REFOLDING AND CHARACTERIZATION OF METHIONINE
ADENOSYLTRANSFERASE FROM *Euglena gracilis*

Francisco Garrido¹, Sylvie Estrela¹, Claudia Alves¹, Gabino F. Sánchez-Pérez², Antonio Sillero¹,³, María A. Pajares¹,*

¹Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC-UAM), Arturo Duperier 4, 28029 Madrid (Spain).
²Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Canada B3H 1X5.
³Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid (UAM), Arzobispo Morcillo 2, 28029 Madrid (Spain).

*To whom correspondence should be addressed at: Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain. (Phone: 34-915854414; FAX: 34-915854401; email: mapajares@iib.uam.es)

**Keywords**
Methionine adenosyltransferase, inclusion bodies, refolding, characterization, secondary structure, structural model.

**Abbreviations**
MAT, methionine adenosyltransferase; cMAT, methionine adenosyltransferase from *E. coli*; BsMAT, methionine adenosyltransferase from *B. subtilis*; MAT I, mammalian methionine adenosyltransferase tetramer; MAT III, mammalian methionine adenosyltransferase dimer; MAT II, mammalian methionine adenosyltransferase heterotetramer; Mj-MAT, methionine adenosyltransferase from *Methanococcus jannaschii*; MATα1, MAT I/III catalytic subunit; AdoMet, S-adenosylmethionine.
ABSTRACT

Methionine adenosyltransferase from *Euglena gracilis* (MATX) is a recently discovered member of the MAT family of proteins that synthesize S-adenosylmethionine. Heterologous overexpression of MATX in *Escherichia coli* rendered the protein mostly in inclusion bodies under all conditions tested. Therefore, a refolding and purification procedure from these aggregates was developed to characterize the enzyme. Maximal recovery was obtained using inclusion bodies devoid of extraneous proteins by washing under mild urea (2 M) and detergent (5%) concentrations. Refolding was achieved in two steps following solubilization in the presence of Mg$^{2+}$; chaotrope dilution to <1 M and dialysis under reducing conditions. Purified MATX is a homodimer that exhibits Michaelis kinetics with a $V_{\text{max}}$ of 1.46 µmol/min/mg and $K_m$ values of approximately 85 and 260 µM for methionine and ATP, respectively. The activity is dependent on Mg$^{2+}$ and K$^+$ ions, but is not stimulated by dimethylsulfoxide. MATX exhibits triphosphatase activity that is stimulated in the presence of S-adenosylmethionine. Far-UV circular dichroism revealed β-sheet and random coil as the main secondary structure elements of the protein. The high level of sequence conservation allowed construction of a structural model that preserved the main features of the MAT family, the major changes involving the N-terminal domain.
INTRODUCTION

Reactions using S-adenosylmethionine (AdoMet) are among the most abundant processes taking place in any cell [1,2]. The routes in which the AdoMet-consuming reactions are involved allow the synthesis of a large variety of compounds, as well as the control of cell function (i.e. epigenetic modifications). This wide use of AdoMet derives from the variety of groups that this molecule is able to donate, being methyl group donation the main consumer of the compound [2-5]. In contrast, methionine adenosyltransferases (MATs) are the only enzymes known to synthesize AdoMet in a rather unusual reaction that occurs in two steps [6,7]. First, the substrates methionine and ATP are combined to obtain AdoMet and triphosphate that is hydrolyzed in the second part of the reaction to render pyrophosphate and inorganic phosphate as side products. The catalytic mechanism followed is of the S\textsubscript{N}2 type and most of the inorganic phosphate obtained derives from the γ-phosphate of ATP [6,7]. MATs require divalent cations (Mg\textsuperscript{2+}) for catalysis and are activated by monovalent ions (K\textsuperscript{+}). In mammals, AdoMet has a dual behavior on MATs, inhibiting (MAT I and II) and activating (MAT III) its own synthesis, and enhancing the tripolyphosphatase activity [2,8,9].

MAT enzymes have been found in almost every organism from *Mycoplasma genitalium* to mammals with the exception of a few parasites that obtain AdoMet from their hosts [10]. For this purpose, AdoMet transporters are required and proteins exhibiting such function have been identified in some organisms, as well as in yeast and mammalian mitochondria [11-13]. Most organisms express several MAT isoenzymes that differ in their affinities for the substrates (30 µM to 1 mM for methionine in mammals), despite the high conservation of the sequences of their catalytic subunits [2,7,14]. Most members of the MAT family are homo-oligomers except for mammalian MAT II, and the majority are tetramers with the exception of a few dimers (i.e. mammalian MAT III and archaeal
MATs) [7,15]. All the crystal structures known to date (mammalian and E. coli MATs) show monomers organized in three domains formed by nonconsecutive stretches of the sequence, and the subunits interact through a large flat hydrophobic surface to form the dimers [7,16-18]. Active sites, two per dimer, locate between monomers with residues of both subunits contributing to them, thus the minimum association level of active MATs is the dimer. Sequence and structural restrictions to maintain the relative positions of the active site residues, and the unusual reaction mechanism, impose that the insertions and deletions observed in specific lineages take place mostly in the loops [10]. Moreover, these indels are conserved in the lineages in which they occur as observed in the analysis carried out in Bacteria and Eukarya. Recently, a different type of eukaryotic MAT (MATX) was identified in dinoflagellates, haptophytes, diatoms and euglenids [19,20]. This new MAT preserves the catalytic residues, its sequence contains insertions/deletions in loops and it is able to complement the corresponding Saccharomyces cerevisiae mutant, thus demonstrating its capacity to synthesize AdoMet [21]. Several species express both a canonical MAT and this new MATX, hence raising the possibility that the characteristics of each isoenzyme favor adaptation to the growth conditions or the diverse needs during the life cycle of the organism. The purpose of this article is to fully characterize MATX and compare its properties with those of other members of the MAT family. Differences in the active sites among MATs may provide the basis for the use/design of inhibitors for selective growth control of certain organisms, and hence the infections they produce that may have important pathological or commercial consequences.
MATERIALS AND METHODS

