Ageing Research Reviews
2 (2003) 263–285

ageing
research
reviews

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# Aging as war between chemical and biochemical processes: Protein methylation and the recognition of age-damaged proteins for repair

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Received 24 December 2002; accepted 27 December 2002

**Abstract**

Deamidated, isomerized, and racemized aspartyl and asparaginyl residues represent a significant part of the spontaneous damage to proteins that results from the aging process. The accumulation of these altered residues can lead to the loss of protein function and the consequent loss of cellular function. However, almost all cells in nature contain a methyltransferase that can recognize the major damaged form of the L-isoaspartyl residue, and some of these enzymes can also recognize the racemized D-aspartyl residue. The methyl esterification reaction can initiate the conversion of these altered residues to the normal L-aspartyl form, although there is no evidence yet that the L-asparaginyl form can be regenerated. This enzyme, the protein L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77), thus functions as a protein repair enzyme. The importance of this enzyme in attenuating age-related protein damage can be seen by the phenotypes of organisms where the gene encoding has been disrupted, or where its expression has been augmented.


*Keywords:* Spontaneous protein damage; Isoaspartyl residues; Protein methyltransferases

## 1. Biological aging—chemistry versus biochemistry?

It is clear that almost all measures of physiological function decline in human aging. How can we decipher the molecular basis of this loss of functional capacity with age? Genetics may play a role in programming gene expression for declining function with age. However, a fundamental part of aging may be simply reflected by unwanted chemical

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doi:10.1016/S1568-1637(03)00011-4

processes resulting in the spontaneous appearance of side products of normal metabolism—the formation of mutated, less active, and potentially toxic species of DNA, RNA, proteins, lipids, and small molecules. To the extent that organisms can minimize the accumulation of these altered biomolecules, they can endure.

In a sense then, aging may be seen as a battle between biochemistry and chemistry. Organisms have evolved biochemical systems where just the right DNA sequences encode just the right sequences of RNA and protein, which fold in just the right way to make both catalysts and architectural structures. The catalysts combine speed and specificity to ensure that thermodynamically favorable but kinetically unfavorable reactions occur that lead to metabolic pathways for energy generation, biosynthesis, and signal transduction. Since there are a number of possible chemical reactions with each metabolic intermediate, the provisions of enzymes that catalyze just one of the possible reactions can lead to a rapid and smooth metabolic conversion of reactants to products with few side products (Fig. 1). All of this represents the beauty of biochemistry in making life possible. Why should not such life last forever?

What works against the beauty of biochemistry is chemistry itself. While enzymes can speed up reactions, it is more difficult to slow down reactions. Side reactions still go on, and the more time that elapses, the more unwanted side products are formed. Importantly, these side products are not just small molecules, but all types of biomolecules including nucleic acids and proteins. Almost all of the molecules that make up living systems, from small metabolites to proteins, are not thermodynamically stable (carbon dioxide and water may be the exceptions). Thus, from the moment that biomolecules are synthesized they begin the process of being slowly but surely converted non-enzymatically to decomposition products. These spontaneous chemical reactions (or side reactions) over time thus result in the modification of the biochemical species required for the orderly processes of life

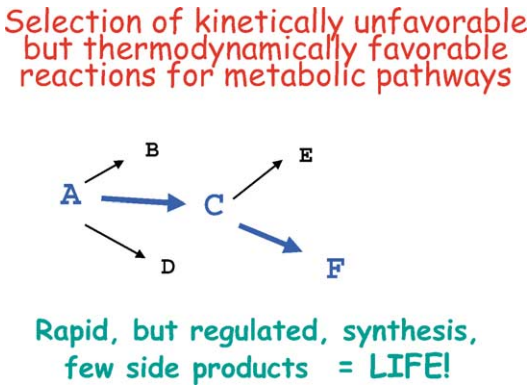


Fig. 1. Efficient enzyme catalysis can reduce, but cannot eliminate the accumulation of potentially toxic side products with age. In this example, a smooth transition of metabolite A → F is insured by enzymes that catalyze the conversion of A → C and C → F (blue pathway). However, it is generally not possible to limit the rate of the spontaneous chemical conversions resulting in the formation of compounds B, D, and E (black arrows). As an organism ages, these potential anti-metabolites would be expected to accumulate unless specific pathways were present to ensure their removal.

described above into less functional species. Perhaps the clearest example of this is the decomposition of DNA, including photochemical alterations, the hydrolytic loss of bases, and oxidative modifications, all of which lead to altered structures and mutation. To combat this, the genome encodes an army of DNA repair enzymes that can efficiently reverse the effects of the spontaneous degradation reactions (Gilchrest and Bohr, 2001).

