METHIONINE ADENOSYLTRANSFERASE (S-ADENOSYLMETHIONINE SYNTHETASE)

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OUTLINE

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1. INTRODUCTION

S-adenosylmethionine (abbreviated AdoMet or SAM) is the main methyl donor for the multitude of transmethylation reactions that take place in all organisms. The trivalent bonding of its cationinc sulfur atom, however, also allows donation of two other groups, hence broadening the catalogue of reactions in which AdoMet participates; amongst these are decarboxylation followed by propylamine donation in polyamine biosynthesis, and reductive cleavage of the bond between the sulfur and the ribosyl carbon to yield the 5'-deoxyadenosyl radical which is catalyzed by radical SAM proteins (see Figure 1A) [1, 2]. It has been estimated that AdoMet participates in as many reactions as ATP. In contrast AdoMet can be synthesized only through the process catalyzed by methionine adenosyltransferase (MAT also called S-adenosylmethionine synthetase; E.C. 2.5.1.6) in the reaction shown in Figure 1B. The MAT family of enzymes has been shown to be present in all cell types through archaea, bacteria and eucarya, except for a few obligatory parasites [3]. Moreover, a high level of sequence conservation exists between bacterial and eukaryotic MATs, whereas archaea express a distinct class of MAT. In all the cases the preferred quaternary structure is an oligomer, at least a dimer. Amongst MATs, the *E. coli* (cMAT) and mammalian MATs (MAT I/III and MAT II) are the most studied, hence most of the reported structural and regulatory data have been obtained from these proteins.

MAT catalysis of AdoMet synthesis is the rate limiting step of the methionine cycle (Figure 2). The use of AdoMet in transmethylation reactions leads to production of S-adenosylhomocysteine (AdoHcy), which is a potent inhibitor of methyltransferases. AdoHcy is typically metabolized by S-adenosylhomocysteine hydrolase (SAHH), in a reversible reaction that favors resynthesis of AdoHcy. Thus, homocysteine (Hcy) and adenosine, the products of AdoHcy hydrolysis, have to be eliminated in a coordinated manner in order to avoid accumulation of the transmethylation inhibitor, which would lead to a reduction in the methylation index (the AdoMet/AdoHcy ratio). Adenosine is removed by adenosine deaminase catalyzed conversion to inosine, whereas Hcy is consumed in several processes, which vary

amongst tissues and organisms. In mammalian liver, three are three reactions involved in Hcy utilization, thus making a branch point in the cycle. These are: i) synthesis of cystathionine by cystathionine-β-synthase (CBS), which initiates the trans-sulfuration pathway; ii) remethylation of Hcy, catalyzed either by methionine synthase (MS) or betaine homocysteine methyltransferase (BHMT); and iii) Hcy exportation to the plasma. Remethylation reactions allow recovery of methionine for AdoMet resynthesis, thus playing an important role when there are low levels of this amino acid. Moreover, MS links the methionine cycle to the folate cycle, and BHMT allows recovery of one of the methylation equivalents used for choline synthesis. On the other hand, export to the plasma and CBS catalyzed entrance in the trans-sulfuration pathway allows elimination of excess Hcy, and in the last case cysteine is formed that can be used for protein and glutathione (GSH) production. The flux through the cycle is modulated at different levels by some of the metabolites mentioned above; see previous reviews such as [4].

The last decade has produced a large increase in the understanding of the structural, functional and regulatory properties of MATs, including the elucidation of the three dimensional structures of several of these enzymes and unexpected findings on evolutionary conservation and diversity. This review of the MAT family of enzymes attempts a complete overview of the achievements of the last decade. Lastly, important new results relating MAT function to the development of certain diseases are also discussed.

2. MAMMALIAN MATs

Early studies on mammalian MATs showed the existence of this activity in all the tissues studied, the highest being observed in liver. However, complex kinetics were obtained which made the results difficult to interpret until the presence of several isoenzymes was realized. Separations carried out on phenyl Sepharose columns showed elution of three peaks with different hydrophobic character; these were named according to the order of elution as MAT I, II and III. All these isoenzymes share some characteristics such as the Mg^{2+} dependence of their

activity ($S_{0.5}$ ~1 mM), stimulation by K^+ ions, and their AdoMet-inducible tripolyphosphatase activity [4, 5].

2.1. MAT I/III isoenzymes

Since their discovery, these isoenzymes were known as liver-specific forms of MAT, until Lu *et al*. in 2003 [6] showed their presence also in pancreas. Several laboratories have reported in depth studies of MAT I and III which have been carried out using adult liver. The differences can be summarized as follows: i) MAT I is a tetramer whereas MAT III is a dimer, with respective Mr appearing as 200 and 110 kDa upon gel filtration chromatography, ii) their Km values for methionine are in the micromolar range for MAT I (60-120 μM) and in the millimolar range for MAT III (1 mM) [7, 8]; iii) MAT III can be activated by DMSO at physiological concentrations of methionine (60 μM) and is highly hydrophobic (eluting from phenyl Sepharose columns at 50% DMSO (v/v)); and, iv) AdoMet inhibits MAT I and activates MAT III. Some of these differences are normally used to distinguish between isoenzymes in activity assays. Both purified proteins show a common single band (designated α1) of approximately 48 kDa on SDS-PAGE, and antibodies raised against the dimer form recognized both, thus suggesting that a single type of subunit arranged in two assemblies could explain the presence of both oligomers [7]. Further experiments that supported this hypothesis included peptide mapping of the purified isoenzymes [7], dissociation of the tetramer to the dimer using LiBr [9] or N-ethylmaleimide (NEM) modification [10]. The final confirmation came upon cloning of a single cDNA [11, 12] followed by overexpresion in *E. coli* cells [13], which showed *in vivo* production of dimers and tetramers. This single subunit type is a product of the *MAT1A* gene that contains nine exons and eight introns spanning ~20 kb [14], and has been localized by fluorescence *in situ* hybridization to the human chromosome 10q.22 [15].

Animal models, as well as the analysis of human samples, led to the identification of changes in the MAT I/III ratio in pathological conditions [16], which has stimulated interest in the mechanisms that regulate this interconversion. Several *in vitro* approaches have indicated the

importance that cysteine residues have both in the regulation of the association state, and in controlling their activity [8, 17-19]. N-ethylmaleimide (NEM) modification of two sulfhydryls/subunit leads to inactivation and dissociation of the tetramer to inactive dimers [10]. Moreover, only 7-8 out of the 10 cysteines present in each subunit were modified by either NEM or nitric oxide (NO), even after denaturation [20, 21], but denaturation in the presence of DTT allowed full labeling of the sulfhydryl groups [22]. These observations along with data on SDS-PAGE mobility changes after thiopropyl Sepaharose chromatography, and the mechanisms of GSH/GSSG regulation involving the production of an intrasubunit disulfide [8], prompted deeper study of the oxidation state of MAT I/III cysteines. Thus, the presence of a disulfide bond linking C35 and C61 within a subunit was identified [22]. Once available, the MAT I crystal structure showed that these residues are the only cysteines properly oriented, and at distance suitable for formation of an intrasubunit disulfide bridge [23]. Although it is not common for a cytosolic protein to contain a disulfide, examples exist, and their formation is highly dependent on a protein conformation that provides a high local concentration of sulfhydryls to allow formation of the disulfide even in the highly reducing environment of the cytosol [24]. The role of this disulfide in MAT I/III stability is discussed in the Folding section (10, below). In addition, site directed mutagenesis studies demonstrated that changes of any of the cysteines located between residues 35 and 105 modify the MAT I/III ratio; most interesting was the C69S mutant which appears mainly as dimers [19]. Within this region, C121 was found to be of great importance for control of activity, as deduced from the experiments using NO and hydrogen peroxide that are discussed in the MAT regulation Section (2.3, below).

Early estimates of MAT I/III subunit size obtained by SDS-PAGE indicated molecular weights of 48 kDa for the α1 subunit [7]. This value was higher than the theoretical size calculated from the sequence, and which led several authors to suggest post-translational modifications as responsible for these differences. Examination of the sequence revealed the presence of putative phosphorylation sites for several kinases, but only the case of PKC has been explored *in vitro* [25]. Incorporation of ^{32}P-phosphate onto threonine-342, the most

exposed residue according to hydrophobicity profiles, was obtained mainly in the dimer with a concomitant delay in its elution on gel filtration chromatography. These results suggested that MAT III is a partially phosphorylated form, and implied that complete phosphorylation was responsible for oligomer dissociation. These interesting data still await confirmation *in vivo*.

2.2. MAT II isoenzyme

MAT II has been especially studied in lymphocytes and in cancerous cells in which it is located in the cytosol [26]. Immunohistochemistry revealed that MAT II is located in distal tubules of kidney [27] and in liver stellate cells [28]. MAT II was purified to apparent homogeneity from human chronic lymphocytic leukemia cells; the purified enzyme showed a single band on native PAGE gels which was coincident with the MAT activity in gel slices [29]. Surprisingly, the preparation showed three bands in SDS-PAGE, with sizes of 53 (α2), 51 (α'2) and 38 (β) kDa. Peptide maps were identical for α2 and α'2, leading to the suggestion that they represent different post-translationally modified forms of the same subunit [29], the nature of this difference remains unknown to date. Subsequently, the *MAT2A* and *MAT2B* genes have been identified as encoding the α2 and β subunits. The *MAT2A* gene consists of nine exons and eight introns covering ~6 kbp [30], and is located on human chromosome 2p.1.1 [31]. Further information about the β subunit and its gene is provided in Section 2.2.1 (below). The native molecular weight estimates obtained by both gel filtration chromatography and sedimentation velocity indicate an oligomer of approximately 185 kDa, a value compatible with several association arrangements ($\alpha 2_2\beta_2$, $\alpha' 2_2\beta_2$ or $\alpha 2\alpha' 2\beta_2$). To date it has not been determined which of these defines the correct association state, but a tetramer is the accepted form. In addition, the presence of a 68 kDa precursor protein (λ) for α2 and α'2 has been proposed, based on recognition of such a band by monoclonal antibodies raised against α2 subunits [32]. This protein, called λ, is not detectable in continuously proliferating cells, and is lost upon induction of resting cells, wherein a parallel increase in α2 subunits occurs. The λ subunit showed MAT activity in cells where the α2, α'2 and β proteins were undetectable. Such a precursor protein

has not been observed in the non-mammalian organisms that express unusually large MAT α subunits [3], and its sequence has not been reported.

The kinetic properties of MAT II from human chronic lymphocytic leukemia cells have been characterized in detail, see Table 1 [29]. The enzyme has an optimal pH of 7-8.5, and is inhibited by AdoMet (with a K_i of 60 μM), PPP_i, PP_i, P_i, S-carbamyl-L-cysteine, polyamines and 10% DMSO [29, 33-35]. The Km values have been reported as 31 and 3.3 μM for ATP and methionine, respectively. The kinetic mechanism, deduced from product and dead end inhibition patterns, is a steady-state ordered Bi-Ter with ATP adding first and AdoMet being the last product released [29].

2.2.1. The regulatory β-subunit

The presence of a non-catalytic β-subunit in MAT II was found by Kotb *et al*. [29]. This subunit was cytosolically localized by immunofluorescence visualization in transiently transfected Huh7 cells [36]. Peptide maps [29] and immunoreactivity [37] studies showed that the β protein is structurally unrelated to the α2 subunit. Expression of the β-subunit has been observed in all the extrahepatic tissues examined, and at very low levels in normal adult liver [36]. Purified MAT II from bovine brain [38], Ehrlich ascites tumor cells [38] and human mature erythrocytes [39], showed two types of bands on SDS-PAGE gels, one of them being 38 kDa. The sequence of a full length cDNA for the β-subunit of human MAT II isolated by LeGros et al. [40] showed that it encoded a protein of 334 amino acids (an ORF of 1002 bp), a calculated Mr of 37552 and a pI of 6.9. No sequence differences were found in the cDNA obtained from different cell types from several subjects; the ORF always started with the sequence Met-Val. Analysis of the deduced protein sequence revealed a 28% homology with bacterial enzymes that catalyze the reduction of TDP-linked sugars, several nucleoside-diphosphate sugar epimerases and other proteins involved in the synthesis of polysaccharides. Kyte-Doolittle hydrophobicity plots for β revealed two regions of prominent hydrophobicity with much higher scores than the three segments described for α2. Expression in *E. coli* of a His-tagged β

showed a protein of the expected molecular weight which was recognized by antibodies raised against N-terminal peptides of the β-subunit. These antibodies did not crossreact with α2 or any protein from either *E. coli* or yeast, confirming the absence of this type of subunit in these organisms.

The *MAT2B* gene consists of seven exons and six introns, spanning ~6.8 kbp [41]. Exon 1 contains 203 bp of the 5'-noncoding region in addition to 63 bp of the coding region, whereas exon 7 contains 117 bp of the coding sequence and 802 bp of the 3'-untranslated region. This gene was located in the long arm of the human chromosome 5, in an area that corresponds to the interphase between bands 5q34 and 5q35.1. A sequence of 1.1 kbp of the 5'-flanking region (along with several deletions) was tested for promoter activity in COS-1 and Jurkat T-cells, with little differences in behavior between the systems. Primer extension analysis allowed identification of the transcription start site 203 bp upstream the translation initiation site. The minimal promoter corresponds to the region +52/+93, a GC-rich sequence, the TATA box appears at -32/-8, and a Sp1 site was identified at +9/+15. Mutations of this Sp1 site and the TATA box reduced the activity 35-50% and 25%, respectively, whereas combination of both mutations rendered a 60% decrease. Supershift assays using anti-Sp1 and anti-Sp3 antibodies suggested Sp3 as one of the main factors that bind to the Sp1 site at +9/+15. Moreover, chromatin immunoprecipitation showed the involvement of Sp1 and Sp3 in complexes at the *MAT2B* promoter [41].

The role of the β-subunit was not initially clear after its discovery. However, cloning of the α2 subunit and its expression in *E. coli* revealed important differences between the kinetic behavior of the homo-oligomer compared to the hetero-oligomer [42], leading to the suggestion of a regulatory role for the β-protein. This role was further supported by kinetic data obtained for MAT from peripheral blood mononuclear cells (PBMC) stimulated with superantigen (SEB), where the β-subunit expression was negligible and a 3-fold increase in the Km for methionine (55-67 μM) was observed in addition to a decrease in the sensitivity to AdoMet feedback inhibition [43]. Moreover, transient transfections of COS-1 cells with either α2, β or both subunits

showed changes in the kinetics for methionine saturation [44]. Expression of a His-tagged α2 subunit showed two kinetic forms with Km values of 15 and 75 μM; expression of the β-subunit did not have an effect on Km, but increased the Vmax. Moreover, coexpression of both subunits rendered an increase in specific activity along with a single Km of 20 μM for methionine. Spontaneous association of the α2 and α'2 with β subunits was observed in both COS-1 [44] and Huh7 cells [36] and in cell free systems [44], which was reflected in changes in sensitivity to AdoMet inhibition. The purified recombinant β-subunit shows no MAT activity, but is able to modulate α2 kinetics, as well as those of the cMAT and MAT I/III α1 subunits [40, 44].

2.3. MAMMALIAN MAT REGULATION

Studies on MAT regulation in mammals have been carried out at different levels using animal and cell models, as well as purified proteins. The main achievements in the last decade probably correspond to the understanding of transcriptional regulation as described below.

2.3.1 Transcriptional regulation

Sequences for *MAT1A* [45], *MAT2A* [30, 46, 47] and *MAT2B* promoters [41] have been obtained, allowing their analysis in different cell types.

Promoter analysis. The genomic clone obtained for rat *MAT1A* contained 1557 bp of the promoter, showing 88% identity to that of the mouse [45]. Its main characteristics are depicted in Figure 3. Briefly, the transcription initiation site is located 251 nt upstream of the ATG, a possible TATA box appears at -29/-23, and two canonical CAAT boxes are located at -379 and -1514. Several consensus sites for binding of specific trans-activating factors were also identified: 1) two putative AP-1 sites; 2) two PEA3 sites; 3) four glucocorticoid responsive elements; 4) two IL-6 binding sequences; 5) two NF-1 putative sites; and 6) one for each HNF-1, HNF-3 and HNF-4. Expression in hepatoma cells of promoter constructs linked to a luciferase reporter gene showed positive acting elements located at -1251/-958 and -193/-87, whereas regions -958/-727 and -375/-193 contain those elements in CHO cells. Footprinting analysis

using rat liver nuclear proteins showed eight DNase protected areas; the fragment -1251/-996 contained binding elements for liver enriched factors at -1132/-1229, as judged from comparison with the results obtained using kidney and lung proteins. Negative regulatory elements were also identified between -1154/-1134 in both H35 and CHO cells, an area where gel mobility shift assays revealed HNF-3 and HNF-1 binding. Moreover, functional analysis of this promoter demonstrated its activity in hepatic and non-hepatic cells, suggesting that liver restricted expression is probably mediated by DNA methylation or genomic organization [45]. Demonstration of *MAT1A* promoter hypermethylation in non-hepatic cells was obtained by Torres *et al*. [48], who also showed stronger histone acetylation in liver as expected for methylation silencing. This same methylation pattern was observed in fetal hepatocytes, in livers from CCl_4-induced cirrhosis and transformed cells [48]. Treatment of hepatoma cells with demethylating agents or histone deacetylase inhibitors increased MAT1A expression.[48].

Analysis of the human *MAT2A* promoter sequence revealed several putative sites for transcription factor binding. Among these factors, two sites for NF1 and AP-2, and one each for AP-1, c-Myb, NFκB and SpI were found [49]. Footprinting of the -163/+5 fragment showed three to five DNase protected areas, which varied depending on the type of cell extract used; phytohemaglutinin (PHA) stimulation did not induce the same pattern. Four additional protected areas were identified using shorter promoter fragments, including three putative SpI sites in the -163/+5 fragment occupied as shown by supershift analysis [30, 46]. Positive elements responsive to TNFα have been also located in an area including NFκB and AP-1 consensus sites (-352/-314) [47]. Blocking NFκB with IκB superrepressor did not influence the capacity of TNFα to induce AP-1 binding to this promoter, but blockage of the AP-1 site with dominant negative cJun prevents induction of NFκB binding. Electrophoretic Mobility Shift Assays (EMSA) using normal liver and HCC proteins identified c-Myb binding (-354/-328) in both cases, whereas differences were observed in the -60/-40 area. One or two bands were shown in these cases for normal and malignant extracts, respectively, the differing band being ascribed to SpI binding [49]). Both c-Myb and SpI are known to increase their expression levels in HCC.

Effect of hormones and growth factors. Transcriptional regulation of MAT has been shown in several models. Thus, *MAT1A* and *MAT2A* expression is modified in the presence of growth factors, such as HGF and TNFα, as discussed in the **MAT and Cancer** and **MAT and Ethanol** sections, 12.3 and 12.4 respectively [47, 50]. Partial hepatectomy (PH) also induced a decrease in *MAT1A* mRNA levels, whereas those of *MAT2A* increase due to a rise in transcriptional activity and in mRNA stability [51]. In addition, hormones have been shown to modulate expression; adrenalectomy induces a reduction in *MAT1A* mRNA which correlates with decreases in protein and activity levels [52]. These effects were counteracted by administration of glucocorticoids, with synthetic forms being more effective [52]. Moreover, administration of triamcinolone to hepatoma cells increased *MAT1A* mRNA expression, with the protein levels following a similar trend. Increases in MAT activity were also observed upon alloxan treatment, whereas the combined action of insulin and triamcinolone on H35 cells reduced this parameter [52]. On the other hand, insulin alone had negligible effects on *MAT1A* mRNA levels. Confirmation of these effects was obtained using *MAT1A* promoter constructs and H35 cells treated with glucocorticoids; an increase in the decay rate of this mRNA was observed. Development provides another model where alterations in expression can be related to hormonal changes. In rat liver, *MAT2A* expression is reduced immediately before delivery, while a corresponding increase in *MAT1A* occurs [53].

