The relationship between human MAT1A mutations and disease: a folding and association problem?

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Abbreviations: MAT, methionine adenosyltransferase; AdoMet, S-adenosylmethionine; GSH, glutathione reduced form; GSSG, glutathione oxidized form; AdoHcy, S-adenosylhomocysteine; Hcy, homocysteine; CBS, cystathionine β-synthase; MTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; NO, nitric oxide; PKC, protein kinase C.

ABSTRACT

Methionine adenosyltransferases (MATs) are a family of highly conserved oligomers that catalyze the only known reaction for the synthesis of S-adenosylmethionine (AdoMet), the main cellular methyl donor. Their catalytic subunits exhibit a characteristic structure, organized in three domains formed by nonconsecutive stretches of the sequence. The active sites locate at the interface between subunits in the dimer with amino acids of each monomer contributing to catalysis. Changes in activity, oligomerization level and expression have been detected in several hepatic diseases; the knockout mouse for *MAT1A* spontaneously developing hepatocellular carcinoma (HCC). However, none of the patients with persistent hypermethioninemia caused by mutations in this gene exhibits hepatic problems, instead a few cases showing demyelination have been described. This chapter discusses aspects related to the structural features of these enzymes and the impact that the mutations found in the human *MAT1A* gene may have in the final protein structure. The influence of the redox environment in MAT folding and association is also analyzed, in light of the effects that drugs and metals that alter the GSH/GSSG ratio produce in the activity and association level. The recent report of the nuclear localization of the MAT I/III isoenzymes, along with their presence in tissues other than liver opened the option to MAT moonlighting. The possibility exists that disease development is related not only to a decrease in AdoMet production, but also to the role of these particular isoenzymes in different subcellular compartments. Therefore, the influence of *MAT1A* mutations, especially those leading to protein truncations, on folding and subcellular localization is discussed, paying special attention to the Hazelwood's hetero-oligomerization hypothesis to explain the demyelination process in patients with persistent hypermethioninemia.

1. Introduction

The first mention of an "active methionine" capable of transmethylating in the absence of ATP was made in 1951 by Giulio L. Cantoni in a paper where the biosynthesis of N^1-methylnicotinamide from methionine and nicotinamide was studied [1]. The nature of this compound was ignored at that moment, although it was described as a product resulting from the reaction between ATP and methionine. In 1953, a new paper by this author described the reaction catalyzed by the "methionine-activating enzyme" and the chemical nature of the "active methionine", S-adenosylmethionine (AdoMet)[2]. This compound was defined as an addition product of methionine and the adenosine moiety of ATP, having the configuration of a sulfonium compound in which an energy-rich bond links the methyl and the onium groups. This sulfonium ion is also involved in carbon-sulfur bonds with two additional groups, the 3-amino-3-carboxypropyl and 5´-deoxyadenosyl groups that can be donated as well. In the three cases donation of either of these groups requires the nucleophilic attack of the corresponding carbon-sulfur bond [3, 4]. Additionally, AdoMet can provide other groups for the synthesis of biological compounds (Figure 1), a fact that favors its implication in a large variety of reactions. Such a versatility let authors calculate that the number of reactions in which this donor participates is as large as that involving the use of ATP [5].

Methylation reactions are of special importance due to the large number of such processes that take place in any cell, and that result in the main consumption of AdoMet (~90-95%). These processes serve either for the synthesis of new compounds (methylation of small molecules, phospholipids, etc.) or for the regulation of cell function (methylation of DNA, RNA or proteins), and in all cases S-adenosylhomocysteine (AdoHcy) is produced [6]. AdoHcy is a potent inhibitor of most transmethylation reactions, but serves also as the intermediate for the synthesis of homocysteine (Hcy) and an important bacterial signaling molecule, Autoinducer-2 [7]. The aminocarboxypropyl group of AdoMet can be donated for the synthesis of the hypermodified tRNA nucleoside 3-(3-amino-3-carboxypropyl) uridine [8], but previous decarboxylation at this group is required for aminopropyl donation for the synthesis of polyamines (spermidine and spermine in mammals), plant hormones (ethylene), or signal molecules of Gram negative bacteria (N-acyl-L-homoserine lactones) [9]. Recently, a new class of proteins collectively named as SAM radical proteins was uncovered [10]. This family of enzymes uses the 5´-deoxyadenosyl

radical of AdoMet for the synthesis of vitamins (biotin), antioxidants (lipoic acid) and photosynthetic pigments. Other uncommon reactions involving AdoMet include the donation of amino groups for the synthesis of 7,8 pelargonic acid (a precursor of biotin) [11], ribosyl groups for queuosine synthesis [12], and carboxylic groups to lipophilic acceptors [13]. AdoMet has also been shown to undergo biohalogenation reactions leading to 5′-deoxy-5′-fluoroadenosine or its chloro-, bromo- or iodo- analogues [9].

AdoMet has additional roles as regulator of its own production and elimination through its effects on several enzymes of the methionine and the folate cycles, as well as in the trans-sulfuration pathway. In mammals, the essential amino acid methionine is mainly metabolized in the liver (~48% of the ingested amino acid in humans), where it can be used either for protein synthesis or by the methionine cycle (Figure 2). The first and rate-limiting step in this last pathway is AdoMet synthesis catalyzed by the methionine adenosyltransferase (MAT) isoenzymes, some of which are inhibited by the product. In human adult liver up to 8 g of AdoMet are produced by MATs daily [14], most of which is used as methyl donor for the transmethylation reactions leading to AdoHcy generation. This compound is metabolized by AdoHcy hydrolase leading to Hcy and adenosine, the only reversible step of the cycle that favors AdoHcy accumulation. Thus, elimination of the products of this reaction, Hcy and adenosine, controls the levels of this transmethylation inhibitor. Four are the enzymes involved in this removal: i) adenosine deaminase that transforms adenosine into inosine; ii) cystathionine β-synthase (CBS) that uses Hcy and serine in a condensation reaction to render cystathione, a step that is activated by AdoMet; iii) methionine synthase that remethylates Hcy using as methyl donor 5-methyltetrahydrofolate (MTHF); and iv) betaine homocysteine mehyltransferase that remethylates Hcy utilizing as methyl donor the betaine provided by choline oxidation, in a step inhibited by AdoMet [15]. These reactions allow connection of methionine metabolism with other pathways such as trans-sulfuration through the CBS reaction and the folate cycle that includes methionine synthase. The trans-sulfuration pathway renders cysteine that is used among others for protein and glutathione synthesis, the GSH/GSSG ratio modulating MAT activity [16]. Folate recycling is also controlled by AdoMet, which inhibits methylenetetrahydrofolate reductase (MTHFR), and hence MTHF production. This last compound is also an inhibitor of glycine-N-methyltransferase, the transmethylating enzyme responsible of the largest

consumption of AdoMet in a key step for controlling the levels of this metabolite [17]. In addition to these data, AdoMet has been also involved in the regulation of several processes, among them apoptosis and growth [18]. The role exerted in apoptosis (protector or inducer) depends on the cell type under study (hepatocytes or hepatoma cells) and can be mimicked by one of AdoMet derivatives, methylthioadenosine (MTA). All this condensed information allows the reader to get a glimpse of the importance of AdoMet for all kind of cells, and hence of the only known route for its synthesis catalyzed by the MAT family of enzymes to which this article is committed.

2. Methionine adenosyltransferases

MAT enzymes use methionine and ATP to render AdoMet and triphosphate that is excised into pyrophosphate and inorganic phosphate in the second part of the reaction. This catalysis follows a S_N2 displacement mechanism, in which 95% of the P_i derives from the γ-phosphoryl group of ATP [19]. MATs need divalent cations (Mg^{2+}) for catalysis, and many of them are also activated by monovalent cations (K^+). AdoMet is needed for cell survival, and hence MATs have been found in almost every single organism (from *Mycoplasma genitalium* to mammals) except for a few parasites that obtain the compound from their hosts [20]. Sequences for the catalytic subunits are very well conserved between *Bacteria* and *Eukarya*, whereas *Archaea* express highly divergent forms in which catalytic amino acids are preserved [21]. All the enzymes of the family characterized to date are homo-oligomers, except for mammalian MAT II hetero-oligomers that consist of catalytic (α2) and regulatory subunits (β) [22]. This β-subunit, however, is also able to interact with mammalian α1 and *E. coli* catalytic subunits, as demonstrated upon its overexpression in cell culture lines and bacterial systems. Most of the oligomers found are tetramers, with two notable exceptions, the archaeal MATs and one mammalian isoenzyme known as MAT III that form dimeric associations [23].

Several MAT isoenzymes are expressed in diverse organisms (from *Eukarya* to *Bacteria*), the larger number of variants being found in plants [20]. Probably the most interesting, and best characterized, case is that found in mammals, where three isoenzymes exist, their main feature being the differences in K_m values for methionine exhibited by each isoform: MAT I (homo-tetramer) ~100 μM, MAT II (hetero-oligomer) ~30 μM and MAT III (homodimer) ~1 mM [5,

24]. These mammalian MATs are also regulated in a dissimilar way by the reaction product, thus whereas MAT I and II are inhibited by AdoMet, MAT III is activated [5, 25, 26]. Since their discovery it was thought that MAT I and III expression was restricted to normal liver, but recent papers showed first their expression in pancreas [27] and later in many other tissues [28]. On the other hand, MAT II was known to be ubiquitous, as well as the hepatic fetal form and the isoenzyme expressed in hepatoma [5]. Analysis of the β-subunit expression revealed that this subunit appears in most tissues and that its splicing variants show a differential expression pattern [29].