## 2. Protein damage as a root cause of aging: what can be done?

It has been thought that such repair processes would not occur with proteins, since it is generally possible to utilize degradation pathways to convert a damaged protein to its amino acid constituents and then resynthesize it. While this may be the case in most tissues, it is not the case in red blood cells or eye lens where the biosynthetic capacity is severely limited and cells must survive largely without any new protein synthesis (Takemoto and Boyle, 2000). It is also not the case where it is important to maintain specific covalent modifications on proteins, such as the enzyme-catalyzed phosphorylation events associated with learning and memory in the brain (Chain et al., 1999). Significantly in the last few years, at least three protein repair processes have been identified (Fig. 2). One of these involves a family

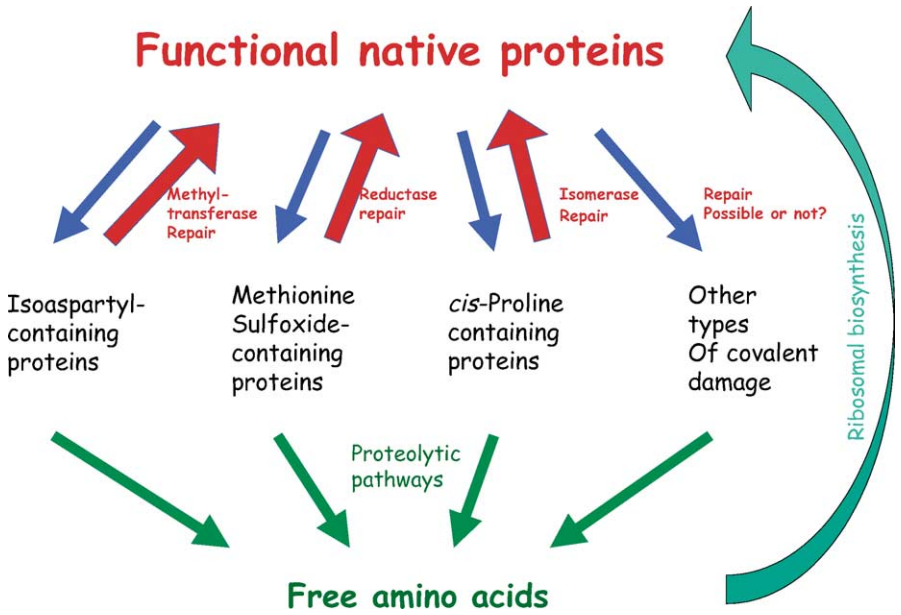


Fig. 2. Pathways of spontaneous degradation, repair, and replacement of aged proteins. Native proteins can be covalently altered by a number of pathways (blue arrows). Enzymes are present that can directly repair some of these types of damage (red arrows), but no repair pathways have been described for many types of damage. Altered proteins can be proteolytically digested to free amino acids (green arrows) and native proteins regenerated by new ribosomal biosynthesis (cyan arrow).

of protein proline isomerases that can convert generally abnormal *cis*-proline residues to *trans*-proline residues (Schiene and Fischer, 2000). A second group of enzymes catalyzes the conversion of oxidatively-modified methionine sulfoxide residues to normal methionine residues (Grimaud et al., 2001; Ruan et al., 2002). The third repair system is the focus of this chapter and involves a methyltransferase that can recognize L-isoaspartyl (and sometimes D-aspartyl) residues for repair.

It should be stressed that for many observed types of spontaneous protein damage no repair systems have been found. At this point, it is unclear whether this reflects the fact that we have not yet discovered these systems, or whether in fact no repair pathways exist for these reactions. However, as mentioned above, proteins containing damaged amino acid residues can all be potentially proteolytically degraded to free amino acids, which can then be used to resynthesize the protein (Fig. 2).

In this review, I summarize recent results on the generation of one type of spontaneous damage to proteins, reactions that result in altered aspartyl and asparaginyl residues. I also review advances in our understanding of the pathways for their metabolism and repair of these proteins. Recent reviews that summarize much of the older literature include Galletti et al. (1995), Visick and Clarke (1995), Clarke (1999), Aswad et al. (2000), and Lindner and Helliger (2001).

## 3. Spontaneous deamidation, isomerization, and racemization of aspartyl and asparaginyl residues

It is now clear that asparagine and aspartyl residues represent hot spots for spontaneous protein degradation under physiological conditions. For both types of residues, nucleophilic attack of the peptide-bond nitrogen atom of the following residue on the side chain carbonyl group results in the formation of a five-membered succinimide ring intermediate (Fig. 3; Geiger and Clarke, 1987). The succinimidyl residue is itself unstable, hydrolyzing with half-times under cellular conditions of hours to give a mixture of aspartyl and isoaspartyl forms. The latter residues represent kinks in the polypeptide chain where the main chain is rerouted through the side chain at the isoaspartyl site. The succinimide is also racemization-prone (Radkiewicz et al., 1996) and generates the D-succinimidyl, D-aspartyl, and D-isoaspartyl forms. Thus, from the original L-aspartyl and L-asparaginyl residues encoded by protein biosynthesis reactions, spontaneous aging results in the formation of at least five altered forms—the D-aspartyl form, the D- and L-isoaspartyl forms, and the D- and L-succinimidyl forms. Of these, the L-isoaspartyl form is generally formed in the largest amount.

Spontaneous direct hydrolysis of asparagine residues by water attack on the side chain amide group can also result in aspartyl residue formation. However, at neutral pH, the rate of this reaction appears to be much less than that of the succinimide pathway. Glutamine and glutamic acid residues are also capable of undergoing similar degradation reactions, but the rates of these reactions are much slower than at those of asparagine and aspartic acid residues. There is no information to date on whether deamidated, isomerized and racemized forms of glutamyl residues can be recognized by enzymes for either repair or proteolytic degradation reactions.