Effects of methionine and related metabolites. The levels of methionine in the growth medium for hepatoma cells also influence expression. Thus, deficiency of this amino acid in the medium induces *MAT2A* mRNA expression and increases its half-life, while AdoMet levels decrease [54]. AdoMet and methylthioadenosine (MTA) down-regulate *MAT2A* mRNA levels in the absence of transcription; the effects of MTA and methionine are carried out through their metabolites. The dependence of tumor cells on methionine for growth has been associated with defects in MTA phosphorylase expression which suppress this salvage pathway [55-58]. In addition, the influence of *MAT1A* expression on other pathways has been studied using a knockout (KO) mouse [59]. Disappearance of *MAT1A* expression correlates with up-regulation

of genes involved in cell communication, control of cell growth/maintenance, cell death and development, whereas down-regulation of genes for metabolic functions occurs.

Effect of oxygen levels. Hypoxia can also modulate the glucocortocoid effect on *MAT1A* mRNA. It also produces decreases in *MAT1A* transcriptional activity, but not in mRNA stability. Concomitant GSH decreases and a reduction in MAT I/III protein levels, with no changes in isoenzyme ratio, were also observed [60, 61]. These effects are exerted through a heme-binding protein, and not through respiratory chain production of ATP, as judged from the response obtained using appropriate inhibitors. H35 cells are resistant to *MAT1A* down-regulation by hypoxia [62]. The role of oxygen tension is noteworthy among the factors that condition the functional heterogeneity of periportal and perivenous hepatocytes [63].

Other effects. SEB induced an increase in $\alpha 2/\alpha' 2$ mRNA and protein levels, whereas β-subunit expression was reduced and almost disappeared after 72 hours of treatment [43]. This down-regulation in β-subunit expression occurs with a 6-10-fold increase in AdoMet intracellular levels [43]. The opposite effect on AdoMet levels upon stimulation of DNA synthesis was observed for *MAT2B* expression in Huh7 cells[54].

2.3.2. Post-transcriptional regulation

Post-transcriptional regulation for MAT I/III was demonstrated in several models, with most studies confirming the important role of cysteines.

Regulation by glutathione levels. *In vivo* studies using rats showed that changes in GSH levels produced either by carbon tetrachloride treatment or by inhibition of glutathione synthesis by buthionine sulfoximine (BSO) were linked to decreases in MAT I/III activity [64, 65]. A direct effect of glutathione on the enzyme was later demonstrated [8], showing inhibition by GSSG that could be modulated by GSH. The mechanism for this effect excluded production of a mixed disulfide, and detection of monomers after GSSG inhibition suggested formation of an intrasubunit disulfide. MAT III was shown to be more sensitive to GSH/GSSG action, and hence easier to oxidize. *In vitro* modulation occurs in the 0-50 range of the GSH/GSSG ratio, as

expected for a strongly oxidizing environment, whereas physiological ratios between 10-300 are normal. Inclusion of thioltransferases in the assay revealed that a thiol-disulfide exchange catalyzed by a protein disulfide isomerase-type enzyme could permit MAT regulation by disulfide formation in conditions of mild to severe oxidative stress [66]. The question remains whether C35-C61 is the only disulfide formed in the protein, or if other disulfides can partake in this type of regulation, and which thiol transferases are involved in such a regulatory mechanism *in vivo*.

***Nitrosylation*.** Intraperitoneal injection of rats with bacterial lipopolysaccharide (LPS) induced serum accumulation of nitrites and nitrates, as well as expression of inducible nitric oxide synthase (iNOS) [18]. NO production induced by this treatment coincided with MAT I/III inhibition, but no reduction in protein levels or mRNA was observed. In hypoxia, L-NAME (an inhibitor of iNOS) and N-acetylcysteine prevented MAT inactivation, but not changes in GSH levels. Treatment of hepatocytes with BSO had no effect on iNOS protein or mRNA, but induced a time-dependent increase in MAT III nitrosylation and inhibition [67]. L-NNA (N^G-nitro-L-arginine), an inhibitor of NOS with higher affinity for the constitutive enzyme, did not exert any effect on MAT activity or nitrosylation, nor on the GSH depletion caused by BSO. However, recovery of GSH levels using glutathione monoethyl ester (EGSH) caused the loss of most of the NO groups incorporated on MAT III, and hence led to recovery of activity. Simultaneous administration of BSO and EGSH prevented the effect of BSO, thus suggesting that MAT inactivation induced by BSO takes place through S-nitrosylation of the protein.

Indirect data relate nitrosylation of MAT I/III with control of proliferation. Intracellular levels of AdoMet are related to the differentiation state of the hepatocyte, with low levels of this metabolite in growing cells. Knockout mice for iNOS show abnormal hepatic regeneration, and KO mice for MAT I/III have low AdoMet levels and induced expression of genes related to growth and differentiation [68]. The HGF and TGFα proliferative responses of isolated hepatocytes are inhibited by L-NAME, an effect which, for HGF, was overcome by concomitant administration of the NO donor SNAP. This effect of L-NAME was dependent on L-methionine

concentration, and since inhibition of NO production activates MAT I/III, AdoMet levels also increased [69].

Using purified MAT I/III and NO donors such as SIN-1, S-nitrosylated glutathione (GSNO) and S-nitroso-N-acetylpenicillamine (which has no structural relationship to glutathione), a direct inhibition of the enzyme was obtained that did not affect the oligomerization state of the protein [18, 21]. This inhibition was reversed by either GSH or β-mercaptoethanol. GSNO reduced the Vmax for both isoenzymes, and increased the $S_{0.5}$ of MAT III for both methionine and ATP [21]. In the search for NO the reactive residues, cysteine mutants of MAT were used, which revealed that C121S was resistant to SIN-1 action, whereas the C69S, C105S and C150S mutants were more sensitive than the wild type enzyme. This regulation by nitrosylation should be specific for MAT I/III, since the thiol of C121 has no equivalent in MAT II which has glycine at that position.

The mechanism proposed for protein S-nitrosylation involves an acid-base catalyzed nitrosothiol exchange reaction, where the target cysteine is localized next to acidic and basic residues [70], requirements that were met by MAT I/III. Site-directed mutagenesis allowed identification of the residues involved, D355, R357 and R363, and confirmed their role [71]. Incubation of recombinant MAT III with peroxynitrite also inactivates MAT without nitrosylation, an effect that was prevented in the C121S mutant, whereas D355S, R357S, R363S and R357S/R363S substitutions had no effect. Residue 121 is a glycine in MAT II, however it was possible to create an equivalent nitrosylation site by the mutation G120C, as demonstrated using recombinant MAT II and GSNO [72]. Equivalent acidic and basic residues are preserved in MAT II (D354, R356, R362), thus allowing modification of position 120 in the G120C mutant. No effect of nitrosylation on the basal tripolyphosphatase activity of MAT I/III was detected, but its stimulation by AdoMet was reduced in the presence of NO donors [73]. These authors suggested the presence of two MAT III isoforms, one of low tripolyphosphatase activity which is insensitive to NO, and another of high activity which is inhibitable by this agent. Interconversion

between the two conformations would be a slow process that results in a lag phase detected in kinetic studies.

Hydroxylation. Treatment of a stable CHO clone overexpressing MAT I/III (ST-C) with hydrogen peroxide showed enhanced cell death as compared to the wild type CHO cells [74]. Analysis of these cells showed that H_2O_2 further depletes the NAD and ATP levels that were already 50% reduced in the ST-C clone. This effect could be prevented using an inhibitor of poly (ADP-ribose) polymerase, 3-aminobenzamide (ABA), but not with the antioxidant N,N'-diphenylphenylenediamine (DPPD). *In vitro,* purified MAT I/III was inactivated by hydrogen peroxide in a dose dependent fashion, the effect being exerted at the Vmax level [75]. This inactivation was reversed in the presence of GSH, and had no effect on the oligomeric state of the isoenzymes. However, the GSH concentration needed for this reversal of MAT I (25 mM) is far beyond the physiological levels of this metabolite (~5 mM), and hence oxidation was suggested as the cause for the low tetramer activity observed in cirrhosis. Site-directed mutagenesis allowed identification of C121 as the site of oxidation by hydrogen peroxide. However, inhibitory effects were also observed related to other cysteine residues (69, 105, 312 and 377). Hydrogen peroxide treatment of ST-C cells had no effect on the amount of MAT protein and reduced the activity in this clone, but not in wild type CHO cells or CHO-T_{121}, a clone overexpressing the C121S MAT mutant. This effect was mediated by hydroxyl radicals as demonstrated by the use of desferrioxamine, an inhibitor of the Fenton reaction. Again this type of modification should be specific for the MAT I/III isoenzymes since C121 is not present in other MATs. Free radical mediated MAT inactivation was suggested as an adaptive response to a nitrosative or oxidative stress, aimed at preserving ATP cellular levels [61]. On the other hand, a long lasting interruption of MAT activity would stop many important cellular functions.

Other regulatory models. *MAT1A* changes upon partial hepatectomy (PH) are regulated at a post-transcriptional level through factors that do not need *de novo* protein synthesis. These alterations cause decreases in AdoMet levels, increases in those of AdoHcy, and hence reductions in the AdoMet/AdoHcy ratio. In addition, DNA methylation is decreased,

whereas GSH levels rise; this last effect could help maintain MAT I/III activity [51]. This type of regulation is also observed as an early effect upon hepatocyte incubation under low oxygen concentrations [61]. The initial MAT inactivation with concomitant AdoMet and GSH reduction correlates with MAT I/III protein decreases after 24 hours. Inactivation under these conditions is mediated by nitric oxide (NO), as judged from increases in inducible nitric oxide synthase (iNOS) transcripts and the blockage of MAT inhibition in the presence of L-NAME, an inhibitor of iNOS. The partial protection observed with N-acetylcysteine also suggests a role for GSH in this regulation. Ceramide stimulation of hepatocytes or H35 cells induces a reduction in MAT I/III expression [76]. Such induction is also observed upon IL-6 addition, either alone or in combination with ceramides. This decrease in protein correlates with the reduction in activity [76].

Regulation of MAT II isoenzyme. Changes in the relative amounts of the different MAT II subunits have been observed upon stimulation with T-cell mitogens [32]. PBMC cells incubated with phytohemagglutinin (PHA) sequentially increase their IL-2 production, followed by their MAT activity and lastly DNA synthesis [32]. Analysis of the amount of MAT II subunits by Western blot revealed an increase in α2, and more dramatically in α'2, whereas β-subunit levels remained constant. Kinetic analysis by Hanes-Woolf plots indicated the presence of two enzymatic forms catalyzing AdoMet synthesis in activated cells. Increases in activity preceding protein synthesis could be due to post-translational modifications, leading to alterations in the relative amounts of α2/α'2 and, hence in their oligomerization with β-subunits. These changes in the amount of MAT II subunits during activation were not observed in other cell lines, such as Jurkat or freshly isolated PBMC cells from acute lymphocytic leukemia ALL-2 patients. In those cases activation induced IL-2 production, but no increase in MAT activity or change in the relative amount of MAT II subunits was observed [77]. Moreover, the use of SEB as stimulator also caused activation of PBMC cells and a 2-fold increase in MAT II activity, but the changes in the relative amounts of the subunits differed from those observed with PHA treatment [77].

3. MAT IN PLANTS.

Plant cells need AdoMet to carry out a variety of specific AdoMet dependent processes, such as ethylene formation [78] and lignin polymerization [79], as well as the functions essential for their viability that are common to other organisms [80]. To accomplish these needs the presence of up to seven MAT genes has been described in some plants [81-91]. Some of these genes lack introns [87] and contain short leader sequences (9-13 nucleotides) [81, 82]. Most studies have been carried out using *Arabidopsis thaliana* as a model. Comparison of the *SAM1* and *SAM2* genes of this plant revealed a high divergence among the sequences flanking the ORFs [81]. However, the promoters possess three regions of conservation that are candidates for the presence of cis-acting elements responsible for organ-specific expression [82]. The ORFs encode proteins of approximately 394 residues and are highly homologous to each other (up to 96% identical at the amino acid level) and to the *E. coli* and yeast enzymes (50-60%) [3]. However, exceptions to this conservation were observed in wheat embryos and dwarf pea epicotyls, where subunits exhibited 84-87 kDa molecular weight on SDS-PAGE gels [92-94]. The native enzyme chromatographed normally as dimers [92, 94], except for the *Caranthus roseus* isoenzymes which were recovered as monomers after gel filtration chromatography [87]. In addition, associated tripolyphosphatase activity has been observed in MATs purified from wheat embryos [92], and, as occurs with other MATs, the importance of cysteine residues in three *Arabidopsis thaliana* isoenzymes has been shown using NEM and DTNB [95].

Expression varies among different parts of the plant, for example *SAM1* of *Arabidopsis* has higher mRNA levels in stem and root as compared to leaves, and this pattern is directly correlated with MAT activity levels [81]. Such data suggest that regulation takes place mainly at the transcriptional level. Moreover, expression is primarily located in vascular tissues, sclerenchyma and root cortex. The strong cellular preference in the expression of the *SAM1* gene seems to be, at least partly, correlated with the extent of lignification the tissues are undergoing. In addition, several models showed up-regulation of MAT gene expression, protein levels and activity at different stages during development : i) during germination [92, 96]; ii) in

early stages of development of leaf and root [85]; iii) in young stems, sepals and corollas [97]; iv) during initiation of fruit development [86]; v) in steps preceding anthesis [98]; vi) during adventitious root development [99]; and vii) in the pre-storage phase of endosperm development during early desiccation [100]. On the other hand, down-regulation occurs in other steps, such as after anthesis [86, 101]. Hormonal, nutritional and stress regulation of plant MATs is linked to the above mentioned changes. Thus, gibberellic acid induces expression of two isoenzymes in dwarf pea epicotyls, an effect blocked by cycloheximide [94], and reduces expression in ovaries after anthesis [86, 101]. Auxins induce MAT during fruit development [86]. Salt stress and drought induce differential expression of MAT isoenzymes in tomato seedlings, an effect that can be due to a combination of ion toxicity and osmotic stress, the latter inducing synthesis of abscisic acid (ABA) [84, 85, 102, 103]. Such differential expression is also obtained with mannitol and ABA treatment, as well as upon wounding. The reason for such induction may be the need for larger AdoMet amounts for either cell wall synthesis or modification. Moreover, these stimuli also increase ethylene biosynthesis, a common process for plant response to environmental stress. MAT genes are also among the genes whose expression increases in relation to pathogen defense upon exposure to fungal elicitor [104]. Several methylation reactions are known to be associated with pathogen defense, most notably the formation of diverse classes of phenylpropanoid derivatives. Ethylene biosynthesis is also stimulated in elicitor-treated leaves [104].

Regulation by nitrosylation has been studied in *Arabidopsis thaliana* isoenzymes [95]. Only recombinant SAM1 was inhibited by GSNO, the modified residue being cysteine 114. This residue is located close to the putative substrate binding site, at the loop of access to the active site according to a structural model, and flanked by amino acids that promote nitrosylation (T**K**C̲P**EE**). Crosstalk between ethylene and nitric oxide (NO) signaling has been proposed at the level of MAT regulation through nitrosylation of C114 of the SAM1 isoenzyme, whereas the other isoenzymes are not affected significantly [95, 105]. Production of NO regulates key plant defense networks, and thionitrosyl (SNO) formation and turnover are required for multiple

modes of disease resistance. Finally, overexpression of Arabidopsis *SAM1* in tobacco plants has phenotypic effects such as the appearance of dark green sectors in leaves [106]. The dark sectors have reduced MAT activity and silencing of the transgene. On the other hand, transgenic plants with suppressed MAT activity produce the methanethiol associated with cabbage odor, as do methionine treated plants [106].

4. MAT IN YEAST

The understanding of MAT has greatly benefited from the use of yeast as a model organism. The observation that some yeast strains accumulate large intracellular concentrations of AdoMet in UV-dense vacuoles [107-109], which also have high levels of polyphosphate as counter ion, has led to the use of yeast fermentation as a route to commercial production of AdoMet. The pioneering mechanistic work of Mudd in the 1960s utilized bakers yeast preparations [110-114]; it is unknown whether these contained mixtures of the isozymes that were only discovered a decade later by Chiang and Cantoni [115]. These authors obtained the first preparation of pure, catalytically active MATs, and found two isozymes in the genetically heterogeneous bakers yeast. SDS-PAGE gels showed that each isozyme had two distinct types of subunits with apparent molecular weights of 55 and 60 kDa, forming hetero-dimers with a native molecular weight of 110 kDa. Both MATs had tripolyphosphatase activity and displayed requirements for divalent and monovalent cations. The steady state kinetic data showed downward curvature in double-reciprocal plots for both ATP and methionine; this was interpreted as negative cooperativity, although the same observations could result from the simultaneous action of two enzymes with different kinetic parameters. The kinetic parameters are listed in Table 1 along with those of other purified MATs.

Using the yeast *Saccharomyces cerevisiae*, Surdin-Kerjan and coworkers identified mutations in two genes that caused reductions in MAT activity; these genes are denoted *SAM1* and *SAM2* (originally called *ETH10* and *ETH2* because the mutations were selected by their conference of resistance to ethionine) [116, 117]. The *SAM1* and *SAM2* encoded MAT subunits

are 92% identical in polypeptide sequence. The genes are differently regulated, with methionine inducing *SAM2* expression while repressing that of *SAM1* [118]. The same authors constructed a strain that combined mutations in *SAM1* and *SAM2*; this strain had no MAT activity and required AdoMet for growth, providing the first evidence that this metabolite is essential for any organism. The construction of deletion mutants that are totally defective in AdoMet synthesis was possible due to the ability of *S. cerevisiae* to import AdoMet from the media via the specific transport protein encoded by the *SAM3* gene [119]. More recently several obligate intracellular parasitic organisms have been found to have AdoMet transport systems which compensate for the absence of an encoded functional MAT (see **MAT and Parasites**, Section 8).

5. MAT IN BACTERIA

Most bacteria have a single copy of a structural gene for MAT, which is highly homologous to the eukaryotic form [3]. The protein appears as a homotetramer in which early studies demonstrated the importance of cysteine residues [120]. The modification of two cysteines per subunit of the *Escherichia coli* MAT (cMAT) led to inactivation and dissociation to inactive dimers, followed by aggregation. Identification of the modified cysteines revealed cysteines 90 and 240 of cMAT as the labeled residues. There are few biochemical data regarding the regulation of the activity of bacterial MATs, with the exception of the potent product inhibition of cMAT by AdoMet [121]. The transcriptional regulation of MAT in both Gram positive and Gram negative bacteria has been extensively studied. In both cases, the expression of the structural gene for MAT, generally denoted *metK*, is regulated in conjunction with the genes of the enzymes of methionine biosynthesis [122-125].

