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Review

Structure-function relationships in methionine adenosyltransferases

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Abstract. Methionine adenosyltransferases (MATs) are the family of enzymes that synthesize the main biological methyl donor, *S*-adenosylmethionine. The high sequence conservation among catalytic subunits from bacteria and eukarya preserves key residues that control activity and oligomerization, which is reflected in the protein structure. However, structural differences among complexes with substrates and products have led to proposals of several reaction mechanisms. In parallel, folding studies begin to explain how the three intertwined domains of the catalytic subunit are produced, and to highlight the importance of certain intermediates in attaining the active final conformation. This review analyzes the available structural data and proposes a consensus interpretation that facilitates an understanding of the pathological problems derived from impairment of MAT function. In addition, new research opportunities directed toward clarification of aspects that remain obscure are also identified.

Keywords. Methionine adenosyltransferase, *S*-adenosylmethionine synthetase, crystal structure, reaction mechanism, folding, mutants, hepatic disease.

Methionine is a nonpolar amino acid characterized by the presence of a methyl group attached to a sulfur atom located in its side chain. In addition to its role in protein synthesis, large amounts of this amino acid are used for the synthesis of *S*-adenosylmethionine (AdoMet) by methionine adenosyltransferases (MATs) in a reaction that is the rate-limiting step in the methionine cycle (Fig. 1) [1, 2]. The MAT catalyzed reaction combines methionine, ATP, and water to produce AdoMet, pyrophosphate, and inorganic phosphate; the enzyme requires both Mg^{2+} and K^{+} ions for maximal activity [1, 2]. AdoMet participates in a large number of reactions, due to its ability to donate all the groups surrounding the sulfur atom. SAM radical proteins use the 5′-deoxyadenosyl moiety of AdoMet to synthesize biotin, among other compounds. Also, after decarboxylation AdoMet participates in polyamine synthesis, rendering 5′-deoxy-5′methylthioadenosine (MTA) [3] that is recycled for methionine synthesis through the methionine salvage pathway [4]. Methyltransferases use AdoMet to obtain the methyl groups used to synthesize a large number of compounds, such as phospholipids and neurotransmitters, and in each case *S*-adenosylhomocysteine (AdoHcy) is formed. AdoHcy can act in many cases as a potent inhibitor of these enzymes. The AdoMet/AdoHcy ratio is known as the methylation index, with its normal value in mammalian cells being approximately three [1]. Maintenance of this ratio depends on

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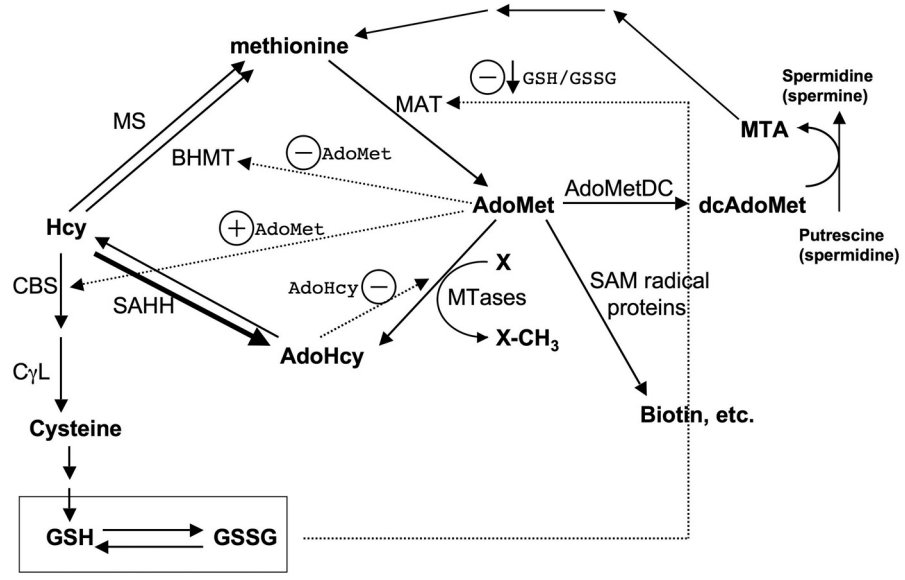

Figure 1. A scheme of the mammalian hepatic methionine cycle and some of the related pathways. Methionine is converted to *S*-adenosylmethionine (AdoMet) by methionine adenosyltransferases (MATs; EC 2.5.1.6); this compound can be used by a multitude of enzymes such as methyltransferases (MTases; EC 2.1.1.X), SAM radical proteins (EC 2.8.1.6 for biotin synthesis) and AdoMet decarboxylase (AdoMetDC; EC 4.1.1.50). Polyamine synthesis occurs with methylthioadenosine (MTA) production, a compound that can be reused for methionine synthesis by the methionine salvage pathway. On the other hand, the action of MTases renders methylated products and *S*-adenosylhomocysteine (AdoHcy) that can be hydrolyzed by AdoHcy hydrolase (SAHH, EC 3.3.1.1) to adenosine and homocysteine (Hcy). This reaction is reversible and favors AdoHcy synthesis. Hcy can be metabolized through the trans-sulfuration pathway by the consecutive action of cystathionine β synthase (CBS; EC 4.2.1.22) and cystathionine γ lyase (CγL; EC 4.4.1.1) rendering cysteine for glutathione synthesis, among other purposes. In addition, Hcy can also serve in resynthesis of methionine by two reactions catalyzed by methionine synthase (MS; EC 2.1.1.13) and betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5). Some of these steps can be modulated by metabolites synthesized in these pathways and dashed lines indicate the most relevant.

