S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions
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A PROTEIN CARBOXYL METHYLTRANSFERASE THAT RECOGNIZES AGE-DAMAGED PEPTIDES AND PROTEINS AND PARTICIPATES IN THEIR REPAIR

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The protein L-isoaspartate (D-aspartate) *O*-MTase (E.C. 2.1.1.77) is an unusual enzyme in that its substrates are isomerized and racemized aspartyl residues generated by spontaneous aging processes in peptides and proteins. Normal L-aspartic acid and L-asparagine residues are susceptible to intramolecular reactions under physiological conditions that lead, via a succinimide intermediate, to the formation of isomerized L-isoaspartate residues (in which the peptide backbone is routed through the side chain β-carboxyl group rather than the main chain α-carboxyl group) and, in smaller quantities, to racemized D-aspartate and D-isoaspartate residues. While the bacterial, plant, and worm enzymes catalyze the AdoMet-dependent methylation of L-isoaspartyl residues (the predominant damaged species), the human and bovine enzymes can also catalyze the methylation of D-aspartate residues, albeit with lower affinities and rates. The affinities of the mammalian enzymes for L-isoaspartyl peptide substrates are five to one hundred-fold greater than the non-mammalian enzymes. The methyl esterification of L-isoaspartate residues leads to spontaneous succinimide formation at a much higher rate than for the unmethylated side chain. Spontaneous hydrolysis of the succinimide regenerates L-isoaspartate residues but also forms normal L-aspartate residues in significant amounts. This combination of enzymatic and nonenzymatic steps can lead to the net conversion of L-isoaspartate residues to L-aspartate residues in good yield, effectively leading to the repair of age-damaged proteins. Recently, gene knockouts of this enzyme have been made in the bacterium *Escherichia coli*, the nematode worm *Caenorhabditis elegans*, and mice. While the effects of the gene knockout were not severe in *E. coli* or *C. elegans*, the MTase-deficient mice were found to accumulate damaged proteins and to die prematurely at an average age of about 6 weeks. These results suggest that different organisms may have distinct strategies for dealing with the problem of the accumulation of damaged proteins in the aging process.

1 Introduction

In the search for MTases that could modulate protein function by catalyzing the reversible methyl esterification of carboxyl groups on proteins, a prominent candidate in the 1970s was an enzyme that utilized a variety of proteins as

substrates [61, 62, 63, 54, 76]. I was fond of referring to this enzyme as the "Grant-Getting MTase" not only because it was present in almost all cell types, but because it was relatively easy to show that it would methylate your favorite protein, opening the possibility that such protein was indeed regulated by a MTase-mediated modification in analogy to the system found to operate upon bacterial chemoreceptor proteins [109, see chapter 6]. However, this "regulatory hypothesis" was severely weakened when the broad specificity of the enzyme was found to result from its recognition of L-isoaspartyl and D-aspartyl residues in polypeptides in a variety of sequence contexts [72, 82, 1]. These are not formed during ribosome-mediated protein synthesis or subsequent enzymatic post-translational modifications, but instead result from the age-dependent spontaneous isomerization of L-aspartyl and L-asparaginyl residues in normal proteins (Fig. 1). Since virtually all proteins are affected by this process, all proteins are likely to have some amount of altered aspartyl residues and thus can be substrates for what was initially a puzzling reaction. When it was realized that the methyl esters were spontaneously hydrolyzed in a pathway leading to the regeneration of normal L-Asp residues, a new paradigm was established that this MTase is involved in the repair of aging proteins [72, 46, 73] (Fig. 2). Thus the general physiological role for this type of MTase more closely resembles that of the DNA repair enzymes, rather than that of the enzymes that catalyze protein phosphorylation reactions or the reversible methylation of protein L-glutamic acid and C-terminal amino acids [19, 20].

In this review, I will focus on recent work that has played a major part in establishing the physiological role of these enzymes. Previous reviews that document the earlier developments in the understanding of this MTase include: Gagnon and Heisler [31], Paik and Kim [96], O'Dea et al. [89], Clarke and O'Connor [21], Billingsley and Lovenberg [8], Clarke [15, 17, 18, 19, 20], Aswad and Johnson [4], van Waarde [112], Aswad et al. [5], Galletti et al. [33, 34], Aswad [2], Barten and O'Dea [6], Johnson and Aswad [44], Ota and Clarke [94], Billingsley [7], Lowenson and Clarke [66].

2 Distribution of the L-isoaspartyl MTase in nature

Genes coding for the L-isoaspartyl MTase have been found in almost all organisms studied [51, 40]. Phylogenetic trees have been constructed recently for a variety of animal, plant, and bacterial enzymes and a rate of amino acid substitutions of 0.39 per 10^9 years has been calculated [51]. This represents a very high degree of sequence conservation, similar to that seen in the lactate dehydrogenase and cytochrome b_5 families, and is only about 77% greater than the substitution rate of the very well conserved cytochrome c family. It appears that this conservation may reflect the structural constraints of maintaining an enzyme that can recognize altered aspartyl residues, but not normal L-Asp residues, in many different species.

Recently, the completion of a number of bacterial genomes has allowed us to look more for the presence of the gene for this enzyme in the eubacterial and archaeal

bacterial worlds [40]. Searches of the open reading frames of both completed and partially completed genomes have identified likely candidates for L-isoaspartyl MTases. Such orthologs have been found in all of the Gram-negative bacteria in the gamma subdivision with the exception of *Haemophilus influenzae*. However, no corresponding sequences were found in the Gram-positive bacteria *Mycoplasma genitalium*, *Mycoplasma pneumoniae* or *Bacillus subtilis*. It is possible that the ability of these organisms to sporulate under starvation conditions may alleviate the problem of the accumulation of altered proteins in stationary phase in non-sporulating cells. Direct analysis of MTase activity in various bacterial extracts confirmed its wide distribution in the gamma subdivision of the Gram-negative species (with the exception of the plant pathogen *Erwinia chrysanthemi*) and its absence in the Gram-positive species *Bacillus subtilis*, *Bacillus stearothermophilus*, *Lactobacillus casei*, and *Streptomyces griseus* [56].