In Gram positive bacteria, such as *Bacillus subtilis,* the "S-box" riboswitch metabolite-sensing RNA binds AdoMet and regulates the expression of at least 26 genes, including the *metK* gene [126-129]. The dissociation constant for AdoMet binding to this riboswitch RNA is 10 nM; the complex acts as a transcriptional terminator [129]. In contrast, Gram-negative bacteria regulate *metK* expression through use of the *metJ* repressor protein in a complex with

AdoMet as corepressor [130]. Expression of the *metK* gene of *E. coli* is also repressed by *Lrp* (leucine regulatory protein) [131].

6. ARCHAEAL MATs

The existence of an archaeal MAT activity was first demonstrated in *Sulfolobus solfataricus* extracts [132]. The subsequent purification of MAT from *Methanococcus jannaschii* enabled identification of the gene within the completed genome sequence [133]. The sequence of *M. jannaschii* MAT is widely diverged from that in eucarya or most bacteria, retaining ca. 18% sequence identity [133]. Close homologues of the *M. jannaschii* MAT are found in both *Crenarchaeota* and *Euryarchaeota*. Thus, a dramatic phylogenetic differentiation in MATs occurred early in evolution. It is unclear whether the difference in archaeal vs. bacterial/eucaryotic sequences reflects convergent or divergent evolution. The biochemical properties of archaeal MATs are generally similar to those from bacteria and eucarya providing no clue as to a functional meaning of the sequence divergence [132-134]. Thus, the *M. jannaschii* MAT is a dimer, has tripolyphosphatase activity, and requires both divalent and monovalent metal ions for activity [133, 134]. Recombinant, highly thermostable, *M. jannaschii* MAT is readily produced in *E. coli* and its ability to use a variety of nucleotides in addition to ATP may make it a useful synthetic tool [134]. How MAT activity is regulated in archaea remains unknown.

7. BACTERIA WITH ARCHAEAL-TYPE MATs.

The finding that the sequence of MATs in the archaea is substantially different from that in eubacteria or eucarya enabled computational identification of organisms with both types of MAT in their genomes [133]. The archaeal type of MAT has not been found in any eucarya, nor has the eucaryotic/bacterial class of MAT been discerned in any archaea (unpublished results). However several bacteria have been identified as possessing both the expected type of MAT and the archaeal type. These bacteria are widely scattered throughout phlyogengy. These

organisms include: *Aquifex aeolicus*, a deeply branching bacterium which encodes both types of MAT in its small 3 Mb genome; the gram positive human pathogen *Streptococcus pyogenes* (but not other *Streptococci)*; several *Chlorobaicaes* such as *Chloribium tepidum* and *Pelodictyon lutelum*; the *Alphaproteobacterium Bradyrhizobium japonicum*; the *Betaproteobacteria Ralsotnia eutropham*, *Burkholderia vietnamiensis and Pelobacter carbinolicus*; and several *Delatproteobacteria (Delta proteobacterium MLMS-1*, *Pelobacter* propionicus, *Syntrophus aciditrophicus* and several members of the *Geobacter* group). Proteomic studies of *S. pyogenes* [135] and *G. sulfurreductans* [136] show that both types of MAT proteins are indeed expressed, implying functionality. The metabolic reason for the presence of two types of MATs in these organisms remains to be elucidated.

8. MAT IN PARASITES

Many parasitic organisms have MATs with atypical properties, and even in a few cases have no MAT and are thus AdoMet auxotrophs (recently reviewed in [137]). The parasitic fungus *Pneumocystis carnii* is unable to synthesize AdoMet, but has two AdoMet transporters with Km values of 4.5 and 333 μM [138, 139]. Trypanosomes of the *Trypanosoma brucei* group are amongst organisms that both have MAT and are able to transport AdoMet from the media via a specific transport system [140]. These organisms lack the capacity for *de novo* purine synthesis. It is relevant that the AdoMet level is reported to be 70 nM in human sera [141], whereas the Km for the transporter is in the 4–10 millimolar range [142]. Thus, transport will be inefficient *in vivo*. Trypanosomal MATs are unusual in that they are not regulated by AdoMet, with K_i values in the millimolar range, in contrast to the micromolar values for AdoMet inhibition of MAT from most organisms [143]. Amongst the *Leishmania*, *Leishmania infantum,* a trypanosomatid protozoan parasite, has two MAT genes in a tandem array. Two AG dinucleotide trans-splicing sites are located in its 5'-UTR, and the +420/+905 region of the 3´-UTR contains cis-regulatory elements [144]. These genes are expressed as a single transcript [145]; the resultant protein forms a dimer. Surprisingly selenomethionine and cycloleucine did

not inhibit their MAT activity, and AdoMet had a remarkably high K_i of 1.5 mM. *Leishmania dovanii*, also has two MAT genes [146]. The properties of the purified enzyme have been studied with particular attention to the importance of cysteine residues in folding and catalysis [137, 146]. These authors also showed that truncations at F382, D375 and F368 of MAT sequence resulted in loss of MAT activity and reduction of tripolyphosphatase activity [147]. An internal deletion, E376ΔF382, preserved a high tripolyphosphatase activity that is not stimulated by AdoMet [147].

The MAT from the protozoan parasite that causes malaria, *Plasmodium falciparum*, has been cloned and a molecular model was constructed based on the cMAT crystal structure [148]. The authors proposed this enzyme as a target for the development of new anti-malarial drugs. The enzyme had a high K_i for cycloleucine as compared to a human hepatic MAT (17 mM vs. 10 mM); little additional characterization was reported.

The *Amoeba proteus* xD strain harbours symbiotic X-bacteria; these bacteria become obligatory endosymbionts in a process that is accompanied by a change in the MAT isozyme expressed by the amoeba [149, 150]. The lack of expression of the ameoba's primary MAT is due to lack of transcription rather than mutations to the gene itself. Apparently the bacterium becomes essential to the survival of the amoeba by modification of the expression of the host MAT gene. The mechanism of AdoMet exchange between the two organisms has not been reported.

Obligate intracellular bacteria have been found in which the *metK* gene encoding MAT is present but not functional [151, 152]. Among these are *Chlamydia trachomatis* [153], the α proteobacterium *Rickettsia prowazekii*, and other members of the spotted fever group of *Rickettsia*. The *metK* gene in these *Rickettsia* is regarded as a marker for the process of the degradation of genes that have become unnecessary as a result of changes either in the habitat of the organism or the gain of redundancy accompanying horizontal gene transfer [154]. In contrast, a functional MAT is present in *Rickettsia* of the typhus group [155]. *R. prowazeiii* have a characterized transporter than can import AdoMet from the host [155], and which is functional

when expressed in *E. coli* [156]. Finally, *Nanoarchaeum equitans* is the only known archaeal parasite; it also lacks a gene for MAT in its small 0.5 Mb genome [157], but the nature of its acquisition of AdoMet remains unknown.

9. STRUCTURE AND CATALYTIC MECHANISM

9.1. Structures of MAT

Crystal structures have been reported for the rat MAT I (cf. pdb code 1QM4 (2.7 Å resolution) [23]), the human MAT IIa (pdb code 2PO2 (1.03 Å) [158]) and cMAT (cf. pdb codes 1XRA, 1XRB and 1XRC, 1FUG) [159, 160]. As might be expected from their sequence similarity, the overall topology is the same for each protein (RMSD for alpha carbons = 1.3 Å between rat and human; 0.78 Å between *E. coli* and human). The structure of MAT is unique among all protein structures determined to date. The topology is described in the SCOP database (http://scop.berkeley.edu/data/scop.b.htm) as belonging to the Alpha + Beta protein class, and there are no other members of the MAT superfamily. Three repeats of *beta-alpha-beta(2)-alpha-beta* secondary structure are found; notably there is no significant sequence conservation within the structural repeats (illustrated for the human MAT IIa in Figure 4a). These repeats constitute the three domains of the subunit which are formed by non-consecutive stretches of the sequence. The dimer of the enzyme forms in an inverted arrangement so that the two active sites are located in a deep cavity between the subunits (Figure 4b). The tilt between the dimers of MAT I is distinct from that of cMAT; the human MAT II structure has not yet been analyzed in detail. A tetramer forms from the association of two dimers at right angles at their central tips (Figure 4b). The hydrophobic interface between monomers in the tight dimers is much more extensive than is the polar interface between pairs of dimers, consistent with interconversion of the dimeric and tetrameric forms of the mammalian MAT I and III and the stability of the dimer.

The majority of the reported MAT structures share a disordered loop segment (residues 117–128 in rat MAT I, 102–107 for cMAT) that forms a dynamic lid over the active site. The only

structures in which this lid is ordered are of the human MAT II complex with AdoMet (pdb code 2PO2 [158]), the cMAT both in a ligand free form (1FUG [160]) and in structures with the active site filled with methionine and the ATP analogue AMPPNP (or the products of the reaction in the crystal, AdoMet and PPNP (1RG9, 1P7L [161]). Other structures in which only some of the ligand binding sites are occupied have the a lid segment disordered, suggesting that motion of the lid is involved in allowing access of substrate and products to the active site, an interpretation supported by kinetic and spectroscopic studies of cMAT [162, 163].

Structures for several MAT complexes have been published. These include: i) cMAT in complexes with ADP, BrADP, PP_i and the ternary complex with AMPPNP and methionine [159, 161, 164]; ii) rat MAT I in binary complexes with L-cisAMB or aminoethylphosphonate (AEP), and ternary complexes including ATP plus methionine, ATP plus AEP, and ADP plus L-cisAMB [23, 165]; and iii) the AdoMet complex of human MAT II [158]. The ADP binding site observed in binary complexes with cMAT (1MXB, 1MXC) is essentially the same as that found in MAT I ternary complexes (1O93 and 1O9T). Moreover, both enzymes hydrolyze ATP to ADP and P_i, and hence in some of the complexes where ATP was added ADP was observed. The methionine binding site determined for MAT I in complexes with the methionine analogues L-cisAMB and AEP is also similar, with stacking against Phe-251 (Figure 5a). In addition, the three phosphates appear also in analogous positions for rat and *E. coli* enzymes [23, 159], the P_γ being oriented against a putative P-loop previously identified by photoaffinity labeling [166]. However, differences arise between ligand locations in ternary complexes. Thus, in the rat MAT I•ATP•methionine complex (1O9T, 2.70 Å) additional density compatible with AdoMet appears in a different orientation than ATP. The cMAT•AMPPNP•methionine complex (1P7L, 2.50 Å) shows interchanged positions for the methionine and adenine moieties which is observed also in active sites containing AdoMet + PPNP (which were formed by reaction of AMPPNP and methionine in the crystal (Figure 5b; pdb codes 1PL7 and 1RG9)). Figure 5c shows the ligand positions when the *E. coli* and rat MAT I protein structures are superimposed (see the active site histidine (residue 14 or 30, respectively) at lower right). This AdoMet location in the *E. coli*

complex superimposes on that for AdoMet in the MAT II-AdoMet structure (2PO2) Figure 5b). In the structures of the MATII•AdoMet and cMAT•AdoMet•PPNP complexes the adenine ring stacks against the phenylalanine in the position occupied by L-cisAMB and AEP in the MAT I complex structures. The location of two phosphoryl groups (which might be expected to be relatively well located in the X-ray data due to the grouping of heavy atoms) is preserved throughout. At present the origin of the differences between AdoMet locations in MAT I and *E. coli* or MAT II structures are unclear, but could either reflect the various other ligands bound (see the legend to Figure 5), or represent the positions for AdoMet as a product and as an inhibitor. Available mutagenesis data for the two proteins do not discern which is the functional configuration. Resolution of this difference will be of major importance in understanding the enzyme mechanism.

Among the questions that await a structural solutions are the structure of the regulatory β subunit and its sites of interaction within MAT II isozyme. The high sequence similarity of the β subunit to nucleotide sugar reductases of known structure, such as dTDP-rhamnose reductase (26 % identity, 49 % similarity over 235 residues) suggests that the protein fold can be recognized by homology. The structure of dTDP-rhamnose reductase (pdb code 1vl0 with bound NADH, 2.05 Å resolution) shows a NAD-binding Rossman fold. No available data indicate the sites at which the MAT II α and β subunits interact, or if NAD(H) is involved. It will also be intriguing to see the structure of an archaeal type MAT since the sequence similarity to the eukaryal/bacterial MATs is very low, and differences in circular dichroism spectra suggest that the proteins have different α-helical content, perhaps implying different topologies. Nevertheless the few residues which are conserved in a sequence alignment are primarily located in the active site of the bacterial/eucaryotic MATs [134]. There are no reported structures of proteins with significant sequence homology to the archaeal MAT.

9.2. Kinetics and mechanism

Studies of the kinetic mechanism of MAT catalysis date to the pioneering work of Mudd in the 1960's (reviewed in [167]. Many of the seminal reports are unfortunately complex because before the studies of Chiang and Cantoni [115] it was not realized that in many organisms there are co-purifying isoforms. Even in systems with pure isozymes kinetic studies have been challenging, due several unusual behaviours: 1) low concentrations of AdoMet activate its own synthesis by yeast MATs [112, 168], 2) the rat MAT III shows complex hysteretic behavior with a temporal lag phase before the steady state rate is attained [73] and 3) severe non-competitive inhibition by the product AdoMet, particularly with cMAT [121]. Nevertheless the overall picture that has emerged for MAT from various organisms is remarkably consistent. A common overall physical basis for the kinetic mechanism appears applicable, however among MATs there are physiologically significant variations in the detailed rate constants (and,equally importantly from an overall perspective, the ratios of the rate constants and thus relative K_M values).

The two yeast MATs show negative cooperativity for both ATP and methionine [115], the non-hepatic rat MAT II isozyme displays negative cooperativity for methionine [169], while the rat MAT III shows positive cooperativity with a Hill coefficient of 1.8 [169]. Hence in many cases the concentration dependences of substrate saturation are referred to $S_{0.5}$ values rather than K_M values.

All studies point to a sequential kinetic mechanism, as illustrated in Figure 6. There is 1) formation of a E•ATP•Met complex, 2) reaction to form an E•AdoMet•PPP_i complex as an obligatory tightly enzyme-bound intermediate and 3) the subsequent hydrolysis of PPP_i before regeneration of the free enzyme [112, 121, 170]; reviewed in [167, 171]. Variations in rate constants among MATs from different organisms, combined with a diversity of experimental conditions, can account for the observed differences in steady state kinetic mechanisms. These differences are seen in the apparent order of substrate binding for example, with ATP being first (most eucaryotic MATs and the archaeal *M. jannaschii* MAT) vs. random addition (e.g. *E. coli*, although in this case in the preferred order of binding ATP also binds first [170]). The order of

product release is also variable with the most obvious path being taken by the *E. coli* enzyme, where the products are liberated in order of diminishing affinity, i.e. P_i before PP_i, before AdoMet.

In terms of interconversion of enzyme-bound substrates and products, extensive early work by Mudd and others demonstrated that the only detectable reaction intermediate was PPP_i, [111]; no covalent enzyme-intermediates have been identified, either in AdoMet formation or in PPP_i hydrolysis. PPP_i is hydrolyzed in a predominantly oriented fashion; > 95% of the P_i originates from the γ-phosphoryl group of ATP, and PP_i from the α, β groups [110]. This demonstrates that PPP_i is tightly bound during turnover so that the rate of escape from the enzyme, or even of reorientation of this symmetric compound within the active site, is slow compared to hydrolysis. The position of the γ-phosphate is preserved in the crystal structures to date, showing the active site structure at different steps of the reaction in which movement of the α- and β-phosphates, as well as on the ribose moiety is observed [161, 164, 165]. The site of bond cleavage as $O_{\beta\gamma}$ -Pγ was shown by the incorporation of ^{18}O from H_2O^{18} solely into P_i [110].

The AdoMet activation of its own synthesis in the reaction catalyzed by the yeast MATs [111, 168] and MAT III [169]; deserves note: this phenomenon appears to reflect the escape of AdoMet from the E•AdoMet•PPP_i complex before PPP_i hydrolysis, which results in E•PPP_i hydrolysis becoming rate limiting in turnover. The rate of exogenous AdoMet binding to E•PPP_i becomes comparable to the rate of E•PPP_i hydrolysis and the hydrolysis rate accelerates after AdoMet re-binding to form E•AdoMet•PPP_i complex. In general MATs catalyze the hydrolysis of added PPP_i, and the rate of this reaction is increased by added AdoMet. Most MATs show the anticipated product inhibition of the overall reaction by AdoMet.

9.3. Chemistry of Catalysis

A significant contribution to understanding the chemical mechanism of catalysis was the determination of the stereochemical course of the yeast MAT reaction using chiral [5'-^{2}H]-ATP

[172]. The chiral [5'-^{2}H]-AdoMet was formed with inversion of stereochemical conversion at carbon-5'. This result demonstrated that an odd number of displacements takes place at carbon-5', which is most simply attributed to a direct displacement of the PPP_i chain from ATP by the sulfur of methionine. Subsequent kinetic isotope effect measurements with the *E. coli* enzyme showed a 13% reduction in catalytic rate when ^{14}C replaced ^{12}C at the C5' of ATP which demonstrates that the bonding change at C5' is involved in the rate limiting step of the reaction [173]. The magnitude of the ^{14}C effect was near the theoretical maximum, and there was no detectable secondary isotope kinetic effect from 5'-^{3}H; the observed isotope effects from ^{35}S and [methyl-^{3}H]methionine showed the involvement of methionine in the displacement step. The combined data demonstrate that AdoMet is formed in a classical S_N2 displacement mechanism with simultaneous attack of the sulfur of methionine on the C5' of ATP and displacement of the PPP_i chain [173].

The unique catalytic task of MAT is thus to facilitate the attack of methionyl sulfur on ATP to form AdoMet, a reaction in which charge separation occurs to yield the sulfonium cation and an additional negative charge on the polyphosphate chain. It is remarkable that when bound to the enzyme the equilibrium for the formation of AdoMet and PPP_i lies far towards the products, i.e. at the active site the PPP_i hydrolysis step is not needed for product accumulation [111, 121, 170]. Rather PPP_i hydrolysis facilitates product release by the cleavage of the tightly bound PPP_i intermediate to the more weakly bound PP_i and P_i.

The mechanism of hydrolysis of the PPP_i intermediate appears to be similar to that of the myriad of metal ion dependent ATPases and other phosphatases [174]. However the chemical means by which water is activated to react with the PPP_i and thus split the polyphosphate chain into PP_i and P_i is unknown. The hydrolysis reaction requires Mg^{2+} and in many MATs it is stimulated by K^+, although the effect of K^+ is typically not as large as is seen for AdoMet formation. It is not clear from the available crystal structures whether a metal activated water (or hydroxide ion) is involved in this step of the reaction, or whether a protein group activates water. The active site arginine (R244 in cMAT, R265 in MAT I) is critically important

for orienting the PPP_i for hydrolysis [175]. It is an intriguing possibility that one of the residues in the conserved Lys-Arg sequence may be unprotonated and acts as the catalytic base; an unprotonated arginine has been recently proposed to be the catalytic base in other hydrolytic reactions [176]. However, the R244L mutant of cMAT had less than a 10-fold decrease in tripolyphosphatase activity [175]; the K245M mutant had >100-fold decrease in PPPase activity, but CD spectra showed that it was incorrectly folded precluding definitive interpretation [177].

