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Subunit association as the stabilizing determinant for archaeal methionine adenosyltransferases

Francisco Garrido¹, Carlos Alfonso², John C. Taylor³, George D. Markham³, and María A. Pajares¹

¹ Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain

² Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid

³ Institute for Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA19111, USA

SUMMARY

Archaea contain a class of methionine adenosyltransferases (MATs) that exhibit substantially higher stability than their mesophilic counterparts. Their sequences are highly divergent, but preserve the essential active site motifs of the family. We have investigated the origin of this increased stability using chemical denaturation experiments on *Methanococcus jannaschii* MAT (Mj-MAT) and mutants containing single tryptophans in place of tyrosine residues. The results from fluorescence, circular dichroism, hydrodynamic, and enzyme activity measurements showed that the higher stability of Mj-MAT derives largely from a tighter association of its subunits in the dimer. Local fluorescence changes, interpreted using secondary structure predictions, further identify the least stable structural elements as the C-terminal ends of β -strands E2 and E6, and the N-terminus of E3. Dimer dissociation however requires a wider perturbation of the molecule. Additional analysis was initially hindered by the lack of crystal structures for archaeal MATs, a limitation that we overcame by construction of a 3D-homology model of Mj-MAT. This model predicts preservation of the chain topology and three-domain organization typical of this family, locates the least stable structural elements at the flat contact surface between monomers, and shows that alterations in all three domains are required for dimer dissociation.

Keywords

S-adenosylmethionine synthetase; stability; structural model; thermophile; tryptophan mutants; methionine adenosyltransferase

1. INTRODUCTION

Methionine adenosyltransferases (MATs) are a family of enzymes that catalyze the synthesis of the universal metabolite S-adenosylmethionine (AdoMet) from methionine and ATP [1]. Phylogenetically these proteins appear in all types of organisms, except some parasites that

Address correspondence to: María A. Pajares, Instituto de Investigaciones Biomédicas “Alberto Sols” (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain. (Phone: 34-915854414; FAX: 34-915854401; email: E-mail: mapajares@iib.uam.es).

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seem to obtain AdoMet from the host [2]. Catalytic MAT subunits present a very high degree of sequence conservation (~80%) among Bacteria and Eukaryotes [2,3], whereas for archaeal MATs the conservation is reduced to ~20% [4]. However, archaeal MATs preserve most of the characteristics of the family including their Mg^{2+} and K^{+} dependence for AdoMet synthesis, their tripolyphosphatase activity and their presence as oligomers, in this case homodimers. Their affinity for methionine is slightly below millimolar [5], between that of mammalian homotetramers (~0.1 mM) and homodimers (1 mM). Differences arise in the larger stability of the archaeal protein, which exhibits its maximal catalytic activity at $\geq 55^{\circ}C$, the T_m for mammalian MAT I/III inactivation [6].

Crystal structures for *Escherichia coli* [7] and mammalian MATs [8 and PDB code 2P02] have been reported. Two active sites per dimer are located between pairs of monomers that interact through a flat hydrophobic surface. The subunits have an α/β topology, and show a common organization in three domains formed by non-consecutive stretches of the amino acid sequence [9]. This complex organization seems to be achieved *in vivo* through assisted folding, as indicated by the identification of *E. coli* MAT among the GroEL/GroES substrates within the bacterium [10]. However, the size of the protein precludes association of the subunits in the chaperonin, thus suggesting the possibility of folding intermediates, whose existence has been detected *in vitro* for mammalian MAT I/III [11–13]. In fact, during unfolding of MAT I/III a high population of one apolar monomeric intermediate accumulates and favors the final folding step and subunit association [11,14]. Similar structural information about archaeal MATs is however lacking, and hence questions about structural preservation and about the key elements that provide additional stability to the enzyme have emerged. To approach these problems we have analyzed the stability of an archaeal MAT through the use of denaturants in conjunction with single tryptophan mutants and the results have been interpreted in the context of a 3D-structural model of the protein.

2. MATERIALS AND METHODS

2.1 Analysis of Mj-MAT secondary structure

In order to overcome the lack of structural information concerning Mj-MAT, secondary structure predictions were carried out. For this purpose, the Mj-MAT sequence (Q58605) and several programs and servers were used. These included PHD [15], SSPro (associated with the Prime software), PsiPred [16] and the NNpredict servers (http://alexander.compbio.ucsf.edu/_nomi/nnpredict.html). The results obtained differed slightly in the length of the predicted elements, but not in their location. These elements were only considered when the reliability score was $\geq 70\%$ for α -helix and $\geq 50\%$ for β -sheet. Thus, predictions obtained with SSPro and PsiPred [16], programs which interface with the chosen 3D-homology modeling package, were preferred in preparation of a structural model.