efficient removal of AdoHcy by either a bacterial nucleosidase [5] or eukaryotic AdoHcy hydrolase, rendering adenine and ribosylhomocysteine or adenosine and homocysteine (Hcy), respectively. The eukaryotic hydrolysis reaction is reversible and the equilibrium favors synthesis, thus to preserve the methylation index a rapid elimination of both products is required. Adenosine is primarily metabolized to inosine by adenosine deaminase, whereas in mammals Hcy can be used in three different reactions or can reach the blood. These reactions include remethylation to methionine by either methionine synthase or betaine homocysteine methyltransferase, or alternatively entry into the trans-sulfuration pathway through the action of cystathionine β synthase [6]. Regulation of the mammalian methionine cycle is exerted at several levels by hormones (*i.e.* glucocorticoids) [7], products of the cycle (*i.e.* AdoHcy) [1], and compounds produced in related pathways (glutathione, MTA) [8–10]. These regulatory mechanisms provoke changes in the expression levels of several enzymes of the pathway [7, 11], variations in enzyme activity through changes in the oligomerization state (*i.e.* interconversion of MAT I/III in liver, see below) [12, 13], or the introduction of post-translational modifications (*i.e.* nitrosylation) under a variety of stimuli [14]. A detailed description of these mechanisms is beyond the scope of this review, and hence for additional information on enzyme regulation we refer the reader to other reviews [1, 15].

The methionine adenosyltransferase family

The MAT family is expressed in almost every organism identified to date, with the exception of some parasites that seem to obtain AdoMet from their hosts [16–18]. The presence of several genes encoding isoenzymes of the catalytic subunit (α) is quite common, whereas the existence of single copy genes for regulatory subunits (β) has only been described in mammals [19–21]. A high level of conservation is displayed among α-subunits from bacteria and eukarya (typically 30% identity) [16]. Five clusters of special sequence preservation can be identified, which include substrate-binding motifs such as GHPDK and GAGDQG. On the other hand, the major differences occur among loops connecting secondary structure elements [16]. A clearly divergent class of MATs was, however, identified in archaea (~18% identity at the amino acid level), with preservation focused apparently in the catalytic residues [22]. Most MAT

isoenzymes appear as homotetramers, except for the archaeal class and mammalian MAT III isoenzymes, which are both dimers, and the mammalian hetero-oligomer MAT II [23].

To date, most of the structural studies have been carried out using the *E. coli* (cMAT) and rat liver MATs (*rl*MATs), thus most of the details given in this review will concern these isoenzymes. cMAT is encoded by the *metK* gene that contains an ORF for a 384-residue protein, although *in vivo* the N-terminal methionine is removed (differences in cMAT numbering among publications derive from this fact) [24, 25]. In contrast, mammalian isoenzymes are encoded by three genes, *MAT1A*, *MAT2A*, and *MAT2B* [26]. The catalytic subunits are 85% identical at the amino acid level, and derive from the *MAT1A* and *MAT2A* genes, which specify ORFs for proteins of 396 (MATα1) and 395 (MATα2) residues, respectively. The *MAT2B* gene includes an ORF for a 335 amino acid protein, the regulatory β-subunit [21]. MATα2 and β-subunits form the hetero-oligomer MAT II with a putative $(\alpha 2\beta)_2$ stoichiometry, whereas MATα1 subunits associate into dimers (MAT III) and tetramers (MAT I). These isoenzymes differ in their K_m for methionine, which are ~30 μM for MAT II [23], ~100 μM for MAT I, and ~1 mM for MAT III [1]. Additionally, MAT III is substantially activated by DMSO through an unknown mechanism [1]. Changes in which isoenzyme is preferentially expressed, as well as changes in the ratio of MAT I/III, have been observed in both animal disease models and liver biopsies of cancer and cirrhotic patients [11, 13, 27]. These observations raised the interest in understanding the protein structure and its changes through different regulatory mechanisms as will be discussed thereafter.

Insights into structure and function through chemical modification

Early structural studies reported information obtained through chemical modification of cysteine residues using N-ethylmaleimide (NEM). Labeling of two cysteines/subunit in both cMAT and *rl*MAT I/III produced total inactivation [28, 29], although modification of one of these residues was primarily responsible for the activity loss. This cysteine was identified as C89 in cMAT and C150 in *rl*MAT I/III [28, 30]. In addition, NEM-modification of the tetramers led to inactive dimers, thus suggesting a role for cysteine residues in both enzymatic activity and subunit association [28, 29]. Moreover, under denaturing conditions only 8 out of the 10 cysteines of the *rl*MAT I/III sequence were modified, whereas complete labeling required denaturation in the presence of DTT [30]. In addition, SDS-PAGE showed no differences in mobility under reducing conditions as compared with the nonreduced monomer (~48 kDa), but changes in electrophoretic mobility could be observed after elution from thiopropyl Sepharose columns [8]. In this case, the monomer band exhibited a calculated M_r of 43100 on SDS-PAGE, closer to its theoretical value (43600). Altogether, these data suggested the presence of an intrasubunit disulfide, which was later localized between residues C35 and C61 of the *rl*MAT I/III [31]. The role of this disulfide was subsequently analyzed using site-directed mutagenesis and *in vitro* folding systems as will be discussed in the next sections. Although the presence of disulfide linkages in cytosolic proteins is unusual, the number of reported cases is increasing and includes a variety of proteins in addition to those involved in redox regulatory mechanisms [32, 33].

The importance of cysteines in mammalian MAT activity was further demonstrated upon treatment with nitric oxide donors. The action of these reagents on *rl*MAT I/III reduced its specific activity in a process involving C121 nitrosylation [34]. This type of post-translational modification seems to be specific to these mammalian isoenzymes, as a glycine occupies an analogous position in MATα2 [35]. The *in vivo* relevance of this type of reversible regulation of *rl*MAT I/III function was later demonstrated using animal and cellular models of lipopolysaccharide (LPS) [36] and buthionine sulfoximine (BSO) toxicity [14].