Kinetic and spectroscopic studies of cMAT have revealed that protein conformational changes are coordinated with catalysis [163, 170, 178]. Pre-steady state and solution viscosity dependence kinetic studies of cMAT show that a conformational change occurs in conjunction with the formation of AdoMet and PPP_i, and a second change occurs after PPP_i hydrolysis [163, 170, 178]. These changes probably involve movement of the protein loop that covers the active site in some crystal structures but is disordered in others [23, 159-161, 165]. This loop is the least conserved region of the protein sequence among eucayal/bacterial MATs and varies in length from 6 to 19 residues [3]. The closure of the active site loop would prevent release of the PPP_i intermediate before hydrolysis. The movement of protein loops to sequester reaction intermediates while allowing access of substrates and release of products is a common feature in enzyme catalyzed reactions [179-181]. Blockage of substrate access to the active site of MAT I/III by nitrosylation has been suggested as a physiological regulatory mechanism [18]. Nitric oxide modification of C121 in this flexible loop may stabilize its closed conformation thus impeding the entrance of the substrates for catalysis.

9.4. Metal Ion Activation

MAT from all sources requires Mg^{2+} or another divalent metal ion for activity, and where it has been examined, a monovalent cation such as K^+ has been found to enhance the reaction rate on the order of 100-fold (studies that have specifically address the cation activation include those in [29, 113, 115, 121, 182]. The monovalent cation effect has not always been noted because other ions such as Na^+ and NH_4^+ can substitute for K^+, and these cations are commonly

present as counter ions of ATP or buffer components. Crystal structures show that the active site of MAT is highly polar, with many charged residues. This is consistent with the course of the reaction in which additional charges accumulate as first AdoMet and PPP_i form, and then upon creation of PP_i and P_i, presumably with release of one proton from water. Thus, a role for cations in stabilizing the new negative charges is easily rationalized.

Binding studies in conjunction with EPR and NMR spectroscopic investigations of cMAT have shown the presence of two divalent cations and one monovalent cation at the active site [183-186]. EPR studies of the Mn^{2+} activated enzyme showed that two Mn^{2+} ions bind very closely together, which crystallographic studies confirmed, showing two divalent cations 5.2 Å apart, with both binding to the phosphate groups; one ion coordinates all three α, β and γ groups. while the second coordinates the α- and γ-phosphoryl groups. The coordination of two metal ions to the leaving α-phosphoryl group could play a substantial catalytic role by stabilizing the negative charge formed upon C-O bond rupture. A monovalent cation binds at distances of 3.8 and 8.1 Å from the two divalent ions. The sole protein ligand to this ion is the carboxylate of E42, and mutation of this residue to glutamine abolished monovalent cation activation [187]. Neither crystallographic, solution spectroscopic nor mutagenesis studies have indicated that the monovalent cation directly binds to the substrates, and this ion appears to play an indirect role in organizing the active site structure [187]. The crystal structure of the *E. coli* enzyme revealed an additional monovalent cation bound between two subunits in the tight dimer; this ion appears to play a role in dimer stability and is conserved in the MAT I structures. The crystal structures of the rat MAT I enzyme have shown as many as three Mg^{2+} at the active site [23, 165]. One of these cations interacts with the carboxylate of the substrate methionine or an analogue such as L-cisAMB or AEP; such an interaction is not observed in any of the cMAT structures. The two additional PO_4 are each within 4 Å of the phosphates of ATP (1O9T) in the MAT I structures; the significance of these phosphate ions are unclear.

9.5. Mutagenesis Probes of the Mechanism

The crystal structures of MAT have directed mutagenesis studies of active site residues. In these it has been advantageous to examine possible selective effects of the mutation on both AdoMet synthesis and on the hydrolysis of added PPP_i. The high evolutionary conservation of the active site residues implies little tolerance for variation, therefore it is not surprising that most mutations impair the enzyme function. For the *E. coli* MAT, all mutations of polar active site residues have had larger effects on the AdoMet formation step than on the PPP_i hydrolysis reaction [163, 175, 177, 187, 188], which is consistent with the singular active site architecture known for the former activity and the variety of known ways that enzymes catalyze hydrolysis of polyphosphate chains. The only mutations which affected the tetrameric state of the cMAT were C89A and C90S from which separate and non-interconverting dimers and tetramers were isolated, with a ca. 10–fold reduction in k_{cat} for the tetramer and an additional 20-30 fold reduction in the dimer [189].

The most informative mutagenesis result for the *E. coli* enzyme may be that mutation of the active site arginine-244 to leucine (see Figure 5b) resulted in a reduction of 1000-fold in k_{cat} for AdoMet formation, and a remarkable ability of the PPP_i intermediate to reorient within the active site before hydrolysis, showing the importance of the guanidinium in stabilizing the intermediate conformation [175]. In contrast to the R244L and R244H mutants of cMAT which remained tetrameric and retained wild type secondary structure as indicated by CD spectra, the R265H mutant of MAT I eluted in gel filtration chromatography with a calculated Mr of 41000, corresponding to a monomer [190]. This mutant was reported to have no AdoMet synthesis capacity (2% of wild type), but preserved the tripolyphosphatase activity, its V_{max} for hydrolysis being similar to that shown by the wild type MAT (128 vs. 156 nmol/min/mg). A R265S mutant was also found to be a monomer, but in this case along with a 99% reduction in AdoMet synthesis a 5-fold decrease in tripolyphosphatase activity was also detected. These results together suggest the involvement of the positive charge at 265 in hydrolysis. No association of the mutant monomers was observed under assay conditions, as judged by gel filtration chromatography (41.6 kDa). However, coexpression with the wild type protein produced hetero-

oligomers (90 kDa on gel filtration chromatography) with tripolyphosphatase activity. This arginine 244 (or 265 in rat) residue is involved in a salt bridge with E58 of the opposite subunit, one of the few polar interactions formed at <3.5 Å between monomers in the dimer, and this interaction is preserved in MAT I crystal structure [23, 164].

Active site mutants (D180G, K182G, F251D and F251G) prepared on rat MAT I and overexpressed in *E. coli* showed no AdoMet synthesis capacity, but preserved PPP_i hydrolysis activity. It is noteworthy that the PPPase activity of F251 mutants is double than that shown by the wild type protein. A possible explanation for this fact may be that the reduction in size of the lateral chains in those mutants facilitates accessibility for PPP_i. Moreover, all these mutants were able to bind ATP with Kd values similar to that of the wild type (10.54 ± 0.85 mM) [23]. Thus, mutations at the methionine binding residue (F251) only prevent AdoMet synthesis.

9.6. Solvent effects on MAT activity- practical consequences

A phenomenon that has aided discrimination among the mammalian MAT forms is the ≈10-fold DMSO activation of the rat MAT III isozyme [191, 192], the molecular basis for which has not been resolved. The ability of molar concentrations of mercaptoethanol, acetonitrile and other compounds to alleviate the potent non-competitive inhibition of the *E. coli* MAT by AdoMet has facilitated synthetic applications [193]. Significant changes in the far-UV CD spectra of cMAT are associated with the addition of these co-solvents, suggesting substantial alterations in protein folding. No analogous structural data have been reported for the mammalian MATs.

10. MAT FOLDING

Studies on MAT folding have been carried out mainly *in vitro* using rat MAT I/III with urea and thermal denaturation. The high conservation in sequence and structure found between α subunits of eucarya and bacteria make these results extrapolable to most of the known MATs, probably with the exception of the large subunits observed in some plants. *In vivo* data are

restricted to the identification of cMAT as one of the proteins folded by the *E. coli* GroEL/GroES complex [194].

10.1. MAT III folding under equilibrium conditions Folding of MAT III, the rat liver MAT dimer, was studied using urea as the perturbing agent. The enzyme used for these studies was either the DTT-refolded protein from *E. coli* inclusion bodies at concentrations below 0.2 mg/ml [195] or the rat liver purified dimer [196]. In both cases the equilibrium experiments showed the reversibility of the process that occurs through a three-state mechanism (Figure 7). Data were obtained by activity measurements, fluorescence spectroscopy, CD, sedimentation velocity and gel filtration chromatography. These techniques allowed following of the process at different levels, i.e. the active site, the tertiary structure, the secondary structure and the subunit association level. The loss of activity preceded the changes detected by other techniques, and occurs earlier for AdoMet synthesis ($D_{50\%}$ = 0.3M) [195] than for tripolyphosphate hydrolysis ($D_{50\%}$ =1M) [196]. ANS binding showed a similar trend, exhibiting a single transition at these low urea concentrations ($D_{50\%}$ of 1 M) [196]. Both fluorescence spectroscopy and far-UV CD showed two transitions with a plateau at 1-2 M urea, an interval at which monomers where detected by both gel filtration chromatography and sedimentation velocity [195, 196]. Maximal fluorescence emission for MAT III appears at 334 nm, with intensity decreasing by denaturation as the maximum is displaced to 355 nm [195]. Thus, in the three-state mechanism for MAT III unfolding the activity is lost before dissociation to a monomeric intermediate (I).

The structure of intermediate (I) allows limited proteolysis that produces a 3 kDa fragment, which as deduced from mass spectroscopy data, corresponds to a N-terminal cut at lysine 33 [196]. Other characteristics of intermediate (I) include: a 2.5-fold decrease in tryptophan fluorescence intensity; preservation of a 70% of the secondary structure of native MAT III; and, a 75% lower ANS binding as compared to that of the native dimer [195, 196]. All together these data indicate that this intermediate (I) shows features of a molten-globule. Accumulation of the intermediate was estimated to be maximal between 2-2.5M urea as

deduced from tryptophan fluorescence and CD data. Unfolding data obtained using the active site monomeric mutant R265H [190, 196] also show the presence of an intermediate, thus confirming its monomeric structure [196]. However, this intermediate seems less compact than monomeric R265H as judged from its larger hydrodynamic volume. Data obtained with this mutant however should be considered with care, since analogous mutants obtained for cMAT behave as tetramers [175].

The apparent stoichiometry of ANS binding was determined to be 1 mol ANS per MAT III subunit, suggesting its binding to a specific site [196]. This location appears to correspond to the start of the loop at entrance to the active site, where there is a similar environment to that found in the crystal structure of another ANS-protein complex [197]. Monomers in the crystal structure of MAT I [23] have a large flat hydrophobic contact surface, thus solvent exposure of this interface would lead to a rearrangement in the subunit for hydrophobic burial. Such a rearrangement is suggested by the different ANS binding properties shown by the dimer and monomeric intermediate (I), as well as by the high value of the m_1 constant calculated for the dissociation process in urea denaturation. Unfolding of the intermediate would further require minimal changes to render the unfolded protein.

Analysis of all these data using models for different unfolding mechanisms showed the best fit for a three-state process involving a monomeric intermediate [195, 196]. The global m coefficient for urea dependence was 7.25 kcal/mol/M, whereas the value obtained for the global free energy change was calculated to be 15.7 kcal/mol. This low dimer stability toward urea denaturation is mainly derived from the low $\Delta G_1(H_2O)$ value that represents 50% of the global stabilization energy for MAT III (subunit association). The importance of the monomeric intermediate (I) is highlighted by refolding experiments that include a two step process in which the 2 M urea intermediate is favoured before the total elimination of denaturant. Such a two-step process allows not only a 100% recovery of the activity, but also provides a better yield when using inclusion bodies as the enzyme source [198].

MAT III is one of the many proteins made up of two identical subunits. The proliferation of such an arrangement may involve some advantage for the cell. Recently, it has been proposed that the oligomerization process itself might tune the enzymatic function [199]. These authors indicate that the formation of intersubunit contacts influences the biological activity by allowing very subtle conformational changes in the active site in such a way that oligomerization can indeed activate the monomeric subunits. Moreover, the fact that a 50% of the free energy for unfolding represents dissociation processes is consistent with the idea that contacts at the surface hidden between the monomeric subunits plays a fundamental role in the stabilization of oligomeric proteins. For dimers of a given size, those proteins that have a folding intermediate displayed less empty volumes [199]. The role of dimerization in proteins of class B ($N_2 \leftrightarrow 2I \leftrightarrow 2U$), such as MAT, is mainly structural according to Mei *et al* [199]. The dimerization process for this type of proteins might find a rationale in the protection and stabilization of those molten globule states that alone are not able to complete their self-assembly process.

10.2. Kinetic intermediates

Two kinetic intermediates have been identified during MAT I unfolding by fluorescence spectroscopy (Figure 7). The first one (I_k^2) occurs during the MAT III-intermediate I transition [196]. The data fit to a single exponential, corresponding to a monomolecular process, thus indicating that the folding pathway includes at least one kinetic intermediate (Figure 7). More evidence for the presence of such intermediate was obtained by ANS binding in the presence of tripolyphosphate, where a transient binding of the dye was observed at 1-2.3 M denaturant. These results suggest that even though I_k^2 is not well populated it must bind a large amount of dye to allow detection. No direct information about the association state of I_k^2 was obtained, and hence its monomeric state was inferred from other results [196]. Moreover, Sánchez del Pino *et al*. speculated that the shape of I_k^2 could be that of a folded or a well structured monomer, and hence have a large solvent-exposed hydrophobic interface which should be energetically unfavoured, leading to a very fast association. In addition, such a conformation would also

explain the high unspecific binding of ANS to I_k^2. This kinetic intermediate is also shared by the monomeric R265H mutant, prior to attaining its final folding, thus suggesting its monomeric character.

Another kinetic intermediate was identified in the tetramer-dimer association step using DTT-refolded MAT [17]. DTT-refolded tetramers and dimers appear in a concentration dependent equilibrium and are not distinguishable by either tryptophan fluorescence or CD [198]. However, MAT I dissociation could be followed by ANS fluorescence [17], showing an exponential decay to ANS-dimer fluorescence in a process that yields the best fit as a single exponential. Calculations of the half-life of MAT I render a value of 14.69 ± 0.5 s [17], in contrast to the 858 s previously obtained for the cytosolically overexpressed oligomer [200], but the studies differed in the temperature used. Again, the association state of this new kinetic intermediate (I_k^1) cannot be determined directly due to its short lifetime. However, the fact that its ANS fluorescence intensity is the same as that shown by the tetramer suggests that it might be a dimer that has just separated from the tetramer, before undergoing the changes needed to achieve the MAT III conformation. These changes cause reduction of its affinity for methionine [7, 8], as well as acquisition of DMSO activating capacity, and a higher hydrophobic character [191]. Moreover, in order to be detected by this technique these structural changes should occur close to the ANS-binding site that, as postulated by Sánchez del Pino et al., is located close to the active site loop [196]. The free energy change for MAT I dissociation was calculated as -6.99 kcal/mol, which along with the data derived from MAT III unfolding, give a global free energy change for MAT I unfolding of 24.41 kcal/mol [17]. Further analysis looking for the presence of additional intermediates in this dissociation process will rely on the use of stopped-flow techniques.

10.3 Thermal unfolding

Two-dimensional infrared spectroscopy (IR), activity and tryptophan fluorescence spectroscopy were used to follow thermal unfolding of MAT [201]. The high protein

concentrations needed for IR precluded the study of dimers, since the equilibrium between DTT-refolded MAT forms was clearly displaced towards the tetramer. On the other hand, the low protein concentrations needed for activity and fluorescence measurements allowed analysis of the dimer behaviour. IR spectra in H_2O and D_2O are dominated by two bands at 1652 and 1636 cm^{-1} attributed to α-helix and β-sheet, respectively. A band at 1624 and 1626 cm^{-1} in D_2O and water, respectively, agrees with MAT I existence as an oligomer. The secondary structure composition was calculated to be 44% α-helix, 37% β-sheet, 18% β-turns and a very low percentage of unordered structure, in agreement with structural and CD data. Thermal unfolding was irreversible by all the techniques used in this study, showing a Tm of 47-51ºC [201]. Activity and tryptophan fluorescence measurements revealed one transition for dimer unfolding that occurs in the same temperature range as the IR changes for the tetramer. Such a result indicates that MAT I stability is highly dependent on that of the dimer. Moreover, the IR data show no change in the β-sheet band (1635 cm^{-1}) with temperature, whereas the corresponding α-helix band is split in two, with a reduction in the helical percentage [201].

Analysis of synchronous and asynchronous 2D IR maps gives information about which structures are changing and the order of events taking place at each temperature, respectively. As deduced from this study, native MAT I starts its unfolding at 37ºC, the dominant crosspeak being centered at 1622 cm^{-1}, which corresponds to changes in protein contacts and beginning of aggregation. The initial changes, according to asynchronous maps, correspond to peaks of α-helix, unordered structure and β-turns, structural elements located at the external surface of the subunits [23]. Moreover, changes in the 1624 cm^{-1} band indicate alterations in the oligomerization state that could be assigned either to T-D-M or T-M processes [201]. The unfolding continues, showing aggregation at the expense of the remaining secondary structures, in coincidence with changes in activity and tryptophan fluorescence that could be ascribed either to dimer dissociation and monomer aggregation or just aggregation of the unfolded dimer. No further changes are detectable after complete activity loss. Thus, MAT I thermal unfolding starts with changes in the structures of the hydrophilic surfaces, followed by loss of tertiary structure to

render a dimer, possibly enzymatically active in a narrow range of temperature, and leading, finally, to unfolding and aggregation. A special characteristic shown by MAT is the ability of some structures to interconvert during the process, a new aspect of denaturation events [201].

10.4. Association of MAT III to render MAT I: role of the disulfide C35-C61

As mentioned before, rat liver MAT appears in a protein concentration dependent equilibrium upon overexpression in *E. coli* [17, 198, 200], a fact that has not been observed in the MAT I and III forms purified from liver. On the other hand, GSH/GSSG-refolding renders a mixture of tetramers and dimers that do not interconvert, and that can be isolated as stable entities [198]. These results raised the question as to what feature modulates the association properties of MAT. The obvious difference among both refolding procedures is their different redox potential which might be crucial for a protein that contains 10 cysteine residues per subunit. Thus, analysis of the number of free sulfhydryl groups for each species was performed by chemical modification, and mass spectrometry showed 10 free sulfhydryls/subunit in DTT-refolded MAT, whereas only 8 were detected for GSH/GSSG-refolded MAT I and III [17]. The previous identification of the disulfide linking C35-C61 in rat liver purified MAT [22] prompted the analysis of a possible role for such a disulfide in stability. Such a study was performed using both refolding systems and cysteine mutants (C35S, C57S, C61S and C69S) located in the central domain of the subunit and at disulfide bonding distance according to the MAT I crystal structure [23]. Analysis of their behavior by analytical phenyl Sepharose chromatography, showed persistence of the dimer-tetramer equilibrium for GSH/GSSG-refolded C35S and C61S [17], thus suggesting a role for the residues involved in the disulfide bond in the MAT III to MAT I association process. Mass spectrometry analysis of the oxidized residues in GSH/GSSG-refolded MAT confirmed the existence of the C35-C61 disulfide, and hence that this modification stabilizes both tetramers and dimers.