2.2 Construction of a model for Mj-MAT structure

A search for Mj-MAT homologues with known structures was performed using BLAST and PSI-BLAST and the non-redundant NCBI database. For three-dimensional model building the program Prime v. 1.6 with its associated graphical interface Maestro v. 7.5 (Schrodinger LLC, NY) and the secondary structure predictions mentioned above were used. The protein family that includes Mj-MAT was identified via a hidden Markov model using the program Hmmer v. 2.3 [17]. The model was built using the 1.2 Å resolution structure of the human MAT α 2 protein (PDB code 2P02) as template, which preserves the topology of the highest homologue, *Escherichia coli* MAT (30% sequence homology). The consensus of the secondary structure predictions was used in determining the main chain conformation; sequences not belonging to regular secondary structure were roughly modeled by the loop modeling routines in Prime, and are considered only as guides. The dimer model was constructed using the same symmetry

operations that apply to eukaryotic MATs. The resultant model was subjected to 5000 steps of restrained energy minimization to remove bad atomic contacts; heavy atoms were constrained by a flat-bottomed harmonic potential without penalty for movement within 0.3 Å of their initial positions and with a 25 kcal/(mol·Å²) penalty for larger deviations. The energy was minimized using the OPLS (2001) force field in MacroModel v. 9.0 (Schrodinger LLC, NY). Solvent accessible surface areas for the side chains (including the β-CH₂ group) were calculated using the program Maestro v. 7.5 (Schrodinger LLC, NY).

2.3 Protein expression and purification

Wild type and mutant Mj-MATs were expressed in *E. coli* BL21(DE3) cells transformed with pMJ1208-1 or the mutant plasmids as described [4]. Lysis was performed by sonication (30 cycles, 30 s on/off) in an ice bath. The soluble fraction was used for Mj-MAT purification as previously described [5]. Protein concentration was determined, unless otherwise stated, using the A₂₈₀ of the sample after 30 minutes incubation in 6 M guanidine hydrochloride (Calbiochem, La Jolla, CA) and the calculated extinction coefficients for wild type and single tryptophan mutants (24920 M⁻¹ cm⁻¹) or for W387F (19370 M⁻¹ cm⁻¹).

2.4 MAT activity measurements

The enzyme activity assays measured S-adenosylmethionine formation from [methyl-¹⁴C]-L-methionine (Moravek Biochemicals, Brea, CA) and ATP. Measurements were performed at 55°C with protein concentrations of 60–300 µg/ml as determined by the Bradford Assay Kit (Bio Rad, Hercules, CA)[5]. Methionine (Sigma Chemical Co., St Louis, MO) concentrations ranged from 0.05–1mM for K_m^{Met} determinations in the presence of 2.5 mM ATP (Sigma Chemical Co., St Louis, MO), whereas K_m^{ATP} measurements were performed using 0.5 mM [methyl-¹⁴C]-L-methionine and ATP concentrations between 0.05 to 2.5 mM. Data were analyzed by fitting to the Michaelis-Menten equation using the program SigmaPlot v. 10.0 (Systat Software, Point Richmond, CA).

2.5 Denaturation experiments

Denaturation was carried out using urea (0–8 M; Merck, Darmstadt, Germany) in 50 mM Tris/HCl pH 8, 50 mM KCl, 10 mM MgSO₄ (buffer A). Preliminary experiments were carried out to determine the incubation period necessary to reach the reaction endpoint; these were established to be overnight incubations at 23°C, since longer incubation times (up to 48 hours) or higher temperatures (up to 37°C) rendered identical results. Refolding experiments were performed after dilution of denatured Mj-MAT samples (in 8 M urea) to the desired final denaturant concentrations using buffer A. Mj-MAT concentrations in the assay varied according to the sensitivity of the technique as specified below. Characteristic concentrations at the midpoint of the transitions (D_{50%}) were calculated using GraphPad Prism v. 5.0 (GraphPad Software, San Diego, CA). A minimum of three protein batches were used in denaturation experiments.

2.5.1 Denaturation followed by MAT activity—Protein samples treated with 0–8 M urea were used to evaluate the denaturant effect on Mj-MAT activity. Measurements were carried out in triplicate at 55°C, using a protein concentration of 0.05 mg/ml, essentially as described previously [18]. In this case, the standard MAT reaction mixture contained the corresponding denaturant concentrations (0–8 M) in order to keep them constant in the final reaction volume of 250 µl.

2.5.2 Intrinsic fluorescence measurements—Mj-MAT samples (5–100 µg/ml), after incubation with the denaturant, were used for fluorescence measurements upon excitation at 295 nm with a slit width of 2.5 nm. The exception to this protocol was the case of W387F, a

mutant that only contained tyrosine residues, and hence its excitation was performed at 275 nm using the same slit width. Fluorescence emission spectra were recorded between 300 and 400 nm using a 5 nm emission slit width and 0.5×0.5 cm cuvettes in a photon counting SLM-8000 spectrofluorometer at 25°C. The fluorescence signal for the protein was corrected by subtraction of the solvent signal. The ratio of fluorescence intensities I_{330}/I_{355} was determined to obtain insight into the average exposure of the tryptophan residues during unfolding.

2.5.3 ANS binding—A stock solution containing 100 mM ANS (Sigma Chemical Co., St Louis, MO) in methanol was prepared, and its concentration was determined as described previously [10]. Mj-MAT unfolding reactions (1 ml, 50 µg/ml, 0–8 M urea), and control reactions without protein, received ANS (0–40 µM) and were incubated for an additional hour; the final methanol concentration in the samples was 0.4% (v/v). Changes in ANS emission at 470 nm were monitored from the corrected fluorescence spectra recorded between 400–600 nm using excitation at 380 nm with a 2.5 nm slit width.

2.5.4 Circular dichroism determinations—Urea denaturation reactions containing Mj-MAT samples were used to record far- and near-UV CD spectra on a Jasco J-720 spectropolarimeter at 25°C. Far-UV measurements were carried out using 0.2–0.3 mg/ml protein concentrations and 0.1 cm pathlength cuvettes, whereas near-UV spectra were recorded using 1 mg/ml protein samples and 1 cm 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. The secondary structure composition was calculated using the Convex Constraint Analysis (CCA) with the original set of reference proteins [19]. A minimum of five spectra was averaged for each sample.