Materials

Methionine, ATP, S-adenosylmethionine (AdoMet) dithiothreitol (DTT), ampicillin, molecular mass standards for gel filtration chromatography and protease inhibitors were obtained from Sigma Chemical Company (St. Louis, MO). [2,8-^3^H]-ATP (28.7 Ci/mmol), Pfu DNA polymerase and restriction enzymes were products of Perkin Elmer (Boston, MA), Biotools (Madrid, Spain) and Invitrogen (Carlsbad, CA), respectively. Q-Sepharose HiTrap and Superose 12 10/300 GL columns were purchased from GE Healthcare (Uppsala, Sweden). Isopropyl β-D-thiogalactopyranoside (IPTG) and E. coli strains were products of Calbiochem (La Jolla, CA) and Stratagene (La Jolla, CA), respectively. Electrophoresis reagents and the BioRad protein assay kit were obtained from BioRad (Richmond, CA). YM-30 ultrafiltration membranes were purchased from Amicon Corp. (Beverly, MA). Urea, DMSO and Triton X-100 were products from Merck (Darmstadt, Germany). The rest of the buffers and reagent were from the best quality commercially available.

Construction of MATX expression plasmids

The ORF of MATX was amplified by PCR from the pEXT5-MATX plasmid provided by Dr. A. Rogers’s laboratory. For this purpose, primers that include Ndel (5’-GGAATTCCATATGGCTGAATCTGCTTCAAAG-3’) and EcoRI (5’-GCGAATTCCTAGTCTCCACTTCTG-3’) restriction sites (underlined) were designed. Following an initial denaturation step at 95°C for 2 min, amplification was performed for 30 cycles at 95°C for 30 s, 55°C for 1 min and 72 °C for 1 min using Pfu DNA polymerase. The PCR also included a final elongation step at 72°C for 10 min. The PCR fragment was then
purified, digested and cloned into the pT7.7 vector [22] using the NdeI and EcoRI restriction sites. The resulting plasmid was designated pT7.7-MATX. The sequence was verified by automatic sequencing at the facilities of the Instituto de Investigaciones Biomédicas “Alberto Sols”.

**MATX overexpression in E. coli**

The pT7.7-MATX plasmid was transformed into BL21(DE3) Codon Plus competent cells and single colonies were grown in LB medium (500 ml) containing 50 µg/ml ampicillin at 20°C, 30°C and 37°C. The cell density was measured and aliquots (25 ml) taken and induced at $A_{600}=0-0.8$ using 0.5 mM IPTG for 0-22 h. Cells were harvested by centrifugation and stored at -80°C until processing.

**Analysis of soluble and insoluble fractions**

Cell pellets (1 g wet wt) were lysed by sonication at 4°C in a Soniprep 150 sonifier (15 cycles, 30 s on/off at an output power level of 8 microns) in 25 ml of 50 mM Tris/HCl pH 8, 10 mM MgSO$_4$ (buffer A) containing 5 mM EDTA, 0.1% 2-mercaptoethanol, 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 2.5 µg/ml antipain, 0.5 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Soluble and insoluble fractions were separated by centrifugation at 13000g for 15 min. The insoluble fraction, containing the inclusion bodies, was solubilized in 10 ml of 50 mM Tris/HCl pH 8, 75 mM MgSO$_4$ containing 8 M urea and aliquots prepared for SDS-PAGE. In parallel, soluble fractions were used for both SDS-PAGE and activity measurements.

**MATX refolding from inclusion bodies**
The insoluble fraction obtained from 1 g of cells was resuspended in 25 ml of buffer A containing 1 mM EDTA and sonicated again 6 cycles (10 s on/off, output power 8 microns) on ice. Pellets were recovered by centrifugation for 30 min at 48000g and 4°C. These pellets were used to test washing conditions using 100 mM Tris/HCl pH 7 buffers including 5 mM EDTA, 0-4 M urea, 0-5 % Triton X-100 and 0.1 mM benzanidine. These washes were carried out twice, followed by a final wash with the same buffer devoid of urea and detergent.

One washed pellet was resuspended in 10 ml of 50 mM Tris/HCl pH 8 containing 8 M urea and 75 mM MgSO₄ and incubated for 2 hours at 10°C. Thereafter the sample was diluted to reach 0.5-4 M urea by addition of buffer A. Each diluted sample was immediately dialyzed against buffer A containing 10 mM DTT (total volume 1.5 l). Refolded MATX was recovered by centrifugation of the dialyzed samples for 30 min at 48000g and 4°C, and checked for MAT activity and protein.