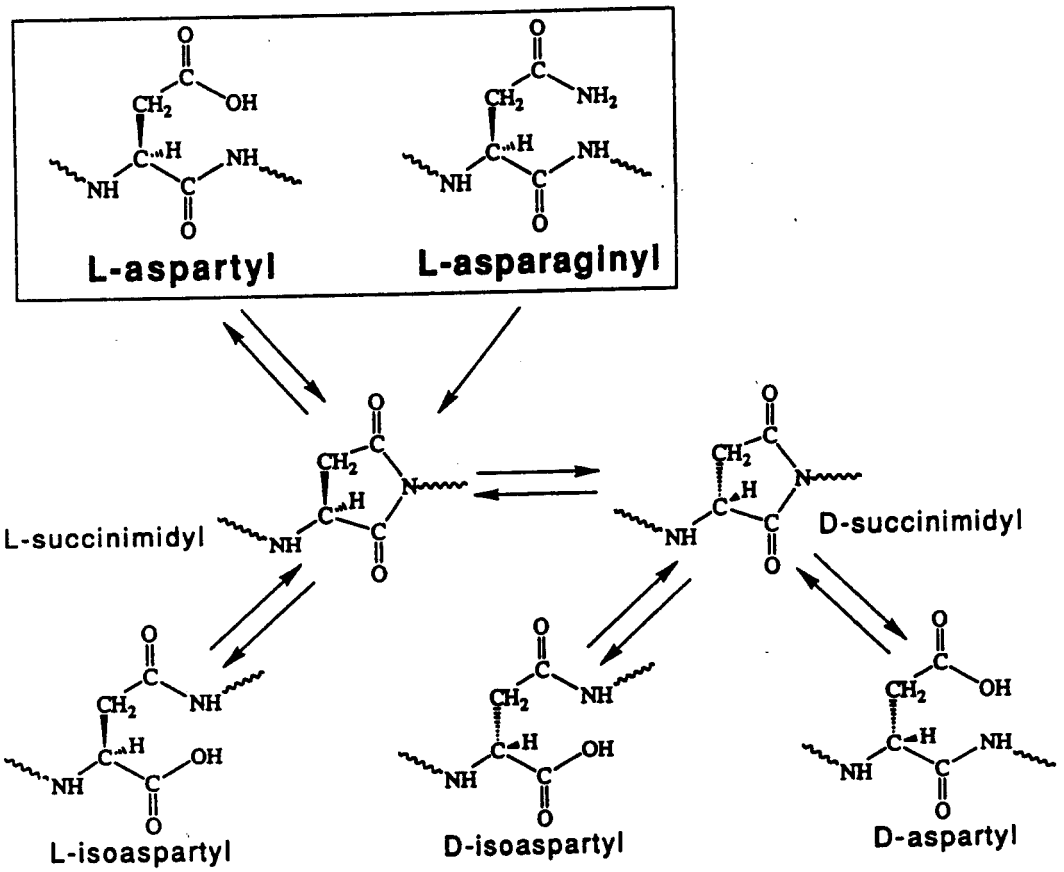

Figure 1. Spontaneous degradation pathways that result in the formation of isomerized and racemized derivatives of L-Asp and L-Asn residues in proteins via succinimidyl intermediates.

Interestingly, the gene for this MTase is also not found in the only eucaryotic organism whose genome has been sequenced to date, the yeast *Saccharomyces cerevisiae* (see below). This yeast is also capable of sporulation. The enzyme has also not been found in a number of non-seed plants such as certain algal species, diatoms, and dinoflagellates, although it is present in other non-seed plants such as mosses and ferns and in almost all seed plants tested [80].