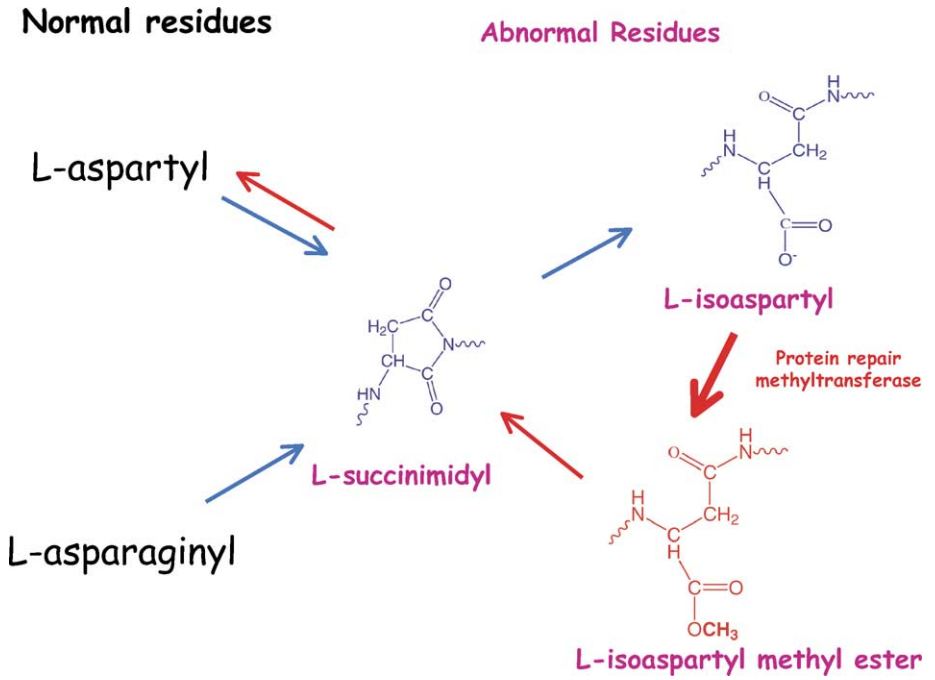


Fig. 3. Pathways of spontaneous chemical degradation of aspartyl and asparaginyl residues in proteins and methyltransferase-mediated repair. Spontaneous degradation reactions of normal L-aspartyl and L-asparaginyl residues lead to the formation of a ring succinimidyl intermediate, which can spontaneously hydrolyze to either give the L-aspartyl residue or the abnormal L-isoaspartyl residue. The L-isoaspartyl residue is specifically recognized by the protein L-isoaspartate (D-aspartate) *O*-methyltransferase which results in the formation of an unstable methyl ester that is rapidly converted back to the L-succinimidyl form. Net repair occurs when the L-succinimidyl residue is hydrolyzed to the L-aspartyl form. Normal protein residues are given in black; altered forms in magenta. Degradation reactions are given in blue arrows; repair pathway reactions are given in red arrows. All of the reactions here are non-enzymatic with the exception of the repair methyltransferase step (heavy red arrow). A side reaction in this pathway (not shown) is the racemization of the L-succinimide intermediate to a D-succinimide residue (Radkiewicz et al., 1996) that can result in D-aspartyl and D-isoaspartyl side products. The methylation of the D-aspartyl residue by the mammalian methyltransferase can result in its net conversion to L-aspartyl and D-isoaspartyl residues by pathways similar to that shown above (Lowenson and Clarke, 1992; adapted from Griffith et al., 2001).

Which aspartyl and asparaginyl residues in proteins are most labile to degradation? It appears that two major factors are at work here. In the first place, the sequence context of the residue, particularly the nature of the following amino acid residue, plays a major role in determining the rate-limiting step of succinimide formation. Secondly, the configuration of the polypeptide chain, particularly in conformations that allow the peptide-bond nitrogen to either approach or not approach the side chain carbonyl carbon atom, also plays a major role.

The effect of sequence context on succinimide formation is now fairly well understood from studies of synthetic peptides where there is much conformational flexibility. It is clear

that the first step is the deprotonation of the attacking peptide-bond nitrogen to form a more nucleophilic anion. Interestingly, for Asp-Pro and Asn-Pro sequences where such succinimide formation cannot occur, an alternate reaction pathway can occur where the side chain can attack the main chain and result in peptide-bond cleavage (Geiger and Clarke, 1987). The acidity of the nitrogen atom depends for most residues on the electron-withdrawing power of the side-chain of the following residue—the more the nitrogen anion is stabilized, the higher its concentration and the higher the rate of succinimide formation (Radkiewicz et al., 2001). The only residue that does not follow this pattern is the glycine residue; asparagine-glycine and aspartyl-glycine sequences are much more labile than would be expected from the stability of the glycine nitrogen anion. This phenomenon has previously been ascribed to steric hindrance effects, but it is now clear that the rate enhancement is due to the increased flexibility of the peptide chain at the glycine residue that allows stabilization of the anion by downstream peptide-bond dipoles (Radkiewicz et al., 2001). The recently measured deamidation rates of 306 pentapeptides of sequence Gly-Xxx-Asn-Yyy-Gly (Robinson and Robinson, 2001) have confirmed the observations made previously on smaller groups of peptides—there is little or no effect of the Xxx residue and the effect of the Yyy residue follows that expected from the results of its side-chain electron-withdrawing ability and the flexibility inherent in the glycine residue. In general, the half-times of aspartyl and asparaginyl peptide degradation under physiological conditions (pH 7.4, 37 °C) vary between about 1 and 1000 days (Brennan and Clarke, 1995). Asparagine residues form succinimides about ten times more rapidly than comparable aspartyl residues (Stephenson and Clarke, 1989).