3. Human MAT genes and their mutations.

The human genome contains three MAT genes, *MAT1A*, *MAT2A* and *MAT2B* that encode for the two catalytic subunits, α1 and α2, and the regulatory β-subunit, respectively. The *MAT1A* gene (~20 kbp) localizes to the 10q22 human chromosome [30], the *MAT2A* gene (~6 kbp) to the 2p.1.1 chromosome [31] and the *MAT2B* gene (~6.8 kbp) was identified in the long arm of chromosome 5 at the interphase between 5q34 and 5q35.1 bands [32]. Both *MAT1A* and *MAT2A* genes consist of eight introns and nine exons that codify for proteins of 395 amino acids [33, 34]. On the other hand, the *MAT2B* gene consists of six introns and seven exons that encode for a protein of 335 residues [32].

Mutations in the *MAT1A* gene have been characterized in patients showing isolated persistent hypermethioninemia with methionine levels reaching 1.3 mM in some cases (35-fold over control levels) (Table 1)[35]. Most of these patients did not show important pathological symptoms, although a few cases of demyelination have been described [30, 36]. The mutations identified to date follow mostly a recessive autosomal inheritance trait, although Mendelian dominant inheritance for the G to A transition leading to the R264H[h1] mutation has been described [37-39](Table 1). Some of the mutations reported led to early stop codons rendering subunits of 91, 184, 349, 350 and 386 residues [30, 35, 36, 40, 41]. The possibility of longer proteins has also been reported as a result of one mutation at the stop codon [42], and a change in the last nucleotide of exon III that alters the splice-donor site [35]. Some of these mutated

[1] The superscript indicates the species: rat (r), human (h), *E. coli* (c), Methanococcus (Mj)

MATα1 forms have been expressed in COS-1 and/or *E. coli* cells to study their activity and association state as has been reviewed elsewhere [34, 35, 39].

Neonatal screenings for early detection of metabolic alterations are carried out throughout the world. One of these procedures carried out in Galicia, a region in the northwest of Spain, revealed a high incidence of persistent hypermethioninemia among the galician population (1/23470 newborns), the most common mutation identified being *MAT1A* R264H^{h} [43]. However, to date no mutations in the human *MAT2A* and *MAT2B* genes have been reported, although the existence of splicing variants for *MAT2B* in hepatocellular carcinoma (HCC) and HepG2 cells has been shown [29]. Four variants have been described to date, the main splicing forms being V1 and V2 that differ in their transcriptional initiation site. These sites locate at positions -203 and -2372 from the ATG codon, respectively. Thus, V2 uses a different exon I and the resulting proteins diverge in the 20 initial amino acids of their N-terminals. In addition, V2a misses the first 51 bp of exon VII, whereas V2b lacks the information of exons III-VI. Available functional data have been obtained only with the V1 variant, and hence information concerning association of the new forms with the catalytic α2 subunits, their effects on methionine affinity, or their putative actions on α1 subunits is lacking.

4. MAT structure and impact of the human mutations.

Recently a review concerning the MAT structural data available has been published [23], and readers are referred to that publication for detailed structural information. Therefore, this section will focus on the location and potential structural effects of the human mutations described to date. The crystal structures that have been reported show common features concerning the α-subunit domain organization, the dimer association and the catalytic sites. The sequence of the α-subunits is arranged in three domains formed by non-consecutive stretches of the amino acid chain (Figure 3). Deletions originated by human *MAT1A* mutations include truncations leading of the loss of part of the C-terminal domain elements (386X and 350X) to proteins that lack information even for one complete domain of the three that are present in the wild type subunit (92X and 185X) (Figure 4). In addition, other truncated forms not only loss ~40% of the C-terminal domain, but also change the sequence of most of the remaining part of this domain, as well as of ~14% of the central domain (351X) (Figure 5). The longer α-subunits

resulting from the stop codon mutation, should in contrast contain the information to build the three wild type domains, but will have a C-terminal domain ~20% longer (X396Y-464X) (Figure 3). On the other hand, those derived from the mutation in the splice donor site should include a large change in the protein sequence, and hence their structure being drastically altered.

Two subunits associate to form the dimer through a large flat hydrophobic surface to which amino acids of the three domains contribute [44, 45](Figure 6). Thus, severe truncations (92X and 185X) will lack most of this interaction surface, whereas more moderate effects could be expected for those mutations rendering shorter C-terminal domains (350X and 386X). The exception should be the 827insG (351X) that may impose additional difficulties for association derived not only from the lack of part of the C-terminal domain, but also from the change in the sequence starting on residue 276 (Figure 5). The effect that a longer C-terminal (X396Y-464X) could have on the subunit association may range from no interference with the normal interaction pattern to steric hindrance of part of the interacting residues. Single amino acid substitutions detected in patients could also affect the interaction pattern indirectly, by changing the spatial orientation of the chains or directly by disrupting them. This last case could be that of $L42P^{h}$ and $L305P^{h}$ mutations that occur at α-helixes H1 and H6, respectively. In addition to the large number of hydrophobic interactions linking monomers, there are a few polar bonds contributing to dimer stability [44]. Among the residues involved in this type of interactions is $R264^{h}$, the equivalent $R265^{r}$ appearing bonded to $E58^{r}$ and $T263^{r}$ in the crystal structure of rat MAT I [44]. Several patients have the $R264H^{h}$ or $R261C^{h}$ mutations, which will clearly block interaction with $E58^{r}$ that involves the side chain amino group of the arginine residue. Disruption of this bond seems will have important consequences for dimerization as judged from studies with the mutated recombinant proteins. Characterization of the recombinant $R264H^{h}$ mutant showed that the protein is a monomer exhibiting tripolyphosphatase activity [46], although analogous mutants prepared by others and in MAT^{c} indicated its oligomeric association [39, 47].

The two active sites per dimer locate between both subunits that contribute the residues for substrate binding and catalysis [44, 48]. Such an organization thus needs the production of a precise arrangement that allows placing of the catalytic residues in the correct positions for catalysis to proceed. Essential residues have been identified by chemical modification and in crystal complexes including substrates (ATP and methionine) or their analogues (AMPPNP, L-

cisAMB, AEP)[23]. In most of the cases their role was later verified in recombinant proteins after site directed mutagenesis. Among them, $F251^r$ against which methionine analogues are found and that locates at the initial position of a short loop in the active site [44]. No single mutations at key residues for AdoMet synthesis have been identified to date, although some substitutions may indirectly affect catalysis because of their sequence or spatial proximity to the catalytic residues, thus explaining the reductions in activity reported. In contrast, the human mutations involving $R264^h$ directly perturb the phosphatase activity, as this residue has been show to orient the PPP_i chain for hydrolysis [47].

Interaction between dimers in the tetramer occurs through the central domains of the subunits. A few polar interactions are responsible of tetramerization, the number of which is lower in mammalian MATs than in the *E. coli* protein [44, 45]. Moreover, the interaction pattern seems quite different, the bacterial tetramer exhibiting a central interaction core, surrounded by a ring of interactions that in mammalian MAT I^r is lacking [49]. The existence of these additional bonds could be responsible for the higher stability exhibited by bacterial tetramers.

Other studies have shown cysteines as key residues for both MAT activity and dimer-dimer association in both rat and *E. coli* enzymes [50, 51]. The α1-proteins contain 10 such residues per monomer, from which $C121^r$ locates in a loop that has been proposed to regulate the access of substrates to the active site [44, 52]. Chemical modification of two cysteine residues/monomer in MAT I/III^r let to dissociation into inactive dimers [51, 53], a fact that has been further explored using single cysteine to serine mutants [54]. The results obtained indicated that the five residues located in the central domain ($C35^r$-$C105^r$) are key in dimer-dimer association, a result later supported by crystallographic data that showed the interface between dimers provided by this single domain. The sulfhydryl groups of residues $C35^r$ and $C61^r$ are facing each other and at disulfide bond distance, an important fact for oligomerization as it will be explained in the following sections [44, 49]. No human mutations at cysteine residues have been reported, although a couple of the substitutions identified to date include new cysteines at positions 264^h or 199^h [30, 35, 42], and the sequence change due to 827insG ($351X^h$) adds 6 cysteine residues more to the remaining C-terminal domain (Figure 5). The impact that inclusion of a larger number of this type of amino acid could have on protein folding may be responsible for the low activity exhibited by the recombinant mutants. The relevance of cysteines in α2

proteins cannot by extrapolated from the results with α1 oligomers, since they differ in the cysteine content and lack equivalent residues at some positions in the central domain, among them $C61^r$.