2.5.5 Sedimentation velocity experiments—Denatured Mj-MAT samples (0–8M urea) containing 0.3–0.4 mg/ml protein concentrations were used in these studies. The experiments were performed at 188000 g and 18°C in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) equipped with absorbance optics, using an An50Ti rotor. Absorbance scans (0.005 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 [20,21]. 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 [22].

2.5.6 Gel filtration chromatography—Urea denatured protein samples (500 µl) at 0.5 mg/ml protein concentrations were loaded on a Biogel A 1.5m column (45 ml; Bio Rad, Hercules, CA) equilibrated in buffer A containing the same denaturant concentration. The flow rate was 7.5 ml/h and 0.5 ml fractions were collected for protein detection using the BCA kit (Pierce, Rockford, IL). The standards (Sigma Chemical Co., St Louis, MO and GE Healthcare Europe GmbH, Barcelona, Spain) used were: blue dextran (2000 kDa), tyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66.2 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ATP (507 Da).

2.6 Site-directed mutagenesis

Mutagenesis was carried out using the oligonucleotides included in Table 1 and the QuikChange site-directed mutagenesis method (Stratagene, Cedar Creek, TX), following manufacturer's instructions. The first step was the preparation of the W387F mutant in the pMJ1208-1 vector containing the wild type sequence. Thereafter the W387F mutant plasmid was used as template to construct the tyrosine to tryptophan mutants. Mutations were verified by automatic sequencing.

3. RESULTS AND DISCUSSION

Methanococcus jannaschii MAT (Mj-MAT), the best studied member of the archaeal class of MATs, was chosen to obtain insight into the determinants that provide higher stability to these isoenzymes. The wild type protein was overexpressed in *Escherichia coli*, purified and characterized before attempting this task. Recombinant Mj-MAT elutes with a calculated mass of approximately 80.2 kDa upon gel filtration chromatography and has a $s_{20,w}$ of 5.75, as expected for a globular dimer. Upon excitation at 295 nm, the single tryptophan of the molecule (W387) exhibits a fluorescence maximum at 336 nm (Figure 1A), suggesting its presence in an apolar environment. Minima at 222 and 210 nm were features of the native Mj-MAT far-UV CD spectrum (Figure 2A), deconvolution of which indicates that the major secondary structure (SS) elements consist of α -helix and β -sheet (53% α -helix, 27% β -sheet, 13% β -turns and 7% random coil). Comparison with CD data for eukaryotic MATs reveals a larger percentage of helicity in Mj-MAT (53% vs 20%), but a similar β -sheet content [5,14]. Differences in helicity content, according to far-UV CD data, also occur between Mj-MAT and BsMAT (53% vs 81%), the MAT isoenzyme of *Bacillus subtilis*, a mesophilic organism able to survive under extreme conditions through spore production [23]. The Mj-MAT near-UV CD spectrum showed a characteristic signature between 280–295 nm (Figure 2B). Changes in these parameters in the presence of urea and comparison of their values with previous data for mesophilic MATs were examined to identify determinants that provide increased stability to Mj-MAT.

Urea denaturation titrations (0–8M) were carried out and the effects on the protein were followed by several techniques that allowed analysis of the changes induced by the denaturant at the active site (activity measurements), the secondary (far-UV CD), the tertiary (near-UV CD and fluorescence spectroscopy) and quaternary structures (hydrodynamic measurements). Data were fit to the presence of two- or three-state processes. Good correlation coefficients ($R^2 \geq 0.99$) were obtained for the presence of two transitions when monitored by activity (Figure 3), sedimentation velocity (Figure 4) and fluorescence spectroscopy (Figure 1B), whereas far-UV CD data presented only one transition (Figure 2C). Alterations in fluorescence curves (Figure 1B) could also indicate a two-state process with a sloping native baseline, however a three-state fit is more consistent with the data ($R^2 = 0.99$ vs $R^2 = 0.97$). The native state is defined by the initial plateau of the curves which encompasses an urea range that depends on the protein concentration used. Maximal denaturation was obtained at 8 M urea with a reduction in the $s_{20,w}$ to < 2.3 and preservation of 40% of the θ_{220} , as well as a 45% decrease in the fluorescence intensity ratio and a partial red-shift of the fluorescence emission $\lambda_{\max}$ to 345 nm. These data suggest incomplete unfolding of Mj-MAT even at the highest urea concentration tested, although monomer production is obtained according to the $s_{20,w}$ value. Comparison with the available data for other members of the MAT family revealed that mesophilic isoenzymes, MAT III [11,12] and BsMAT [23], show complete loss of CD signature and total red-shift of their $\lambda_{\max}$ at 8M urea. Moreover, Mj-MAT retains activity even in 6 M urea, whereas complete loss of AdoMet synthesis and PPP_i hydrolysis was already observed at 1 M urea with MAT III [11,12] and BsMAT [23]. Thus, these results indicate that the differences between Mj-MAT and mesophilic MATs result not only in increased thermostability, but also in higher resistance to chemical denaturation.