**Purification of refolded MATX**

Refolded MATX was purified using Q-Sepharose HiTrap (5 ml) equilibrated in buffer A containing 0.1 % 2-mercaptoethanol and 0.1 mM benzanidine. The flow rate was 1 ml/min and 5 ml fractions were collected. Following sample loading an extensive washing step (100 ml) was carried out using buffer A until A₂₈₀=0. Elution was performed with a gradient from 0-0.5 M KCl in buffer A (150 ml) at the same flow rate. The presence of MATX was detected by activity measurements and the corresponding peak collected and dialyzed against buffer A to eliminate KCl excess or against 50 mM Tris/HCl pH 8 for Mg²⁺ kinetics. In some cases, the peak was concentrated by ultrafiltration using YM-30 membranes until the desired protein concentration was reached.
Far and near UV circular dichroism

MATX samples were prepared at 0.25 mg/ml and 1 mg/ml protein concentrations for far- and near-UV CD, respectively. Spectra were recorded on a Jasco J-720 spectropolarimeter at 25°C, using 0.1 cm (far-UV) or 1 cm (near-UV) pathlength cuvettes. After baseline subtraction the observed ellipticities were converted to mean residue ellipticities (θ_{mrw}) on the basis of a mean molecular mass per residue of 110 Da. A minimum of five spectra was taken for each sample. Analysis of secondary structure was performed using the deconvolution software CDNN (© G. Böhm).

Sedimentation velocity

MATX samples (0.3-0.5 mg/ml) were used for sedimentation velocity runs that were carried out at 48000 rpm and 4°C in an XL-1 analytical ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) with a UV-Vis detection system, using an An50Ti rotor and 12 mm double-sector centerpieces. Absorbance scans (0.003 cm step size) were taken at 280 nm. Differential sedimentation-coefficient distributions, c(s), were calculated by least squares boundary modeling of sedimentation velocity data using the program SEDFIT [23,24]. The values obtained from this analysis were corrected for solvent composition and temperature to obtain s_{20,w} using the public domain software SEDNTERP, retrieved from the RASMB server [25].

Analytical gel filtration chromatography

MATX samples (100 µl containing a minimum of 50 µg) were injected on a Superose 12 10/300 GL gel filtration column equilibrated in buffer A containing 150 mM KCl. The flow rate was 0.3 ml/min and 210 µl fractions were collected while A_{280} was recorded. Elution and MAT activity measurements (100 µl) were performed as previously
described [26]. The protein standards used and their elution volumes were as follows: Dextran Blue (2000 kDa), 8.69 ml; apoferritin (443 kDa), 9.87 ml; β-amylase (200 kDa), 11.34 ml; alcohol dehydrogenase (150 kDa), 12.07 ml; carbonic anhydrase (29 kDa), 15.12 ml; and ATP (0.551 kDa), 19.26 ml.

Electrophoresis

Denaturing gel electrophoresis was performed on 10% SDS-PAGE gels under reduced conditions, using the Laemmli buffer system. Samples of soluble (150 µg), inclusion bodies (30 µg) and refolded MATX (6 µg) were loaded per lane and stained using Coomassie blue R250. Densitometric scanning of the bands was carried out using the ImageJ software 1.37v (http://rsb.info.nih.gov/ij/).

MAT activity measurements and kinetics

Activity assays were carried out at 37ºC as previously described [27], using a protein concentration of 0.05 mg/ml and the standard MAT reaction mixture containing 5 mM methionine and 5 mM ATP in a final volume of 250 µl. Kinetics were performed using standard MAT reaction mixtures containing: i) 1-600 µM of the amino acid and 5 mM ATP, for methionine kinetics; ii) 1-1500 µM of the nucleotide and 5 mM methionine, for ATP kinetics; iii) 0-13 mM MgSO₄, for Mg²⁺ kinetics; and iv) 0-85 mM KCl, for K⁺ kinetics. DMSO stimulation was tested using reaction mixtures containing 60 µM methionine and 10% (v/v) DMSO.

Tripolyphosphatase activity measurements

MATX tripolyphosphatase activity was measured following absorption at 750 nm as previously described [17]. Tripolyphosphate kinetics were carried out using 0-5 mM
triphosphate in the presence or absence of 50 μM AdoMet and using 0.05 mg/ml protein samples.

*Theoretical calculations of the pI and extinction coefficient.*

Information from the amino acid sequence was used for theoretical calculation of the pI using a method previously described by Ribeiro and Siller [28]. In addition, the extinction coefficient was estimated from the number of tryptophan and tyrosine residues in the sequence, and using extinction coefficients for these amino acids of 5550 and 1490 M$^{-1}$cm$^{-1}$, respectively.

*Protein concentration determinations*

The protein concentration of the samples was measured routinely using the BioRad protein assay kit and bovine serum albumin as the standard. On the other hand, for CD and sedimentation velocity experiments protein concentration was determined from the A$_{280}$ of the sample after 30 min incubation in 8 M urea using a calculated extinction coefficient of 48610 M$^{-1}$cm$^{-1}$.