5. Actual knowledge on MAT folding.

Most of the available data have been obtained *in vitro* using MAT I/III^r, *Bacillus subtilis* and *Methanococcus jannaschii* MATs in unfolding and refolding experiments using denaturants such as urea and temperature [55-57]. Here we will refer mainly to MAT I/III^r due to its high homology to the human enzyme. Refolding results indicated the need to populate an intermediate state to achieve the final active dimer and highlighted the importance of Mg^{2+} ions in the process [58]. Moreover, the last steps of the monomer refolding and their association need a reducing environment provided by DTT or appropriate GSH/GSSG molar ratios (10:1). Under these conditions fully active dimers were produced, although those obtained in the presence of GSH/GSSG contained a single disulfide bond ($C35^r$-$C61^r$) as well as several oxidized methionine residues [49]. Production of this single disulfide blocks the dimer-tetramer concentration-dependent equilibrium observed in the fully reduced forms, thus providing further evidence of the key role of cysteines in the central domain for association. The presence of copper during refolding has also been shown to reduce the production of active MATα1 and MATα2 oligomers, although no effect on the association state attained was observed [59].

Unfolding experiments revealed a rapid loss of AdoMet synthesis at very low urea concentrations, a process that preceded dimer dissociation [55]. In addition, structural information indicated that urea unfolding is a two-step process in which a monomeric intermediate (I) can be identified [55, 60]. The main characteristics of this state are: i) its lack of activity; ii) the conservation of a 70% of the secondary structure composition; and iii) some features of a molten globule. In addition, this intermediate exhibited reduced ANS binding capacity as compared to the native dimer, therefore suggesting exposure of a smaller hydrophobic surface to the solvent. The reversibility of MAT III^r folding allowed calculation of the free energy of the process that is 65.69 kJ/mol. Kinetic experiments showed later the existence of a second short-lived intermediate (I_k^2) between I and the dimer that is supposed to be monomeric, although its precise association state has not been demonstrated [60]. Furthermore, tetramer to

dimer dissociation also occurs through a kinetic intermediate (I_k^1) that was detected following ANS fluorescence. This new state was suggested to be a dimer just before undergoing the conformational changes that differentiate MAT I and MAT III dimers [49]. The fact that this process can be followed by ANS fluorescence indicates that the dye binds near the tetramerization area, this site being identified as the ^{358}PG^{359} dipeptide^r and the ^{131}VGA^{134} stretch^r located to the beginning of the active site loop. The free energy of tetramerization was calculated to be 29.24 kJ/mol and that of the overall process 102.14 kJ/mol, thus showing that the main contribution to the oligomer stability derives from monomer-monomer association.

Recently the stability of the highly divergent MAT^{Mj} has been explored using urea and single tryptophan mutants at several secondary structure elements [56]. Despite its highly divergent sequence, MAT^{Mj} seems to preserve the topology and structural features of the rest of the family, and hence some data can be extrapolated. Thus, although MAT^{Mj} exhibits substantially higher stability than the mesophilic MATs, the structural elements more susceptible to urea denaturation are the exposed areas of three β-sheets at the flat contact surface between monomers, dimer dissociation requiring a wider perturbation involving the three domains of the molecule. In order to achieve higher stability the access of the denaturant to the monomer interface should be limited. Structural predictions show a tighter binding between monomers of MAT^{Mj} as compared to MAT^r [56], and crystal structures illustrate that the active site cavity is much wider in MAT^r than in the *E. coli* counterpart [23]. Extrapolation of these data to MAT^r suggests that urea access to analogous secondary structure elements is easier and that a larger portion of the β-sheets is exposed to the denaturant because of a wider active site. Thus, a more important perturbation of the β-sheets can be expected, and hence the rapid loss of activity observed in a protein with a low affinity for the substrate K_m^{Met} (~1 mM) and its dissociation at inferior urea concentrations.

On the other hand, thermal denaturation of MAT^r is an irreversible process in which changes in activity and structure occur in the range 37-55ºC [61]. According to data obtained by two-dimensional infrared spectroscopy the first elements to undergo unfolding are the most solvent exposed, α-helixes and β-turns. Exposure of hydrophobic residues and intersubunit hydrogen bonds are the second events that lead to final aggregation. These results could be a

consequence of two phenomena, dimer dissociation and monomer aggregation or direct aggregation of unfolded dimers.

The only *in vivo* folding data available refer to MAT^c, which has been identified among the substrates of the GroEL/GroES chaperonin system [62]. Several authors have indicated that the capacity of the active site cavity of this chaperonin would be able to admit proteins no larger than ~50 kDa, thus precluding the folding of MAT dimers in its inner space. The combined data available suggest that a monomeric intermediate (analogous to I) can be folded by the chaperonin, and such a monomer, according to ANS binding results, may expose a smaller hydrophobic surface to the solvent than the final dimer. Thus, this intermediate will need to accomplish some additional conformational changes to allow the establishment of the large number of hydrophobic contacts observed among monomers in the dimer. This association step may require additional chaperones that remain unidentified, the effects of copper accumulation on MAT oligomers suggesting a role for metallochaperones in this step [59].

6. Post-translational modifications.

The identification of post-translational modifications that could regulate MAT behavior has been the subject of study for several years, although the information obtained to date is quite limited. Most of the data relate to the redox regulation of the protein and have been attained with rat MAT I/III. Both *in vitro* and *in vivo* studies showed that MAT activity is decreased under oxidative conditions due to reductions in the GSH/GSSG ratio [16, 51, 63, 64]. Moreover, redox stress induced the accumulation of inactive dimers in animal models [65]. Analysis of the agents that let to such behavior revealed MAT inhibition by GSSG, H_2O_2 and NO, and related this inactivation to the production of an intrasubunit disulfide and $C121^r$ hydroxylation or nitrosylation, respectively [16, 52, 66, 67]. The use of mutants and refolding systems allowed identification of $C35^r$ and $C61^r$ as the residues involved in the disulfide, and showed that production of this covalent modification blocks dimer-tetramer exchange [49, 68]. The presence of these two cysteines in the central domain suggests that the disulfide may contribute to the stabilization of the β-sheet through which interactions for tetramerization are established. Two additional cysteines exist in this sheet, $C57^r$ and $C69^r$ that according to the structural data appear at disulfide bond distance but with their sulfhydryl groups facing opposite directions [44].

Although not demonstrated yet, it is possible that folding intermediates in which anomalous disulfide patterns are established are favored during oxidative stress, and hence rendering inactive species that may or may not associate into dimers. The accumulation of inactive or poorly active dimers described during disease or intoxication processes that reduce the GSH/GSSG ratio may relate to the production of this kind of oxidized species, an aspect that remains unsolved.

Regarding nitrosylation and hydroxylation, the presence of $C121^r$ in a flexible loop at the active site entrance, has led authors to postulate the putative role of this modification in the control of the access of substrates to the catalytic site. This modification has been suggested to block the loop in a closed conformation, thus impeding catalysis [52, 66, 67]. The presence of acid and basic residues in the proximity of the cysteine to be nitrosylated is a requirement for the modification to take place [69]. Such residues have been identified in rat MATα1 as $D355^r$, $R357^r$ and $R363^r$ and its key role further demonstrated by mutation to serine, a substitution that led to decreased NO modification. Some of the mutations identified in human persistent hypermethioninemia affect the equivalent $R356^h$ residue introducing a glutamine or proline in this position, and change $P357^h$ to leucine. Therefore, impairment in NO-induced regulation of MAT I/III activity is expected to occur in these patients. Both $C121^r$ nitrosylation and the $C35^r$-$C61^r$ disulfide provide regulatory mechanisms apparently restricted to α1 oligomers, as the catalytic α2 subunits lack the equivalent cysteine residues (G120 and A60).

In vitro refolding assays in the presence of GSH/GSSG allowed identification of methionine residues susceptible to oxidation (amino acids 20^r, 65^r, 139^r and 151^r) [49]. However, the impact of methionine modification on activity and oligomerization has not been explored further. The introduction of additional methionine residues due to human mutations ($I322M^h$) may provide the enzyme with a new oxidation target with higher affinity for modification. The location of this residue in the dimer structure and its contribution to the monomer-monomer stability may be affected by this substitution, rendering proteins with low activity as reported [34, 35].

Phosphorylation may be another mechanism through which MATs can be regulated [70]. Analysis of their sequences revealed several putative phosphorylation sites for a variety of kinases, although only PKC modification of MAT I/III at $T342^r$ has been demonstrated *in vitro* [71]. One of the mutations identified to date in humans occurs at $E344^h$, residue that is substituted

by alanine. Such a change of charge near the phosphorylation site could be key for kinase recognition, thus altering the modification pattern. This effect could be also expected to occur in other human mutations that also locate next to putative phosphorylation sites, these include: $R264H^h$, $K289N^h$ and $G336R^h$. However, both $R264H^h$ and $K289N^h$ may not be at truly phosphorylation sites due to their location at the active site cavity.

7. Alterations in MAT behavior in disease.

Changes in expression, oligomerization and MAT activity have been described in several pathologies and animal models of disease (Figure 7). Probably the largest amount of data published refers to hepatopathologies related to ethanol abuse and hepatomas, although results concerning leukemia and cognitive diseases are also available. Several authors have reported reductions in MAT1A mRNA levels during human alcoholic cirrhosis, although discrepancies about MAT2A transcript levels have been published. Thus, Avila et al. showed no induction of MAT2A by ethanol [72], whereas other studies reported increases in MAT2A expression, an effect mediated by TNFα [73]. In parallel, decreases in MAT activity and accumulation of dimers have been described that correlate with the corresponding reductions in AdoMet concentration [65, 74-77]. In contrast, differences in the effects on AdoHcy production among models occur, although the net effect in the methylation index (AdoMet/AdoHcy ratio), a decrease, is the same. Liver damage during ethanol consumption may be derived from the impairment of methylation reactions and GSH-dependent detoxification capacity. Reductions in GSH levels and increases in protein nitrosylation are known to take place in these cases, effects to which MAT I/III is susceptible. Other protein modifications caused by products of ethanol catabolism cannot be excluded, although this aspect has not been analyzed. Several reports have shown also that treatment with AdoMet attenuates ethanol liver injury [75], thus highlighting the importance of MAT function in the development of cirrhosis.