In proteins, structural constraints on succinimide formation are imposed, and it becomes very difficult to predict for a given site on a protein whether it will be particularly susceptible or resistant to succinimide formation without knowledge of the three-dimensional structure (Clarke, 1987). For the most part, asparagine and aspartyl residues are arranged in three-dimensional protein structures so that succinimide formation is not readily possible. The conformation of the side chain generally puts the carbonyl carbon atom out of reach of the peptide-bond nitrogen, and the peptide-bond nitrogen atom is itself generally pointed away from the side chain (Clarke, 1987). These two effects are probably most responsible for the fact that proteins are as stable as they are. So when can peptide-bond nitrogen atom attack occur on the side chain carbonyl? It appears that the crucial factor is the flexibility of the polypeptide chain in the region of the aspartyl or asparaginyl residue so that the peptide-bond nitrogen can rotate around to attack the side chain carbonyl when it is transiently exposed. Empirical methods have been suggested to predict deamidation rates from an estimation of the flexibility that may occur at any given aspartyl or asparaginyl residue, and these methods appear to be able to predict degradation rates with some accuracy (Robinson, 2002).

It has been of interest to try to examine why some asparagine/aspartyl residues in proteins appear to be particularly labile, and others particularly stable. For example, tubulin (Najbauer et al., 1996), synapsin I (Paranandi and Aswad, 1995), histone H2B (Young et al., 2001), and bacterial ribosomal protein S11 (David et al., 1999) all appear to have residues that are particularly prone to L-isoaspartyl formation based on their reactivity with the protein L-isoaspartyl methyltransferase described below. On the other hand, some residues are particularly stable, such as specific residues in long-lived lens crystallin proteins (Takemoto and Boyle, 2000). It is tempting to speculate that these results depend largely on the

degree of flexibility of the peptide chain at the aspartyl/asparaginyl residue, either allowing peptide-bond nitrogen attack or preventing such attack.

One important result of such considerations is that conditions in the cell that result in increased flexibility of the polypeptide chain should result in enhanced degradation at aspartyl/asparaginyl residues. This has been shown to be the case for simple heat shock, which can result in transient unfolding of proteins (Ladino and O'Connor, 1992). Additionally, photochemical (D'Angelo et al., 2001) or oxidative (Ingrosso et al., 2000, 2002) damage to proteins, conditions that can also result in protein unfolding, also give enhanced rates of formation of isomerized aspartyl residues. A recent study has suggested that the presence of D-isoaspartyl-containing peptides in elastic fibers of skin can be a marker for the ultraviolet light-induced loss of skin function in aging (Fujii et al., 2002). In fact, increases in the methylation of damaged aspartyl forms in hereditary spherocytosis, where the cytoskeleton of the red cells is disrupted, have been ascribed to the additional flexibility of proteins not held properly in place by the cytoskeletal network (Ingrosso et al., 1995).

Recent studies have also reported the effects of aspartyl/asparaginyl spontaneous degradation reactions in age-dependent human diseases. While no correlation of isoaspartyl residues with the scrapie form of the prion protein has been found (Weber et al., 1998; Sandmeier et al., 1999), altered aspartyl residues are found in the τ-protein of paired helical filaments in (Watanabe et al., 1999) and the β-amyloid peptide (Fukuda et al., 1999; Shimizu et al., 2000, 2002) in Alzheimer's disease. In particular, it has been shown that isoaspartate formation at residue 23 of the β-amyloid peptide can increase fibril formation (Shimizu et al., 2002).

Finally, although it seems clear that the major route of L-isoaspartyl formation in proteins are the spontaneous reactions described above, the possibility exists that mischarging of an aspartyl-tRNA with L-aspartate at its β-carboxyl group (rather than its α-carboxyl group) might result in protein L-isoaspartyl formation if the charged tRNA was allowed into the peptidyl reaction center of the ribosome and utilized for peptide-bond formation. Here, damaged proteins would result from their initial synthesis rather than aging reactions. The level of such mischarging has been estimated using the *Escherichia coli* amino acyl synthetase (Momand and Clarke, 1990). No mischarging was found under conditions where 1 mischarged tRNA in 10,000 would have been detected. Thus, it appears that the great majority of L-isoaspartyl residues in proteins do occur through the succinimide pathway and would be expected to accumulate in the aging process.

## 4. Specificity of the protein L-isoaspartate/D-aspartate *O*-methyltransferase for damaged polypeptides

The unusual property of this methyltransferase to recognize damaged aspartyl residues was first shown for the human and bovine enzyme (McFadden and Clarke, 1982; Murray and Clarke, 1984; Aswad, 1984). When it was realized that the methyl esters were spontaneously hydrolyzed in a pathway leading to the regeneration of normal L-aspartyl residues, a new paradigm was established that this methyltransferase is involved in the repair of aging proteins (McFadden and Clarke, 1982; Johnson et al., 1987a,b; McFadden and Clarke, 1987).