*MATX structural model and sequence alignment.*

A structural model of the MATX monomer was prepared using the Swiss-Model server ([http://swissmodel.expasy.org/](http://swissmodel.expasy.org/)), the protein sequence and the 2.05 Å resolution structure of the human MATα1 protein (PDB ID: 2OBV) as template (49.5% sequence identity)[29-31]. The model extends from residue 13 to 444 of the sequence and preserves the three-domain organization of all the previously crystallized MATs [7,16,17]. Sequence alignment of the MATs mentioned in the text was performed using the COBALT Multiple Alignment Tool ([http://www.ncbi.nlm.nih.gov/tools/cobalt](http://www.ncbi.nlm.nih.gov/tools/cobalt)).
RESULTS AND DISCUSSION

MATX from *Euglena gracilis* belongs to the MAT family of enzymes characterized by the high level of sequence identity exhibited by its members [10]. Its ORF encodes for a protein of 471 residues, which means a notable increase in length as compared to most MATs (~400 amino acids long). MATX has a theoretical pI of 5.91, similar to the values obtained to date for most members of the family (i.e. 5.9 for rat MAT III) [7,32]. Heterologous expression of the protein was initially carried out in *E. coli* using the plasmid pEXT5-MATX, which includes a His-tag at the protein N-terminal. However, the expression levels obtained were very low under all the conditions tested (growth temperature, cell density for induction, etc.). Addition of tags to other MATs, both at the N- or C-terminal ends, was previously shown to decrease the amount of soluble protein obtained [33], and hence the pT7.7-MATX plasmid that contains the MATX ORF without the His-tag was constructed. Growth and induction conditions of *E. coli* cells transformed with pT7.7-MATX were studied and optimized. The protein appeared in 10% SDS-PAGE gels with a calculated Mr of 57600, according to the mobility of the standards (Fig. 1), which is slightly above the calculated size. MAT activity was higher in soluble fractions of cultures induced at $A_{600}= 0.8$ (Fig. 2), although no significant changes in the protein band intensity were detected by densitometric scanning of the stained gels (Fig. 1A). Overexpression of MAT enzymes produces a large decrease in cellular ATP levels, as its consumption is increased to produce AdoMet. Thus, it is possible that only low levels of expression allow cell survival, and hence that a larger cell number may led to an apparent constant level of MATX once a certain density is achieved, whereas the activity levels increase due to a larger contribution of the endogenous cMAT. The activity was also increased in soluble fractions of cultures grown at 30°C as compared to those grown at 37°C (Fig. 3). Maximum induction with IPTG was already detected after a 3 h induction
period at both temperatures. However, in all the conditions tested most of the protein (~90%) was accumulated in inclusion bodies (Fig. 1B). The use of lower temperatures (20°C) and IPTG concentrations was reported to improve *Bacillus subtilis* MAT (BsMAT) heterologous expression [34]. In our hands none of these conditions improved the results obtained and in fact the soluble protein was almost undetectable (data not shown). Therefore, the standard grown conditions were established to obtain MATX from inclusion bodies using 3 h IPTG induction at *A*$_{600}$=0.6-0.8 and 37°C and refolding from inclusion bodies was attempted.

Several parameters were studied using the inclusion bodies, in order to obtain the maximum amount of protein for refolding. These included the protein concentration, pH of the buffer and the concentrations of the solubilization agents, as previously described for mammalian MAT I/III refolding [32]. Inclusion bodies contain not only the desired overexpressed protein, but also bacterial membrane and cell wall and therefore the need to eliminate these extraneous proteins. For this purpose, the inclusion bodies were subjected to several washing steps with buffers including variable concentrations of urea (0-4 M) and Triton X-100 (0-5%) at pH 7. In contrast to MAT I/III, with a similar pI [32], a large amount of MATX was solubilized when using washing buffers containing >2 M urea, whereas the percentages of detergent examined did not cause such protein loss. Therefore, the standard washing conditions were established as two washes in the presence of 2 M urea and 5% Triton X-100.

Refolding of MATX from the washed inclusion bodies was then attempted. For this purpose, the protein was solubilized using a buffer containing 8 M urea, and the refolding protocol previously developed for rat MAT I/III [32]. However, for this protocol to be adapted to MATX several requirements had to be tested. First, the range of protein concentration at the solubilization step that renders the largest amount of active refolded
MATX. According to the results obtained, this protein concentration was established between 1-3 mg/ml. Secondly, the intermediate urea concentration to be reached in the dilution step. This dilution was found to dramatically improve the yield of active-refolded MAT I/III [32], and hence expected to be crucial for MATX refolding. Our results indicate that achieving a urea concentration between 0.5-1 M before dialysis, and hence a protein concentration of 0.1-0.3 mg/ml, allowed the largest recovery of active protein (Fig. 4). Finally, the presence of 10 cysteine residues in the MATX sequence, and the crucial role that this type of amino acids have in all MATs studied to date [7,35,36], recommends the inclusion of a reducing agent in the dialysis buffer to avoid the production of off-pathway aggregates due to oxidation. Based on the data obtained, an intermediate dilution to 1 M urea followed by dialysis in the presence of 10 mM DTT was used as the standard procedure.

The refolded protein was further purified by ion exchange chromatography, and a single peak exhibiting MAT activity eluted in the presence of 100 mM KCl. The MATX content of this preparation was estimated to be >90% by densitometric scanning of the stained gels (Fig. 5). The purification results appear in Table 1, and show a remarkably lower yield at the dialysis step as compared to MAT I/III (25 vs. 88%) [32]. Given the high sequence identity level between MATs this result was unexpected, and demonstrated how highly homologous proteins can behave quite differently under similar conditions.