A search for additional motifs in cMAT revealed a sequence characteristic of inorganic pyrophosphatases; for several MATs this has the consensus DXGXTGRKII, which contains the residue R244 [37]. Chemical modification of cMAT arginines using phenylglyoxal and *p*-hydroxyphenylglyoxal caused inactivation that was protected by the presence of ATP, PPP_i, or PP_i. Further biochemical studies using 8-azido-ATP photoaffinity labeling of *rl*MAT I/III led to modification of the peptide that contains residues 267–286, which conform to a P-loop signature [38]. These sequences are known to bind tripolyphosphate chains such as those present in both ATP and PPP_i, clearly in contrast with the predicted location for the labeled peptides that are expected to lie in areas at the nucleoside portion of the ATP binding site.

Crystal structures and predictions

Recent years have seen the elucidation of crystal structures for cMAT (pdb code 1XRA) [39, 40], mammalian *rl*MATα1 (pdb code 1QM4) [41], and human MATα2 (pdb code 2HJ2) catalytic subunits, all

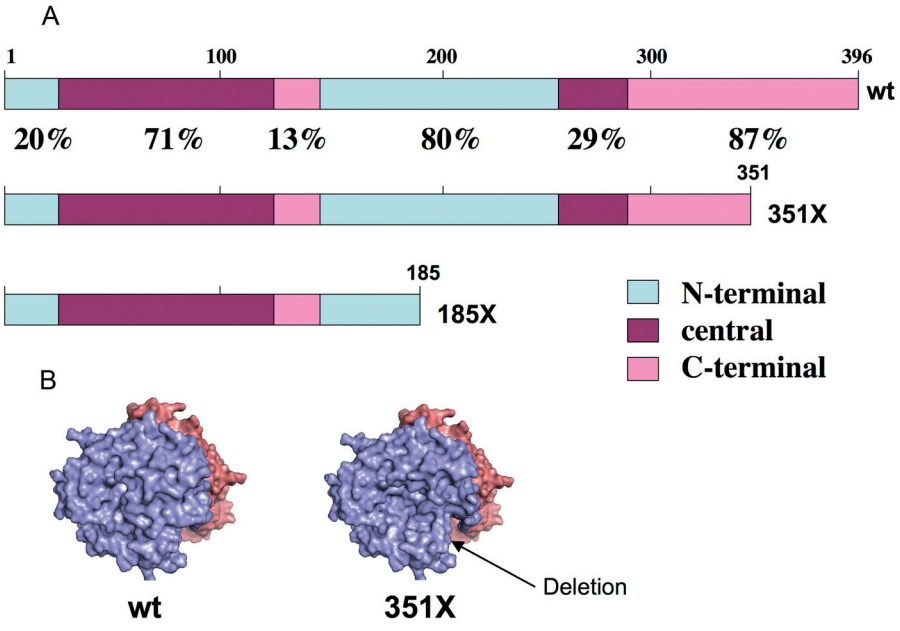

Figure 2. Schematic organization of the domains and the dimer in rat liver MAT (*rl*MAT). (*A*) The distribution along the sequence of the segments composing each domain of the wild-type MAT monomer and comparison with two of the truncated forms derived from mutations detected in the human *MAT1A* gene (351X and 185X). (*B*) Cartoons for the wild-type (wt) and putative 351X truncated dimers detected in patients with hypermethioninemia and demyelination. An arrow indicates the area that differs between the wild type and 351X structures.

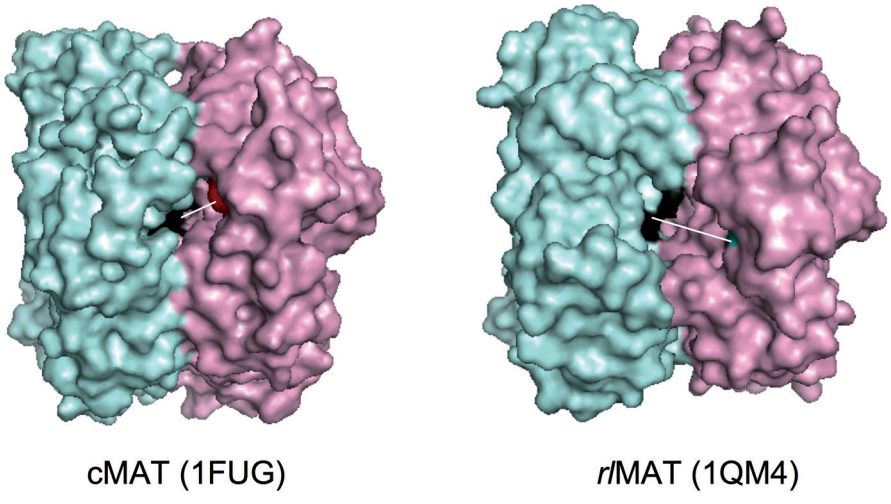

Figure 3. Comparison between dimers of *E. coli* MAT and rat MAT I/III. Colored cartoon forms for dimers of cMAT (1FUG) and *rl*MAT I/III (1QM4). The subunits A and B of each dimer are colored in palecyan and light pink; respectively. The P-loop sequence of subunit A appears in black, whereas the active site phenylalanine residue appears in red for cMAT (F230) and aquamarine for *rl*MAT I/III (F251). The white bars show the distance between both areas in each active site.

in tetrameric assemblies [42]. The structure of the classical MAT catalytic subunits is unique, with the topology belonging to the α/β protein class listed in the SCOP database (http://scop.berkeley.edu/data/scop.b.htm). Consistent with the high sequence homology among eukaryotic and bacterial MATs, the crystal structures of these three isoenzymes conserve both secondary structure elements and their order of appearance, leading in each case to three domains that result from the tertiary structural arrangement of nonconsecutive stretches of the sequence (Fig. 2). Each domain is formed by a βαββαβ repeat with no significant sequence conservation, and named according to its position in the structure as N-terminal, central, and C-terminal domain. The MAT monomer distributes α-helices at what will be the solvent exposed surface of the oligomer, whereas β-sheets are located in a flat hydrophobic surface that will constitute the monomer-monomer contact area in the dimer. Pairs of subunits are arranged in an inverted configuration leading to two deep cavities between them; these cavities contain the active sites, to which amino acids of both monomers contribute (Fig. 3).