The question remained as which is the step of the folding process where this disulfide is established. Two structures could be suitable for this purpose, MAT III and I_k^1 (Figure 7). MAT

III could undergo oxidation, in which case association would be precluded, or evolve to render I_k^1 which then evolves to the tetramer. This kinetic intermediate could also be the subject of oxidation, a process that would then preclude the conformational changes that lead to MAT III, and hence produce the oxidized tetramer. All these events would take place at the central domain that establishes the contact between dimers in the tetramer structure. The tetrameric arrangement is maintained by only five polar interactions between dimers in rat liver MAT, with a small area in direct contact with the solvent. The dissociation constant for these proteins was calculated to be 10^5 M, a value comparable to those described for proteins showing association/dissociation behaviour [200, 202], but much lower than that for cMAT (10^{10} M) [120]. Such a large difference in the constants is in agreement with all the data obtained to date with cMAT and its structure which shows a much larger contact surface between dimers. Thus, the few interactions that maintain MAT I can be easily modified/avoided by just a small rearrangement in the central domain, or by a higher flexibility of its secondary structure. The presence of the C35-C61 disulfide would stabilize this domain and facilitate the correct pattern of interactions between dimers.

10.5 Complementary folding data obtained in MAT proteins of different origins

The critical role of C69 in dimer association was shown by site directed mutagenesis studies of the rat liver protein, where C69S appeared mostly as low activity dimers upon overexpression in *E. coli* cytosol [19]. However, DTT-refolding from inclusion bodies showed an increase in the specific activity of that mutant, reaching a 64% of that of the wild type, as well as production of tetramers [17]. These data indicate a blockage of the folding pathway for C69S in bacterial cytosol that can be overcome *in vitro*. Cysteines at residues 61 and 69 are specifically found in liver MATs, and hence can be related to the special behaviour exhibited by these MAT forms. Both residues, as well as C57, are located in β-sheet B2, the position of C69 being close enough as to form a disulfide bond with C57 upon a slight torsion of the main protein chain [23]. However, it could be possible that under certain circumstances, such as during folding, this

second disulfide is formed either alone or in concordance with the C35-C61 disulfide, a possibility that would be precluded for C69S. Thus, blockage of the normal folding pathway could led to disulfide arrangements that would certainly render alterations in β-sheet B2, the contact area between dimers. Such changes would obviously alter the oligomerization pattern.

Folding studies carried out with *Leishmania donovani* MAT showed proper folding only under reducing conditions, with cysteines 22, 44 and 305 being crucial for the global process [146]. Conformational transitions were observed via fluorescence quenching during refolding from *E. coli* inclusion bodies in the presence of DTT. Such transitions generated fast and large events that took place during the first two hours of reaction, and were found to be essential for activity recovery.

A mixture of tetramers and dimers was also described for the cMAT mutants C89A and C89S [189]; C89 is located at the border between dimers with no involvement in strong interactions [164]. However, C89 is a conserved cysteine residue for MAT proteins of different organisms, thus suggesting an important role for such amino acid in dimer production. If this is the case, the structure of the dimer interface might be changed substantially by cysteine to alanine replacement leading to more labile liver-type arrangement.

11. MAT INHIBITORS.

The multitude of metabolic roles of AdoMet, and its involvement in modulation of cell growth, have resulted in an enduring search for MAT inhibitors with *in vivo* and *in vitro* utility. A selection of the most effective inhibitors produced to date, some of which have been used for structural studies, are shown in Figure 8, and their inhibitory properties are included in Table 2. The most widely used inhibitor has been the amino acid analog cycloleucine, since it is long established and commercially available [206]; with its K_i in the millimolar range and possible additional effects *in vivo* it is clearly not an optimal inhibitor.

The inhibitor design process has included analogues of both substrates ATP and methionine, both which have been tested in cancerous cell lines. Much of this work has been

carried out in the laboratories of Hampton and Sufrin (cf. [203, 204]); the two laboratories had significantly different approaches and have provided much information that we cannot analyze in depth in this review. Their results will be invaluable to the future development of potent, bioavailable, isozyme selective inhibitors. Hampton and co-workers strove for maximal inhibitory potency by linking the methionine methyl to the reactive 5' position of the nucleotide substrate, forming bi-substrate analogs; isozyme selectivity was achieved by further modification of these inhibitors. These compounds had sub-micromolar affinity for the rat isozymes, however the compounds are only available by complex synthetic routes, and this work has not been extended to yield bioavailable compounds.

Sufrin has focused on methionine analogues, which are permeable to many types of cells, and her L-cis-AMB (L-2-amino-4-methyl-cis-but-3-enoic acid) has proven to be a useful tool for *in vivo* inhibition of MAT, notably in trypanosomes [205]. A related analogue, L-2-amino-4-methythio-cis-but-3-enoic acid (L-cisAMTB), is a competitive inhibitor against the amino acid with K_i of 21 and 5.7 μM against MAT I and II, respectively . It is also a substrate with K_m values of 555 and 33 μM. This inhibitor is 2-10-fold more potent against hepatoma MAT than against liver isoenzymes [204]. L-cisAMTB has more structural fidelity to methionine than the oxygen analog L-cisAMB. The highly planar conformation of the hydrophobic enol ether chain of L-cisAMB was anticipated to be exhibited by L-cisAMTB. However, X-ray crystallography demonstrated a slight torsion and a consequent deviation in planarity on the thioenol ether side chain [204], which may be reflected in the difference in affinity.

Among the methionine analogs, cyclic 5-membered ring amino acids bind with optimal affinity to all of the isozymes tested [206]. Recent studies with 4,5-epoxide and 4,5-epithio methionine analogs have provide inhibitors with affinity as high as 7 μM for recombinant rat liver MAT, although none were found to irreversibly inhibit the enzyme by covalent modification [207]. The 2'-hydroxyl of ATP and the dianionic form of the γ-phosphate of ATP may play a role in the reversible binding to these enzymes. The low V_{max} exhibited by 2'-deoxy-ATP in MAT II suggested a possible implication of the 2'-OH in catalytic events [208, 209]. All of the 2-, 3- and

4-mono-C-methyl derivatives of methionine assayed are low affinity selective inhibitors of MAT II and tumor MAT, and the three exhibiting the highest inhibition are competitive inhibitors against methionine [210].

Diimidotriphosphate (O_3P-NH-PO_2-NH-PO_3), which is a nonhydrolyzable analogue of the tripolyphosphate intermediate, is a slow binding inhibitor with nanomolar affinity for the *E. coli* and *M. jannaschii* enzymes; studies with eucaryotic MATs have not been reported [134, 211]. The slow binding and high affinity were associated with a protein conformational change after the initial binding, events that were attributed to ionization of one of the –NH- groups when the compound is bound to two metal ions at the active site [161]. The commercially available imididiphosphate (O_3P-NH-PO_3) is also a slow binding inhibitor of cMAT with a K_i of 0.8 μM [211].

12. ROLE OF MAT IN DISEASES

Data from many studies link changes in MAT activity to the development of different types of disease. Such changes lead to the corresponding alterations in AdoMet levels, and hence in the many reactions in which this compound is involved. Thus, in many cases a direct relationship between the development of the disease and MAT is difficult to establish.

12.1. Cognitive and neurodegenerative diseases

Alterations in transmethylation mechanisms in neurodegenerative diseases such as Parkinson, Alzheimer and subacute combined degeneration of the spinal cord have been demonstrated [212-215]. Similar defects have been suggested to occur in complex psychiatric disorders, such as schizophrenia and dementia [216]. In mice, monkeys and pigs an association has been found between a defect in methionine synthesis and/or MAT activity and the development of myelopathies, ataxia, peripheral neuropathy and subacute combined degeneration of the spinal cord [217-219]. Moreover, Charlton and Way showed in 1978 that AdoMet injection into the lateral ventricle of rats and mice produced tremors, rigidity and abnormal posture [220]. Blood AdoMet and AdoHcy levels have been shown to decrease in

Parkinson's patients, combining to yield a lower AdoMet/AdoHcy ratio, whereas erythrocyte MAT activity was found to be increased [215]. However, no association between AdoMet and/or AdoHcy levels, or MAT activity, and the age or the duration of Parkinson's disease has been described.

Parkinson's disease is a disorder marked by degeneration of nigrostriatal dopaminergic neurons, and consequential depletion of both dopamine in the basal ganglia, and melanin in the *susbtantia nigra pars compacta*. L-dopa administration is the major treatment for this disease, leading to an increase in hepatic and brain levels of AdoMet and AdoHcy at short-term [221]. However, AdoMet depletion occurs upon long-term therapy with this compound, which results in losses of efficacy [222-225]. Short-term treatments also produce increases in brain (48%) and liver (34%) MAT activities that parallel increments in the corresponding protein levels detected by Western blot [221]. Moreover, the levels of MAT and catechol O-methyltransferase (COMT) mRNA are increased by L-dopa, which also induces transcription factors such as NF-1 and cJun/AP-1 that are known to control MAT and COMT promoter activities. Clinical improvements (reduced tremor and rigidity) in these patients have been also reported after treatment with methionine [226]. In the presence of AdoMet, COMT methylates L-dopa to 3-O-methyldopa, and this treatment results in increased MAT activity in brain [227]. The frequency of administration and the duration of the treatment are related to the induction observed. A sudden rise in dopamine may be responsible for hyperkinesias that can occur following L-dopa administration, since motor activity has been associated with such increases [228]. On the other hand, the dimethylated product of dopamine is known to be hypokinetic [229]. Thus, it is proposed that patients from whom L-dopa control of the disease is long-term may be resistant to the induction of MAT activity, whereas those in which the L-dopa treatment is ineffective may initially have enhanced MAT activity at the onset of therapy. There is also substantial evidence which indicates the neurotoxicity of L-dopa, for which the proposed mechanism is the generation of hydrogen peroxide and oxyradicals resulting from oxidation of dopamine in the nigral neurons

and its nerve terminals. Thus, it is possible that L-dopa accelerates the rate of dopamine neuronal degeneration as a result of this direct neurotoxicity [230].

Schizophrenia has been also extensively studied, due to its high incidence (1% of the population worldwide). The rate of whole body methionine metabolism in these patients is reduced to one third that of controls [231]. Significant increases in blood Hcy [216], low MAT activity in erythrocytes [232], regionally selective decreases in MAT's K_m for methionine [233] and low serine hydroxymethyltransferase [233] have also been reported. Some of these alterations were also found in individuals with dementia (Gomes-Trolin 1996). Exacerbation of schizophrenic symptoms was observed in response to large oral doses of methionine [234], whereas AdoMet is a reported antidepressant [235]. Profound defects in membrane phospholipids of lymphocytes have been also detected [236], phospholipid methylation being stimulated by dopamine, epinephrine and norepinephrine, as well as serotonin in neuroblastoma SK-N-MC cells [237].

12.2. Human mutations in *MAT1A* gene: hypermethioninemia and demyelination

Mutations have been characterized by single-strand conformation polymorphism (SSCP) and DNA sequencing for patients showing isolated persistent hypermethioninemia (Table 3). However, MAT deficiency is not the only alteration related to the presence of high methionine levels in plasma. Such an increase could also take place transiently during the neonatal period, disappearing when the dietary intake of proteins is reduced [238, 239], or as a consequence of CBS deficiency, or due to deficieny in fumaryl acetoacetate hydrolase (tyrosinemia type I) or liver disease. Therefore, newborn children are routinely screened for hypermethioninemia [240], a small percentage of those who show isolated persistent hypermethioninemia have deficiencies in hepatic MAT or glycine methyltransferase (GNMT) [241]. Definitive positive diagnosis however requires demonstration of reduced MAT activity in liver biopsies, a procedure that has been precluded in many cases due to the absence of pathological symptoms. Some patients present unusual breath odor, due to large increases in dimethylsulfide levels [242, 243],

while others have neural demyelination [15, 214]. In cases where liver biopsies were available, a reduction in MAT's K_m for methionine was also observed [244, 245]. The plasma levels of methionine in this type of patients may be as high as 1.3 mM (35 μM for normal levels), but the presence of 50-60% MAT activity is enough for maintenance of a normal level of the amino acid [15, 240]. On the other hand, MAT activity in the erythrocytes, lymphocytes or fibroblasts of the patients is normal [14, 243].

The first patient with MAT deficiency was an infant described by Gaull in 1974, [246], and subsequently since more than 60 individuals with mutations in the *MAT1A* gene have been identified (Table 3). Most of the mutations described were found to follow a recessive autosomal inheritance trait. However, gene tracking in families showing the G to A transition at nucleotide 791, leading to R264H, revealed a Mendelian dominant inheritance [245, 247, 248]. Some of the mutations lead to stop codons in the ORF (Table 3), and hence to truncated forms of the protein. Subunits of 91 [249], 184 [15, 250], 349 [15, 240], 350 [15] and 386 residues [251] will result from these changes. In addition, a mutation at the wild type stop codon has also been described, rendering a longer protein [252]. Only truncated forms of 349 and 350 amino acids are present in patients developing demyelination, a result that lead to the hypothesis that the size of these anomalous subunits allows partial folding to an intermediate state that is able to associate with the wild type subunits. Thus, these truncated proteins may sequester MAT2α subunits in inactive heterodimers, whereas shorter proteins (i.e. 184 residues) cannot. Heterodimerization was also suggested for the 386 residue truncated protein, however, no validation was provided [251]. The presence of truncated subunits will lead to a lack of methylated products, among them phosphatidylcholine and creatine which are important for the myelin in neural sheath and neural structure [253]. These data, along with experiments carried out in rats that were given cycloleucine [254], suggested that AdoMet administration may overcome, at least in part, these problems. However, only one of the patients showing demyelination was treated with AdoMet and responded to this therapy [214]. Kim *et al*. [251] also indicate that the physiological effects may be due to lack of AdoMet rather than to

hypermethioninemia, and suggested that elevation of methionine levels may promote a greater flux through the remaining MAT, thus alleviating AdoMet deprivation.

Polymorphisms such as that of T to C at nucleotide 426 have been also detected in *MAT1A* gene [15]. Other silent mutations in exons III (C225T rendering A75A) [248], VIII (C to T in intron 8 and C882T rendering A292A)[255], and IX (T1131C, leading to Y377Y)[248, 255] have also been observed. Another interesting mutation occurs in the last nucleotide of exon III. This change alters the splice-donor site, and hence, could either render normal (G98S) or anomalous splicing. Analysis of both possibilities was carried out, showing normal activity for the G98S mutant, whereas minigenes indicated the presence of abnormally large products [249].

Some of the mutant MATs have been expressed in COS-1 cells and/or *E. coli* in order to study their activity and association state to understand the behaviors shown by the patients. Thus, truncated proteins of 349 and 350 residues, as well as, the mutants R199C, R356Q and G378S were prepared and shown to follow the same trend in both systems. Only R356Q (53%), R199C (10%) and G378S (<0.1%) displayed MAT activity [15]. A more detailed analysis was carried out at the R264 position, the results suggesting the necessity of a positive charge at that position to attain maximal activity [248]. Moreover, some of the mutants at this position were able to homo- or hetero-dimerize [248, 249]. The hetero-oligomerization shown by R264H and wild type subunits explained its dominant effect on MAT activity. Analogous mutants prepared on other MATs show differences in the oligomerization pattern [175, 190]. Thus, MAT I/III R265H mutant behaves as a monomer with tripolyphosphatase activity, whereas c-MAT R244H remained a tetramer. Moreover, R265H was shown to be able to dimerize with the wild type enzyme, giving rise to an enzyme that is unable to synthesize AdoMet, but that hydrolyzes tripolyphosphate. Based on these results, Pérez-Mato et al. [190] suggested that R265H folds approximately to MAT's final structure before association takes place [190]; association then could occur in a late intermediate as deduced from the folding data [195, 196].

12.3. MAT and Cancer

Cancer is one of the most common causes of death in the first world. Its basis depends on genetic and environmental factors, but in all cases the common outcome is anomalous cell growth. For this to occur, cell cycle deregulation must take place. Moreover, another common property of human cancer cells is their dependence on exogenous methionine for growth, whereas normal cells can use Hcy instead [256]. Thus, there is interest in understanding the anomalies in the methionine cycle caused by carcinogenesis, as part of a search for chemotherapeutic drugs.

In this regard, the effects of carcinogens such as 2-acetyl-aminofluorene [257], thioacetamide [192, 258], N-2-fluorenylacetamide [259] and 3'-methyl-4-dimethylaminobenzene [260] on methionine metabolism have been studied in animal models. The collected data indicate reductions in *MAT1A* mRNA[257], protein [258, 261] and activity [192, 257, 258], whereas increases in these parameters were reported for *MAT2A* [258, 260, 262].

Other lines of research have taken advantage of samples from different types of tumors or tumor-derived cell lines [261, 263]. In all the cases, rapid growth has correlated with an increase in MAT II protein levels, while a corresponding decrease in MAT I/III occurs [5]. Moreover, an increase in MAT activity in malignant cells has been reported, using measurements at 50-80 μM methionine [5, 264, 265], thus indicating an alteration of the low K_m forms of the enzyme. Such an effect was confirmed in colorectal carcinoma samples where increases in MAT II protein and activity were observed [266]. Northern blots did not show signals for *MAT1A* mRNA in hepatoma cell lines, a fact that is not due to deletion or gene reorganization, but rather to lack of transcription [265]. Samples from cirrhotic patients and HCC show reduced *MAT1A* mRNA levels, while *MAT2A* is induced only in the carcinoma [267, 268]. These effects correlate with increases in transcription and low methylation of the *MAT2A* promoter only in cancer [268]. The effect of reduced methylation on the *MAT2A* promoter has been studied using SssI methylation of promoter constructs fused to a luciferase reporter gene, followed by transfection of HepG2 cells and AdoMet treatment of transfected cells. In both

cases a reduction in *MAT2A* promoter activity was observed, while no effect on *MAT1A* promoter was detected [268]. AdoMet treatment was shown to induce *MAT2A* promoter hypermethylation, a mechanism that has been shown to involve binding of histone deacetylase and methyl cytosine-binding proteins such as MeCP2 [269, 270]. Comparison of protein binding patterns to the *MAT2A* promoter between normal and malignant tissues has been also carried out, and the observed differences were able to explain the transcriptional up-regulation of *MAT2A* in HCC, as is discussed elsewhere in this review [47, 49].

The observed changes in MAT expression and activity are reflected in AdoMet levels. Thus, in hepatoma cells an increase in AdoMet is observed upon overexpression of MAT I/III [271], whereas a decrease is detected upon thioacetamide treatment [272, 273]. Moreover, this rise in AdoMet is correlated with a boost of the AdoMet/AdoHcy ratio. Such effects, together with an increase in DNA methylation, can also be observed after AdoMet treatment. In fact, it has been suggested that the reduction in expression of several oncogenes during hepatic carcinogenesis may be occur through changes in their DNA methylation [271]. Global DNA hypomethylation seems to be a common property of several diseases in which AdoMet levels and *MAT1A* expression are altered [268]. The role of AdoMet in cancer development was confirmed when a *MAT1A* knockout mouse became available [59]; its chronic AdoMet deficiency led to the spontaneous appearance of HCC. Moreover, AdoMet therapy has been shown to be efficient in preventing HCC [274], since it inhibits HGF mitogenic activity and accelerates resynthesis of IκB, thus blocking NFκB activation of cytokine-stimulated hepatocytes [275]. On the other hand, antisense RNA against *MAT2A* produced cell death either in normal or *MAT1A* overexpressing hepatoma cells [271]. The presence of the regulatory β-subunit makes MAT II more susceptible to AdoMet inhibition [36]. *MAT2B* expression provides a growth advantage to human hepatoma cells, its expression being common in cancer and its induction is frequently associated with hepatic dysfunction [36]. AdoMet and 5-methylthioadenosine induce apoptosis in cancer cells. This effect is mediated by induction of Bcl-x_s and increases in protein phosphatase 1 catalytic subunit which would carry out the protein Ser-Arg dephosphorylation

needed for alternative Bcl-x splicing [276]. Methionine deprivation imposes a metabolic stress that inhibits mitosis and induces cell cycle arrest and apoptosis. cDNA array analysis indicates that several families of transcription factors are affected by this type of stress, among them AP-1 and NFκB. These factors are among those known to regulate *MAT2A* transcription, as discussed in Section 2.3.1 [277].