This highly purified MATX preparation was then used for characterization. The oligomeric state of the refolded protein was analyzed using gel filtration chromatography and sedimentation velocity. The elution volume of MATX (13.2 ml) was determined from MAT activity measurements of the fractions eluted from the gel filtration column (Fig. 6). According to the elution position of the markers on the Superose column, this elution volume corresponds to that expected for a globular protein of ~70 kDa. However, the $A_{280}$
profile indicates elution of the protein as two peaks in positions compatible with tetrameric and dimeric oligomers. In addition, sedimentation velocity experiments identify species with calculated $s_{20,w}$ values of $3.4 \pm 0.1$ and $6.4 \pm 0.1$ S. These hydrodynamic measurements are compatible with the presence in the sample of monomers (compact or globular) and compact dimers, according to the $A_{280}$ measurements of sedimentation velocity experiments, from which only compact dimers were active as deduced from the gel filtration chromatography profile. Detection of tetrameric species could be ascribed to a partial association during sample concentration preceding gel filtration chromatography, whereas that of monomeric species could be due to partial denaturation of the sample during ultracentrifugation, phenomena described to occur with a variety of enzymes [37,38]. As deduced from these data, MATX is a new member of the growing group of homodimeric MATs that includes mammalian MAT III, archaeal MATs and *Mycobacterium tuberculosis* MAT [7,15,35,39,40]. The AdoMet synthesizing activity of some of these dimeric MATs is stimulated by DMSO at low methionine concentrations [7]. Therefore, we analyzed this stimulatory effect on MATX and found that this dimeric MAT is not stimulated under similar conditions ($1.80 \pm 0.15$ vs. $1.67 \pm 0.11 \mu$mol/min/mg).

MAT activity is dependent on divalent ($\text{Mg}^{2+}$) cations and stimulated by monovalent ($\text{K}^+$) ions [7], and MATX is not an exception to this rule. Its dependence on these essential cations was explored and the data obtained appear in Table 2. The affinity for the cations is in the millimolar range, similar values to those measured for refolded MAT III [32]. Kinetics for the substrates, methionine and ATP, showed Michaelis-Menten behavior, the calculated $K_m$ values being about half of those exhibited by MAT III (Table 2)[32]. In addition, the $V_{\text{max}}$ is ~18 fold higher than that of MAT III [32], and similar to that of *E. coli* MAT (cMAT)[6] or *Methanococcus jannaschii* MAT (Mj-MAT)[40]. The
tripolyphosphatase activity exhibited by the MAT family was also evaluated for MATX. Kinetics for PPP$_i$ showed a $V_{\text{max}}$ for this substrate similar to that shown by refolded-MAT III in the presence of 50 µM AdoMet [32]. MATX tripolyphosphatase activity was stimulated by AdoMet ~23 fold, rendering a calculated $V_{\text{max}}$ ~2 fold larger than that of the AdoMet synthesis reaction as occurs for cMAT [6] (Table 2). However, this 2-fold increase in $V_{\text{max}}$ (1464.22 vs 2725.20 nmol/min/mg) is also observed when the AdoMet synthesis is measured as P$_i$ using the colorimetric method (3349.33 ± 71.61 nmol/min/mg). This stimulation occurs without a significant change in affinity for the PPP$_i$ substrate (Table 2), in contrast to the decreased affinity shown by cMAT in the presence of AdoMet (~4 fold)[6].

Structural characterization of refolded MATX was carried out using circular dichroism. Far-UV spectra were obtained (Fig. 7) and the overall secondary structure composition estimated by deconvolution (Table 3). The major elements were found to be the random coil and $\beta$-sheet as previously described for other members of the MAT family. Comparison of these data with the secondary structure composition published for other members of the MAT family showed an increase in the random coil component that is especially important when compared to archaeal or BsMAT [32,34,41]. The difference in subunit length between these MATs (471 vs. 400 residues)(Fig. 8C), and the fact that the insertions occur in loops [19], may account for such increased random coil content. In addition, some differences can also be expected from the diversity of programs used in this type of calculations by each laboratory. Near-UV spectra were also obtained (Fig. 7B), the main feature being the lack of signal at 265 and 295 nm. MATX contains five tryptophans (positions 181, 182, 314, 437 and 469) and fourteen tyrosines (positions 78, 148, 162, 195, 232, 311, 336, 360, 375, 413, 419, 434, 446 and 449) per subunit, two types of residues with transitions in this area of the spectrum. Hence, a compensatory effect between signals
of both types of residues must occur to render this profile. Such a compensatory effect has been previously observed in near-UV CD spectra from Mj-MAT (containing 1 Trp and 13 Tyr), despite the low degree of sequence conservation in archaeal MATs [41]. Moreover, the absence of a significant signal in the 250-270 nm range suggested the lack of disulphide bonds in the molecule, as expected from refolding being carried out under reducing conditions.