Denaturation curves showing two transitions present a plateau between them, where the protein has constant properties. This plateau can be observed between 2.5–5.5 M urea depending on the technique chosen to monitor the changes, and suggests the presence of an intermediate state. Such an intermediate exhibits a 50% reduction in activity, a 15% decrease in the fluorescence intensity ratio without significant changes in $\lambda_{\max}$ and a calculated $s_{20,w}$ of ~ 3.32 . In contrast, no changes in secondary structure (Figure 2C) and hydrodynamic volume by gel filtration chromatography (Figure 4A) were observed in this denaturant range. Therefore, the

results indicate the presence of a species with reduced activity and a slight change in the average exposure of its single tryptophan (I_{330}/I_{355}). Uncertainties about its quaternary structure exist however, as judged by data from hydrodynamic measurements. The calculated sedimentation coefficient corresponds to that expected for a globular monomer, whereas gel filtration chromatography data indicate the presence of dimers until 3 M urea (Figure 4). This difference in hydrodynamic results suggests a weakening of the monomer-monomer interactions that allow dissociation of the oligomer upon ultracentrifugation. Dissociation of oligomers due to centrifugal forces has been reported for several proteins and depends on the strength of the interactions between subunits [24]. Comparison with MAT III and BsMAT, which already dissociate at <1M urea [11,12,23], indicate that the increased resistance of Mj-MAT to denaturation seems to derive from an enhanced stability of the dimer. The extra stability provided by subunit association seems to be one reason for hyperthermophiles to prefer oligomeric proteins [25]. For members of the MAT family, dimers represent a lower level of association than that observed in most mesophiles, but nevertheless the more stable oligomer.

Characteristic denaturant concentrations at midpoints of the transitions were calculated, and are listed in Table 2. Enzymatic activity measurements showed that alterations in the active site occur at lower urea concentrations than those in the tertiary structure near W387 when determined at the same protein concentration (50 $\mu\text{g/ml}$). Comparison with far-UV CD, analytical ultracentrifugation and gel filtration chromatography data was only possible through extrapolation, as these techniques require higher protein concentrations because of lower sensitivity. Fluorescence and far-UV CD data at 100 $\mu\text{g/ml}$ (not shown) indicate modifications in tertiary structure at lower urea concentrations than changes in SS. Additionally, alterations of the dimer are detected by ultracentrifugation at lower denaturant concentrations than those observed by gel filtration chromatography (Figure 4) and before changes in far-UV CD (i.e. SS), each measured at 300 $\mu\text{g/ml}$. Thus, dissociation of the subunits arises before important alterations in ellipticity take place, and differences in hydrodynamic data suggest production of “loose” dimeric species (I_2) in a narrow urea range. The partial inactivation observed at <2M urea also supports dimer alteration without dissociation (Figure 3), as the active sites in all known MATs require the contribution of residues from both subunits [7,8].

The last part of the plateau (4–5.5 M urea) and the second transition detected by activity loss (5–6.5 M urea) correspond to changes in ellipticity, the main alterations in fluorescence, a reduction in the $s_{20,w}$ of the monomer and detection of aggregates by gel filtration chromatography. Thus, in this denaturant range the SS alterations take place together with the largest changes in the tryptophan environment, leading to a monomeric intermediate (J), which is the species detected by sedimentation velocity and that is prone to aggregation according to the gel filtration results. All these results together suggest multi-state denaturation for Mj-MAT, although the number and association state of the intermediates differs from those of MAT III and BsMAT [11,12,23]. Furthermore, MATs also differ in the polar character of their intermediates as judged from their ANS binding capacities. Thus, lack of ANS binding to I_2 and J of Mj-MAT indicates exposure of polar surfaces in these species as also occurs with BsMAT intermediates [23], whereas monomeric MAT III intermediates exhibited apolar characteristics [11,12]. Mj-MAT meets requirements for multi-state unfolding such as a C_p value larger than zero and a size that exceeds the estimated average dimensions [26]. Other differences observed between Mj-MAT and mesophilic MATs include the lack of complete reversibility of the process for the archaeal form.

Further information about the stability of Mj-MAT tertiary structure was obtained from the fluorescence properties of mutants containing single tyrosine to tryptophan replacements on a W387F mutant background, taking advantage of the random distribution of tyrosine residues in the sequence. This procedure allows the introduction of a single intrinsic fluorophore at different predicted SS elements according to the PHD program (Figure 5). The resulting

mutants were characterized both functionally and spectrally, and the results are listed in Table 3. All of them retain substantial MAT activity, the largest decrease in k_{cat} being approximately 32% for W387F-Y49W, whereas a few mutants have increased k_{cat} values, up to 4.9-fold for W387F-Y233W. In addition, the largest changes in K_m values for methionine and ATP are 3.3- and 2-fold, respectively. Far-UV CD spectra do not show alterations in the SS composition for any of the mutants (Figure 2A). However, differences from the wild type protein are observed upon comparison of the near-UV CD spectra, a region that is dominated by the environment of the tryptophan and which lacks a characteristic signature in the mutants (Figure 2B). Analytical ultracentrifugation results show no significant differences in the calculated $s_{20,w}$ of any of the mutants as compared to wild type Mj-MAT, indicating that their globular dimeric association is not perturbed by the mutations (Table 3). Fluorescence emission spectra display different maxima (Table 3), which allow classification of the mutated residues as located in apolar [amino acids 387 (wt), 49, 120, 170, 226, 233 and 323], moderately polar [positions 255, 267 and 344] or polar environments [residues 72, 85, 273, and 371]. Several mutants show reduced fluorescence intensity, especially W387F-Y255W, which retains only 18% of that of the wild type protein, thus suggesting their location in microenvironments that cause quenching of the fluorescence signal.