In the early 70s, Halpern et al. described the high dependence of human cancer cells on exogenous methionine for growth, whereas normal cells can use Hcy instead [78]. Therefore, the interest in understanding the alterations caused in the methionine cycle during carcinogenesis grew in order to search for useful drugs against this pathology. This search was conducted using a variety of carcinogens in animal models in which the effects exerted by the drugs in this

pathway were analyzed [79-85]. The results obtained showed a shift from MAT1A to MAT2A expression, reflected by reductions in MAT1A mRNA, protein and activity, and the corresponding increases in these parameters for MAT2A. The preferential expression of the α2 catalytic subunit renders a decrease in AdoMet levels that correlates with a global DNA hypomethylation [86]. This relationship with hepatocellular carcinoma (HCC) was further confirmed with the production of a MAT1A knockout mouse that spontaneously develops the disease [87], and reinforced by the observation that AdoMet therapy is efficient in preventing HCC [88]. In contrast, silencing of MAT2A expression in normal or MAT1A-overexpressing hepatoma cells produced cell death [89]. MAT2B provides a growth advantage to hepatoma cells, and its overexpression is normally associated with hepatic dysfunction [90]. In all these processes iNOS induction increases NO production leading not only to MAT I/III inactivation, and hence to a reduction in AdoMet concentration, but also to effects on several transcription factors that may control the switch among MATs [79].

Wilson's disease is another interesting pathology in which copper is accumulated in the liver due to mutations in the ATP7B transporter, thus leading to redox stress, among other effects. Several animal models of the disease exist and in one of them, Long Evans Cinnamon rats (LEC), changes in MAT activity and AdoMet levels were described at late stages of disease [91]. Several years later Delgado et al. examined the changes in the methionine cycle in this model but at early stages of copper accumulation, and showed changes in the expression pattern of the MAT isoenzymes [59]. In 9 week-old animals, a switch between MAT1A and MAT2A expression was already detected, but surprisingly MAT2B followed the opposite trend to MAT2A decreasing by 80%. Copper effects on MAT2B expression were also shown to rely on redox mechanisms in experiments carried out in cell culture. The changes in MAT1A expression correlated with decreases in protein, AdoHcy and GSH levels, the net result being an increase in the methylation index and a decrease in the GSH/GSSG ratio. As observed in other pathologies, copper accumulation also modified the MAT I/III ratio, but in this case reductions in dimer content were observed. Analysis of the effect of copper on MAT isoenzymes *in vitro* confirmed inhibition of the enzyme activity and showed the influence of this metal in folding of the subunits. Recombinant MATα2^{h} oligomer activity was more susceptible to metal inactivation than that of MATα1^{r} oligomers [59].

Patients suffering from several neurological diseases are known to display alterations in transmethylation reactions [92-96], and several animal models showed an association between methionine synthesis and/or MAT activity and development of myelopathies, ataxia and other related diseases [97-99]. Parkinson's patients show low AdoMet and AdoHcy levels in blood and increased erythrocyte MAT activity [95], although no relationship with age and duration of the disease has been reported. L-dopa treatment induces a short-term increase in hepatic and brain MAT expression and activity, as well as in AdoMet levels [100], but AdoMet depletion is observed in long therapies [101-105].

Currently, newborn screenings for the detection of hypermethioninemia are carried out all over the world. Most of the cases identified are due to defects in CBS, fumaryl acetoacetate hydrolase (tyrosinemia type I) or liver disease, however in a small number of patients this alteration is due to mutations in the *MAT1A* or *GNMT* genes [105]. The first patient with isolated persistent hypermethionemia due to *MAT1A* mutation was reported in 1974 [106], and ever since new mutations have been identified, a summary of which is included in Table 1. Many patients are asymptomatic, but in a few cases neurological defects have been noticed. The most severe cases present demyelination and correspond to mutations that introduce early stop codons in the *MAT1A* ORF, leading to subunits of ~350 amino acids. One of these patients was treated with AdoMet and an improvement in demyelination was reported, thus suggesting that these symptoms derive from low AdoMet production [94]. In addition, former experiments using cycloleucine, a MAT inhibitor, in rats showed development of myelin abnormalities that were prevented by AdoMet [107]. All these data together and the absence of such symptoms in patients exhibiting shorter forms of the α1 protein led Hazelwood et al. [36] to propose the hetero-oligomerization hypothesis, which will be discussed in the last section of this chapter to explain the origin of the disease. This hypothesis presented a conceptual problem at the time of its proposal that was related to the restricted expression pattern known for MATI/III (considered hepatic only) and alterations in the nervous system, where only MAT II was known to exist.

8. MAT tissular distribution and subcellular localization.

Early data on MAT tissue-specific expression derived mostly from activity measurements carried out in samples from different tissues at two methionine concentrations (to distinguish

among MAT isoenzymes with different K_m^{Met}) and in the presence of DMSO (a known activator of MAT III). The same is true for the subcellular distribution that was analyzed in fractionation experiments. All these data established the extrahepatic presence of MAT II, together with the hepatic expression of MAT I/III in normal tissues. This postulate was reassessed in the last decade using Northern and Western blot and real time PCR. The compiled results confirmed the broad MAT2A and MAT2B expression [90] and a wide distribution for MAT1A that exceeds normal adult liver [28]. The expression levels correlated with early results: i) very high for MAT1A in liver followed by pancreas [28]; ii) high for MAT2B in spleen, heart, lung and very low in normal adult liver [90]; and iii) high in extrahepatic tissues, fetal liver and hepatoma for MAT2A [5]. Subcellular distribution was also thought to be restricted to the cytosol and fluorescence microscopy confirmed this localization for the β subunit [90]. However, recent studies by immunohistochemistry, confocal fluorescence microscopy, subcellular fractionation and western blot showed MATα1 in the cytosol of hepatocytes and pancreatic cells but also in the nucleus of almost every cell type analyzed [28]. Thus, the question arises as to which may be the biological role for this distribution.

Nuclear MATα1^r was mainly monomeric, although a small amount of active tetramers could be detected in this compartment in liver [28]. The protein colocalized with nuclear matrix markers and both nuclear distribution and matrix localization were independent of AdoMet synthesis capacity, the inactive F251D^r mutant showing the same pattern than the wild type protein. Basic residues in the C-terminal domain were shown to be involved in the nucleocytoplasmic distribution, and two partially overlapping areas were identified that are implicated in localization. One area delimited by amino acids 313^r, 368^r, 369^r and 392^r was related to cytosolic retention, whereas that comprised by residues 340^r, 344^r and 393^r was linked to nuclear localization. The subcellular distribution of a truncated protein at residue 351X^r, analogous to some of the mutants detected in human patients with hypermethioninemia, was also examined. This shorter form appeared mainly in the nucleus and colocalized with the nuclear matrix marker SC-35. The use of mutants showing preferential nuclear or cytosolic localization allowed correlation of nuclear MATα1^r distribution with increases in specific epigenetic modifications (H3K27 trimethylation) [28]. This result suggested the need for the presence of active nuclear MATα1 oligomers to guarantee specific methylations, and opened the possibility

of additional unknown functions for the nuclear monomers. Moreover, identification of the areas responsible for nucleocytoplasmic distribution close to the *in vitro* phosphorylated $T342^{r}$ residue deserve additional studies to determine the role that such modification may have in the control of MAT subcellular localization. Additionally, both the nucleocytoplasmic distribution and its putative control by phosphorylation may be altered in patients with mutations in these areas, such as those related to $E344^{h}$ and $G336^{h}$ residues which induce charge changes at the molecular surface (Table 1).

9. Hazelwood's hypothesis of MAT hetero-oligomerization in demyelination.

The observation of demyelination exclusively in patients with *MAT1A* mutations leading to subunits ~350 amino acids long, together with the improvement observed in the symptoms of one of this patients upon AdoMet treatment let Hazelwood et al. propose that truncated α1 subunits may hetero-oligomerize with α2 monomers sequestering them in an inactive oligomer, and hence producing a decrease in AdoMet levels [36]. This decreased concentration of the methyl donor will lead to the corresponding lack of methylation products, among them phosphatidylcholine and creatine, important for neural sheath [108] and neural structure [109]. Thus, administration of AdoMet would allow recovery of normal intracellular levels of the methyl donor, and hence the improvement observed in the patient symptoms [94]. This hypothesis was postulated in 1998 when only the MAT^{c} crystal structure was available [45], and the importance of the R264 residue in dimerization was known. Preservation of this arginine and the high homology among α1 and α2 human sequence (84%) seemed enough to consider hetero-oligomerization plausible. Now when more MAT structures are available [23], it is known that monomers may have slightly different orientations between each other in different species [44], and the impact of these truncations on the monomer subunit and the intersubunit interactions in the dimer can be reexamined. Thus, truncated subunits will lack approximately half of the C-terminal domain, specifically the area mostly related to the monomer surface. The impact this deficiency will have in folding of the remaining C-terminal domain structure, including the β-sheet that participates in monomer-monomer contacts, is unknown. However, this fact will force a rearrangement of the region to hide amino acids that will become otherwise exposed. Moreover, the 351X mutant not only suffers the truncation of part of this domain, but also a change in its

sequence that affects a ~15% of the central domain. This means an almost completely different C-terminal domain, and hence making preservation of its structure and interaction pattern unlikely (Figure 5).