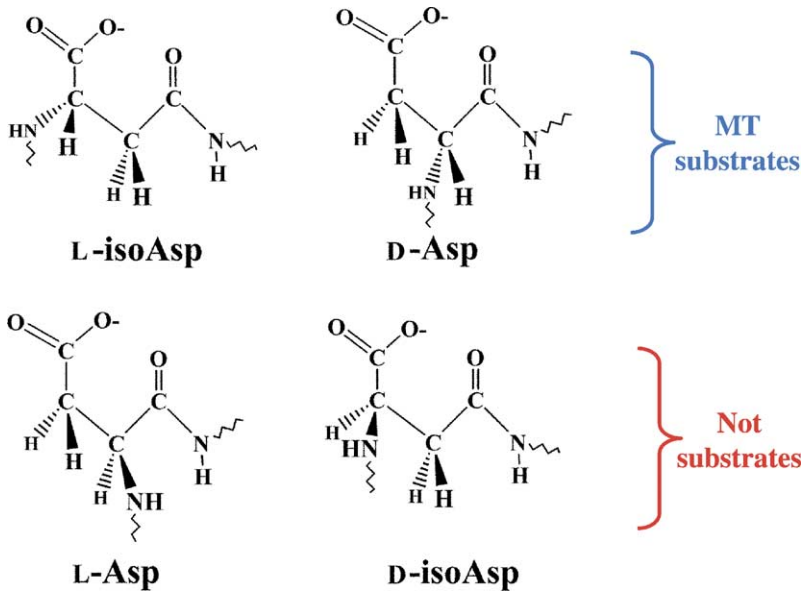


Fig. 4. Top, structure of D-aspartyl and L-isoaspartyl residues, substrates of the protein L-isoaspartate (D-aspartate) *O*-methyltransferase. The carboxyl group that accepts the methyl group from AdoMet is shown at the top left of both structures. Note that the α-nitrogen atoms of these residues are both directed behind the plane of the structure. Bottom, structure of L-aspartyl and D-isoaspartyl residues, which are not substrates of this protein repair enzyme. Note that the α-nitrogen atoms of these residues are both directed in front the plane of the structure.

It is now clear that this enzyme only recognizes aspartyl residues in the L-isoaspartyl and D-aspartyl forms. No methylation of normal L-aspartyl residues, D-isoaspartyl residues, 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-isoaspartyl residues, rather than D-aspartyl residues (Lowenson and Clarke, 1991, 1992).

How can a single enzyme species methylate both L-isoaspartyl and D-aspartyl residues but not recognize L-aspartyl or D-isoaspartyl residues? In Fig. 4, the structures of these four residues are shown 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 methyltransferase would simply require that this nitrogen atom is behind the plane of the structure shown as in the D-aspartyl and L-isoaspartyl residues. When this nitrogen atom is in front of the plane of the structure as for the L-aspartyl and D-isoaspartyl residues shown in Fig. 4, the methyltransferase may not bind the polypeptide and/or may not allow the reaction with AdoMet to proceed. Recently, three-dimensional crystal structures have been obtained for the methyltransferase from the eubacterium *Thermotoga maritima* (Skinner et al., 2000), the archeon *Pyrococcus furiosus* (Griffith et al., 2001), and humans (Ryttersgaard et al., 2002; Smith et al., 2002). Significantly, the *Pyrococcus* enzyme was obtained in a complex with an L-isoaspartyl-containing peptide which has allowed us to understand the details of its specific binding, as well as that modeled for of a corresponding D-aspartyl-containing peptide (Fig. 5; Griffith et al., 2001). These results have confirmed