Association of dimers in cMAT and *rl*MAT occurs at rigid angles through the central tips of the elongated structures, while the tilt between the dimers in either tetramer is different [41, 43]. The hydrophobic contact surface between monomers in the tight dimers is much

Table 1. Summary of the MAT crystal structures reported to date.

MAT type	Ligands	pdb code	Resolution (Å)	References
cMAT	None	1FUG	3.2	[40]
	ADP	1MXB	2.8	[39]
	BrADP	1MXC	3	[39]
	PP_i	1MXA	2.8	[39]
	AMPPNP + Methionine	1P7L	2.50	[45]
	AdoMet + PPNP*	1RG9	2.50	[45]
Rat MAT I	L-*cis*AMB	1QM4	2.66	[41]
	AEP	1O90	3.1	[46]
	ATP + Methionine	1O9T	2.70	[46]
	ATP +AEP	1O93	3.49	[46]
	ADP + L-*cis*AMB*	1O92	3.19	[46]
Human MAT II	AdoMet	2P02	1.21	[42]
Human MAT I	AdoMet	2OBV	2.05	[51]

* Produced by catalysis in the active site.

larger than the polar surface between dimers in the tetramers. Moreover, comparison between cMAT and MAT I revealed that the contact pattern between dimers in both structures is quite different [41, 43]. Indeed, the few interactions observed in *rl*MAT I make a central core that is accessible to the solvent, whereas the *E. coli* tetramer has a central core of interactions surrounded by a ring of additional contacts [44]. Such an organization is consistent with the higher stability observed for cMAT, and probably facilitates the physiologically relevant interconversion between mammalian MAT I and III.

Many of the MAT structures reported to date share a disordered loop that is proposed to serve as a dynamic lid that controls access to the active site. In support of this hypothesis are the kinetic and spectroscopic data, as well as the detection of an ordered loop in only a few of the reported crystal structures, those for MAT II (2HJ2) [42] and some of cMAT (1FUG, 1P7L, and 1RG9) [40, 45]. In addition, location of *rl*C121 on the loop sequence allowed other authors to propose that post-translational modifications, such as nitrosylation [36], may control loop movement. However, such a mechanism would be restricted to MAT I/III isoenzymes that are the only members of the MAT family having a cysteine in such position [16, 35]. Corroboration of this postulate awaits confirmation through the solution of crystal structures including such modifications.

Structures for several MAT complexes have been also published (Table 1). No significant difference in the ATP binding site is observed in cMAT binary and *rl*MAT I ternary complexes, and in both cases ATP is hydrolyzed in some active sites to ADP and P_i [39, 46]. The methionine binding site in *rl*MAT I was identified using two analogues of the amino acid, L-2-amino-4-methoxy-*cis*-but-3-enoic acid (L-*cis*AMB) and (2*S*,4*S*)-amino-4,5-epoxypentanoic acid (AEP), which occupied essentially the same position, stacking against F251 [41, 46]. Conservation exists among positions for the three phosphates, the P_γ interacting with the putative P-loop (residues 259–266 in cMAT) [43]. However, large deviations in ligand positions were found among ternary complexes. Thus, cMAT complexes with methionine and AMPPNP (Table 1), and cMAT and MAT II complexes including AdoMet show adenine ring movement to a site analogous to that of methionine in *rl*MAT I, stacking against the conserved phenylalanine [42, 45]. In contrast, *rl*MAT I structures show a displacement of the AdoMet position to a different orientation than that seen for ATP in those active sites in which ATP and methionine have reacted [46]. To date, the reasons for these differences in AdoMet locations remain unclear, and mutagenesis studies carried out in cMAT and *rl*MAT I proteins did not shed light on this problem [41, 47, 48]. The use of different ligands is one of the plausible explanations for these differences, as they could reflect the presence of AdoMet as a product or as an inhibitor. In this regard, kinetic studies of cMAT found AdoMet to be a noncompetitive inhibitor with respect to both ATP and methionine, suggesting a unique binding mode [49], whereas AdoMet has been shown to act both as an inhibitor for MAT I and an activator for MAT III [50].

Information concerning the structures of other types of MAT subunits is restricted to that obtained from spectroscopic studies of the purified proteins or from sequence comparison. This is the case for both the regulatory β-subunit and the archaeal MATs. Regarding the β-subunit, structural predictions are based on the high sequence similarity exhibited to dTDP-rhamnose reductase, suggesting an analogous fold, including the NAD-binding Rossman fold found in

the reductase [21]. On the other hand, the low similarity exhibited by archaeal MATs, along with the differences in α-helical content deduced from its circular dichroism spectra, suggest variations in its topology as compared with classical α-type catalytic subunits [22]. Thus, numerous questions await solution from structural studies, including: i) clarification of the AdoMet binding site in the catalytic subunits; ii) determination of the structure of the β-subunit and its putative NAD binding capacity; iii) elucidation of the structure of the hetero-oligomeric MAT II; iv) evaluation of NAD-binding effects on the MAT II hetero-oligomer; v) solution of the MAT III structure; and vi) determination of an archaeal MAT structure.

The catalytic mechanism

Kinetic studies suffered from misleading interpretations until the presence of copurifying isoenzymes was realized and the forms could be readily separated. Even then interpretations have been complicated by AdoMet activation of its own synthesis by yeast MATs [52, 53], or its potent noncompetitive inhibition of cMAT [49]. Moreover, negative cooperativity for both substrates is observed with the yeast isoenzymes [54], whereas this is only true for methionine in human MAT II (*h*MAT), and in contrast *rl*MAT III displays positive cooperativity [55]. Thus, the concentration dependences for substrate saturation were preferentially expressed as $S_{0.5}$, rather than by K_m values. In addition, MATs from all sources tested required Mg^{2+} or similar cations for activity and monovalent cations such as K^+ strikingly enhance the reaction rate (up to 100-fold) [1].