As mentioned in previous sections, the last years have also seen some *in vitro* folding studies that showed the need of populating a monomeric intermediate I to attain the final active structure. The appropriate conformation of this intermediate will allow the final changes in the subunit structure and the association into active oligomers. The importance of this intermediate may require chaperonin assisted folding, as deduced from the finding of MAT^c as a substrate of the GroEL/GroES system [62]. To date, it is not known if truncated MATs will achieve a similar intermediate state allowing association with the α2 subunits. Moreover, if additional chaperones are needed to catalyze the last folding and association steps, the possibility exists that the modifications concerning the C-terminal domain may preclude recognition or achievement of a structure resembling at least part of the wild type structure (N-terminal and central domains). Even if the truncated monomer is able to oligomerize, two association scenarios can be considered: i) correct hetero-oligomerization, which should render a dimer with one complete active site (from the two in the molecule); and ii) an hetero-oligomerization pattern different from that in the native dimer, an alteration that will be of special importance for the active sites that locate between momoners. Structures of *E. coli* and mammalian MATs are almost identical, and hence overexpression of the truncated forms in bacteria is expected to produce hetero-oligomers with MAT^c. These experiments were performed and no MAT activity was reported by Chamberlin et al. [30]. In our hands, overexpression of a $351X^r$ mutant let to inclusion body production, thus suggesting incorrect folding of this type of subunit [28].

Another problem of Hazelwood's hypothesis at the time of its proposal was derived from the identification of MATα1 oligomers only in liver. Now that expression of *MAT1A* has been proven in other tissues [28], this problem no longer exists, although new aspects have to be considered. First, the putative interaction of inappropriately folded truncated species with proteins other than the α2 subunits should be taken into account. Such anomalous interactions may be responsible for the observed symptoms. Second, MATα1 expression in extrahepatic tissues occurs mainly in the cell nucleus, where most of the protein appears as monomers. A truncated form similar to that of patients with demyelination showed its preferential accumulation

in the nucleus in several cell types, thus suggesting that the effects observed may derive from the functions MAT is exerting in that particular compartment. To date we only know that nuclear accumulation of MATα1 correlates with H3K27 trimethylation, an epigenetic modification related to gene repression. Accumulation of truncated mutants in the nucleus will either favor this modification (i. e. repressing myelin related genes) or interfere with this methylation due to anomalous binding of MATα1 truncated species that are incorrectly folded. Finally, the hypothesis did not consider α2β interactions. No data exist about how these subunits interact, neither of the role of spliced β-subunit variants, and hence of the effect that α2-truncated α1 hetero-oligomerization may exert in recognition of the regulatory subunit.

CONCLUSION

The knowledge on MAT structure, catalytic mechanism and regulation has increased enormously during the last decades. However, this accumulated information has provided no clue to the relationship between some *MAT1A* mutations and the demyelination observed in patients expressing the corresponding truncated forms. The main aspects that remain to be clarified to understand this relationship include: i) the structure of the truncated protein subunits; ii) its oligomerization abilities with either α2 and β subunits; iii) understanding the *in vivo* folding pathway for MATs and the chaperones/chaperonines involved; iv) the effect of nuclear accumulation of truncated subunits in cellular behavior (i.e. myelin production); and, v) elucidation of the nuclear functions of MATα1, and hence of its alterations due to truncations. In addition, the putative existence of MAT I/III moonlighting related to its presence in to date new subcellular locations, and hence the effect that mutations could have on these additional functions cannot be underestimated. Thus, the number of questions related to MATs that await an answer is increasing, despite the large effort made to date to get insight the knowledge of the relationship among this family of enzymes and disease.

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FIGURE LEGENDS

Figure 1. Reactions involving S-adenosylmethionine. The figure summarizes the reactions in which S-adenosylmethionine participates in any kind of cell organized according to the group that is transferred in each case. S-adenosylhomocysteine (SAH) and S-adenosylmethioninamine are intermediates in some of these processes.

Figure 2. The methionine cycle in mammalian liver. The first step of methionine metabolism is the synthesis of S-adenosylmethionine by methionine adenosyltransferases (*MAT*s). This compound is used as methyl donor by a large number of methyltransferases (*MTase*), and among them glycine N-methyltransferase (*GNMT*), leading to methylated acceptors and S-adenosylhomocysteine. This demethylated compound is degraded by S-adenosylhomocysteine hydrolase (*SAHH*), in the only reversible reaction of the cycle, to render adenosine and homocysteine. Methionine can then be resynthesized from homocysteine by methylations catalyzed by methionine synthase (*MS*) and betaine homocysteine methyltransferase (*BHMT*) that use 5-methyltetrahydrofolate (MTHF) and betaine as methyl donors, respectively. MTHF is synthesized by methylene tetrahydrofolate reductase (*MTHFR*) an enzyme inhibited by S-adenosylmethionine. Homocysteine can enter also the trans-sulfuration pathway, where it is converted to cystathionine by cystathionine β-synthase (*CBS*).

Figure 3. Organization of the MAT amino acid sequence among structural domains and impact of human deletions and sequence extensions. The figure shows the sequence stretches involved in each domain, the percentage of contribution in each case appearing below. The impact of human mutations leading to truncated or extended monomers is also shown. Changes from the wild type sequence are indicated in magenta.

Figure 4. Impact of truncations in the monomer structure. The figure shows the structural elements of each domain preserved in the truncated human MATs; N-terminal (blue), central (red) and C-terminal (green) domains.

Figure 5. The human 351X mutant changes a large stretch of its C-terminal sequence. The 827insG in the human *MAT1A* ORF leads not only to an early stop codon, but also to a complete change in the reading frame, and therefore in the sequence starting on residue 276. Comparison of the resulting sequence and the wild type is shown in the upper part of the figure, whereas the lower part depicts the effect of the sequence changes in the side chains (residues 271-stop) is depicted. The last common amino acids (271-275) appear in cyan and blue for the wild type and 351X C-terminals, respectively.

Figure 6. The human MATα1 dimer. The figure shows the expected structure for the human α1 dimer created by mutation in the rat sequence of the 1QM4 pdb file; the sequences of both proteins are 95% identical. Monomer A surface appears in blue, the areas of contact with monomer B (salmon) being shown in gray. The three β-sheets of monomer B (one contributed per domain) appear oriented against the contact areas of monomer A. The arrow indicates the entrance to one of the active sites of the molecule.

Figure 7. Summary of the knowledge on MAT regulation. The scheme represents the different levels (mRNA, protein and activity) reported for MAT regulation during disease or under several treatments. Black dashed arrows indicate increases, whereas red dashed arrows stand for decreases in any of the parameters examined. Abbreviations correspond to: superantigen staphylococcal enterotoxin B (SEB); S-adenosylmethionine (AdoMet); methylthioadenosine (MTA); phytohemagglutinin (PHA); interleukin 2 (IL-2); interleukin 6 (IL-6); lipopolysaccharide (LPS); buthionine sulfoximine (BSO); oxidized glutathione (GSSG); 3-morpholinosydnonimine (SIN-1); S-nitrosoglutathione (GSNO).

TABLE 1

Mutations in the *MAT1A* gene detected in human persistent hypermethioninemia

Exon	Gene mutation	Protein mutation[7]	inheritance	Recombinant protein characteristics[3]	Liver activity[6]	Ref.
I	C65T	S22L	HO[1]			110]
II	G113A	S38N	CHE[2]	No activity[5]	68%	35, 111
	T125C	L42P	HO			110
	C164A	A55D	CHE	17%[4]		34
III	G205A	G69S				42
	255delCA	92X	CHE	No activity[5]		35, 111
		G98S	HO	100% activity[4,5]		35
V	C426T	A142	CHE			34
	539insTG	185X	HO, CHE		7%	30, 36
VI	C595T	R199C	CHE, HO	10% activity[5]		30
	C745T	R249W				42
VII	G791A	R264H		<1% activity[4,5] monomer	low	35, 37-39, 42, 43
	G791T	R264C	CHE	No activity[5]		35
	C790T	R264C				42
	G867T	K289N				42
	G870A	V290	CHE			34
	T914C	L305P	CHE	25%[4]		34
	827insG	351X	HO	No activity[5]		30
VIII	T966G	I322M	HO, CHE	21%[4,5]		34, 35
	G1006A	G336R	CHE	23% activity[5]		35
	A1031C	E344A	CHE	12%[5]		35
	1043,1044del	350X	HO	No activity[5]		30, 40
	G1067A	R356Q	CHE	53% activity[5]	low	30, 112
		R356P				42
	C1070T	P357L	CHE	31%[4]		34, 42
IX	T1131C	Y377	CHE			34
	G1132A	G378S	CHE	0.1% activity[5]		30, 41, 112
	G1161A	W387X	CHE, HO	75% activity[5] dimer		41
	G1188T	X396YfsX464				42