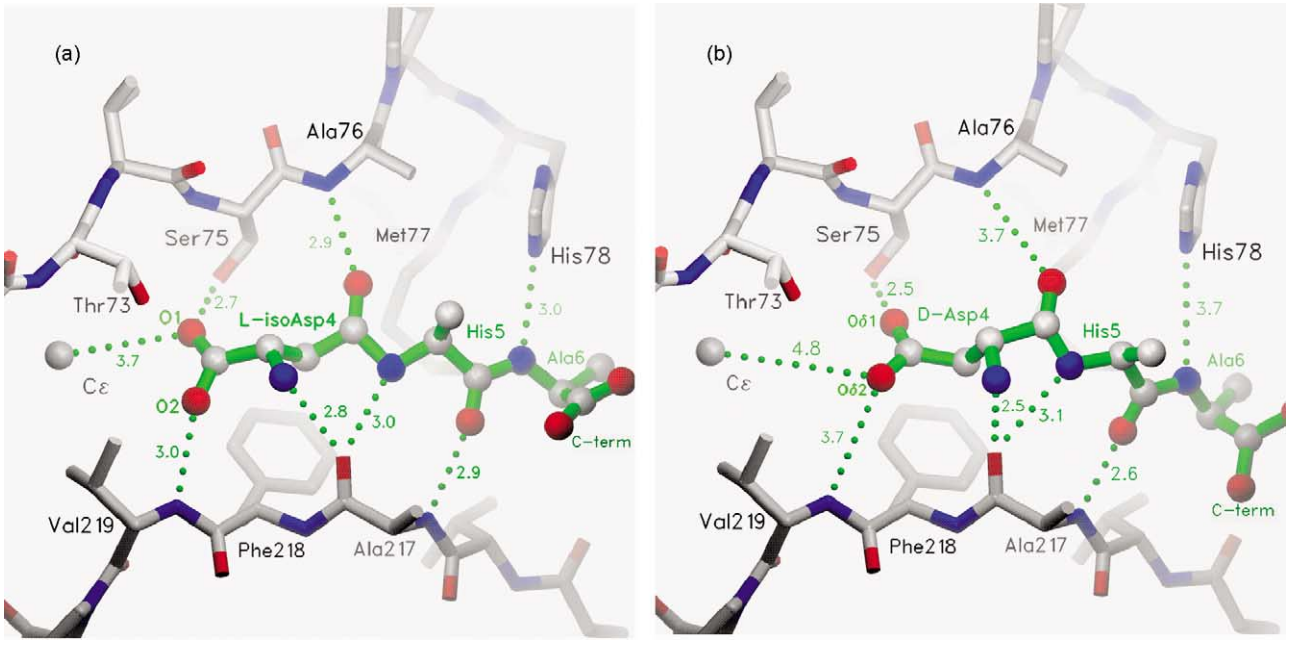


Fig. 5. Specific binding of L-isoaspartyl and D-aspartyl residues to the *Pyrococcus furiosus* protein repair methyltransferase. (a) A portion of the crystal structure of the methyltransferase in complex with the peptide Val-Tyr-Pro-(L-isoAsp)-His-Ala showing the approach of the α-carboxyl group of the isoaspartyl residue to the expected position of the methyl group of *S*-adenosylmethionine (Cε). (b) Calculated binding of the Val-Tyr-Pro-(D-Asp)-His-Ala peptide from a docking program. Note the similar hydrogen bonds formed in each case. It is not possible to accommodate either the D-isoAsp or the L-Asp version of these peptides into the active site due to steric clashes of their amino group with the protein surface (figure from Griffith et al., 2001).

the importance of the role of steric hindrance of the amino group in preventing the binding of the L-aspartyl and D-isoaspartyl forms.

The generally poor recognition of D-aspartyl-containing substrates might indicate that their methylation may represent a relatively unimportant side reaction. However, it is important to note that the best recognition of D-aspartyl residues is found with the human enzyme and the enzyme from the extreme thermophile *P. furiosus* (Thapar et al., 2002). These organisms represent at one end the need to preserve molecules over a period of up to 100 years or more, or under environmental conditions where protein degradation can be very rapid. It should also be stressed that we generally do not know for most in vivo protein substrates of this enzyme whether the methylation site is at a D-aspartyl or at an L-isoaspartyl residue. It may be that D-aspartyl-containing proteins would be significant methyl-acceptors in vivo. The methylation of D-aspartyl residues, in fact, was the first activity discovered in the analysis of methylated proteins from intact red blood cells (McFadden and Clarke, 1982). It has been estimated that a significant fraction of methyl esters in intact red blood cells is present in the form of D-aspartyl methyl esters (Lowenson and Clarke, 1992).

Understanding of the way in which the methyltransferase can recognize both D-aspartyl and L-isoaspartyl residues has suggested that the amino group need not be present on either the α-carbon atom (as in L-isoaspartyl forms) or the β-carbon atom (as in D-aspartyl forms) (Fig. 4). We have thus recently asked whether in fact there may be recognition of peptides where the amino group is replaced entirely by a hydrogen atom, resulting in an *N*-succinyl derivative of a peptide. We found that a variety of *N*-succinyl peptides are in fact substrates for the human and *Pyrococcus* methyltransferases (Thapar et al., 2002). At this point, it is not clear whether this observation has any physiological significance, although one can certainly imagine that enzymatic recognition of proteins spontaneously succinylated from endogenous succinyl-CoA may be a step in the metabolism of these proteins.

## 5. Repair pathways

Cycles of enzymatic esterification by the methyltransferase, 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 (McFadden and Clarke, 1987; Johnson et al., 1987a) and proteins (Brennan et al., 1994) (Fig. 3). It has also been shown that the action of the methyltransferase can initiate the recovery of activity of proteins (Johnson et al., 1987b; Brennan et al., 1994). Such "repair" is not completely efficient—not only are multiple cycles required but a major by-product is the species containing the D-isoaspartyl residue that is not recognized by the methyltransferase and deamidated L-asparagine residues can only be restored to the L-aspartyl form. However, the efficiency of methyltransferase repair in proteins may be better than suggested by the peptide model studies. For example, evidence has recently been provided that for at least one protein succinimide hydrolysis can essentially go completely to the aspartyl rather than the isoaspartyl form (Athmer et al., 2002). In this case, only one cycle of methylation/demethylation would be needed and D-isoaspartyl formation would not be expected to occur.

The efficiency of repair is also dependent upon the affinity of the altered aspartyl residue for the methyltransferase (Lowenson and Clarke, 1991). For the micromolar $K_m$ values seen with many L-isoaspartyl-containing proteins, repair would be expected to work well on the physiological time scale. However, this may not be the case for D-aspartyl-containing proteins, where the affinity can be much less (Thapar et al., 2002). In fact, computer simulations suggest that such "repair" pathways can actually increase the level of D-isoaspartyl residues (Lowenson and Clarke, 1992). It is possible that D-aspartyl residues are more of a problem to cells than D-isoaspartyl residues, where one can argue that they have stereochemical resemblance to L-aspartyl residues (Murray and Clarke, 1984) (Fig. 4). The successful removal of D-aspartyl residues in vivo is attested to by the fact that aspartyl residues 58 and 151 in aging αA-crystallin from the human eye lens are present as a mixture of L-aspartyl and D-isoaspartyl residues (Fujii et al., 1994). The absence of D-aspartyl (and L-isoaspartyl) residues from this protein is consistent with the activity of the human L-isoaspartyl methyltransferase to efficiently convert both L-isoaspartyl and D-aspartyl residues to normal L-aspartyl (and D-isoaspartyl) residues.