The sequence of MATX preserves the main blocks that characterize all the members of this family and that include the residues involved in substrate and cation binding (Fig. 8C) [10]. Residues involved in methionine binding locate to the N-terminal domain, the $^{27}$GHPDK$^{31}$ stretch (block I), and to a loop connecting this domain with the central domain that includes F$^{290}$. This phenylalanine is equivalent to F$^{251}$ of rMATα1 that was shown to have its aromatic ring stacked against the methionine analogue used for crystallization [17]. D$^{141}$ (block III), D$^{186}$ and K$^{188}$ (block IV) are equivalent to the residues involved in Mg$^{2+}$ binding and catalysis. Moreover, residues involved in K$^+$ coordination, G$^{303}$ and R$^{305}$, are also preserved (block V). Overall the sequence presents a high level of conservation, the main difference being the $\sim$30 residue extension at the C-terminal end and an insertion comprising residues 218-248 according to the sequence alignment (Fig. 8C). This high conservation allowed construction of a structural model using the MATX sequence and the structure of human MATα1 (PDB ID: 2OBV), its closest homologue. This model extended from residue 13 to 444 and preserved the three-domain organization of all MATs (Fig. 8). The main differences occur in the N-terminal domain, where the strands of the β-sheet seem longer, as well as the loop (residues 259-281) preceeding the last strand. In addition, one of the α-helixes in the outer side of this domain is also longer (residues 234-259), thus protruding to the environment. However, these changes do not
seem to affect positioning of the active site residues as compared with rMATα1 (Fig. 8A)[17,41], and hence the high activity observed comparable to that of cMAT.

In summary, we have been able to produce and refold MATX using an heterologous system. The enzyme is a homodimer showing AdoMet synthesizing capacity, but its $V_{\text{max}}$ and the affinities displayed for ATP and methionine are closer to those of the cMAT homotetramer rather than to Mj-MAT or rMAT III dimers. MATX activity is also dependent on divalent and monovalent cations. Moreover, the enzyme exhibits the triplyphosphatase activity typical of the MAT family that is stimulated in the presence of AdoMet.

ACKNOWLEDGEMENTS

S. E. and C. A. were fellows of the Erasmus Program from Porto University (Portugal). This work was supported by grants of the Ministerio de Ciencia e Innovación (BFU2008-00666 and BFU2009-08977 to M.A.P.). The authors wish to thank Dr. A. Martínez del Pozo from the Dept. of Bioquímica y Biología Molecular I of the Universidad Complutense de Madrid for his help with CD spectra, Dr. A. Rogers from the Dalhousie University (Canada) for the pEXT5-MATX plasmid and Brenda Ashley Morris for style and grammatical corrections.

REFERENCES


FIGURE LEGENDS

Fig. 1. SDS-PAGE electrophoresis of soluble and insoluble fractions of MATX overexpressing cells. Cultures of E. coli cells transformed with pT7.7-MATX were grown at 37°C to reach several cell densities (A$_{600}$). At this point cultures were divided into two samples and grown for 3 additional hours in the absence (lanes 1, 2, 4 and 6) or presence (lanes 3, 5 and 7) of IPTG. The cells were harvested by centrifugation and lysed to obtain soluble and insoluble fractions. (A) Samples of the soluble (30 µl containing ~150 µg) and (B) insoluble (20 µl containing ~30 µg) fractions were loaded in SDS-PAGE gels. Induction was done at A$_{600}$=0 (lane 1), 0.3, (lanes 2 and 3), 0.6 (lanes 4 and 5) and 0.8 (lanes 6 and 7). The figure shows results of a typical experiment.

Fig. 2. MAT activity in soluble fractions of cultures induced at different cell densities at 37°C. Cultures of E. coli cells transformed with pT7.7-MATX were grown at 37°C to reach several cell densities (A$_{600}$). At this point cultures were divided into two samples and grown for 3 additional hours in the absence (●) or presence (■) of IPTG. The cells were harvested by centrifugation and lysed to obtain soluble and insoluble fractions. MAT activity was measured in the soluble fractions. The figure shows the mean ± SEM of a typical experiment carried out in triplicate.

Fig. 3. MAT activity in soluble fractions of cultures induced at 30°C or 37°C. Cultures of E. coli cells transformed with pT7.7-MATX were grown at 37°C to reach A$_{600}$= 0.8. At this point cultures were divided into two samples and grown for 3 additional hours in the absence (●) or presence (■) of IPTG at 30°C (A) or 37°C (B). The cells were harvested by centrifugation and lysed to obtain soluble and insoluble fractions. MAT
activity was measured in the soluble fractions. The figure shows the mean ± SEM of a typical experiment carried out in triplicate.

**Fig. 4. Two step refolding of MATX from inclusion bodies.** Washed inclusion bodies were solubilized in 8 M urea buffer and incubated for 2 h at 10°C. The samples were then diluted to achieve final urea concentrations between 0.5-4M and immediately subjected to dialysis in the presence of 10 mM DTT. Samples were recovered and clarified by centrifugation and the soluble fraction assayed for MAT activity. The figure shows the mean ± SD of a typical experiment carried out in triplicate.