Analysis of the mutants by urea denaturation was carried out using activity (Table 4) and fluorescence measurements (Table 5), the lack of a near-UV CD signature precluding additional studies using this technique. Activity measurements showed the existence of two transitions for all the mutants. Differences in their characteristic $D_{50\%}$ values from the wild type were observed during the first transition for mutants at residues 120, 226, 233, 255, 267, 344 and 371, whereas alterations in the second transition were seen for those at positions 49, 233 and 344. On the other hand, when denaturation of the mutants was followed by fluorescence the displacement of the tryptophan emission maxima to 355 nm was induced at 8M urea for most mutants, indicating their exposure to a polar environment. Exceptions to this rule were mutants at positions 49, 233 and 323, for which the environment becomes moderately polar as judged by an incomplete red-shift of their emission. The urea titration curves exhibited two transitions except for W387F-Y371W and W387F-Y233W (Figure 1C), which showed one and three transitions, respectively. Higher susceptibility to the denaturant as compared to the wild type is seen during the first transition for tryptophan probes at positions 49, 72, 85, 170, 233 and 273, whereas proteins with substitutions at residues 120 and 226 are more resistant at this transition. In the second transition, higher susceptibility to urea is observed for mutants at residues 49, 273, 323 and 344, whereas only the third transition for W387F-Y233W showed a larger $D_{50\%}$. In general, alterations in the active site take place at lower denaturant concentrations than changes in tertiary structure around the corresponding tryptophan, except for the alterations at low urea concentrations observed for mutants at positions 72, 85 and 170. Sedimentation velocity experiments carried out with these three mutants showed ~30% reductions in the $D^1_{50\%}$ values for mutants at residues 72 and 170, but no change in this parameter for the mutant at position 85. This decrease in $D^1_{50\%}$ is significant as compared to the wild type value, but still in the range between the changes in tertiary structure and those in activity for these mutants. Therefore, it can be deduced that a higher susceptibility to dissociation exists when positions 72 and 170 are changed.

In view of the urea denaturation results and to aid in their interpretation, we prepared a 3D-homology model of Mj-MAT (Figure 6). The search for homologues with known structure rendered other members of the MAT family, which share low sequence homology (17–30%) with Mj-MAT, but preserve active site motifs and predicted SS. The use of homologues with such characteristics, despite low sequence conservation, has been shown previously to produce structural models with a high accuracy level [27]. Therefore, the α -subunit of human MAT II (MAT α 2), for which a 1.2 Å resolution crystal structure is available (PDB code 2P02), was utilized as the closest homologue. Sequence and structural changes are expected to occur

between mesophilic and thermophilic homologues in order for the later to acquire their higher thermostability, and a combination of factors typically seem to contribute to this global effect [28–30]. Among the various stabilizing determinants that have been proposed for proteins from thermophiles, Mj-MAT only meets the increased percentage of charged residues (glutamic acid and lysine especially), whereas a slight decrease in hydrophobic amino acid content is also observed and the average length for MAT chains is maintained [2]. The use of MAT α 2 as template fulfills additional considerations such as functional conservation, with preservation of the active site residues (Figure 6B and C), and hence potential maintenance of the reaction mechanism [5]. Conservation of the active site structure need not be accompanied by structural conservation at other levels, but correlation of both the length and order of appearance of SS elements with that predicted for Mj-MAT is also observed (Figure 5). Based on its calculated SS composition, the predictions classified Mj-MAT as an α/β protein (37% α -helix, 22% β -strand and 41% loop plus random coil) in agreement with data for other MATs. Differences observed between far-UV CD (53% α -helix) and theoretical percentages may derive from assignment of random coil to segments where the templates show short helices. Moreover, calculations of SS composition from experimental data may vary among the techniques used and important divergences can be obtained depending on the set of reference proteins used for analysis. In fact, previous divergences among crystal structure and far-UV CD data (up to 14%) for MAT III have been reported [6].

The Mj-MAT model includes residues 17–387, organized in the SS elements listed in Table 6, leading to the typical three-domain organization of MAT monomers. The overall structure shows α -helices at the surface of the oligomer, whereas β -sheets are part of the monomer-monomer interface (Figure 6). The arrangement of monomers in the proposed dimer model differs from that in the template, but changes in the relative orientation of the subunits in MAT dimers were previously reported upon comparison of *E. coli* and MAT I crystal structures [8, 9]. Thus, although the programs used impose some limitations on the proposed arrangement, alterations in subunit orientation compared to the mesophilic forms can be anticipated.

The positions of the aromatic residues used as reporters in this study were found to be distributed among domains in the model and to occur within different SS elements. Inspection of the model revealed amino acids 72, 170 and 344 located at the interface between monomers (Figure 6B), whereas residues 85, 226, 233, 255, 273 and 371 appear at the dimer surface, close to the subunit interface (Figure 6D and E). The remaining mutants (49, 120, 267, 323 and 387) are located at the external surface of the monomers (Figure 6). The aromatic rings of tyrosines 120, 170, 226, 233, 255, 267, 323, 344 and 371 appear in apolar environments oriented to either the inside of the molecule or in the monomer-monomer contact area, whereas those of residues 49, 72, 85, 273 and 387 (the last residue in the model) appear to be at least partially exposed to the solvent or are in the active site. Most of the mutations that cause important kinetic changes are located in the interface between subunits in the model, where the active sites are found or, as occurs with Y233W, in elements placed near the entrance to the catalytic sites. Moreover, the characteristics of the environments of aromatic residues proposed by the model roughly coincide with the $\lambda_{\max}$ for fluorescence emission exhibited by the corresponding mutants. Exceptions are the $\lambda_{\max}$ of mutants at positions 72 and 85 that correspond to polar environments, probably due to their presence in the active site cavity. Furthermore, the model shows the tryptophan of W387F-Y255W in the proximity of charged residues (E256 and R178), thus providing an explanation for its quenched fluorescence signal [31]. Thus, all these data together support the validity of the proposed model, and hence the conservation of the overall structure of MAT proteins.