The repair methyltransferase is itself subject to the formation of D-aspartyl/L-isoaspartyl residues and these sites can also be recognized and methylated by other molecules of the methyltransferase (Lindquist et al., 1996). It is tempting to speculate that this may reflect one more case where the decline in organismal function with age may be linked to damage of the repair system itself. Nevertheless, it is interesting to note that active methyltransferase has been detected in "fossil" seeds of lotus plants over 1000 years old (Shen-Miller et al., 1995).

The activity of the L-isoaspartyl methyltransferase repair system is altered in at least two human diseases and appears to be affected by the nutritional status of the organism. As mentioned above, in hereditary spherocytosis disorganization of the cytoskeleton may lead to increased damage at aspartyl and asparaginyl residues and increased methylation (Ingrosso et al., 1995). In uremia, erythrocyte *S*-adenosylhomocysteine increases to levels that significantly inhibit the repair methyltransferase (Perna et al., 1997). A similar picture may be present in homocystinuria, where increased *S*-adenosylhomocysteine may also inhibit the L-isoaspartyl methyltransferase (Clarke and Banfield, 2002). Here, elevated plasma total homocysteine levels are associated with the *S*-adenosylhomocysteine-based inhibition of the large group of *S*-adenosylmethionine (SAMe)-dependent methyltransferases, and the L-isoaspartyl methyltransferase is especially sensitive to such inhibition (Clarke and Banfield, 2002). Experimental evidence that this may be the case is suggested by a recent report that folate-deficient rats, which would be expected to have elevated homocysteine and *S*-adenosylhomocysteine levels, demonstrate the accumulation of L-isoaspartyl residues in their liver proteins, as one would expect from the inhibition of this enzyme (Ghandour et al., 2002). These studies suggest the importance of dietary status in maintaining protein repair in the aging process.

## 6. Repair, proteolytic degradation, autoimmune disease, and apoptosis

It is interesting that almost all proteolytic enzymes are specific for normal α-peptide linkages of L-amino acid residues. What happens to proteins containing unrepaired D-aspartyl

and L-isoaspartyl residues, including species located in compartments inaccessible to the cytoplasmic- and nucleoplasmic-localized L-isoaspartyl methyltransferase (Clarke, 1999)? Do the altered peptides accumulate? The facile recognition of the L-isoaspartyl methyltransferase of short peptides (Lowenson and Clarke, 1991, 1992), however, may represent a mechanism to "repair" such peptides so that their proteolytic degradation to free amino acids can continue. 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 proteasome cleavage near isomerized or racemized aspartyl residues and their display on the cell surface by the major histocompatibility complex. Evidence has been presented that peptides containing L-isoaspartyl residues can trigger autoimmune responses. For example, the L-isoaspartyl form of a peptide corresponding to residues 90–104 of cytochrome *c* results in strong B and T cell autoimmune responses in mice, while the peptide with an L-aspartyl residue gives no response (Mamula et al., 1999). A similar picture occurs where some murine T cells can specifically recognize the isoaspartyl form of a hen egg white lysozyme peptide but not the normal asparagine form (Cirrito et al., 2001). Finally, the discovery that histone H2B is not only a major L-isoaspartyl (D-aspartyl) methyltransferase-acceptor protein in the cell but a major antigen in autoimmune diseases such as lupus links protein damage and immune recognition (Young et al., 2001). Overall, it now appears that the failure of an organism to repair L-isoaspartyl (or D-aspartyl) residues can lead to the generation of peptides that are not seen as "self" and auto-immunity.

The possibility that proteins containing altered aspartyl residues might be specifically targeted for degradation rather than, or in addition to, repair has recently been addressed. It has been found that calmodulin damaged by in vitro aging and expected to contain L-isoaspartyl and D-aspartyl residues can be selectively degraded by proteasomes in amphibian oocytes and human cultured cells in a process that does not depend upon ubiquitination (Tarcsa et al., 2000). It is not clear, however, whether such degradation reflects the specific-recognition of isomerized or racemized aspartyl residues or simply the presence of unfolded conformations that may result from the modification. In the absence of repair methyltransferase activity, evidence has been presented for enhanced proteolysis of mouse proteins containing altered aspartyl residues (Lowenson et al., 2001; see discussion below). Additionally, proteolysis and aspartyl residue degradation has been linked in plant systems (Kumar et al., 1999).

A recent paper has now correlated the L-isoaspartyl protein methyltransferase repair system with apoptotic cell death (Huebscher et al., 1999). These workers found that the methyltransferase mRNA formation was induced by the drug CGP3466, which is structurally and functionally related to the anti-Parkinsonian drug *R*-(−)-deprenyl which blocks apoptosis induced by nerve-growth factor deprivation of neuronal cells. They then showed that overexpression of the methyltransferase by transient transfection of the cDNA in mouse primary cortical neurons results in resistance to apoptosis induced by the expression of the *Bax* gene. The biological significance of these findings is not yet clear, but the suggestion has been made that apoptosis may be correlated with increases in *S*-adenosylhomocysteine levels and the inhibition of methyltransferases, as seen in homocystinuria (Huebscher et al., 1999; Kruman et al., 2002).

