obtained upon activation of PKC in T-cells, leading to the dissociation of the complex CD4–p561ck [26]. *In vitro*, the nuclear factor-$_\kappa$B (NF-$_\kappa$B) is normally present as an inactive form, that is activated upon dissociation of an inhibitory protein (I_κB). This dissociation occurs when PKC phosphorylates this inhibitory protein [27]. In our case, a change in the oligomeric state of the dimer is obtained by PKC phosphorylation, leading to the production of an active monomer. Either dimers or monomers have been previously obtained *in vitro* by oxidation [2] or *N*-ethylmaleimide-modification [16] of some protein sulphydryl groups, but in those experiments only inactive enzyme forms were produced. Moreover, in patients with cirrhosis, where a reduction in AdoMet synthetase activity has been detected, the predominant enzyme form that can be measured is the dimer [28,29]. In fact, the only reference to the presence *in vivo* of an active monomer has been detected in one of these patients [30].

The monomer obtained by PKC phosphorylation has the

same affinity for its substrates as the dimer, but its $V_{max.}$ is slightly increased, around 1.4-fold, when tested under saturating conditions of methionine (5 mM) and ATP. On the other hand, the phosphorylated tetramer showed identical kinetic parameters with the non-phosphorylated form. Therefore, the tetramer, either phosphorylated or non-phosphorylated, is the only form that has a K_m^{Met} in the physiological range (60 μM) [2,3]. Based on these data, most of the AdoMet synthetase activity *in vivo* should be due to the high-M_r form of the enzyme. Moreover, since monomer and dimer have very similar affinities for methionine, their contribution to the synthesis *in vivo* of AdoMet should be in the same range, approximately 10-fold less than that of the tetramer [3].

Phosphorylation did not alter the sigmoidal kinetics that the dimer shows before being reduced on a thiopropyl Sepharose column or after elimination of GSH [2], and the kinetic plots of the monomer are also sigmoidal. In these studies, the performance of such a purification step to obtain AdoMet synthetase gave the enzyme forms in the presence of a high concentration of GSH [2]. Compound that was eliminated before any experiment was made, since both, PKC [31] and AdoMet synthetase [2] are sensitive to the GSH/GSSG ratio.

The fact that the stoichiometry of the labelling is low, approximately one subunit per tetramer and one per dimer (0.2 versus 0.5) could be due to the presence of the enzyme in an already phosphorylated state. Therefore, we have studied the phosphorylation of AdoMet synthetase after alkaline phosphatase treatment. Modulation of enzyme activities by dephosphorylation has been described in glycogen metabolism among others, where phosphorylase kinase activity decreases upon dephosphorylation [32,33]. Alkaline phosphatase dephosphorylation of AdoMet synthetase produces a reduction of activity in the tetramer and dimer forms, but no alteration of their oligomeric state. When rephosphorylation experiments are carried out, a higher incorporation of ^{32}P was obtained in the dimer (stoichiometry 1 versus 0.2), and again this enzyme form is dissociated to a monomer. Comparison of the monomer-labelling obtained by phosphorylation after alkaline phosphatase treatment versus the incorporation without this treatment (stoichiometry 1 versus 0.5), reveal that the treated form incorporates double the amount of ^{32}P. These results suggest the presence of the purified dimer in a partially phosphorylated form (one subunit/dimer), and that labelling of the second subunit in the same Thr produces the monomer. Finally, the observation that the rephosphorylated monomer does not recover the initial activity may be because alkaline phosphatase could have eliminated phosphate groups included by another type of kinase.

The ^{32}P incorporation in the tetramer is identical before and after alkaline phosphatase treatment. These results may be explained by differences in structure between dimer and tetramer, that in the latter case allows only the PKC phosphorylation of one subunit. Moreover, changes of conformation in the subunits after the introduction of new charge are a possible explanation of the transient recovery of the initial activity observed by rephosphorylation of the tetramer.

In summary, this is the first time that a fully active monomer of AdoMet synthetase has been obtained *in vitro*. This new enzyme form has the same affinity for the substrates as the dimer, and therefore, under physiological conditions both forms would contribute to the same extent to AdoMet production; i.e. 10-fold less than the tetramer [3]. All the data available at the moment suggest that dimers and monomers may have arisen as a consequence of a deactivation mechanism or serve as precursor forms of AdoMet synthetase. Oxidation/reduction and phosphorylation/dephosphorylation *in vivo* may therefore be the signals that determine the route that AdoMet synthetase forms follow, either degradation or aggregation to render the more active enzyme forms, the tetramers. All these data lead to a more complicated equilibrium of enzyme forms that would include tetramer, dimer and monomer, the factors regulating the conversion of tetramer to dimer and vice versa remaining obscure.

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