**Fig. 5. SDS-PAGE of the MATX purification steps.** The figure shows a scheme of the purification steps, where the number indicates the corresponding lane of the SDS-PAGE gel. Samples (40 µl) of representative purification steps were prepared for SDS-PAGE electrophoresis under reducing conditions and loaded on a 10% acrylamide gel. The samples correspond to: washed inclusion bodies solubilized in 8 M urea buffer (lane 1); refolded MATX (lane 2); the peak eluted from the Q-Sepharose cartridge (lane 3). MATX appear as the main band stained in all the fractions with an apparent Mr of 57600 according to the standards: phosphorylase B (97400), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000) and soybean trypsin inhibitor (21500).

**Fig. 6. Gel filtration chromatography of refolded MATX.** MATX was refolded from inclusion bodies following the two-step protocol described under Materials and Methods, purified by ion exchange chromatography and dialyzed. A sample (100 µl containing ~80 µg) was injected on a Superose 12 10/300 GL column equilibrated in 50 mM Tris/HCl pH 8, 10 mM MgSO₄, 150 mM KCl. Elution was carried out at a flow rate of 0.3 ml/min, the A₂₈₀ was recorded and 210 µl fractions were collected for MAT activity measurements. The elution volume of the markers was: Dextran Blue (8.69 ml); apoferritin (9.87 ml); β-
amylase (11.34 ml); alcohol dehydrogenase (12.07 ml); carbonic anhydrase (15.12 ml); and ATP (19.26 ml). The figure shows results from a typical experiment.

Fig. 7. Far- and near-UV circular dichroism spectra of refolded MATX. Samples of purified refolded MATX were used to obtain (A) far- and (B) near-UV circular dichroism spectra. The protein concentration of the samples was 0.25 mg/ml and 1 mg/ml for far- and near-UV spectra, respectively. After baseline subtraction the observed ellipticities were converted to mean residue ellipticities using a mean molecular mass per residue of 110 Da.

Fig. 8. Comparison of monomer structures for rat MATα1 (crystal) and MATX (structural model) and alignment of MAT sequences. (A) The structural model constructed using the Swiss-Model server and human MATα1 (PDB ID: 2OBV) as template is shown. The arrows indicate extensions in the N-terminal domain that affect a loop and α-helix. The active site residues appear as sticks (greencyan) over which the sequences are indicated. (B) The structure of the rat MATα1 monomer (PDB ID: 1QM4) is depicted for comparison. The position of the N-terminal residue for each monomer is also indicated. (C) Alignment of the MAT sequence mentioned in the text, including that of human MATα1 the higher homologue used for construction of the structural model.
# TABLE 1
Refolding and purification of MATX<sup>a,f</sup>

<table>
<thead>
<tr>
<th></th>
<th>Total protein (mg)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Total enzyme units&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea solubilization&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91.89</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis + DTT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22.99</td>
<td>24.88 ± 3.98</td>
<td>1153.71 ± 208.02</td>
<td>25.02</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>9.08</td>
<td>1.12 ± 0.39</td>
<td>1553.04 ± 238.61</td>
<td>9.88</td>
</tr>
</tbody>
</table>

<sup>a</sup>Washed inclusion bodies were used for refolding.
<sup>b</sup>Solubilization in the presence of 8 M urea for 2 h at 10ºC.
<sup>c</sup>The diluted sample was dialyzed against buffer A containing 10 mM DTT.
<sup>d</sup>Protein concentration was determined by Bradford protein assay.
<sup>e</sup>One unit is the amount of enzyme that catalyzed the production of 1 µmol AdoMet per minute.
<sup>f</sup>The table shows results of a typical purification.
<table>
<thead>
<tr>
<th><strong>TABLE 2</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kinetic parameters of MATX activities</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>MAT activity</strong>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><strong>MATX</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (nmol/min/mg)</td>
<td>$1464.22 \pm 164.26$</td>
</tr>
<tr>
<td>$K_m$ methionine (µM)</td>
<td>$85.35 \pm 28.42$</td>
</tr>
<tr>
<td>$K_m$ ATP (µM)</td>
<td>$260.36 \pm 38.90$</td>
</tr>
<tr>
<td>$K_m$ $K^+$ (µM)</td>
<td>$591.31 \pm 76.64$</td>
</tr>
<tr>
<td>$K_m$ Mg$^{2+}$ (mM)</td>
<td>$1.72 \pm 0.27$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Tripolyphosphatase activity</strong>&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (nmol/min/mg)</td>
<td>$117.28 \pm 15.73$</td>
</tr>
<tr>
<td>$K_m$ PPP&lt;sub&gt;i&lt;/sub&gt; (µM)</td>
<td>$82.75 \pm 22.24$</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/min/mg) + 50 µM AdoMet</td>
<td>$2725.20 \pm 115.15$</td>
</tr>
<tr>
<td>$K_m$ PPP&lt;sub&gt;i&lt;/sub&gt; (µM) + 50 µM AdoMet</td>
<td>$130.65 \pm 25.95$</td>
</tr>
</tbody>
</table>

<sup>a</sup>MAT activity measurements were carried out using 0.05 mg/ml protein samples for 30 min.