The effect of several chemotherapies on methionine metabolism behavior has been also studied. Among them, fenretidine has been shown to counteract both the induction of NFκB mRNA and protein levels, as well as the reduction in levels of IκB upon 2-acetylaminofluorene administration. In parallel, the carcinogen induces iNOS while decreasing *MAT1A* expression [257]. NFκB activation is involved in the entry to the G1 phase of cells that are primed to proliferate, maintains cells in early G1, and ensures progression throughS phase by transcriptional activation of c-Myc and cyclin D1. On the other hand, AdoMet increases IκB synthesis, and hence raises the levels of the NFκB/IκB complexes and reduces MAT I/III activity. In addition, an increase in NO levels may also collaborate by inactivating MAT I/III and reducing AdoMet levels. However NO can also inactivate NFκB [278], and hence lead to a more complicated picture. Resistance to chemotherapy has also been linked to methionine metabolism in some cases, probably through epigenetic effects on DNA methylation [279]. In others cases, i.e. doxorubicine resistance, overexpression of multidrug resistance gene (MDR) is the primary factor involved.

Fetal, regenerating and transformed hepatocytes are able to proliferate. Their progression through the mid/late check point at G0/G1 is regulated by growth factors such as HGF, insulin, EGF and TGFα; among these HGF has been shown to induce *MAT2A* [50]. Moreover, inhibition of the MAPK and PI3K pathways prevents this up-regulation with concomitant induction of *MAT1A*. Treatment with the growth arrest factor TGFβ attenuates mitogenesis and *MAT2A* up-regulation, while inducing *MAT1A*. In addition, AdoMet has been shown to inhibit HGF induction of cyclin D1 and D2 expression, leading to the suggestion that this metabolite is a negative modulator of cell cycle progression [54]. Moreover, samples of

human HCC always showed expression of the β-subunit at higher levels than those shown in cirrhotic samples [36]. The same is also true for HCC induced by diethylnitrosamine treatment. Analysis of expression in several hepatoma cell lines revealed the presence of the β-subunit in HepG2, PLC and H3B, but its absence in Huh7. Expression of *MAT2A* in cultured hepatocytes led to an increase in *MAT2B* levels.

AdoMet inhibits the mitogenic activity of HGF and prevents *MAT2A* induction. The mitogenic activity of HGF depends on iNOS and on the L-methionine concentration in the medium which exerts its effects through its conversion to AdoMet. A transitory decrease in AdoMet may liberate factors such as HGF from its inhibition, thus allowing the proliferative response, such as that observed during regeneration, whereas chronic AdoMet reduction would lead to malignant degeneration [276].

12.4. MAT and Ethanol

Intragastric ethanol feeding of rats in combination with a high fat diet increased the steady-state levels of both *MAT1A* and *MAT2A* mRNAs [280]. In the rat model only MAT II protein levels rose noticeably, and in concordance MAT activity (measured at 20 μM methionine) doubled [280]. Ethanol induction of *MAT2A* is mediated by TNFα stimulation, which exerts its action on the *MAT2A* promoter through NFκB and AP-1 binding [47]. On the other hand, micropigs showed a decrease in total MAT activity upon ethanol feeding (measured at 5 mM methionine) [281], and *MAT1A* transcripts decreased under these diets, either combined or not with folate deficiency [281]. Reductions in AdoMet levels were observed during ethanol feeding of rats [280], baboons [282] and micropigs [281], as well as in patients with alcohol hepatitis [283]. However, differences in the effects on AdoHcy levels were observed among the models. Thus, while rat intragastric ethanol feeding produced no change in AdoHcy [280], micropigs fed ethanol in combination with a folate deficient diet showed an increase in the levels of this metabolite [281]. In both cases the net result is a decrease in the AdoMet/AdoHcy ratio

that in rats correlates with a reduction in the global DNA methylation. In addition, increases in c-Myc expression and genome-wide DNA strand break accumulation are observed.

Alcoholic liver cirrhosis is associated with increased risk of liver cancer [280]. A reduction in *MAT1A* mRNA levels was observed in samples from cirrhotic [267] and alcoholic hepatitis patients [283], while *MAT2A* is not induced. Hypermethylation of *MAT1A* promoter was also detected. A high percentage of samples of cirrhotic patients (84%) express the β-subunit, but no correlation between expression and etiology was established [36]. In addition other enzymes of the methionine cycle, such as GNMT, MS, BHMT and CBS have very low levels in these patients, the percentage of livers with low or very low levels is higher in HCV cirrhosis samples than in alcoholic cirrhosis specimens [267]. Moreover, Hcy can potentiate the fibrogenic effects of ethanol by inducing expression of tissue inhibitor metalloproteinase-1 (TIMP-1) and α1 (I) procollagen [4], and reducing GSH, plasma taurine and urine sulfate levels. Thus, defects in Hcy remethylation may contribute to HCC development in cirrhosis [267]. Cellular levels of AdoMet seem to be related to the differentiation status of the hepatocyte, being lower in growing cells [271].

Liver damage during short- and long-term ethanol consumption [284-286] has been associated with a reduced oxygen supply to this organ. Sustained hypoxia such as that observed in liver cirrhosis and long-term ethanol consumption would induce down-regulation of MAT expression; this situation may promote irreversible tissue damage through the impairment of methylation reactions, and GSH-dependent detoxification capacity [61]. In this regard, the reversible inactivation of MAT regulated by the intracellular levels of GSH and NO [67] may exert a fundamental role in the effects derived from ethanol consumption. Both GSH reduction and increases in protein nitrosylation are known to occur in these cases. Other protein modifications, due to products of ethanol catabolism, such as the irreversible covalent binding of acetaldehyde to lysine residues, have not been explored yet, and may also be involved in the described effects.

The *MAT1A* knockout mouse displays alterations common to ethanol treatment, such as induction of CYP2E1; these effects make it more susceptible to injury (i.e. by CCl_4) and reduction in GSH levels [59].

13. Conclusions

This review has attempted to provide an overview of the methionine adenosyltransferase research, an area that has vastly expanded in the past decade. Numerous opportunities remain before a comprehensive understanding of this unique enzyme is obtained and can be exploited for therapeutic purposes. New crystal structures that clarify the mechanism of the reaction are needed, as well as structural data for dimeric and large-molecular weight isoenzymes. Moreover, discerning the role of MAT in diseases as complex as hepatoma or Parkinson's may relate, not only to AdoMet synthesis, but also reveal interactions with other pathways that thus far remain elusive.

Figure legends

Figure 1. **A)** MAT and AdoMet metabolism The many ways in which AdoMet is used contrast with the single way in which it is formed.; **B)** The MAT catalyzed reaction illustrating the intermediate complex with bound tripolyphosphate (PPPi).

Figure 2. The methylation cycle in which is predominantly found in mammalian liver.

Figure 3 Schematic representation of MAT promoters. The upper scheme represents MAT1A promoter. The elements and DNase protection areas are identified as: +1 indicates the transcription initiation site, the TATA box is located at -29/-23, two CAAT box are also indicated. Boxes A-D indicate the mocations for positive acting elements, A and B identified in H35 cells and C and D in CHO cells. The lower scheme shows the MAT2A promoter, where E-G represent SpI sites and H the area of NFkB and AP-1 binding.

Figure 4 A) Ribbon diagram of the monomer of the human MAT II illustrating the near three fold symmetry of the fold and the central loop (pdb code 2PO2); **B**) the tetramer of cMAT (pdb code 1RL7) in complex formed with AMPPNP and methionine; half of the active sites had bound products AdoMet and PPNP. The active site is buried at a subunit interface.

Figure 5 Active site structures of MATs A) MAT I B in complex with ATP and methionine; Mg^{2+}, K^{+} and Pi are also in the active site (pdb code 1O9T) **B**) *E. coli* MAT in complex with AMPPNP and methionine; the 2 $Mg^{2+}$and 1 K^{+} are not shown (pdb code 1RL7) **C**) overlay of selected regions of the active sites of cMAT and rMAT. The exchanged orientation of the substrates is shown when the protein portion of the structures Is aligned, illustrated by the position of the active site histidine.

Figure 6 Kinetic Mechanism A) The mechanism for the *E. coli* MAT is shown. Other MATs prefer to bind ATP first and have different orders of product release. The protein conformational change, probably due to loop movement, is shown by the change in font of **E** to "E*". **B)** The free energy changes associated with each step are illustrated for physiological concentrations of substrates and products; the chemical reaction steps are included in the box.

Figure 7. Folding pathways for MAT I/III. The figure shows a schematic representation of the data available for MAT I/III unfolding. Free energy changes calculated for each step are also included. The different intermediates are denoted I, I_k^1 and I_k^2, whereas M represents the monomeric mutant R265H, and U the unfolded state.

Figure 8 Structures of selected inhibitors of MATs. Ki values for various MATs are listed in Table 2.

TABLE 1

Enzymatic properties of MAT from different sources.

source	Oligomeric state	K_m^{ATP} or $S_{0.5}$ (μM)	K_m^{Met} or $S_{0.5}$ (μM)	K_i^{AdoMet} (μM)	V_{max} (μmol/min/mg)	pH optimum	reference
E. coli	tetramer	110	80	10	1.2	6-9	[121]
M. jannaschii	dimer	290	240	120	3.0		[152]
Bakers yeast isozyme 1	heterodimer	74	110		0.6		[115]
Bakers yeast isozyme 2	heterodimer	47	140		0.36		[115]
Rat MAT I*	tetramer	252	125			7.5-8.8	[8]
Rat MAT III*	dimer	950	650			7.5-8.8	[8]
Human MAT II α/β	heterotetramer	31	3.3	18	0.2	7-8.5	[29]
Human MAT II (no β)	dimer		80				De La Rosa, 1995 #182}}
L. infantum	dimer	370 PC	250 PC	1500	0.2		[145]
L. donovanii	dimer	27 PC	250 PC	4000	0.2		[146]
P. falciparum	dimer						[148]
T. brucei brucei mixed isoform	Partial purification	53-1750 NC	20-150 NC	240		7.5-10	[143]

***** Data for rat MAT I and III refer to the reduced forms obtained after thiopropyl Sepharose purification.

** C= cooperative; PC= positive cooperativity; NC= negative cooperativity

Various assay methods have been used. Often radioactivity incorporation into AdoMet is measured, which requires separation of AdoMet from substrates either by filtration on ion exchange filters or on ion exchange columns. Phosphate production, either during AdoMet formation or in hydrolysis of added PPP_i, can also be monitored with purified enzymes. Much of the variation in literature values of kinetic constants can likely be traced to different assay conditions and methods of product determination.

Table 2

Selected Inhibitors of MAT.

Analogues of the substrates which have been tested as possible MAT inhibitors. Selected structures are shown in **Figure 8.**

The table shows some of the most effective among them.

Analogue	characteristics	K_i (μM)	references
Cycloleucine, 1-amino-cyclopentane carboxylic acid		209-1633	[206]
1-aminocyclobutane carboxylic acid	Less potent than cycloleucine	1.5-12.4 mM*	[206]
1-aminocyclo-hexane carboxylic acid		8.2-57.4 mM*	[206]
(±)- 2-aminobicyclo [2.1.1]-hexane-2-carboxylic acid	K_i 2-7 fold lower than cycloleucine	80-680	[206, 287]
L-2-amino-4-hexynoic acid	As potent as cycloleucine against control enzyme or Novikoff hepatoma, and 2-3-fold more potent than cycloleucine against normal isoenzymes.	1500	[288]
Z-L-2-amino-5-chloro-trans-4-hexenoic acid		550	[288]
L-ethionine*	All of them have lower affinity than cycloleucine to the active site. * the most potent in this group, also	790-15.4 mM*	[206]
S-n-propyl-DL-homocysteine		39-113 mM*	[206]
S-n-butyl-DL-homocysteine		149-234 mM*	[206]

Se-DL-ethionine*	substrate for normal and tumor isoenzymes, as well bacterial MAT	1.98-11.1 mM*	[206]
L-2-amino-4-methylthio-cis-but-3-enoic acid	Less potent than the ether analog due to non-planrity of the sulfur with the olefin	5.7-21	[204]
L-2-amino-4-methyl-cis-but-3-enoic acid	The most potent methionine analog inhibitor	5.7-21**	[204] [289]
6-(n-Butylthio)-9-[5'(R)-C-(L-homocystein-*S*-yl-methyl]-β-D-ribofunanosyl]purine 5'-β,γ-imidotriphosphate	Bisubstrate analog, selective for tumor isozyme	0.1 – 1.7	[203] [203]
Diimidotriphosphate	slow tight binding intermediate analog; tested only with bacterial and archaeal enzymes	2 nM	[211]

- *The range indicates the lower and higher values for liver MAT isoenzymes.
- ** Assayed against L1210 murine leukemia cells

Table 3

Mutations of *MAT1A* detected in individuals with hypermethioninemia

Exon	Gene	Protein	character	activity	Association state	Liver activity	reference
	C65T	S22L	HO**				[290]; [255]
I	G113A	S38N	CHE*	none		68%	[244], [249]
	T125C	L42P	HO				[290]; [255]
	C164A	A55D	CHE				[14]
	G205A	G69S					[252]
II	255ΔCA	92X	CHE	none			[244], [249]
III		G98S	HO	normal			[249]
	C426T		CHE				[14]
V	539insTG	185X	HO, CHE			7%	[15], [250]
	C595T	R199C	CHE, HO	10%			[15]

VI	C745T	R249W					[252]
	G791A	R264H		<1%	monomer	low	[245, 247], [248, 249], [252]
	G791T	R264C	CHE	none			[249]
	G867A	R264C					[252]
		K289N					[252]
VII	G870A		CHE				[14]
	T914C	L305P	CHE				[14]
	827insG	351X	HO				[15]
	T966G	I322M	HO, CHE	Low			[14], [249]
	G1006A	G336R	CHE	23%			[249]
	A1031C	E344A	CHE	low			[249]
	1043,1044del	350X	HO				[240], [15]
	G1067A	R356Q	CHE	53%		low	[291], [15]
		R356P					[252]

VIII	C1070T	P357L	CHE				[14],[252]
IX	T1131C		CHE				[14]
	G1132A	G378S	CHE	0.1%			[291], [15],[251]
	G1161A	W387X	CHE, HO	75%	dimer		[251]
	G1188T	X396Yfs X464					[252]