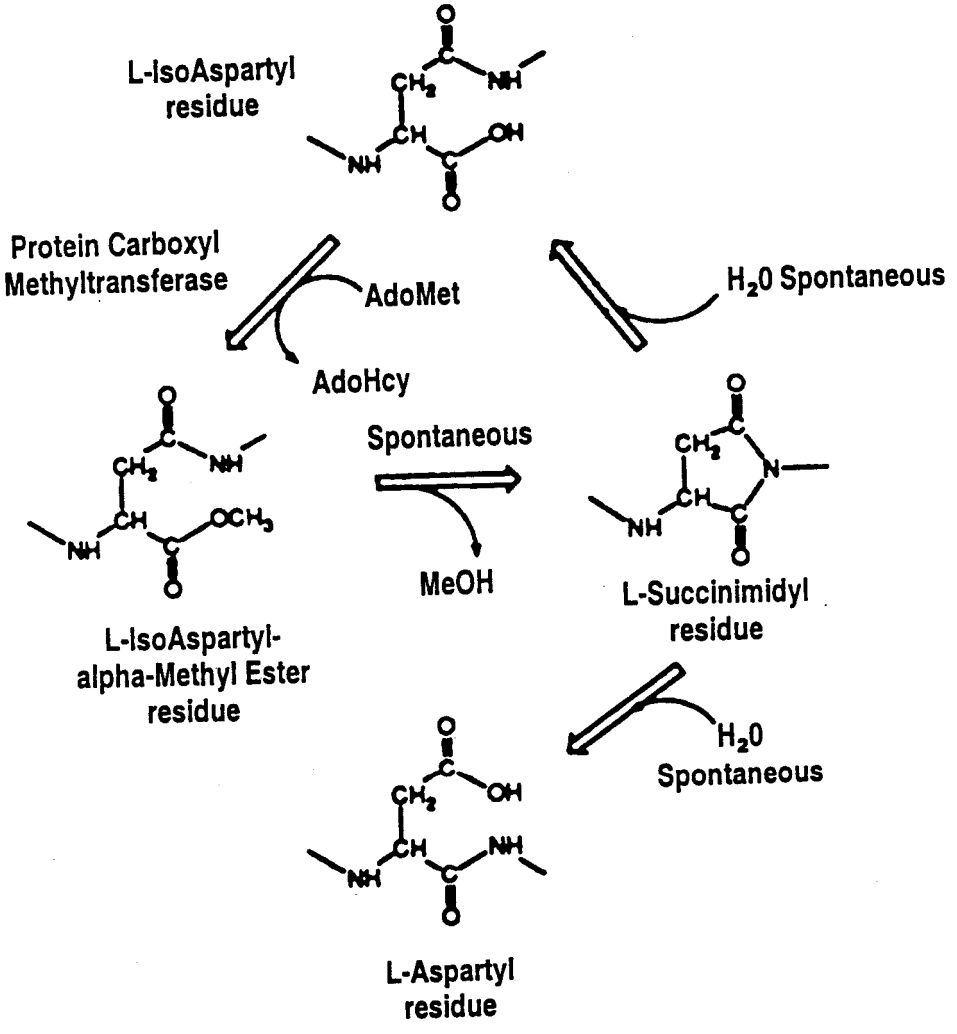

Figure 2. Role of the protein L-isoaspartate (D-aspartate) *O*-MTase in initiating the conversion of L-isoaspartyl to L-aspartyl residues. Enzymatic methylation is followed by spontaneous reactions that convert the methyl ester to a succinimide and then result in its hydrolysis to form either L-Asp or L-isoAsp residues which then re-enter the cycle. A side reaction in this pathway is the racemization of the L-succinimide intermediate to D-succinimide [103] that can result in D-aspartyl and D-isoaspartyl side products. The methylation of D-Asp residues by the mammalian MTase can result in its net conversion to L-Asp and D-isoAsp residues by pathways similar to that shown above and in Fig. 1.

Overall, these results suggest that the protein L-isoaspartyl MTase is one of the "ancient first edition proteins" [28] whose structure and function have been conserved through billions of years of evolution. The identity of amino acids in the human and *Escherichia coli* MTases at 69 out of 224 comparable sites attests to the close-knit relationship of these enzymes [29].

3 Structure of the L-isoaspartyl MTases

3.1 Genes specifying L-isoaspartyl MTases

With one probable exception to date, there appears to be a single gene that codes for this enzyme in any given organism (the exception is the archaeal bacterium *Archaeoglobus fulgidus*, where there appear to be two orthologs of the MTase [40]). The size of the gene varies greatly among bacterial genes without introns (less than 1 kb [29]), a 1.4 kb insect gene with three introns [88], a 1.5 kb plant gene with three introns [79], a 3.2 kb nematode gene with six introns [50], and 26-60 kb mammalian genes with seven introns. The murine gene at 26-31 kb [104, 35, 68] is smaller than the human gene at 60 kb [27]. The sizes of the coding regions of these genes are, however, very similar. The number of amino acids in the encoded gene products (minus the initiator methionine which appears to be generally removed) varies from 207 in *Escherichia coli* to 225 in *Drosophila melanogaster* and *Caenorhabditis elegans* to 226 and 228 in humans to 229 in wheat and *Arabidopsis thaliana* [51]. Most of the orthologs of the enzyme from the recently completed eubacterial and archaal genome projects have coding regions of similar size to the *E. coli* enzyme [40]. However, the enzyme from the hyperthermophilic eubacterium *Thermotoga maritima* is 316 amino acids with the additional C-terminal domain of about 100 amino acids with no similarity to other known protein sequences [40]. The function of this domain is not clear but it may be associated with the thermophilicity of the enzyme (active at temperatures as high as 93 °C), although other putative thermophilic enzymes are of similar sizes as mesophilic species [40].

The human gene has been mapped to the long arm of chromosome 6 at position 6q22.3-6q24, and the mouse gene to a syntenic position on chromosome 10 [69]. The promoters of both of these genes are similar and lack an identifiable TATA box but do contain a CpG island, features characteristic of housekeeping genes with wide tissue distributions [35, 27, see chapter 12]. While it appears that bacterial and animal MTase genes are more or less constitutively active, plant genes appear to be regulated in a more complex manner and involve elements of hormonal, developmental, and environmental controls [78, 79].

3.2 *Diversity of mammalian enzymes due to alternative splicing of transcripts and gene polymorphisms*

In most organisms studied to date, the L-isoaspartyl MTase gene produces a single species of the enzyme. However, in mammalian tissues, multiple isozymes are generated from the single gene. It is now clear that part of this arises from alternative splicing of mRNA transcripts and part arises from the polymorphic nature of the gene, at least in humans.

The initial purification of this enzyme from bovine brain resulted in the separation of two forms by anion-exchange chromatography. Although the polypeptide size and substrate specificity were shown to be identical, the two isoforms differed in their isoelectric point [3]. The more basic isozyme (designated PCM I) has a pI of 6.5, while the more acidic form (PCM II) was shown to separate into two components of pI 5.6 and 5.7. A similar situation was found to occur in bovine and human erythrocytes, although only a single more acidic component was found [95, 38]. Analysis of the structure of the two human erythrocyte isozymes located the differences in the isozymes to the C-terminus [41, 42].

DNA sequences of human brain cDNAs demonstrated that two distinct transcripts were made that differed in the coding region at the C-terminus of the protein. One transcript specified a C-terminal -RWK sequence (corresponding to PCM I) and the other specified a -RDEL sequence (corresponding to PCM II) [70]. The difference between the two transcripts was that a portion of exon 7 was spliced out of the latter transcript so that a DEL termination sequence in exon 8 could be expressed instead of the WK termination sequence in that portion of exon 7 [70]. PCM II from bovine brain was also shown to contain a C-terminal -RDEL sequence [101]. The presence of a -RDEL sequence in the type II enzyme was of considerable interest because secreted proteins with -KDEL or -RDEL C-terminal sequences are marked for retention in the endoplasmic reticulum [99]. Could isozyme II be specifically marked for the endoplasmic reticulum? This does not appear to be the case. In the first place, there is no evidence for a N-terminal signal sequence in these enzymes. Secondly, red blood cells contain no endoplasmic reticulum and both isozymes are cytosolic [95]. Finally, no enrichment of isozyme II was found in the microsomal fraction of rat brain [101]. We are thus left in a quandary to understand why cells would produce two enzymes so nearly alike.