All available data are consistent with a sequential kinetic mechanism including the following steps: i) formation of an E·ATP·Met complex; ii) reaction to form the obligatory intermediate E·AdoMet·PPP_i complex; and, iii) the subsequent hydrolysis of PPP_i before the regeneration of the enzyme by product release [49, 53, 56–58]. Differences in rate constants among MATs, combined with the diversity of experimental conditions that have been used, can explain the discrepancies reported concerning the order of substrate binding and that of product release [57]. The only detectable reaction intermediate is PPP_i and no covalent E·intermediates have been identified for either of the two reactions, AdoMet synthesis and PPP_i hydrolysis. PPP_i is tightly bound to the enzyme, its hydrolysis being oriented so that >95% of the P_i originates from the γ-phosphoryl group of ATP [49]. The P_γ position is preserved in the crystal structures, even in those complexes reflecting different steps of the reaction, where movement of the P_α and P_β, as well as of the ribose moiety is observed [39, 41, 45, 46]. The AdoMet activation of its own synthesis detected in yeast MATs and *rl*MAT III seems to reflect the escape of AdoMet from the intermediate E·AdoMet·PPP_i complex. Thus, E·PPP_i hydrolysis becomes rate limiting and the reaction accelerates as exogenous AdoMet rebinds to form the more rapidly hydrolyzed E·AdoMet·PPP_i complex.

Understanding of the catalytic mechanism was aided by the determination of the stereochemical course of AdoMet formation using chiral [5–^{2}H]-ATP with yeast MATs [59], as well as by the kinetic isotope effect measurements carried out with cMAT [60]. The combined data demonstrated a S_N2 displacement mechanism with simultaneous attack of the sulfur methionine on the C5′ of ATP and displacement of the PPP_i chain. Thus, the enzyme facilitates the attack of the methionyl sulfur on ATP to form AdoMet, and allows the separation of the negatively charged PPP_i from the sulfonium cation. Product release is then facilitated by cleavage of PPP_i to render the less tightly bound species PP_i and P_i, a process in which R244 of cMAT has a critical role in orienting the phosphate chain [37]. The mechanism of hydrolysis of the PPP_i intermediate is similar to that described for other phosphatases and requires Mg^{2+}, and in some MATs it is activated by K^+. How the water is activated for reaction remains unknown, as the available crystal structures do not show any obvious candidates for the basic group to facilitate the reaction.

Several approaches, including NMR and EPR spectroscopy, show the presence of two divalent cations and one monovalent cation in the active site of cMAT [61–63], whereas up to three Mg^{2+} ions have been identified in *rl*MAT I crystal structures [41, 46]. One of the divalent cations of cMAT coordinates the three phosphates, whereas the other Mg^{2+} ion coordinates those at positions α and γ [45]. Such a coordination pattern to the α-phosphoryl group serves to stabilize the negative charge that originates from the C–O bond breakage. On the other hand, the monovalent ion is bound to the carboxylate group of E42 in cMAT, its mutagenesis to glutamine abolishing K^+ activation and suggesting an indirect role related to active site organization [64]. The third Mg^{2+} ion found in *rl*MAT I crystals is coordinated to the carboxylate groups of the methionine analogues L-*cis*AMB and AEP [41, 46], whereas a second K^+ ion found in cMAT seems to stabilize the dimer structure by binding between the two subunits [39].

Protein conformational changes have been detected during catalysis that could involve the loop that gates access to the active site [39–41, 45, 46]. The length of this loop is quite variable among MATs, ranging from 6–19 residues [16], and substitutions in the cMAT

loop show that the catalytic rate is sensitive to both its length and sequence, although loop residues do not contact the substrates [65, 66]. The movement of the loop as an integral part of the catalytic cycle was inferred from the viscosity dependence of the reaction rate. EPR studies of spin-labels placed in the loop show the occurrence of at least two loop conformations, whether or not substrates are present, indicating that loop mobility is an intrinsic property of the protein [65]. The loop's role in the enzyme mechanism is apparently related to the prevention of PPP_i release before hydrolysis. Catalysis may also involve movement of the short loop 251–260 in *rl*MAT I, which contains F251 that is involved in methionine binding [46]. In this case, displacement of the loop could allow shortening the distance between the two reacting groups, the methionine sulfur and the C5′ of ATP (Fig. 3). As mentioned above, regulatory mechanisms may control the movement of these loops through post-translational modification of some of their residues. Data on MAT II catalytic mechanism are scarce and seem to indicate an intermediate behavior between that of cMAT and *rl*MAT isoenzymes. Overexpression of MATα2 subunits gives $S_{0.5}$ values for methionine approaching those of *rl*MAT I [67], and this fact along with its inhibition by AdoMet, the presence of catalytic residues in analogous positions, and an active site loop of a similar length suggests the same reaction mechanism. However, the presence of the β-subunit must induce important changes in the active site, as the hetero-oligomer MAT II increases its affinity for methionine (~3 fold) and is more sensitive to AdoMet inhibition [68]. These results suggest that binding of the regulatory subunit may force local conformational changes at the active site, such as shortening of the distance between ATP and methionine binding sites, which may facilitate the reaction.

Altogether these results still do not establish the precise role of the active site residues or the loops in the catalytic mechanism, nor decipher the differences in the active sites of mammalian isoenzymes that led to a large disparity in $S_{0.5}^{Met}$ values. Additionally, information on the mechanism of MAT III activation by DMSO [69] and the ability of reagents such as acetonitrile, β-mercaptoethanol, and urea [70] to alleviate AdoMet inhibition of cMAT is still lacking. Moreover, the existence of monomeric entities (*rl*R265H) [71] showing at least PPP_i hydrolysis capacity is difficult to understand and poses questions regarding the structure adopted by this mutant.