[1]HO, homozygosis
[2]CHE, compound heterozygosis
[3]Mutant activity levels versus wt protein upon COS-1[4] or *E. coli*[5] overexpression
[6]Measured in liver biopsies
[7]Residue change in the resulting protein

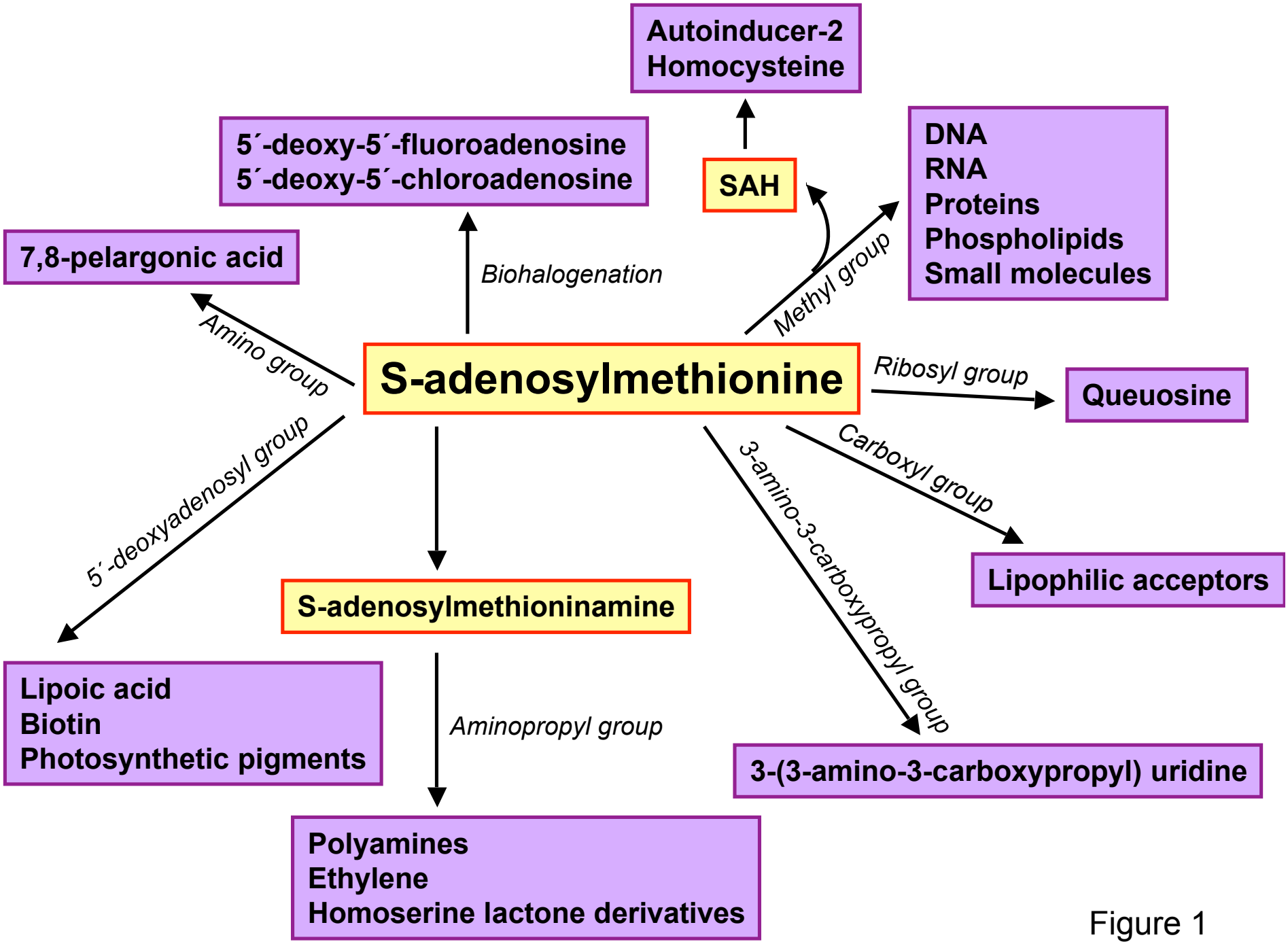

Autoinducer-2
Homocysteine
5´-deoxy-5´-fluoroadenosine
5´-deoxy-5´-chloroadenosine
SAH
DNA
RNA
Proteins
Phospholipids
Small molecules
7,8-pelargonic acid
Biohalogenation
Methyl group
Amino group
S-adenosylmethionine
Ribosyl group
Queuosine
5´-deoxyadenosyl group
Carboxyl group
3-amino-3-carboxypropyl group
Lipophilic acceptors
S-adenosylmethioninamine
Lipoic acid
Biotin
Photosynthetic pigments
Aminopropyl group
3-(3-amino-3-carboxypropyl) uridine
Polyamines
Ethylene
Homoserine lactone derivatives

Figure 1

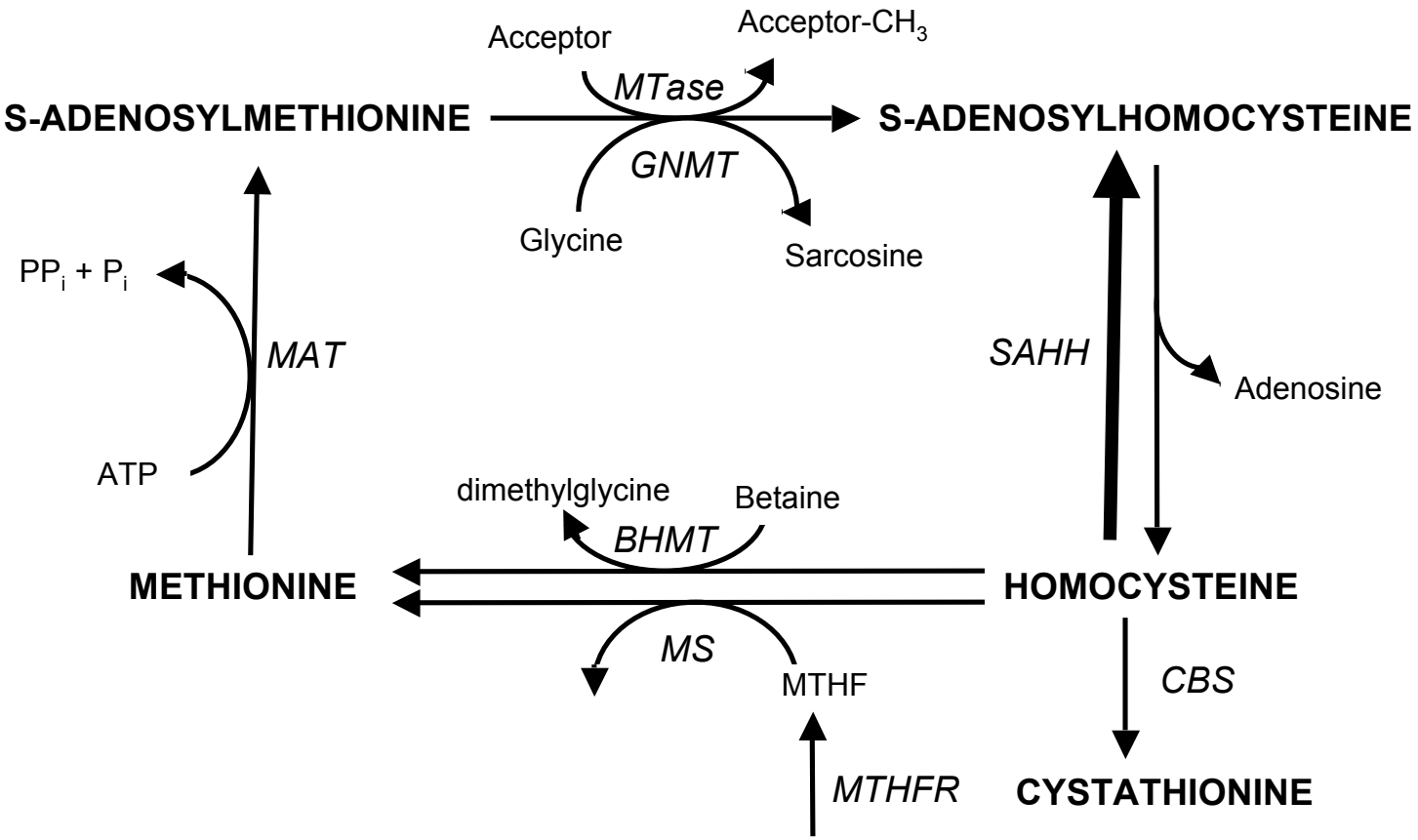

Acceptor
Acceptor-CH3
MTase
S-ADENOSYLMETHIONINE
S-ADENOSYLHOMOCYSTEINE
GNMT
Glycine
Sarcosine
PPi + Pi
MAT
SAHH
Adenosine
ATP
dimethylglycine
Betaine
BHMT
METHIONINE
HOMOCYSTEINE
MS
MTHF
CBS
MTHFR
CYSTATHIONINE