*CHE, compound heterozygosis

**HO, homozygosis

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Figure 1

S-Adenosylmethionine Metabolism

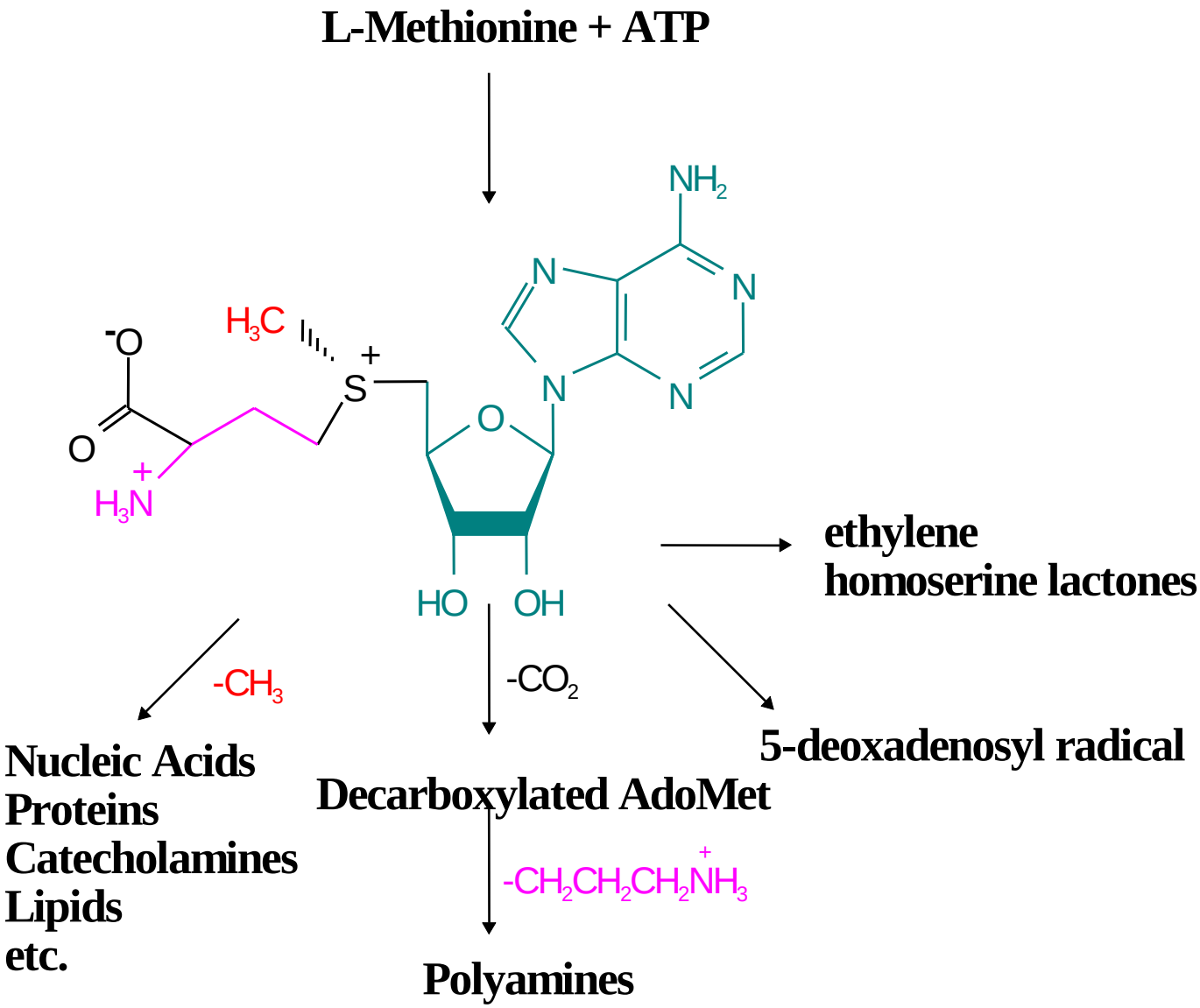


Figure 1B

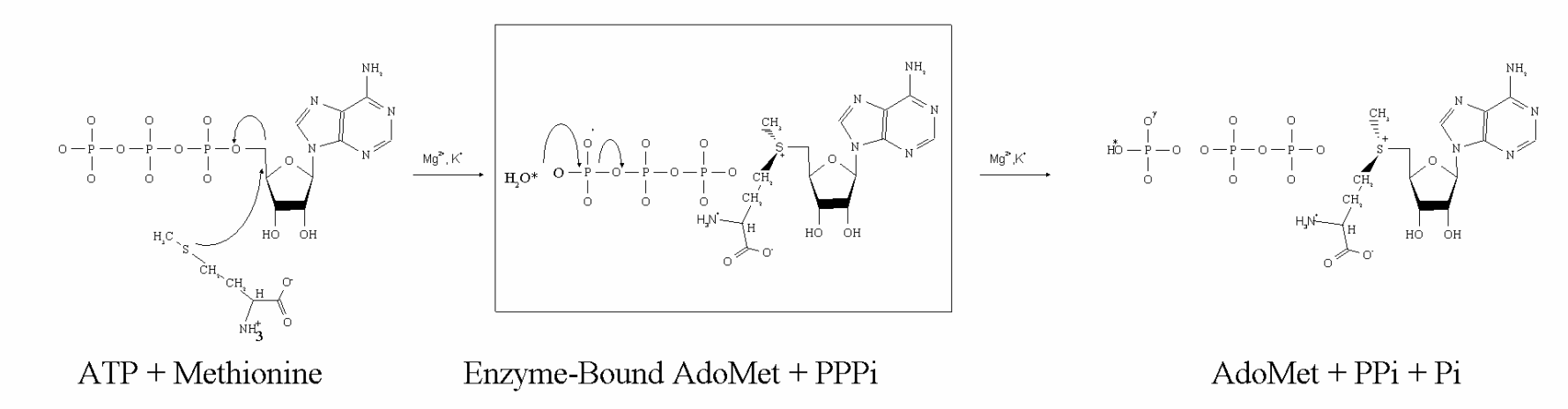

Mg2+, K+
Mg2+, K+
ATP + Methionine
Enzyme-Bound AdoMet + PPPi
AdoMet + PPi + Pi

Figure 2

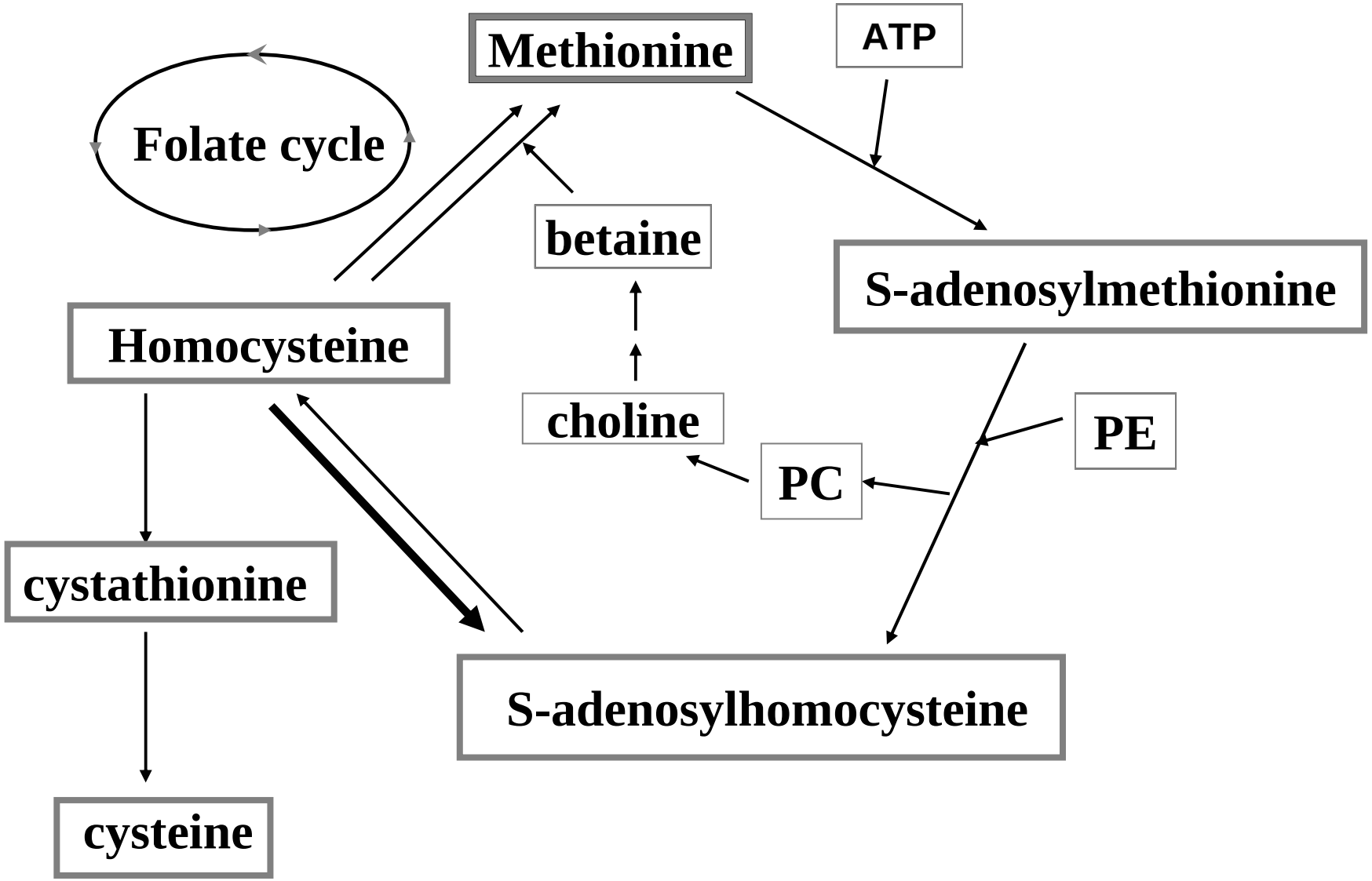
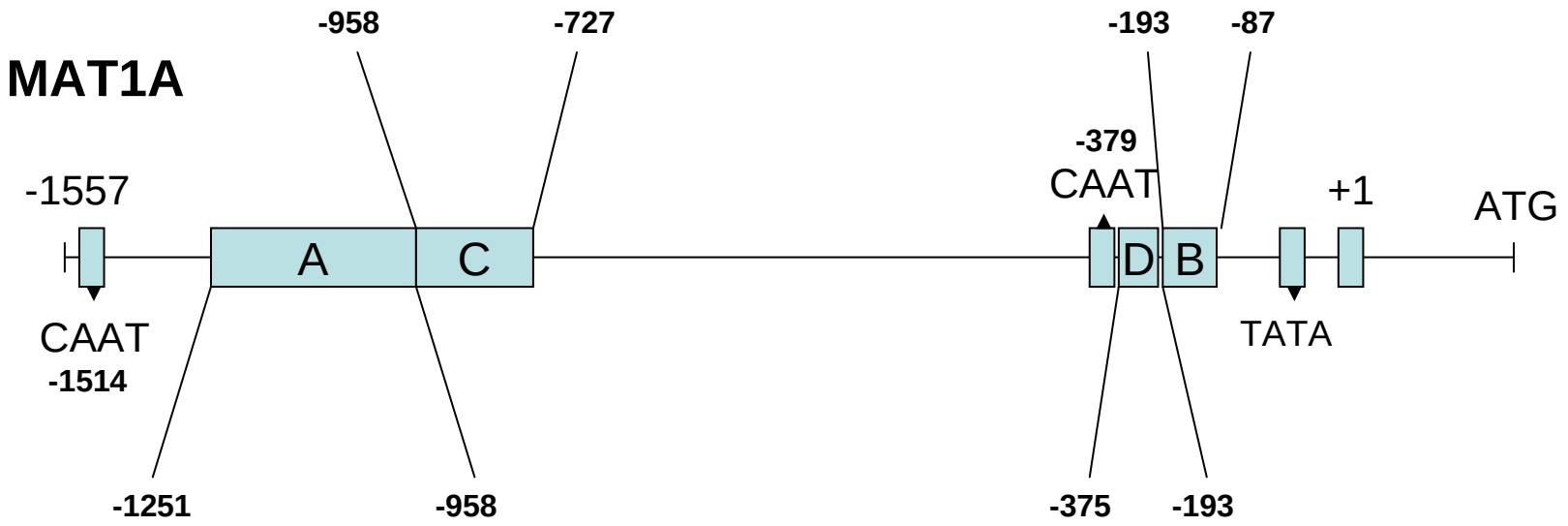

Methionine
ATP
Folate cycle
betaine
S-adenosylmethionine
Homocysteine
choline
PE
PC
cystathionine
S-adenosylhomocysteine
cysteine

Figure 3


MAT1A
-958
-727
-193
-87
-379
CAAT
-1557
+1
ATG
A
C
D
B
CAAT
-1514
TATA
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MAT2A
-571
+60
H
E
F
G
-352
-314