Folding and association of the subunits

Folding studies have been carried out using *rl*MAT III and urea denaturation [72, 73]. Catalytic activity decreases immediately after denaturant addition, $D_{50\%}$ values being 0.3 M for AdoMet synthesis and 1 M for PPP_i hydrolysis [72, 73]. Gel filtration chromatography and sedimentation velocity experiments showed delays in the elution position and decreases in the $s_{20,w}$ values for *rl*MAT III in the 1–2 M urea range, compatible with the presence of monomeric species [73]. Limited proteolysis studies at 1 M denaturant liberate a ~3 kDa peptide corresponding to a cut at K33, which becomes accessible only upon dimer dissociation [72]. Moreover, denaturation titrations monitored by fluorescence spectroscopy and circular dichroism showed a reversible three-state profile with a plateau in the same range as monomer detection. Analysis of all these data using several models rendered the best fit for a three-state mechanism of *rl*MAT III urea unfolding with a global coefficient for urea dependence (m) of 7.25 kcal/mol/M, a global free energy change of 15.7 kcal/mol, and including a monomeric intermediate [73] (Fig. 4).

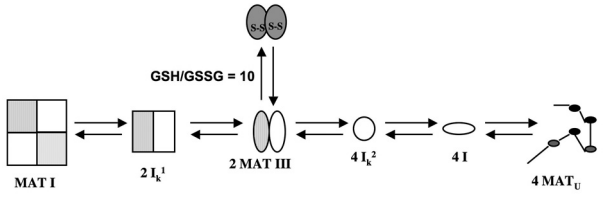

Figure 4. Summary of the *in vitro* folding data. *In vitro* folding studies using *rl*MAT I/III revealed three intermediates: one monomeric equilibrium intermediate (I); and two kinetic intermediates (I_k^1 and I_k^2). I_k^1 is proposed to be a dimer, whereas I_k^2 is predicted to be a monomer. All these species can be detected under reducing conditions, and interconversion among tetramers and dimers is observed in a concentration dependent equilibrium. However, under oxidative conditions (*i.e.* GSH/GSSG = 10) this association/dissociation process is blocked by production of the C35-C61 intrasubunit disulfide.

This monomeric intermediate (I) is characterized by a 2.5-fold reduced tryptophan fluorescence intensity and preservation of 70% of the native secondary structure according to its CD spectrum [73]. In addition, the intermediate exhibits a 75% decrease in ANS binding as compared with the native *rl*MAT III, and its 1:1 molar stoichiometry suggests a specific binding site [72]. Comparison with the structural characteristics previously described for another ANS-protein complex [74], and quenching of the dye signal in the presence of the substrates, suggested that this binding site contains residues P358-G359, as well as the V131-G132-A133 stretch located at the starting

sequence of the active site access loop [72]. Moreover, the differences in ANS fluorescence properties between native and intermediate states and the large m_1 value calculated for the dissociation process indicate structural reorganization, as expected from solvent exposure of the large hydrophobic contact surface between monomers [72, 73]. These data suggest that intermediate I shows features of a molten-globule. Moreover, the importance of this monomeric intermediate is highlighted by refolding experiments in which the yield is improved through inclusion of a step in which formation of the 2 M urea intermediate is favored, before complete elimination of the denaturant [75]. The presence of intermediates in folding pathways for dimers of a certain size seems to be a common feature of those displaying less empty spaces between monomers, the dimerization having a major structural role among those with a B-type folding mechanism [76].

Refolding kinetics of *rl*MAT III starting from the monomeric intermediate I fit to a single exponential, which indicates a monomolecular process including at least one kinetic intermediate (I_k^2) (Fig. 4) [72]. ANS binding kinetics also showed transient binding of the dye between 1 and 2.3 M urea, corroborating the existence of a species binding large amounts of ANS [72]. The association state of this I_k^2 intermediate was inferred from both its spectroscopic characteristics and its rate of production to be monomeric, and this conclusion was further supported by its detection during refolding of the monomeric *rl*R265H mutant [72]. However, the discrepancies observed among the oligomeric state of analogous mutants prepared in cMAT [37], *h*MAT I/III [77], and *rl*MAT I/III (Sanchez-Perez et al., unpublished data), all of which remain oligomers, raise doubts on the definitive association level of I_k^2 and the mutant.

The tetramer dissociation step was also studied taking by advantage the concentration dependent equilibrium shown by recombinant *rl*MAT I/III and the differences in fluorescence intensity between ANS-tetramer and ANS-dimer complexes [44]. The exponential decay of the fluorescence intensity to that of the ANS-dimer complex fits best to a single exponential, preceded by a short lag phase, suggesting a kinetic intermediate (I_k^1). The calculated half-life for *rl*MAT I derived from these data was 14.69 s at 25°C [44], but the association state of I_k^1 could not be directly deduced (Fig. 4). However, the authors [44] proposed that I_k^1 could be a dimer that had just dissociated from the tetramer and had not yet attained the active site changes that lead to its ability to be stimulated by DMSO, and to the large decrease in affinity for methionine. These data allow the calculation of the global free energy change for tetramer unfolding as 24.41 kcal/mol [44], most of it (~65%) coming from dimer dissociation, and hence corroborating the lower stability of the dimer-dimer interactions.

Thermal denaturation studies on recombinant *rl*MAT I/III showed the irreversibility of this unfolding process, which occurs with a T_m of 47–51°C, depending on the technique used to monitor the changes [78]. Measurements carried out at protein concentrations favoring dimer accumulation (activity and fluorescence) revealed one transition for its unfolding. These changes occur in the same temperature range as alterations observed by infrared spectroscopy (IR), which were measured at concentrations typical for tetramer presence. Thus, MAT I stability is highly dependent on that of the dimer MAT III. Moreover, 2-dimensional IR maps indicate that the early changes affect the most exposed secondary structure elements, α-helices and β-turns according to the crystal structures [78]. The changes in the 1624 cm^{-1} oligomerization band could be initially assigned either to tetramer-dimer-monomer or tetramer-monomer processes, whereas the later modifications correspond to aggregation processes that could be ascribed to both dimer dissociation and monomer aggregation or just to aggregation of unfolded dimers. Of special notice is the ability of some structures to interconvert during thermal denaturation as can be deduced from the behavior of the cross-correlation peaks of the 2D maps [78].