Another source of distinct enzymes, at least in humans, is a major polymorphism that results in the production of MTases that either contain an isoleucine or a valine at position 119 [41, 111]. An initial analysis of genomic DNA from 30 individuals of mixed ethnicity at a site corresponding to amino acid position 119 showed that the ATA codon for Ile was present at a frequency of 0.77 and the GTA codon for Val was present at a frequency of 0.23 [111]. Recently, the presence of the Ile allele has been correlated with enhanced thermostability of the enzyme in red blood cell extracts [25]. These results suggest that human isozymes I and II that contain a valine residue at position 119 might be more labile than the

enzyme containing the isoleucine. It remains to be seen whether the valine-containing enzyme may have other properties that might compensate for this heat lability.

A number of mammalian mRNA transcripts of different sizes (ranging from 1 - 4 kb) have been detected in different tissues that presumably differ in the size of the 3' untranslated region [104, 55, 35, 74, 110]. The physiological relevance of the sizes of these transcripts is not clear at present.

3.3 Enzyme structure and localization

The L-isoaspartyl MTase is a monomer in solution for all enzymes tested to date [3, 15, 38, 29, 50, 67, 24, 40]. As described above, the L-isoaspartyl MTase appears to be localized in the cytosolic fraction of human erythrocytes and brain. One would also expect that this enzyme would also readily enter the nucleus through the nuclear pores [26]. However, there have been a number of studies suggesting that a membrane-bound form of the enzyme is present in mammalian cells [9, 39, 10]. It has been reported that the membrane-bound form could be solubilized with detergents, but not by salt, urea or alkaline solutions [39, 10]. Significantly, Triton X-114 fractionation studies indicated a hydrophilic character for the MTase [39]. Purification of the membrane-bound form showed that its properties were essentially identical to those of the soluble MTase [9]. At this point, it is difficult to understand why a subpopulation of this enzyme might interact with membranes. It has been hard to rule out the possibility that the "membrane-bound" enzyme is simply inside membrane vesicles formed during tissue homogenization. Another possibility is that membrane interaction occurs via an intermediate protein. Recent results indicate that the MTase can form a complex with calmodulin that modulates its activity [87]. Other types of transient complex formation are certainly possible in addition to the enzyme-substrate complexes involved in catalysis.

3.4 Availability of purified enzymes

The L-isoaspartyl MTase has been purified to homogeneity from *E. coli* [29], bovine brain (isozymes I and II) [3], and human and bovine erythrocytes (isozymes I and II) [38]. Large scale preparations of enzyme have been produced in *E. coli* using either the human [67] or the rat [24] cDNA for isozymes II and I, respectively. These latter recombinant enzymes differ from the native species in that they lack an acetyl group on the N-terminal alanine residue. All of these enzymes appear to be monomers in solution.

3.5 Three-dimensional structural studies

Crystals of the human recombinant enzyme that diffract to 2.1 Å and that have one molecule per asymmetric unit have recently been reported [107] but no structure is

as yet available. However, the presence of signature sequence motifs shared by the L-isoaspartyl MTase and a wide variety of protein, DNA, RNA, and small molecule MTases from both procaryotic and eucaryotic organisms [41, 49] suggests that the L-isoaspartyl MTase is likely to have a core structure very similar to those enzymes whose structures have been solved to date and that also include these motifs (see chapter 1). Such enzymes include the mammalian catechol *O*-MTase (chapter 3) and bacterial DNA MTases (chapter 11) [105]. Fig 3 shows an alignment of the human L-isoaspartyl MTase with the rat catechol *O*-MTase [119] that emphasizes the probably structural similarity of these enzymes.