Structural data derived from the proposed model allow further interpretation of the experimental urea denaturation data (Figure 7). Tertiary structure changes around residues in β -strands of the central and C-terminal domains (E2, E3 and E6) that compose the monomer-

monomer interface are detected at urea concentrations (< 1 M) where native activity levels are preserved. Local changes near these SS elements may cause softening of intersubunit interactions, and thus result in production of “loose” dimers. In fact, mutants in β -strands E2 and E3 are more susceptible to dissociation upon ultracentrifugation. Mj-MAT dissociation requires urea concentrations > 4 M, much greater than mesophilic MATs [11,12] and even BsMAT [23]. Many changes in tertiary structure can be detected below these concentrations, but most importantly some elements of each of the three domains have attained all their detectable alterations (H1, H2, H6, E9 and E12). Among these are some helices that constitute the most temperature labile elements in MAT III [6], which denature at temperatures where Mj-MAT has not yet reached its maximal enzymatic activity [5]. However, there is no information about specific SS elements affected by chemical denaturation of MAT III, although its dissociation to monomers at < 1 M urea suggests that elements at the dimer interface are among the most labile. Nevertheless, changes in mutated residues at the N- or C-terminal ends of some β -strands indicate initial perturbations in the most exposed areas of these elements, whereas total denaturation and dissociation requires much higher urea concentrations. This requirement for a high denaturant concentration suggests either an increase in the number and strength of intersubunit interactions or differences in urea accessibility that could reflect the proposed divergence in the relative orientation of the monomers. Monomer unfolding and aggregation occur in a denaturant range where the remaining alterations in tertiary structure, the SS changes, and reductions in $s_{20,w}$ take place, thus suggesting global structural modifications.

In summary, denaturation studies show that the higher stability observed for Mj-MAT derives from a tighter association of the dimer. These results, interpreted with the aid of a 3D-model for the protein structure that predicts preservation of the chain topology, further indicate that the less stable elements correspond to the C-termini of β -strands E2 and E6, as well as to the N-terminus of E3. Location of these strands at the dimer interface, where the active sites are formed, is consistent with partial inactivation upon their perturbation. Finally, the complex organization of the monomers, and the detection of intermediates during denaturation, point to a multi-state folding mechanism for this protein, which *in vivo* may need the assistance of archaeal chaperonins [32].

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Abbreviations

MAT	methionine adenosyltransferase
Mj-MAT	methionine adenosyltransferase from <i>Methanococcus jannaschii</i>
BsMAT	methionine adenosyltransferase from <i>Bacillus subtilis</i>
IPTG	isopropyl β -D-thiogalactoside

ANS

8-anilidonaphthalene-1-sulphonic acid

SS

secondary structure

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Figure 1. Denaturation of Mj-MAT followed by fluorescence

The effect of different urea concentrations on the Mj-MAT tryptophan fluorescence was determined. Panel A shows spectra of the native and denatured wild type protein at 50 μ g/ml. Panel B depicts denaturation titration curves (0–8 M) for wild type Mj-MAT at (■) 5 μ g/ml and (▲) 50 μ g/ml protein concentrations. Panel C shows urea titration curves for mutants with behavior clearly differing from the wild type Mj-MAT. The results presented in panels B and C are from a typical experiment for each type of protein, and the lines correspond to fitting of the data to the presence of several transitions using GraphPad Prism.

Figure 2. Unfolding of Mj-MAT followed by circular dichroism

The figure shows CD spectra for wild type Mj-MAT and representative mutants carried out at a protein concentration of 250 μ g/ml. Panel A shows far-UV CD spectra, whereas panel B shows those in the near-UV range. Panel C presents data from urea denaturation of wild type Mj-MAT. The results were fit to the presence of either one or two transitions using GraphPad Prism. The figure shows the results of a typical experiment.

Figure 3. Denaturation followed by activity measurements

The effect of urea denaturation on Mj-MAT activity at different urea concentrations was measured at 50 μ g/ml protein. The results were fit to the presence of either one or two transitions using GraphPad Prism. The figure shows results of a typical experiment carried out in triplicate (mean $\pm$ SD).

Figure 4. Urea denaturation followed by sedimentation velocity and gel filtration chromatography Mj-MAT was incubated in the presence of urea, and samples were used for both gel filtration chromatography or sedimentation velocity experiments. Panel A shows the estimated M_r calculated from gel filtration profiles (■) and the calculated sedimentation coefficients from sedimentation velocity experiments (●). Panel B includes the percentage of the detected species shown by sedimentation velocity. The figure presents results of a typical experiment.