The high sequence and structural conservation detected among α-type subunits suggest that no large differences exist in the folding pathways for MATα2 monomers as compared to MATα1. However, changes in the association step for homo- and hetero-oligomers can be expected, because incorporation of the regulatory subunit into the complex may occur concomitantly with the α-type subunit association or in a later step. The first of these possibilities could include the interaction of the β-subunit with the intermediates, whereas in the second pathway the association of the regulatory subunit will follow α-subunit oligomerization. The fact that tryptophan residues appear in equivalent positions for both MATα1 and MATα2, as well as preservation of the ANS binding amino acids, will help in comparing results of analogous experiments when they become available.

Further analysis of the folding pathway will rely on the use of stopped flow techniques for the detection of new intermediates and the understanding of the dimer-tetramer association process. Moreover, the use of mutants in which their dimer-tetramer association is blocked, or that appear as monomers, should be additionally helpful. The significance of the intermediates and the putative need for the aid of

chaperones/chaperonines to attain the physiologically relevant states, as deduced from cMAT binding to GroEL/GroES complexes [79], also deserves special attention. The fact that the size of the cMAT oligomer exceeds that of the chaperonine cavity suggests only partial folding at this step, possibly to a monomeric intermediate similar to I. The capacity of such a monomer to display tripolyphosphatase activity, as suggested by the *rl*R265H mutant, requires the presence of a phosphatase-like active site in this structure. Determination of how these monomers later associate and achieve their final structure, as well as elucidation of the need for additional assistance in these steps, also requires further studies. This is also the case for the mechanisms that stabilize tetramers and dimers *in vivo*, and the putative impairment of the folding pathway during pathological development.

Insights into the function and regulation through mutagenesis

Recent years have seen research in this field through the analysis of mutants at positions relevant for subunit association or for active site function, and those identified in patients showing hypermethioninemia. The first attempts in this area concerned cysteine residues, which were found important for activity and maintenance of the oligomerization state, according to chemical modification studies. These studies have been carried out in cMAT [28], *rl*MAT I/III [80], and *Leishmania donovanni* MAT (*Ld*MAT) [81], where most of the mutants reduce, but do not lose, their AdoMet synthesizing activity, thus suggesting their lack of direct involvement in catalysis. The exception to this rule is *Ld*MAT C92S that only preserves tripolyphosphatase activity [81]. Dramatic changes in the association state were detected for cMAT C89S and C89A, which render cMAT in a dimer-tetramer equilibrium [82], and *rl*MAT I/III C69S that appears mainly as dimers [80]. Moreover, all the cysteine substitutions carried out between positions C35 and C105 in *rl*MAT I/III altered the dimer/tetramer ratio [80], in concordance with their location at the central domain of the structure through which dimer-dimer contacts are established [41]. In general, the activity changes correlated with alterations in the association state attained, thus suggesting a primarily structural role for the cysteines. This view was further confirmed when the role of the intrasubunit disulfide C35-C61 of *rl*MAT I/III in blocking dimer/tetramer interconversion was deciphered (Fig. 4) [44]. In fact, stabilization of the β-sheet that forms the contact between dimers was proposed to facilitate production of the correct pattern of interactions during the association step. These experiments also suggest that an increased production of this disulfide under oxidative stress may be the cause for the dimer accumulation observed in some pathologies, but do not rule out that other disulfide patterns could occur, leading to nonproductive intermediates with low MAT activity. The role of this post-translational modification would be of relevance only for MAT I/III isoenzymes, which alone include appropriate cysteine residues, one of them being C61 that appears exclusively in this type of MATs [16, 80].

Cysteine mutants have been also used to analyze nitrosylation of *rl*MAT I/III, the results allowing identification of C121 as the target residue [34]. Moreover, the mechanism involved in this modification was analyzed through serine substitution of acidic (D355) and basic residues (R357 and R363) surrounding this position [83]. These mutants reduced NO incorporation into C121, whereas conservative changes did not, thus suggesting an acid/base-catalyzed mechanism of modification. These same residues were not required for peroxynitrite-thiol interaction [83]. A similar study carried out using *h*MAT II demonstrated that this isoform contains all the residues required for nitrosylation except for the cysteine, which is substituted by G120 [35].

Alterations of the association state were also reported in the formerly mentioned *rl*MAT I/III R265H that is reported to be a monomer with tripolyphosphatase activity [71], whereas the analogous cMAT R244H mutant remains as a tetramer [37] and *h*MAT I/III R264H is reported to be a dimer [77]. This arginine is involved in a salt bridge with E58 of the opposite subunit, constituting one of the few polar interactions that occur between monomers in the dimer crystal structures. However, the relevance of this interaction in stabilizing the dimer may be of less importance than suggested, because these mutants are reported to produce hetero-oligomers when coexpressed with the corresponding wild-type (wt) MAT [77]. Moreover, the free energy of association provided by the large number of hydrophobic interactions between subunits in the crystal structures may be enough to compensate for the disruption of this single ionic interaction. Residues involved in this salt bridge have been also implicated in monovalent cation activation, as deduced from reductions in catalytic efficiency and lack of K^+ activation in cMAT R244L and E42Q mutants [37, 64].