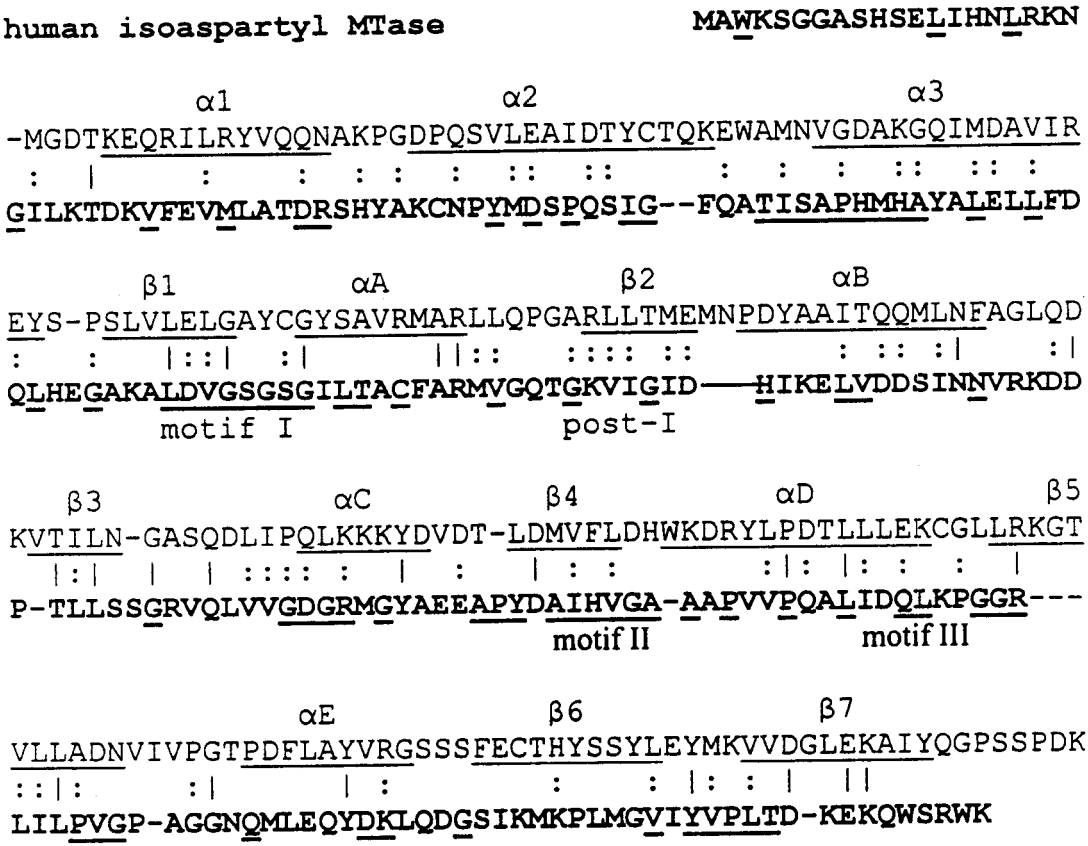

Figure 3. Amino acid sequence alignment of the human L-isoAsp (D-Asp) isozyme I (in boldface type) with the rat COMTase (non-boldface type) [see chapter 3 for the assignment of α helices and β strands (shown in underline) of COMTase]. Identical (|) or chemically-similar (:) amino acids are indicated. The alignment is based on the similarity of the four signature motifs (motif I, post-motif I, motif II, and motif III) shared by many MTases [49, 50], as well as the pattern of conserved amino acids (shown in underline) in the eucaryotic L-isoaspartyl MTases [51].

4 Substrate specificity of the MTase

It is now clear that this MTase only recognizes aspartate residues in the L-isoaspartyl and D-aspartyl forms. No methylation of normal L-Asp, D-isoAsp, or any other structural modifications has been detected. It is also clear that the best substrates for the human enzyme, at least in synthetic peptides, contain L-isoAsp, rather than D-Asp residues [64, 65]. No methylation of D-aspartyl forms by non-mammalian enzymes has been detected to date [50, 40].

How can a single enzyme species methylate both L-isoAsp and D-Asp but not recognize L-aspartyl or D-isoaspartyl residues? In Figs. 4 and 5, I show these four structures with a similar orientation of all atoms except the nitrogen atom attached to the α carbon of the (iso)aspartyl derivative. One might imagine that the active site of the MTase would simply require that this nitrogen atom be behind the plane of the structure as shown in Fig. 4. When this nitrogen atom is in front of the plane of the structure as for L-aspartate and D-isoaspartate shown in Fig. 5, the MTase may not bind the polypeptide and/or may not allow the reaction with AdoMet to proceed.

Kinetic analyses of the purified enzymes from *E. coli* [29], *Thermotoga maritima* [40], *C. elegans* [50], wheat [77], rat [24], cows [45], and humans [66] have revealed that while these enzymes have similar affinities for AdoMet, their affinity to peptide and protein substrates differ considerably (Table 1). Since the efficiency of the repair reaction can be inversely proportional to the K_M for the methyl-accepting substrate [64], these results suggest that the human enzyme may be particularly well designed for protein repair. AdoHcy is a potent inhibitor of these enzymes with a K_i measured for the bovine enzyme of 0.08 μM [45].

Table 1. K_M (μM) values for various IsoD MTases (data from [40]).

Source	Ovalbumin	KASA(isoD)LAKY
Human	35	0.52
Thermotoga	326	2.8
E. coli	727	50.6
Wheat	>1500	12.7
C. elegans	>3000	9.1

The generally poor recognition of D-Asp-containing substrates, even by the human enzyme, suggests that their methylation may represent a relatively unimportant side reaction. However, it may be premature to rule out the importance of methylated D-Asp residues in the function of the mammalian enzyme. The exact localization and chemistry of the methyl-acceptor sites have not been determined for most of the protein (rather than peptide) substrates of this enzyme, and it may be that D-Asp-containing proteins would be significant methyl-acceptors *in vivo*. The

methylation of D-Asp, in fact, was the first activity discovered in the analysis of methylated proteins from red blood cells labeled in their intact form using [*methyl*-^{3}H]methionine [72, 22, 86]. It has been estimated that a significant fraction of methyl esters in intact red blood cells are present in the form of D-aspartyl methyl esters [65].

4.1 *Are some proteins more prone to isomerization and racemization than others?*

It seems clear that many aspartyl and asparaginyl residues in proteins are susceptible to succinimide formation leading to isomerization and racemization (one exception is amino acids followed by prolines where succinimide formation cannot occur). With peptides, the half-times of these reactions under physiological conditions (pH 7.4, 37 °C) vary between about 1 and 1000 days [37, 108, 23, 11, 12, 13]. From these studies, the general rules are that Asn residues form succinimides about ten times more rapidly than Asp residues, and that aspartyl or asparaginyl residues followed by a glycine residue are particularly labile. However, in proteins, structural constraints on succinimide formation are imposed [16], and it becomes very difficult to predict for a given site whether it will be particularly susceptible or resistant to succinimide formation. What does appear to be the most important factor is the flexibility of the polypeptide chain in the region of the Asp or Asn residue. For example, in calcium-liganded calmodulin, the major sites of isoaspartyl formation develop at Asp 2 in the flexible N-terminal region and at Asp 80 in the flexible linker between the two domains [91]. However, in the absence of calcium, increased flexibility of the polypeptide chain results in the generation of additional isoaspartyl residues at the calcium binding sites [92, 93, 102].

Recently, a number of proteins have been shown to be excellent substrates for the MTase, including a high-molecular weight brain polypeptide [90], tubulin [83], and synapsin I [98]. These proteins may contain Asp or Asn residues in particularly labile configurations.

4.2 *Alternative sources of L-isoaspartate residues in proteins*

Although it seems clear that the major reactions resulting in L-isoAsp formation in proteins are the spontaneous reactions described above and in Fig. 1, these products might also arise by other processes. One such process is the mischarging of an aspartyl-tRNA with L-aspartate that would lead to coupling of its β carboxyl group rather than the α carboxyl group. Usage of this charged tRNA would then result in the incorporation of L-isoAsp in the newly synthesized protein. The level of such mischarging has been experimentally approached using the *E. coli* aminoacyl synthetase [75]. No mischarging was found under conditions where 1 mischarged tRNA in 10,000 would have been detected. Although it is possible in special instances that such mischarging would occur, it does not appear to be a general

phenomenon.