Figure 2

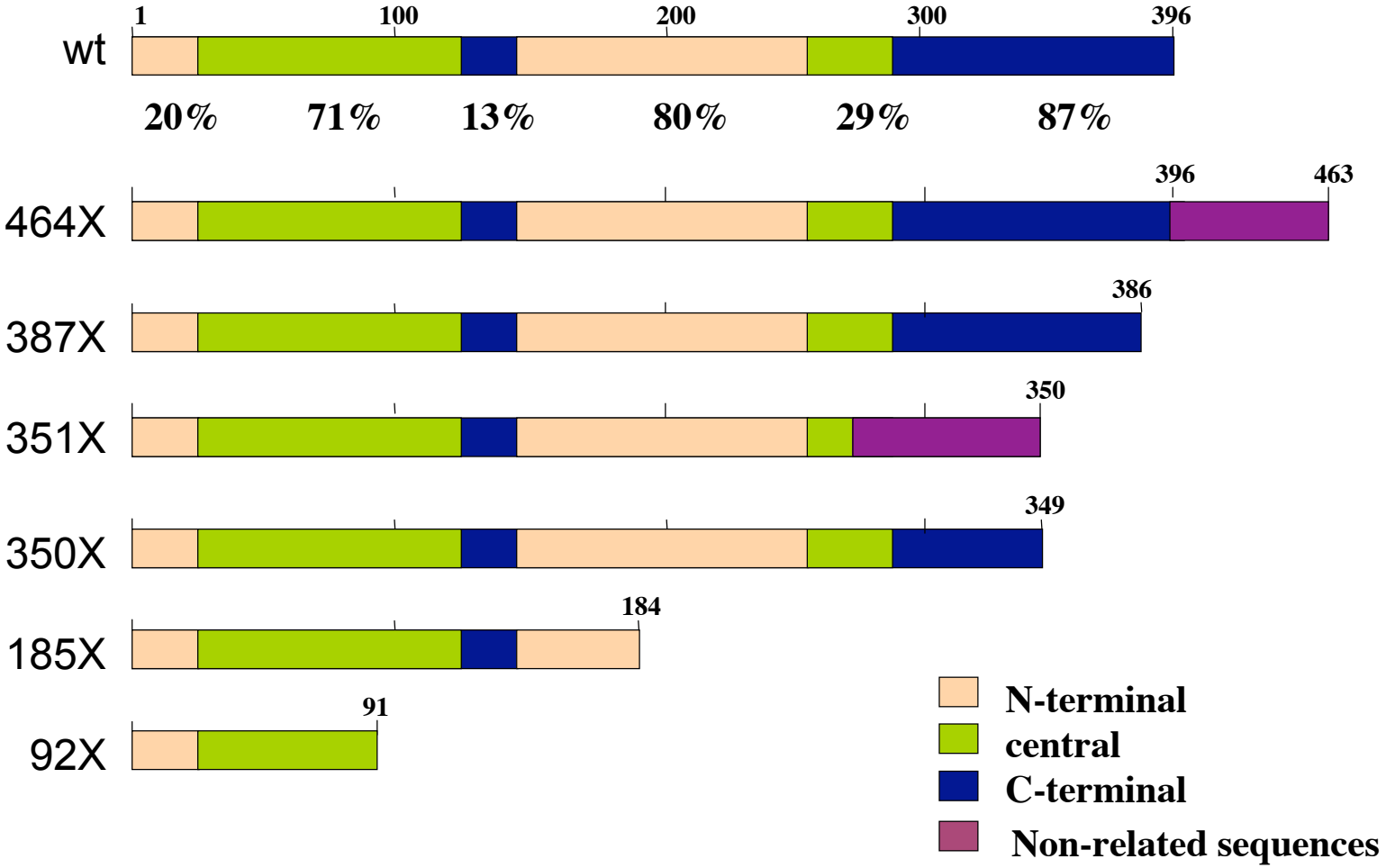
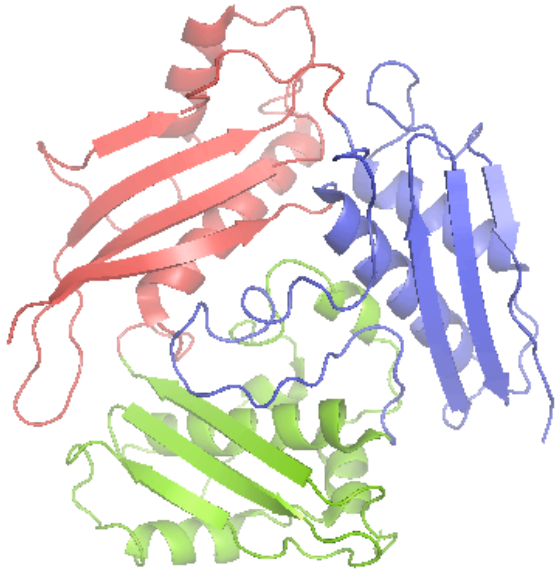
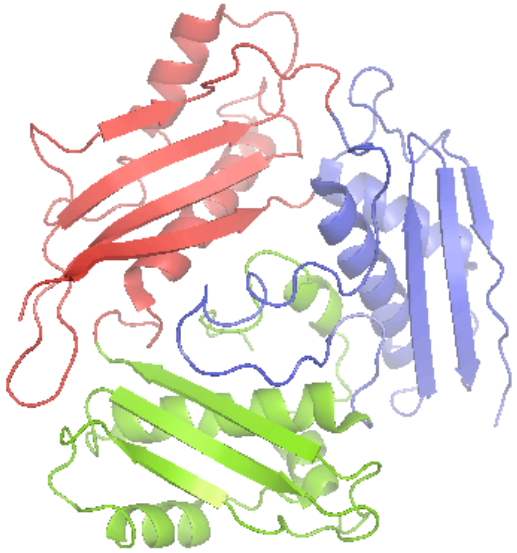

wt
1
100
200
300
396
20%
71%
13%
80%
29%
87%
464X
396
463
387X
386
351X
350
350X
349
185X
184
92X
91
N-terminal
central
C-terminal
Non-related sequences

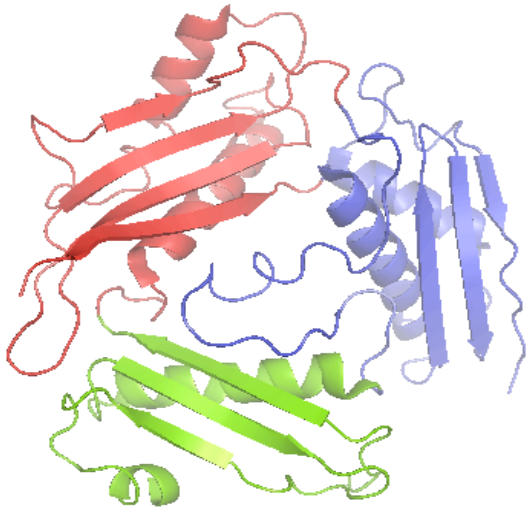
Figure 3



wt



387X



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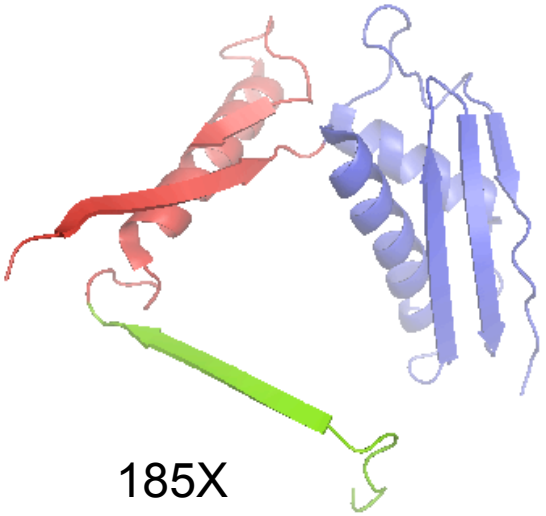

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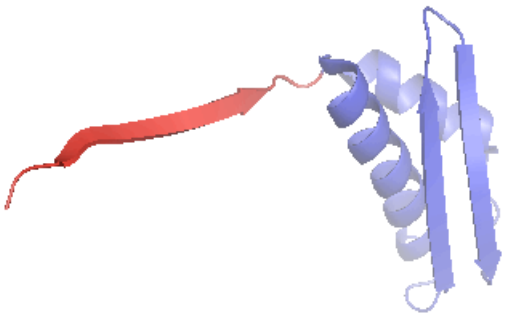