Figure 5. Alignment of Mj-MAT and MATα2 sequences and secondary structure elements
The figure shows the alignment of Mj-MAT and MATα2 sequences with the conserved residues in red, the mutated residues highlighted in yellow and the active site residues are highlighted in magenta. The secondary structure elements identified by PHD prediction [15] and in the proposed 3D-model for Mj-MAT are also shown. Moreover, the secondary structure elements identified in the MATα2 crystal structure used as template for model construction are also depicted. Secondary structure elements containing the mutated tyrosine residues appear with their assigned names. The last part of Mj-MAT sequence that has been excluded from the model appears highlighted in orange.

Figure 6. Prediction of structural elements and illustration of a 3D-model for Mj-MAT

Panel A shows a ribbon picture of the Mj-MAT monomer model with the secondary structure elements labeled and the domains circled. Panel B shows the Mj-MAT monomer interface including the mutated residues (blue) and the active site motifs (cyan). Panel C depicts the MAT α 1 monomer interface with the active site motifs (cyan) for comparison. Arrows indicate the entrances to the active sites for both Mj-MAT (panel B) and MAT α 1 (panel C). Panels D and E depict two different views of the calculated molecular surface of the Mj-MAT dimer model (subunits A and B in salmon and palecyan, respectively) including W387 (blue) and the mutated tyrosines (red).

Figure 7. Schematic representation of the perturbations observed in Mj-MAT domains as a function of denaturant concentration

The figure indicates the changes observed as a function of urea concentration using a schematic representation of the monomer domains: N-terminal (N), central (c) and C-terminal (C). The native dimer (N_2), intermediates (I_2 and J), as well as the unfolded state (U) are presented. The domains that alter their tertiary structure are shown by an increased darkness of the color as the denaturant concentration rises. The points at which activity alterations are detected in the wild type protein appear indicated as “active site”. Changes in the monomer shape that allow aggregation are depicted as flattening of the subunits.

Table 1

Oligonucleotides used for Mj-MAT mutagenesis.

Mutant	Oligonucleotide sequence (sense strand) ^a
W387F	5'-GAGATAGCCAATAAATCTTAGATAACATCATGGAAG-3'
Y49W	5'-GGCTTTATGTAAGATGTGGATGGAGAAGTTTGG-3'
Y72W	5'-GGGGGACATGCATGGCCTAAGTTTGGAGG-3'
Y85W	5'-GGTAAGCCCTATTGGATTTTATTATCTGGAAGAGC-3'
Y120W	5'-GCTGTAAAGCTGCTAAAGAA ^{TGG} TTAAAGAAGTTTAAAG-3'
Y170W	5'-CATTTGGAGTAGGTTGGGCTCCATTATCAACAAC-3'
Y226W	5'-GGCTGTTGTTGATAGGTGGGTTAAAAATATTGAGG-3'
Y233W	5'-GTTAAAAATATTGAGGAATGGAAGGAAGTTATTGAAAAGG-3'
Y255W	5'-GAAGATAGCTGATGGATGGGAGGTTGAAATTC-3'
Y267W	5'-CAGCAGATGATTGGGAGAGGGAGAGTGTC-3'
Y273W	5'-GAGGGAGAGTGCTGGCTAACAGTTACTGG-3'
Y323W	5'-GTAAATCACGTTGGTAAATCTGGAATATCTTAGCAAAC-3'
Y344W	5'-GGAGTTAAAGAGTGCTGGGTTAGAATATTAAGCC-3'
Y371W	5'-CTGAAGATAGCTGGGATATAAAGGATATTGAACC-3'

^aThe bases changed in each case appear underlined.

Table 2

Parameters that define wild type Mj-MAT unfolding.

	Mj-MAT($\mu\text{g/ml}$)	Urea unfolding ^a	
		D ¹ _{50%} (M)	D ² _{50%} (M)
Activity	50	2.01 $\pm$ 0.13	5.79 $\pm$ 0.07
Analytical ultracentrifugation	300	1.82 $\pm$ 0.12	6.09 $\pm$ 0.21
Tryptophan fluorescence	5	1.65 $\pm$ 0.57	6.82 $\pm$ 0.11
	50	3.19 $\pm$ 0.31	6.93 $\pm$ 0.06
	100	3.35 $\pm$ 0.17	7.19 $\pm$ 0.20
Far-UV CD	250	-	6.76 $\pm$ 0.06 ^b
	300	-	7.01 $\pm$ 0.12 ^b

^aValues for the denaturant concentrations at midpoint of the transitions were calculated using equations for a two^b or three-state mechanism using GraphPad Prism v. 5.0. Results are shown as the mean $\pm$ SD.

Table 3

Characterization of Mj-MAT mutants.