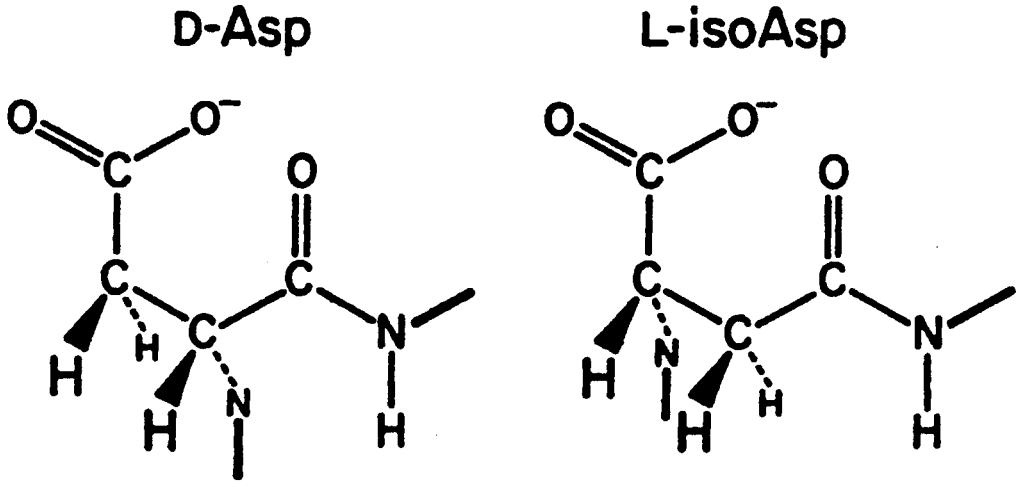

Figure 4. Structure of D-Asp and L-isoAsp residues, substrates of the protein L-isoaspartate (D-aspartate) *O*-MTase. The carboxyl group that accepts the methyl group from AdoMet is shown at the top left of both structures. Note that the α nitrogen atoms are both directed behind the plane of the structure.

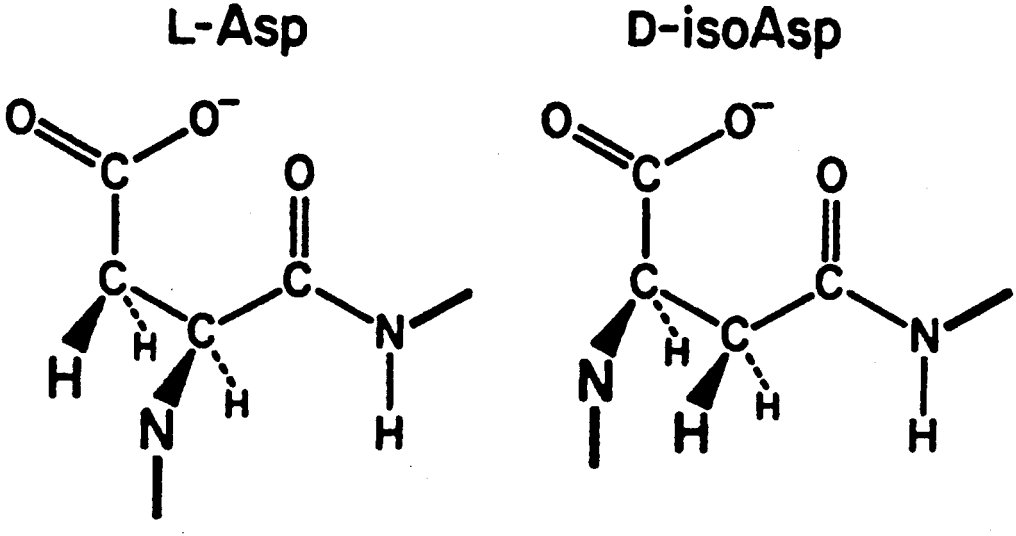

Figure 5. Structure of L-Asp and D-isoAsp residues, which are not substrates of the protein L-isoaspartate (D-aspartate) *O*-MTase. Note that the α nitrogen atoms are both directed in front the plane of the structure, in contrast to the structures shown in Fig. 4.

5 Repair pathways

In several systems, it has been demonstrated that cycles of enzymatic esterification by the MTase, spontaneous demethylation to succinimide derivatives, and hydrolysis to normal and isomerized aspartyl derivatives represent a pathway by which L-isoaspartyl residues can be converted to L-aspartyl residues in peptides [73, 47, 32] and proteins [14]. It has also been shown that the MTase action can initiate the recovery of protein activity [46, 14]. It is important to note that such "repair" is not completely efficient - a major by-product is the species containing the D-isoAsp residue which is apparently not recognized by the MTase [73, 47, 14], and deamidated L-asparagine residue can only be restored to the L-aspartyl form.

5.1 Repair of D-aspartate residues?

Similar repair pathways can be used to convert protein and peptides with racemized D-Asp residues to L-Asp residues [65]. However, the efficiency of these pathways is dependent upon the affinity of the MTase for the D-aspartyl sites. In peptides at least, these affinities are quite low and the conversion to L-Asp residues should also be slow [65]. In fact, computer simulations demonstrate that such "repair" pathways would actually generate more D-isoAsp residues than the number of D-Asp residues they would remove [65]. Unless the D-Asp residue is much more toxic to cells than the D-isoAsp residues it is hard to see how such pathways would be construed as repair pathways. It is interesting that proteins from red blood cell membranes of methylation-deficient patients with uremic diseases actually have lower levels of D-aspartyl derivatives than those of healthy individuals, perhaps reflecting lower levels of D-isoAsp residues resulting from the absence of L-isoaspartyl MTase activity [100]. However, it has been shown that Asp 58 and 151 in aging αA-crystallin from the human eye lens are present as a mixture of L-aspartyl and D-isoaspartyl residues [30]. The absence of D-aspartyl (and L-isoaspartyl) residues from this protein is consistent with the activity of the human L-isoaspartyl MTase to convert both L-isoAsp and D-Asp residues to normal L-Asp (and D-isoAsp) residues.