92X

Figure 4

wt ^{251}VIGGPQGDAG ^{261}VTGRKIIVDT ^{271}YGGWGAHGGG ^{281}AFSGKDYTKV

351X ^{251}VIGGPQGDAG ^{261}VTGRKIIVDT ^{271}YGGWGGSWWW ^{281}GLLWEGLHQG

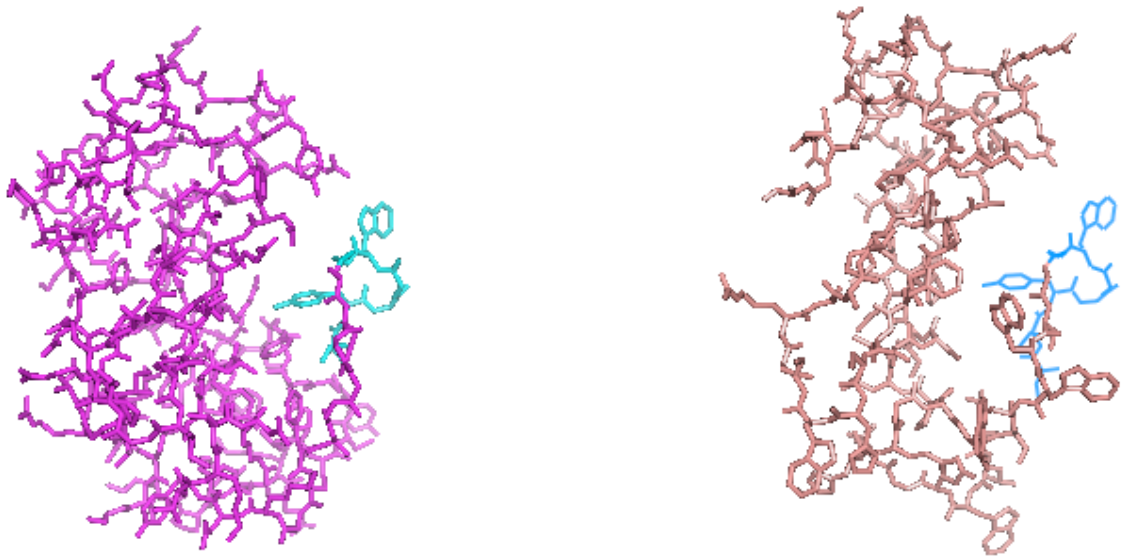
wt ^{291}DRSAAYAARW ^{301}VAKSLVKAGL ^{311}CRRVLVQVSY ^{321}AIGVAEPLSI

351X ^{291}RPLSCICCPL ^{301}GGQVSGESRA ^{311}LPESACPGFL ^{321}CHWCGRAAVH

wt ^{331}SIFTYGTSQK ^{341}TERELLDVVH ^{351}KNFDLRPGVI ^{361}VRDLDLKKPI

351X ^{331}FHLHLRNLSE ^{341}DRARAAGCGA^{350}

wt ^{371}YQKTACYGHF ^{381}GRSEFPWEVP ^{391}RKLVF^{395}



wt **351X**

Figure 5

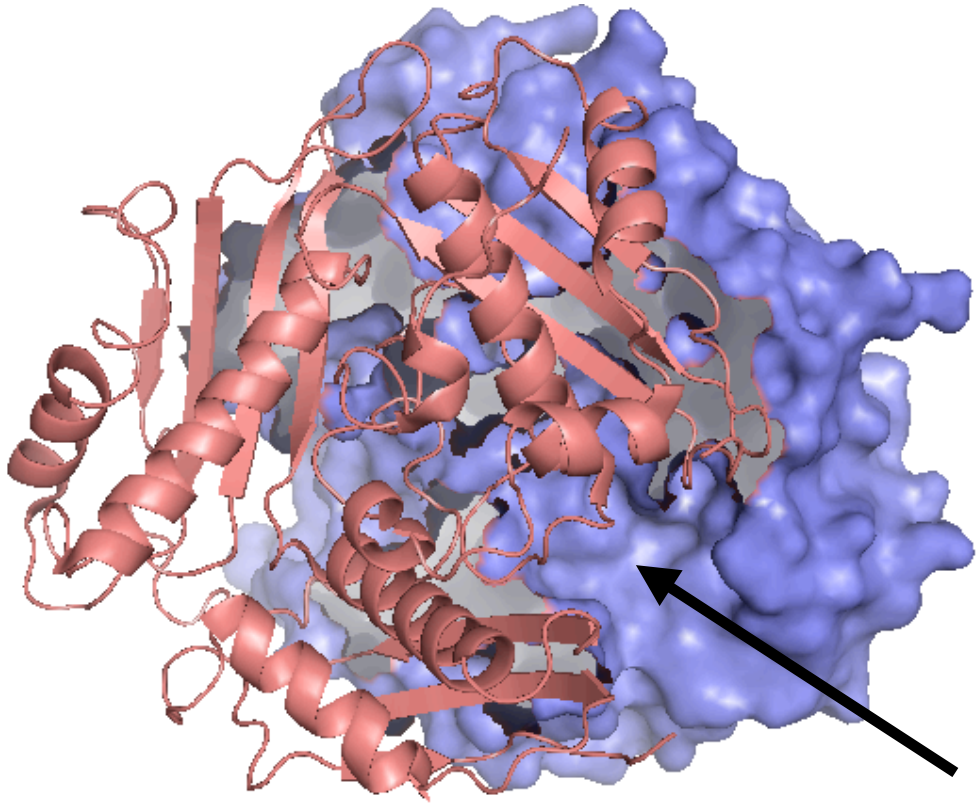


Figure 6

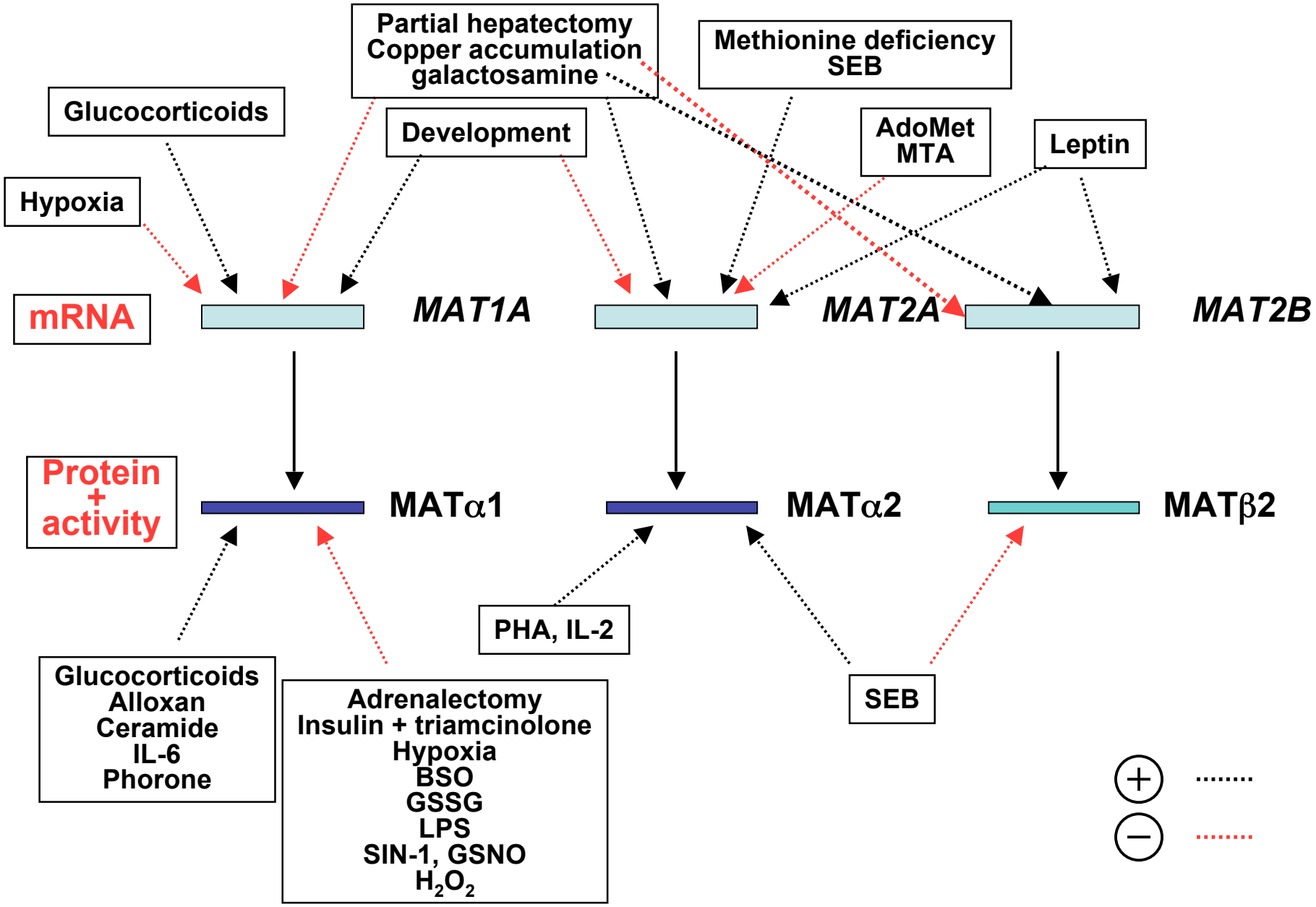

Partial hepatectomy
Copper accumulation
galactosamine
Methionine deficiency
SEB
Glucocorticoids
Development
AdoMet
MTA
Leptin
Hypoxia
mRNA
MAT1A
MAT2A
MAT2B
Protein
+
activity
MATα1
MATα2
MATβ2
PHA, IL-2
Glucocorticoids
Alloxan
Ceramide
IL-6
Phorone
Adrenalectomy
Insulin + triamcinolone
Hypoxia
BSO
GSSG
LPS
SIN-1, GSNO
H_2O_2
SEB

Figure 7