Polar residues within the active site seem to be highly conserved in MAT proteins, and a systematic study of their importance has been performed in cMAT. Substitution of aspartic residues that were postulated to be involved in coordination of one Mg^{2+} ion (D16 and D271) and methionine (D118 and D238) led to

increase in the K_m for the substrates and reduction in the k_{cat} in the presence of the cation. Moreover, D16 mutants do not hydrolyze PPP_i and D271 mutants decrease >1000-fold their affinity for this substrate [48]. In addition, basic residues proposed to be involved in adenine ring (K269) and phosphoryl chain binding (H14, K165, K245, and K265) have been also substituted and found to alter AdoMet synthesis, except for K165M [47]. These same mutants also show important changes in their ability to hydrolyze PPP_i, thus H14N shows a 16-fold decreased tripolyphosphatase activity. Complementary to these data are those derived from *rl*MAT I/III active site mutants, which concern residues that are related directly (F251) or indirectly (D180) to methionine recognition and to β-phosphoryl group binding (K182) [41]. All of them show reduced AdoMet synthesis (k_{cat} <5 % of the wt), while preserving ATP binding and increased ability to hydrolyze PPP_i. In general, active site mutants corroborate the role deduced from crystal complexes for the residues under study, and hence did not aid in the clarification of the differences recently observed in substrate binding locations in the diverse crystal structures.

Analysis of mammalian α-type MAT sequences using several bioinformatics resources suggests the presence of putative phosphorylation sites for different kinases (*i.e.* PKC and casein kinases). However, regulation of MAT activity by this mechanism has not been explored in depth. Only *in vitro* assays using *rl*MAT have reported PKC phosphorylation on threonine 342 [84], but the relevance of such a modification *in vivo* has not been demonstrated. In addition, methionine modification has been also detected *in vitro* under oxidative conditions [44], but its relevance for MAT function remains unknown. Mutagenesis studies on the candidate residues has not yet been carried out, although such an analysis may shed light on the relevance of these type of regulatory mechanisms on MAT function/oligomerization.

Understanding pathologic behavior of mutant proteins through structural data

In spite of the efforts of many groups to obtain a large amount of structural information through Structural Genomic consortia, there are still an important number of proteins and particular problems that need a more specialized analysis. The practical use of these data will rely on a correct interpretation of the results in a physiological context. Many cell biology studies need specific inhibitors that are based on the active site structures and reaction mechanisms, with the next step being the potential application of these compounds in the treatment of diseases. In the present case, the relevance that mutations found in patients with hypermethioninemia could have on MAT behavior can only be deduced through knowledge of the structure and folding pathways. Moreover, the changes in the tetramer/dimer ratio or the inactivation detected in pathologies could only be understood in the light of the structure that provides clues for the effects that post-translational modifications may exert on the protein. In this regard, we can now analyze human mutations such as those leading to early stop codons in *h*MATα1 (350X, 351X, or 185X) [85] and infer their capacity to complete the final folding steps including subunit association. Thus, the 185X truncation could hardly lead to a subunit similar to the native monomer, as this stretch of the sequence does not encode even a single entire domain. On the other hand, in the 350X and 351X truncations only part of the C-terminal domain is lacking, and hence a partially folded structure similar to one of the productive intermediates could be expected. Such an intermediate may allow intracellular oligomerization with MATα2 monomers as was proposed to occur in these patients [85, 86]. The inverted arrangement of the monomers in the dimer may lead to a partially active protein, as one of the active sites may be correctly formed. Another scenario is apparent when we realize that MAT domains are formed by nonconsecutive stretches of the sequence, hence nonproductive intermediates could accumulate and block MATα2 folding at the association step, leading to inactive MAT hetero-oligomers. Thus, in both cases the production of hetero-oligomers may be the cause of the disease, but their structure would be clearly different. Modeling the structural impact of these truncations allows estimation of the magnitude of the surface changes that induce exposure of quite a large area of the buried wild-type structure, making preservation of a native-like monomer structure unfeasible (Fig. 2). Other mutations observed in hypermethioninemia patients also relate to residues that have been shown to be structurally important, as is the case of *h*R264H, which seems to behave as a low active dimer [77]. The importance of this residue could only be appreciated when its role in an intersubunit salt bridge and in K^+ activation became known. Finally, knowledge of the active site structure can allow design of specific inhibitors or activators other than those based on the structures of the substrates or products. So far, this has not been the case for MAT, since the best inhibitors found with applicability to cell biology have been based on the methionine structure (L-*cis*AMB and AEP) [87, 88], whereas the high affinity intermediate analogue diimidotriphosphate is impermeable to cells [89], and no transition state analogues are available.

However, the anticipated availability of new inhibitors may increase the analysis of their *in vivo* effects, and may open new ways of treating a broad number of diseases ranging from cirrhosis to Parkinson's.

Conclusions

Comparison of the structural data obtained to date demonstrates a common topology for the α-type MAT catalytic subunits, as well as similar association patterns in dimer formation. Differences, however, can be observed in the dimer-dimer interactions that lead to less stable arrangements in MAT I tetramers than in cMAT, probably facilitating dimer-tetramer exchange in mammals. Such an interconversion process seems to be crucial for MAT regulation in liver, allowing efficient adaptation to changes in the methionine levels. Catalysis follows a sequential S_N2 mechanism, and the main variations in the kinetic mechanism concern the order of substrate binding and of product release. Moreover, the only reaction intermediate detected is PPP_i, which is hydrolyzed in an oriented fashion. This orientation is maintained by conservation of the γ-phosphate binding site, according to all the available crystal structures.

MAT folding *in vitro* occurs through several intermediates, whose association state remains elusive in several cases. The importance of some of these states for productive folding may require the *in vivo* intervention of chaperones/chaperonines to ensure production of the final active state, as suggested by detection of *E. coli* MAT among the substrates of GroEL/GroES. However, no information of this type concerning mammalian MATs has been published to date, and the possibility of misfolding under pathological conditions has not been explored. The existence of patients with genetic defects in *MAT1A* sequence, which lead to truncated subunits that are expected to render severe structural problems, and the pathologies derived from them, deserve additional studies. Finally, the differences among isoenzymes will be only understood when structures of hetero-oligomers and dimers become available. Thus, numerous research opportunities remain in the areas of protein structure, folding, regulation and inhibitor design, each of which can connect the *in vitro* properties of the proteins to its role in the cell.

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