5.2 Repair of extracellular proteins?

The localization of the L-isoaspartyl MTase to the cytosolic fraction of cells has led to the assumption that enzymatic repair might only occur in this fraction and in the nucleus, where the small size of the MTase may allow it to be taken in through the nuclear pores. This would mean that damaged proteins in internal membrane-bound compartments (mitochondria, endoplasmic reticulum, peroxisomes, lysosomes, etc.) as well as those in the extracellular plasma would either be expected to accumulate or to be metabolized by other processes. This general conclusion has been confirmed by the results seen in mice lacking this MTase, in which the proteins of

the cytosolic fraction, but not plasma proteins, accumulate more damaged proteins than are seen in wild type mice [53]. However, it is certainly possible that the MTase might have access to the proteins in the extracellular environment when cells are lysed. Such a situation has recently been demonstrated in experiments where the MTase is found to be trapped in the extracellular space when rat blood vessels are injured [117] and to catalyze the methylation of aging collagen proteins [118].

5.3 Self-repair of the L-isoaspartyl MTase

It has recently been shown that the repair MTase is itself subject to the formation of D-Asp/L-isoAsp residues and that these sites can also be recognized and methylated by other molecules of the MTase [59, 60].

5.4 Defective repair in human diseases

Recent work has suggested that the activity of the L-isoaspartyl MTase is altered in two human diseases. In the first place, it has been shown that the methylation of red blood cell membrane proteins is increased in individuals with hereditary spherocytosis, where there is some disorganization of the cytoskeleton [43]. These results were interpreted to suggest that the methylation of abnormal Asps on the cytoskeletal proteins bands 2.1 and 4.1 represent a "marker" of membrane damage in spherocytosis, perhaps reflecting either increased accessibility of abnormal Asps or their accelerated formation.

A second model is uremia, where plasma and erythrocyte AdoHcy levels increase to levels that result in the inhibition of MTases, including the L-isoaspartyl MTase [100]. The effect of this inhibition on erythrocyte viability is under investigation.

5.5 A role for repair in proteolysis?

Almost all proteolytic enzymes are specific for normal α peptide linkages of L-amino acids. What happens when proteins containing L-isoAsp (or D-Asp) residues are targeted for degradation? The presence of the isopeptide linkage, or the D-Asp residue, may in fact inhibit normal proteolytic processing and result in the accumulation of abnormal peptides. Isoaspartyl dipeptidases have been described (see [36], for example), but these enzymes may not recognize larger peptide fragments. The ability of the L-isoaspartyl MTase to effectively recognize altered aspartyl residues in peptides [64, 65] and inititate their conversion to normal L-aspartyl containing peptides [73, 46, 32] means that incomplete products of proteolytic digestion may be "repaired" by the MTase system simply so that they can resume their eventual proteolytic processing to free amino acids. The combination of the repair and proteolysis reactions thus insures a smooth removal of altered proteins without peptide by-products. Such a system may also prevent the

formation of distinct peptides resulting from abnormal proteosome cleavage near isomerized or racemized Asp and their display on the cell surface by the major histocompatibility complex [97]. The display of isomerized and racemized peptides could lead to the production of auto antibodies [71].

6 Gene knockout model systems for studying the physiological role of the L-isoaspartyl MTase

6.1 Bacteria

It was initially surprising to find L-isoaspartyl MTase activity in a variety of bacterial species because one would expect that rapid cell division would prevent the accumulation of altered proteins [56]. Outside the laboratory, however, the general condition for most bacterial species is starvation, and periods of rapid growth occur infrequently [113, 84]. During periods of starvation, bacteria must try to preserve their proteins and repair mechanisms might be very important. The apparent simplicity of bacterial metabolism, the large body of both genetic and biochemical data available on these organisms (including the complete genomic sequence of *Escherichia coli* and other species), as well as the facility of making gene specific mutations, has made these organisms important to understanding the physiological role of the L-isoaspartyl MTase.

The isolation of the L-isoaspartyl MTase gene (*pcm*) in *E. coli* [29] allowed the construction of strains where the coding region was either disrupted or deleted to completely eliminate its activity [57]. These strains demonstrated significantly reduced survival in stationary phase where proteins could not be readily replaced, as well as when subjected to heat or osmotic stresses [57]. However, these phenotypes were later called into question when it was found that the construction of these strains resulted in the introduction of a second mutation in the *rpoS* gene, which specifies a stationary phase transcription factor [114]. The *rpoS* gene is only about 1 kb from the *pcm* gene and the P1 phage-mediated transfer of the mutant *pcm* locus to a parent cell background also transferred the mutant *rpoS* gene! This *rpoS* mutation in fact could account for the original phenotypes attributed to the *pcm* mutations [115]. Nevertheless, a careful study of newly made strains with an intact *rpoS* gene did indicate that the absence of the *pcm* gene is detrimental to the survival of stationary phase *E. coli* cells under specific environmental stress conditions [115]. These stresses included incubation in high salt, low concentrations of methanol, or paraquat. This latter agent can result in the continuous generation of destructive oxygen radicals, and all of these treatments can result in protein denaturation. These *pcm* mutants are also more sensitive to repeated cycles of heat stress, another treatment that can result in protein denaturation. These data suggest that cells lacking the L-isoaspartyl MTase are at higher risk under conditions where denaturation might reduce the number of functional protein species.