Protein	k_{cat} (sec ⁻¹) ^a	K_m^{Met} (mM) ^a	K_m^{ATP} (mM) ^a	$s_{20,w}$	λ_{max} (nm) ^b
Wild type	1.46 ± 0.05	0.31 ± 0.06	0.19 ± 0.03	5.85	336
W387F	1.85 ± 0.08	0.31 ± 0.06	0.11 ± 0.03	5.9	305
W387F-Y49W	1.00 ± 0.3	0.66 ± 0.07	0.18 ± 0.03	5.6	335
W387F-Y72W	1.62 ± 0.15	0.13 ± 0.02	0.11 ± 0.03	6	352
W387F-Y85W	2.77 ± 0.06	0.57 ± 0.06	0.21 ± 0.05	5.6	349
W387F-Y120W	3.31 ± 0.08	0.54 ± 0.06	0.13 ± 0.02	5.7	333
W387F-Y170W	2.39 ± 0.03	1.0 ± 0.20	0.10 ± 0.02	5.6	333
W387F-Y226W	5.08 ± 0.06	0.30 ± 0.05	0.10 ± 0.01	5.8	337
W387F-Y233W	7.24 ± 0.08	0.47 ± 0.10	0.20 ± 0.08	5.6	330
W387F-Y255W	2.39 ± 0.06	0.50 ± 0.08	0.19 ± 0.05	5.9	338
W387F-Y267W	2.31 ± 0.06	0.45 ± 0.08	0.20 ± 0.05	5.9	342
W387F-Y273W	1.54 ± 0.06	0.30 ± 0.05	0.24 ± 0.05	5.8	353
W387F-Y323W	1.62 ± 0.09	0.13 ± 0.04	0.22 ± 0.03	5.7	330
W387F-Y344W	2.85 ± 0.08	0.33 ± 0.08	0.22 ± 0.09	5.3	344
W387F-Y371W	4.00 ± 0.08	0.25 ± 0.04	0.11 ± 0.01	5.8	348

^aThe mean ± SD for a typical experiment carried out in triplicate.

^bFluorescence emission maxima upon tryptophan excitation.

Table 4

Effects of urea on Mj-MAT activity.

Protein	Urea unfolding ^a	
	D ¹ _{50%} (M)	D ² _{50%} (M)
Wild type ^b	2.01 ± 0.13 ^b	5.79 ± 0.07 ^b
W387F	2.39 ± 0.18	6.20 ± 0.09
W387F-Y49W	1.85 ± 0.21	4.55 ± 0.12
W387F-Y72W	2.33 ± 0.19	5.70 ± 0.08
W387F-Y85W	1.86 ± 0.14	5.34 ± 0.11
W387F-Y120W	2.57 ± 0.16	5.73 ± 0.08
W387F-Y170W	1.90 ± 0.16	4.51 ± 0.08
W387F-Y226W	2.87 ± 0.17	5.92 ± 0.11
W387F-Y233W	1.61 ± 0.23	3.77 ± 0.09
W387F-Y255W	2.63 ± 0.17	5.83 ± 0.13
W387F-Y267W	1.62 ± 0.12	5.76 ± 0.06
W387F-Y273W	2.19 ± 0.12	5.43 ± 0.10
W387F-Y323W	2.24 ± 0.12	5.79 ± 0.15
W387F-Y344W	1.67 ± 0.11	3.53 ± 0.13
W387F-Y371W	1.15 ± 0.15	5.54 ± 0.06

^aData were fit to the presence of two- or three states to obtain the characteristic denaturant concentrations at midpoint for each transition. The results shown are the mean ± SD of a typical experiment carried out in triplicate at 50 µg/ml protein concentration.

^bData from table 2

Table 5

Data from denaturation curves followed by fluorescence spectroscopy.

Protein	Urea unfolding ^a		
	D ¹ _{50%} (M)	D ² _{50%} (M)	D ³ _{50%} (M)
Wild type ^b	3.19 ± 0.31 ^b	6.93 ± 0.06 ^b	-
W387F	1.72 ± 0.43	6.10 ± 0.19	-
W387F-Y49W	1.57 ± 0.38	5.53 ± 0.07	-
W387F-Y72W	0.44 ± 0.15	6.80 ± 0.57	-
W387F-Y85W	0.83 ± 0.26	7.14 ± 0.13	-
W387F-Y120W	4.10 ± 0.39	6.19 ± 0.12	-
W387F-Y170W	0.78 ± 0.05	6.81 ± 0.04	-
W387F-Y226W	4.72 ± 0.26	6.66 ± 0.08	-
W387F-Y233W	1.12 ± 0.16	4.23 ± 0.05	7.44 ± 0.15
W387F-Y255W	3.13 ± 0.29	6.49 ± 0.09	-
W387F-Y267W	2.64 ± 0.40	6.07 ± 0.05	-
W387F-Y273W	1.03 ± 0.51	5.43 ± 0.17	-
W387F-Y323W	3.18 ± 0.22	5.56 ± 0.05	-
W387F-Y344W	2.69 ± 0.62	5.69 ± 0.09	-
W387F-Y371W	6.81 ± 0.07	-	-

^a Mj-MAT wild type and mutants were incubated in the presence of urea and the fluorescent properties of each protein were then analyzed at 50 µg/ml upon excitation at 275 nm for W387F and 295 nm for the rest of the mutants. The table shows the mean ± SD of D_{50%} from a typical experiment for each case.

^b Data from table 2

Table 6

Secondary structure elements assignments in the proposed Mj-MAT model.

Domain	Secondary structure	Residues ^a
N-terminal	E1	20-26
central	H1	30-49
	E2	64-72
	E3	85-93
	H2	112-124
	E4	133-140
-	H3 ^b	143-150
C-terminal	E5	160-163
	E6	166-171
N-terminal	H4	180-193
	E7	197-205
	E8	211-219
	H5	231-242
	E9	270-275
central	E10	287-289
	E11	311-314
C-terminal	H6	320-336
	E12	342-348
	E13	357-359
	E14	362-368
	H7	376-387

^aThe model includes residues 17-387, as all the structures available lack the initial 16 amino acids and low homology is found after position 387.

^b Helix H3 corresponds to a short stretch of connection between the central and C-terminal domains.