Figure 4A

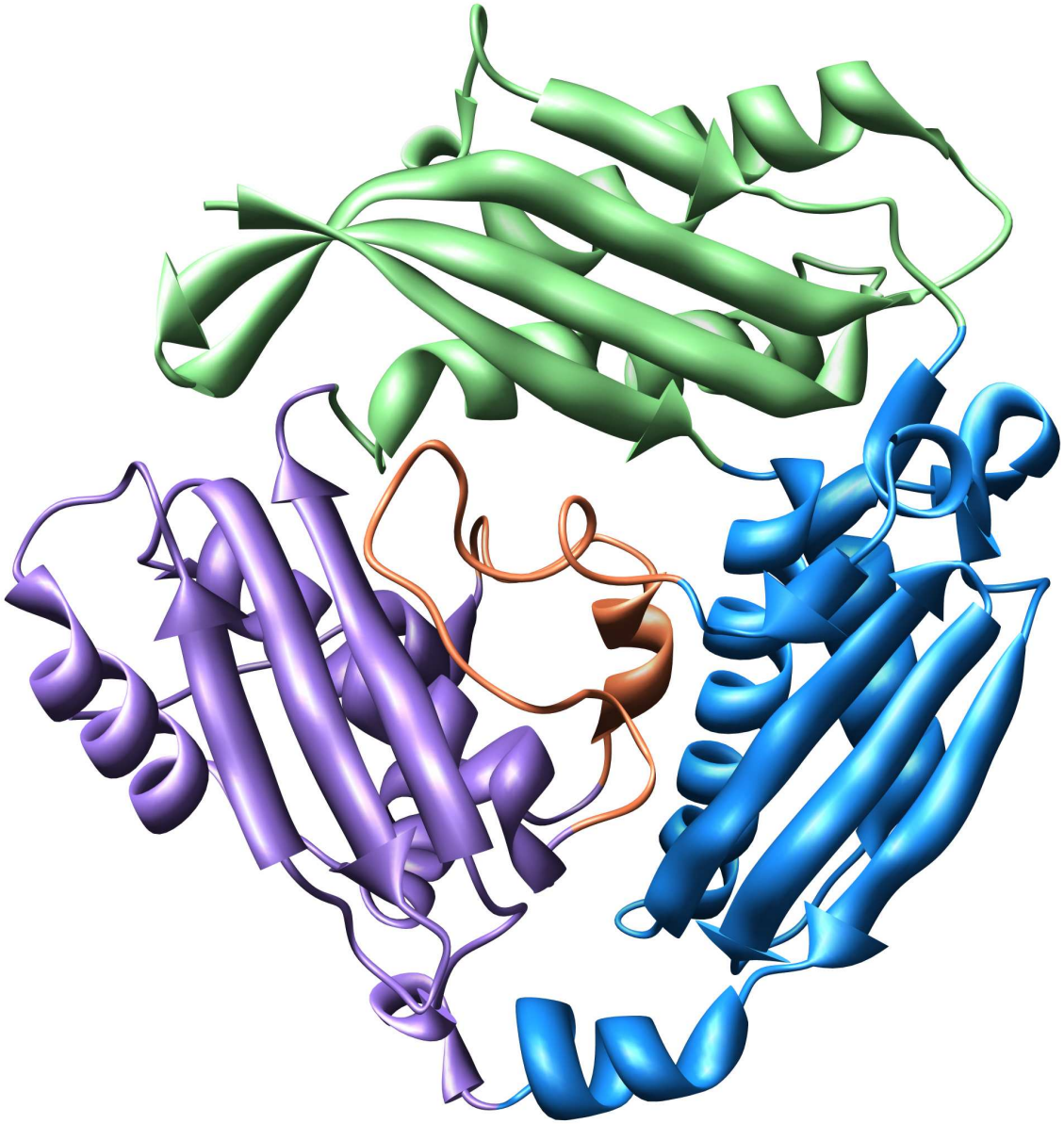


Figure 4B

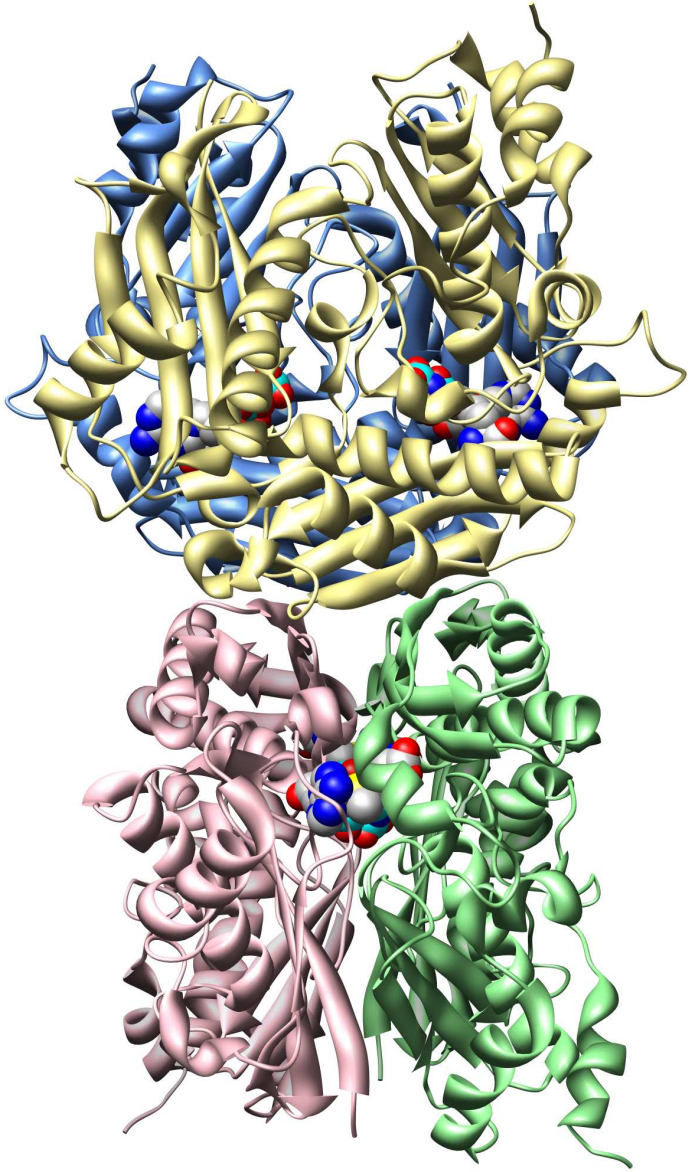


Figure 5A

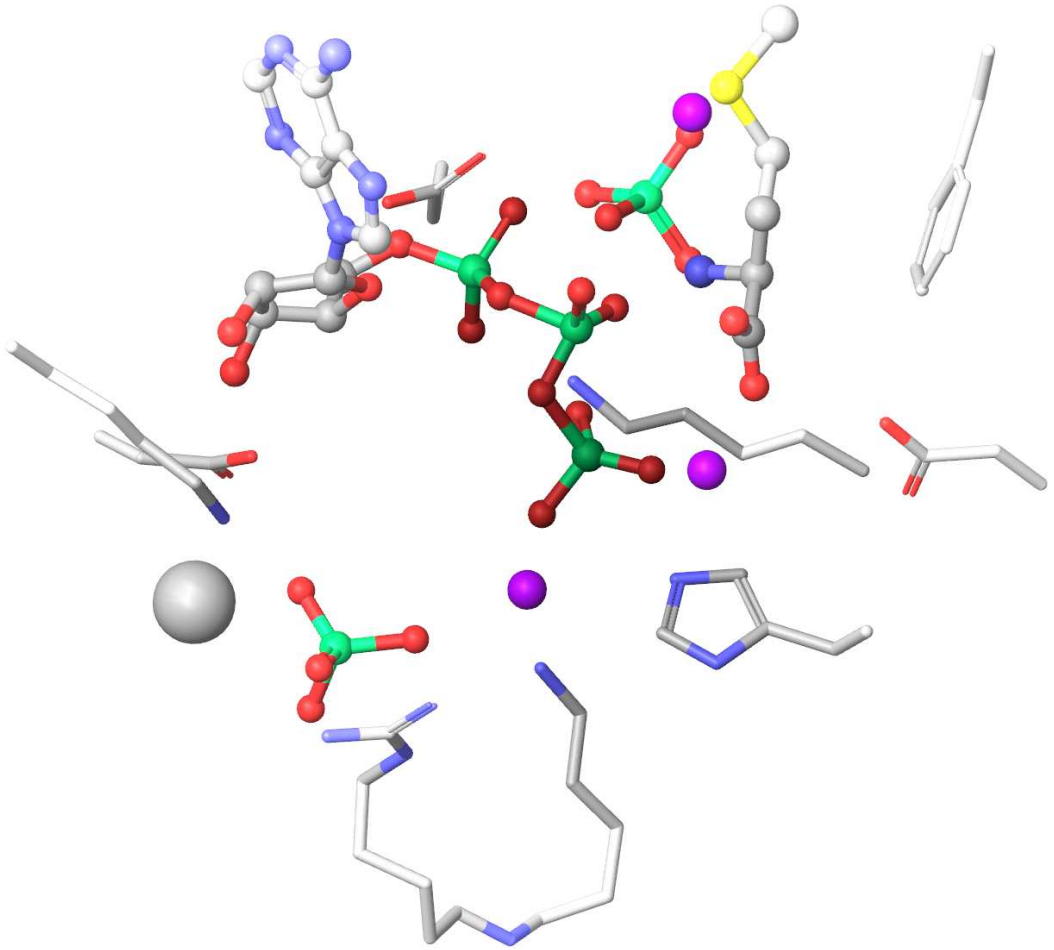


Figure 5B

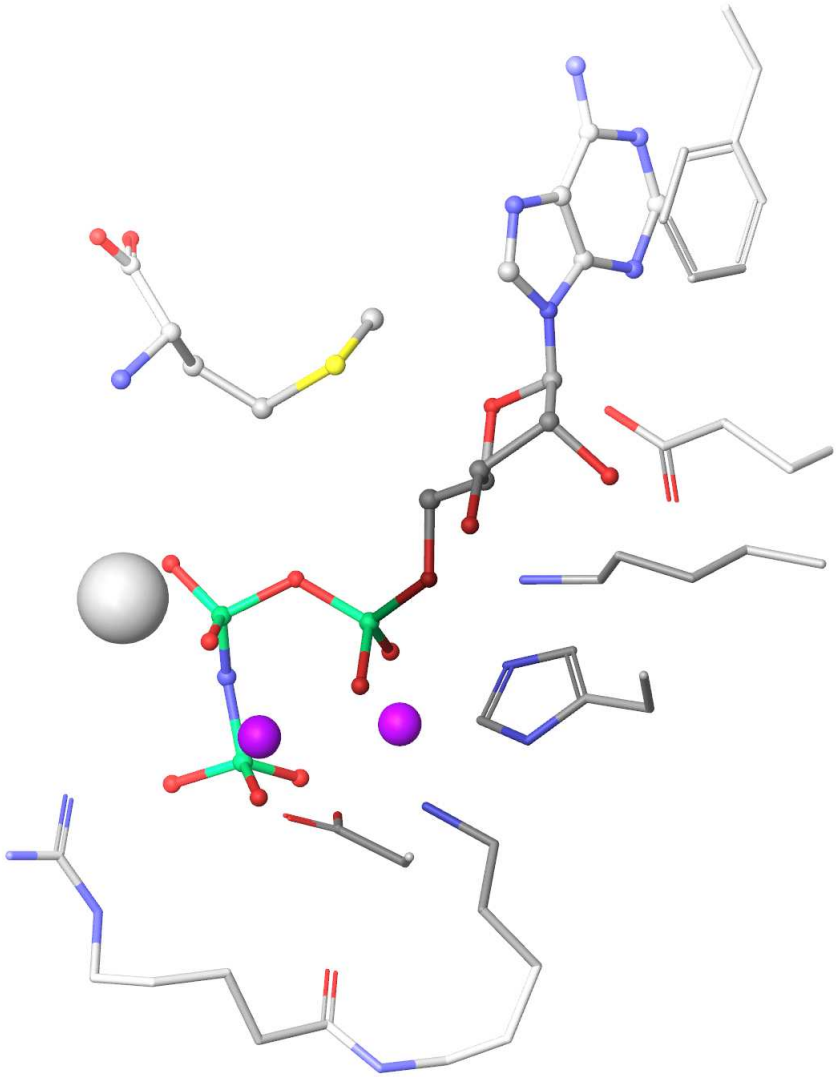


Figure 5C

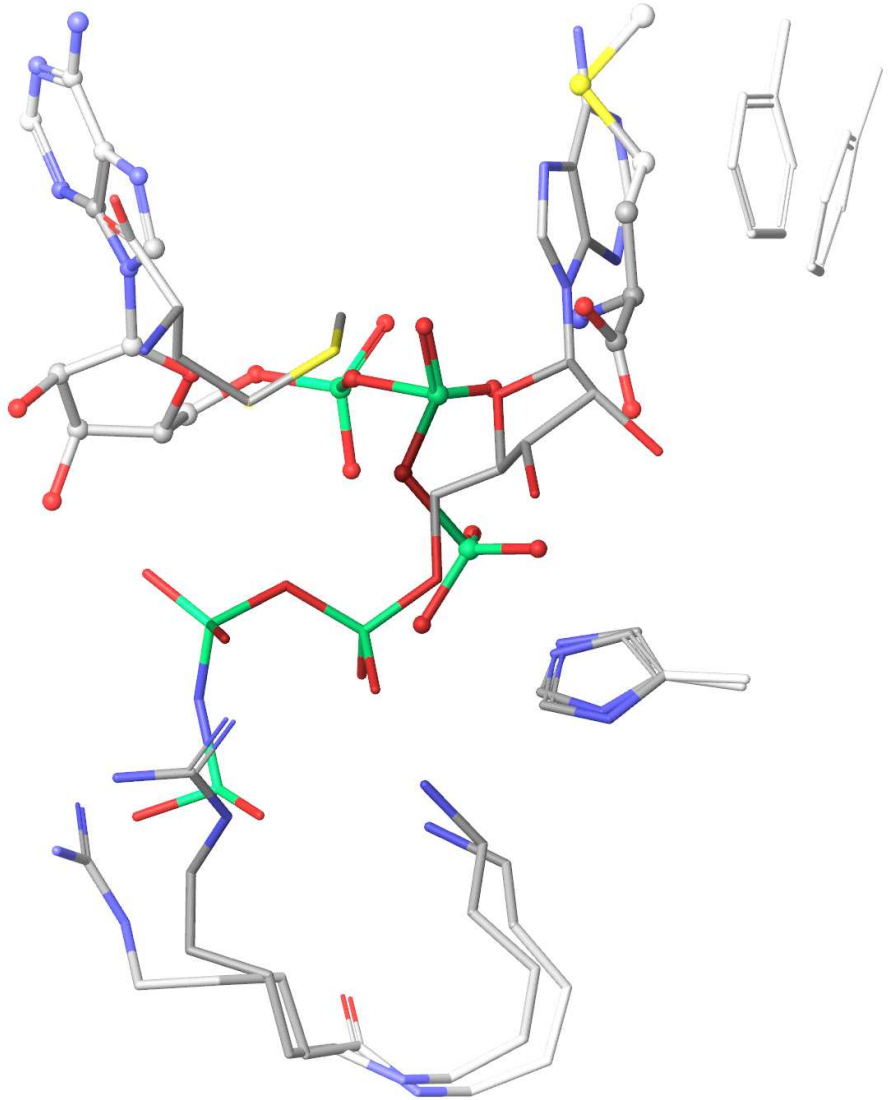


Figure 6A

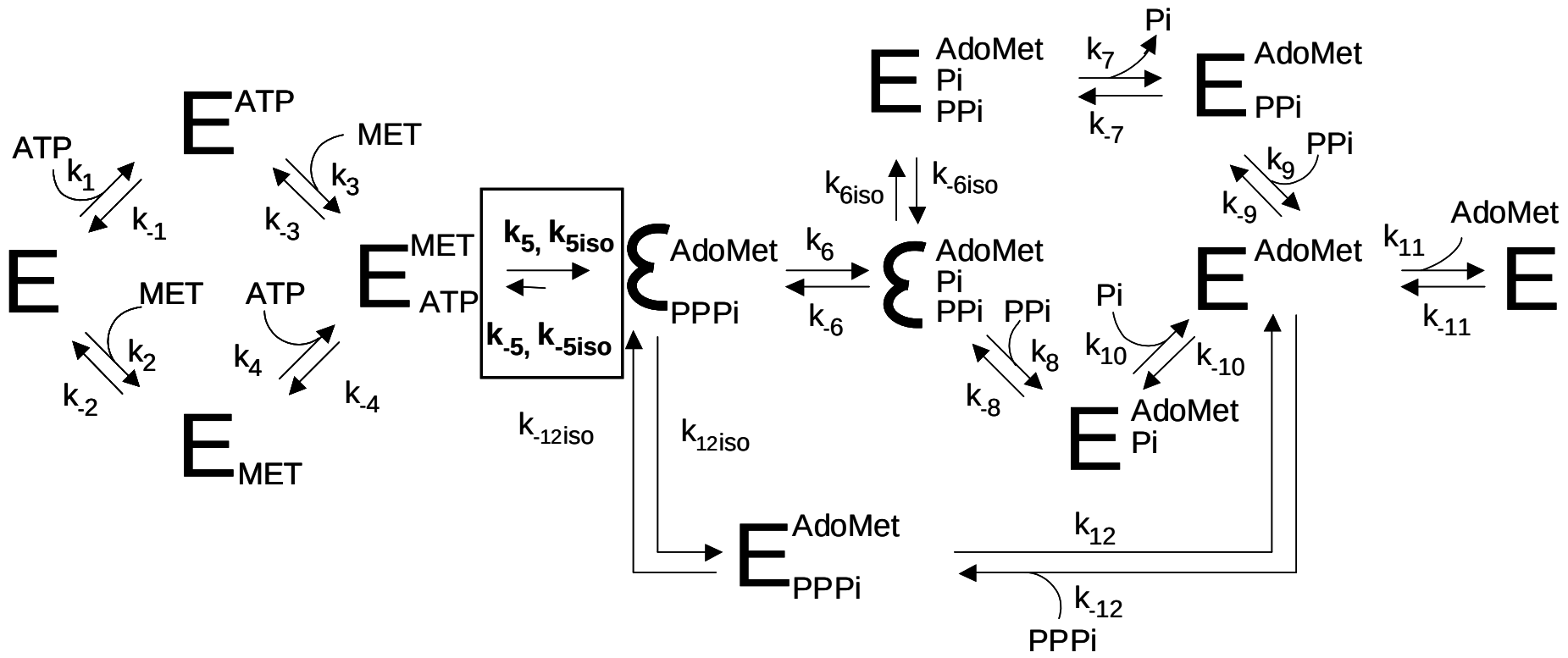

E
ATP
k1
k-1
E ATP
MET
k3
k-3
E MET
MET
k2
k-2
ATP
k4
k-4
E MET
E MET ATP
k5, k5iso
k-5, k-5iso
k-12iso
AdoMet
PPPi
k12iso
k6
k-6
k6iso
k-6iso
E AdoMet Pi PPi
k7
k-7
Pi
E AdoMet PPi
k9
k-9
PPi
E AdoMet
k11
k-11
AdoMet
E
PPi
k8
k-8
Pi
k10
k-10
E AdoMet Pi
k12
k-12
PPPi
E AdoMet PPPi

Figure 6B

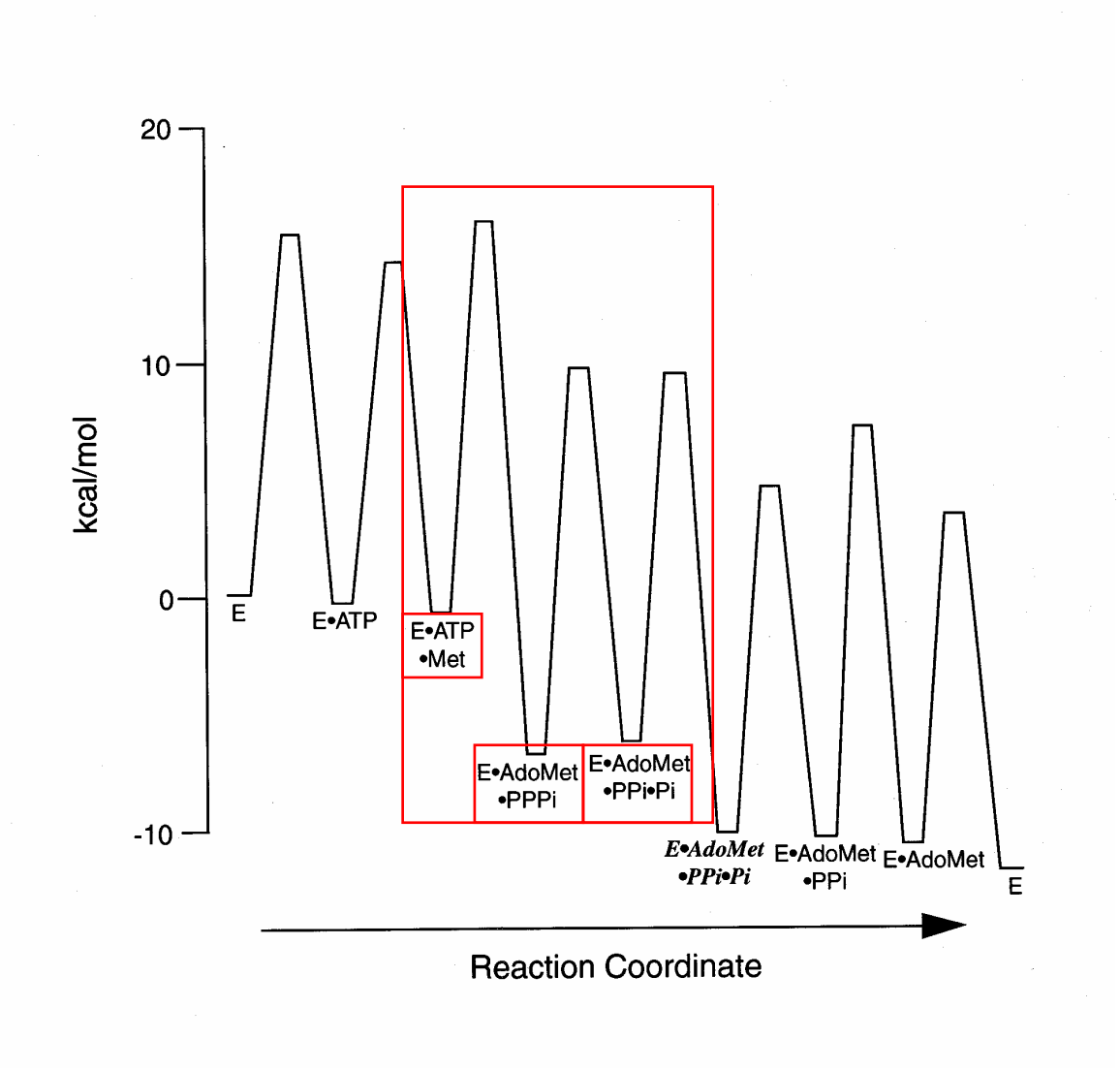
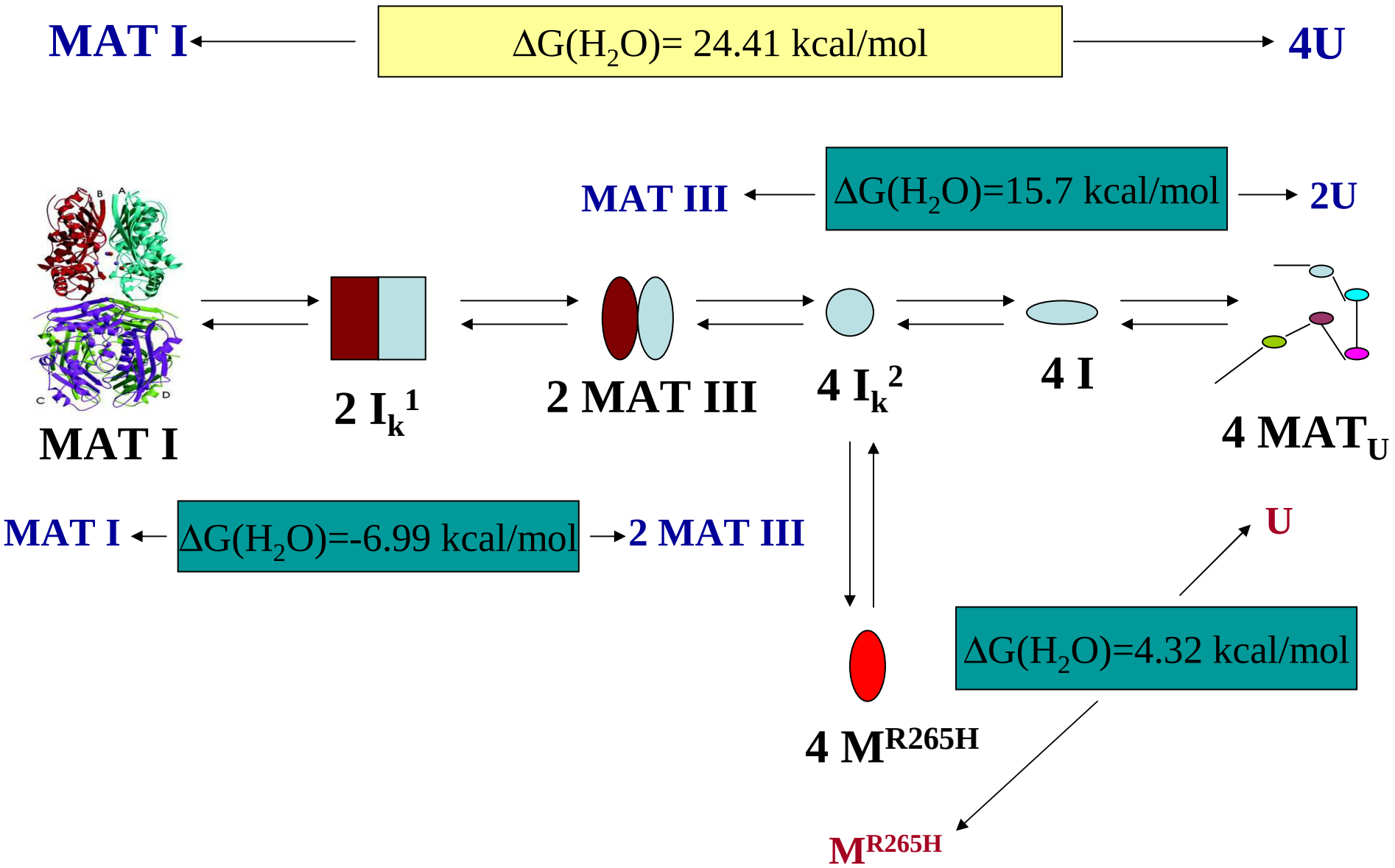
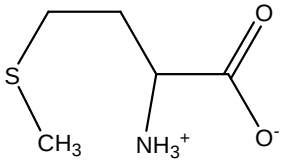

20
10
0
-10
kcal/mol
E
E•ATP
E•ATP
•Met
E•AdoMet
•PPPi
E•AdoMet
•PPi•Pi
E•AdoMet
•PPi•Pi
E•AdoMet
•PPi
E•AdoMet
E
Reaction Coordinate

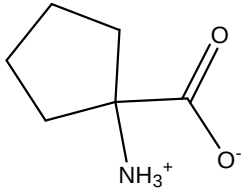
Figure 7


MAT I
ΔG(H2O)= 24.41 kcal/mol
4U
MAT III
ΔG(H2O)=15.7 kcal/mol
2U
MAT I
2 Ik1
2 MAT III
4 Ik2
4 I
4 MATU
MAT I
ΔG(H2O)=-6.99 kcal/mol
2 MAT III
U
ΔG(H2O)=4.32 kcal/mol
4 MR265H
MR265H

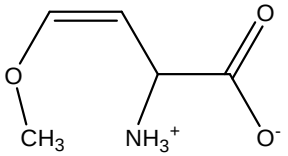
L-methionine



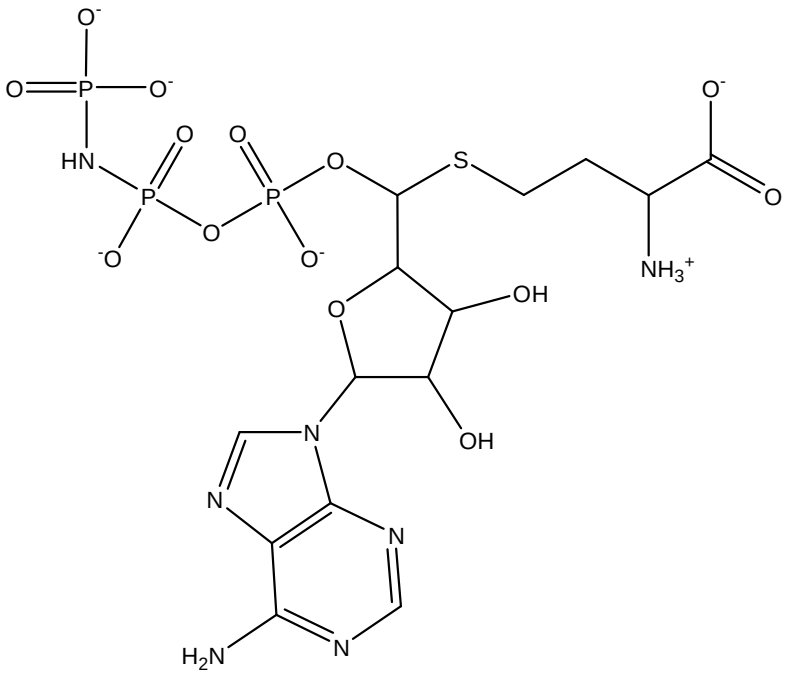
L-cycloleucine



L-cis-AMB



Bisubstrate Analog



Intermediate Analog

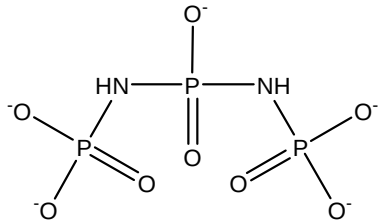


Figure 8