<sup>b</sup>Tripolyphosphatase activity determinations were performed using 0.05 mg/ml protein concentrations for 10 min.
<table>
<thead>
<tr>
<th>Secondary structure (%)</th>
<th>MATX</th>
<th>MAT I&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MAT III&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BsMAT&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>12.7 ± 0.5</td>
<td>23 ± 1.8</td>
<td>19 ± 1.1</td>
<td>81</td>
</tr>
<tr>
<td>β-sheet</td>
<td>25.1 ± 0.5</td>
<td>40 ± 1.2</td>
<td>34 ± 2.3</td>
<td>8</td>
</tr>
<tr>
<td>antiparallel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parallel</td>
<td>5.3 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-turns</td>
<td>21.5 ± 0.5</td>
<td>18 ± 1.0</td>
<td>20 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Random coil</td>
<td>35.9 ± 0.5</td>
<td>19 ± 1.8</td>
<td>27 ± 0.5</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Secondary structure composition was calculated from the far-UV CD spectra using CDNN software (© G. Böhm)

<sup>b</sup>Calculated using the Convex Constraint method, data from reference 32

<sup>c</sup>Calculated using the CDpro software, data from reference 34
Figure 1

A

<table>
<thead>
<tr>
<th>IPTG</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers (kDa)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>97.4-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>66.2-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>45.0-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>31.0-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21.5-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

-MATX

B

<table>
<thead>
<tr>
<th>IPTG</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers (kDa)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>97.4-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>66.2-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>45.0-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>31.0-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21.5-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

-MATX
Figure 3

**A**

MAT activity (pmol/min/mg) over time (h).

**B**

MAT activity (pmol/min/mg) over time (h).
Figure 4

The graph shows the MAT activity (nmol/min/mg) at different urea concentration (M). The activity increases with increasing urea concentration, peaking at 1 M, and then decreases at higher concentrations.
Figure 5

Inclusion bodies

2M urea + 5% Triton X-100

washed inclusion bodies

8M urea solubilization (1)

dilution to 1M urea

dialysis with 10 mM DTT (2)

Q-Sepharose

MATX (3)

Markers (kDa)

1 2 3

- 97.4
- 66.2
- 45
- 31
- 21.5
Figure 6

MAT activity (dpm) vs. elution volume (ml)

A

0.01

0.02

0.03

0.04

0.280

10000

9000

8000

7000

6000

5000

4000

3000

2000

1000

0.01

0.02

0.03

0.04

0.1

10

20

30

40

50

60

70

80

90

100

110

120

130

140

150

160

170

180

190

200

210

220

230

240

250

260

270

280

290

300

310

320

330

340

350

360

370

380

390

400

410

420

430

440

450

460

470

480

490

500

510

520

530

540

550

560

570

580

590

600

610

620

630

640

650

660

670

680

690

700

710

720

730

740

750

760

770

780

790

800

810

820

830

840

850

860

870

880

890

900

910

920

930

940

950

960

970

980

990

1000

1010

1020

1030

1040

1050

1060

1070

1080

1090

1100

1110

1120

1130

1140

1150

1160

1170

1180

1190

1200

1210

1220

1230

1240

1250

1260

1270

1280

1290

1300

1310

1320

1330

1340

1350

1360

1370

1380

1390

1400

1410

1420

1430

1440

1450

1460

1470

1480

1490

1500

1510

1520

1530

1540

1550

1560

1570

1580

1590

1600

1610

1620

1630

1640

1650

1660

1670

1680

1690

1700

1710

1720

1730

1740

1750

1760

1770

1780

1790

1800

1810

1820

1830

1840

1850

1860

1870

1880

1890

1900

1910

1920

1930

1940

1950

1960

1970

1980

1990

2000

2010

2020

2030

2040

2050

2060

2070

2080

2090

2100

2110

2120

2130

2140

2150

2160

2170

2180

2190

2200

2210

2220

2230

2240

2250

2260

2270

2280

2290

2300

2310

2320

2330

2340

2350

2360

2370

2380

2390

2400

2410

2420

2430

2440

2450

2460

2470

2480

2490

2500

2510

2520

2530

2540

2550

2560

2570

2580

2590

2600

2610

2620

2630

2640

2650

2660

2670

2680

2690

2700

2710

2720

2730

2740

2750

2760

2770

2780

2790

2800

2810

2820

2830

2840

2850

2860

2870

2880

2890

2900

2910

2920

2930

2940

2950

2960

2970

2980

2990

3000

3010

3020

3030

3040

3050

3060

3070

3080

3090

3100

3110

3120

3130

3140

3150

3160

3170

3180

3190

3200

3210

3220

3230

3240

3250

3260

3270

3280

3290

3300

3310

3320

3330

3340

3350

3360

3370

3380

3390

3400

3410

3420

3430

3440

3450

3460

3470

3480

3490

3500

3510

3520

3530

3540

3550

3560

3570

3580

3590

3600

3610

3620

3630

3640

3650

3660

3670

3680

3690

3700

3710

3720

3730

3740

3750

3760

3770

3780

3790

3800

3810

3820

3830

3840

3850

3860

3870

3880

3890

3900

3910

3920

3930

3940

3950

3960

3970

3980

3990

4000
Figure 7

(A) 

\[ \theta_{mrw} \quad \text{(deg x cm}^2 \times \text{dmol)} \]

\[ \lambda \quad \text{(nm)} \]

(B) 

\[ \theta_{mrw} \quad \text{(deg x cm}^2 \times \text{dmol)} \]

\[ \lambda \quad \text{(nm)} \]
Figure 8

A

N-terminal domain

13

DGK

281

SGKD

GHPDK

B

N-terminal

N-terminal