The *pcm* gene for the L-isoaspartyl MTase in *E. coli* is co-transcribed in an operon with another gene designated *surE* [58]. Since co-transcribed genes in bacteria often have similar functions, we asked whether mutants in *surE* might also be deficient in stationary phase survival, or whether the survival mutants lacking both the *pcm* and *surE* genes might be even more compromised. We found that not only did mutants in *surE* survive as well as wild type cells, but that the *surE* mutation actually suppressed the decreased stress-survival phenotype seen in the *pcm* mutant cells [116]! To understand what kind of an interaction might be occurring here, we measured the accumulation of altered proteins in *pcm* and *surE* single mutants, as well as the double *pcm surE* mutant. Surprisingly, we found that only in the double mutant was there a significant accumulation of altered proteins recognized by the L-isoaspartyl MTase [116]. Both of these results suggested a functional interaction of the *pcm* and *surE* gene products. We are now examining the hypothesis that these products both work in parallel pathways to eliminate altered proteins from cells. The particular sensitivity of *pcm* mutants might be explained by assuming that the *surE* gene product is a protease that recognizes damaged proteins for degradation leading to amino acids. In the *pcm* mutants, the "runaway" activity of such a protease may deplete the cells of protein species that are essential to cells, even if they may contain isomerized Asp residue [116]. To date, only one protease has been characterized in *E. coli* that is specific for L-isoAsp: a dipeptidase [36].

6.2 Yeast

One of the best eucaryotic organisms for biochemistry and genetic study is baker's yeast (*Saccharomyces cerevisiae*). The complete genomic sequence is available and the active import of AdoMet [81] allows radiolabeling of intact cells. Knockout mutants are also readily made. However, these cells (as well as the yeast *Schizosaccharomyces pombe*) apparently lack the gene and activity of the L-isoaspartyl MTase [51]! It now appears that these organisms represent natural L-isoaspartyl MTase "knockout mutants", and it will be very interesting to see how they can avoid the accumulation of damaged proteins in their aging process.

6.3 Worms

One of the most intensively investigated invertebrates is the soil nematode worm *Caenorhabditis elegans*, whose relatively short life span and amenability to genetic analysis have made it a useful model system for studies of biological aging [48]. We have been able to characterize the gene for this enzyme, designated *pcm-1*, as a 3.2 kb species in the central portion of Chromosome V [50]. The gene contains six introns and displays similar intron/exon splice junctions to five of the seven mouse sites.

We have recently utilized Tc1-transposon-mediated mutagenesis to prepare

a strain of worms that lack exons 2-5 on both copies of the chromosomal DNA [52]. These worms had no detectable L-isoaspartyl MTase activity, but display apparently normal morphology and a normal life span. Mutant worms, however, did not survive as well in the dauer stage, a developmental form exhibited by worms when starved for nutrients. We were also able to measure the reproductive fitness of MTase-containing worms and MTase-deficient worms allowed to grow in the same culture. We found that absence of the MTase resulted in their gradual loss from the population [52]. On a biochemical level, the absence of the MTase was found to lead to a relatively small accumulation of altered proteins in aged dauer worms, and this accumulation was not seen when the population was enriched for viable worms [52].

These results clearly show that the presence of the L-isoaspartyl protein repair MTase is not a crucial component of these nematodes in at least their laboratory environment. Perhaps, similar to the situation in bacteria, alternative pathways are present that can also recognize and metabolize proteins containing damaged Asp.

6.4 Flies

The gene for the protein L-isoaspartyl MTase from the fruit fly, *Drosophila melanogaster*, has recently been reported by O'Connor et al. [88]. This gene is present in the region 83AB of chromosome 3 and consists of four exons. A single transcript of 1.4 kb encodes a polypeptide of 226 amino acids that can be expressed in *E. coli* cells. Well developed methods are present for knocking out genes in this organism and it will be very informative to see the phenotype displayed by MTase-deficient flies.

6.5 Amphibians

Although not readily subject to genetic manipulation at this point, the oocytes of the African clawed frog *Xenopus laevis* provide a unique opportunity to study the role of isoaspartyl methylation in intact cells. Here, it is possible to inject oocytes with inhibitors of MTases as well as substrates and to detect chemical changes [85].

6.6 Mice

Techniques are now well developed for generating mice with disruptions in specific genes. We recently used such gene targeting technology to disrupt the *pcmt-1* gene for the L-isoaspartyl MTase [53]. In mice homozygous for the defective *pcmt-1* locus, abnormal proteins were found to accumulate to relatively high levels in a number of tissues. This result is in sharp contrast to what was found in knockout *E. coli* and *C. elegans* systems (see above) and suggests that mice do not have the effective secondary systems seen in worms or bacteria to remove damaged proteins

containing L-isoAsp or D-Asp residues. What is the consequence of this accumulation of altered proteins? The most dramatic phenotype of these mice is that they die from seizures at an average age of only 42 days, while the normal life expectancy is about 700 days. The simplest explanation is that the accumulation of altered proteins in the brain has interfered with its ability to coordinate its overall electrical activity. However, the possibility exists that the MTase might have an additional function in mammalian brain, possibly in the metabolism of an excitatory or inhibitory neurotransmitter (as in the case of catechol O-MTase, see chapter 3).

6.7 Plants

The protein L-isoaspartyl MTase is present in a variety of plant tissues and is especially concentrated in seeds [77, 80]. We have purified and characterized the L-isoaspartyl MTase from wheat [77] and have shown that its gene is under developmental, hormonal, and environmental regulation [78, 79]. The ability of a plant to regulate its L-isoaspartyl MTase activity in its seeds and vegetative tissues in response to desiccation and environmental stress may allow the plant to efficiently repair protein damage associated with these physiological changes. Significantly, we have been able to detect active enzyme in "fossil" seeds over 1000 years old [106].

We have recently focused our efforts on the meadow weed *Arabidopsis thaliana*, which is amenable to genetic manipulation. We are particularly interested in preparing plants lacking this enzyme activity. The gene for this enzyme has now been identified and the regulation of its expression by hormones, desiccation, and salt treatment has been examined [79]. Current studies are aimed at creating transgenic plants with an antisense MTase construct.

7 Future work

The availability of strains of bacteria, worms, and mice that lack L-isoaspartyl MTase activity allow more precise testing of the role of this enzyme in retarding age-related damage to proteins. These model systems will also allow us to ask whether this MTase may have additional roles in biology. It will be very interesting to obtain a three-dimensional structure of the enzyme to help rationalize its affinities for various peptides and proteins containing both D-aspartyl and L-isoaspartyl residues.

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