## 7. What can polymorphisms of the protein repair methyltransferase tell us about human differences in the aging process?

In humans, a single gene on chromosome 6 can encode several forms of the L-isoaspartyl methyltransferase. Alternative splicing results in two forms of the enzyme with C-termini of either Arg-Trp-Lys or Arg-Asp-Glu-Leu sequences. The similarity of the latter sequence to the endoplasmic reticulum retention signal suggested that this enzyme might be located in the lumen of this compartment, but the experimental evidence suggests that both forms are present in the cytosol of cells at approximate equal concentration (MacLaren et al., 1992; Potter et al., 1992). It is unclear why both forms are made.

Enzyme diversity also originates from a human polymorphism that results in the production of methyltransferases that either contain an isoleucine or a valine residue at position 119, at a surface position in a hydrophobic patch on the face of the enzyme opposite to that of the active site (Fig. 6). It was found that the allele frequency was about 45% Ile/55% Val in a sample of 284 Caucasians, 88% Ile/12% Val in a sample of 92 Asians, and 81% Ile/19% Val in a sample of 40 African-Americans (DeVry and Clarke, 1999). When the Ile-119 and Val-119 forms were analyzed in red blood cell extracts, the Ile-form was found to be more thermostable and the Val-form to have a higher apparent affinity for damaged

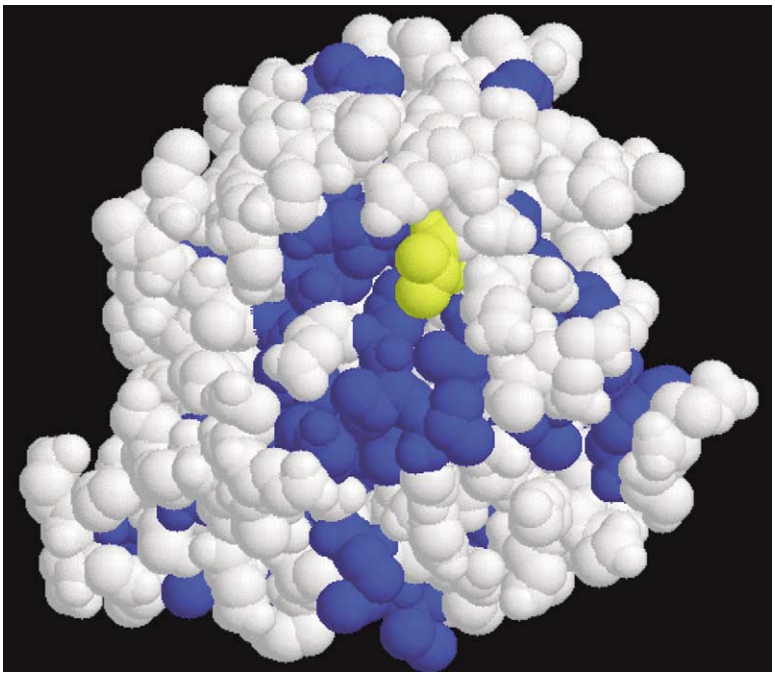

Fig. 6. View of the "back side" of the human L-isoaspartyl methyltransferase showing the location of the valine 119 residue in yellow and other hydrophobic residues (valine, isoleucine, leucine, methionine, phenylalanine, and tryptophan) in blue. The active side of the enzyme is on the other side of the molecule. Visualized using RasMol software and the Protein Data Bank coordinates for accession number 1KR5 (Ryttersgaard et al., 2002).

protein substrates (David et al., 1997; DeVry and Clarke, 1999). The biological rational for the presence of the polymorphic site in a surface hydrophobic patch is not clear. However, yeast two-hybrid analysis has suggested that the mammalian methyltransferase can bind the calcium ion regulatory protein calmodulin, which is known to affect the function of target proteins by binding to hydrophobic patches (O'Connor and O'Connor, 1998).

We then asked whether the presence of one or the other type of enzyme might be associated with successful longevity in humans (DeVry and Clarke, 1999). Because the potential accumulation of damaged proteins may have detrimental effects over the human lifespan, an analysis of a group of "successfully aged" individuals was initiated with the assumption that this population might be enriched in the most beneficial alleles (including those of the PCMT1 methyltransferase) for a long and healthy life. A preliminary study was carried out with 40 individuals of Ashkenazi Jewish descent that met our "successfully aged" old phenotype by being age 75 years or older and in good mental and physical health. The 40 healthy older individuals in this study ranged in age from 75 to 104 years with an average age of 87.2 years, and were compared to a younger control population of the same ethnic background consisting of 40 individuals ranging in age from 21 to 74 years and averaging 36.2 years. We found that the overall allele frequencies were nearly identical between the two groups and very similar to the expected values for the Caucasian ethnic background (Table 1). Surprisingly, however, the distribution of genotypes revealed that within the older population the heterozygous genotype was represented at an unusually high frequency of 65%, given the 46% frequency in ethnically-matched younger controls. While comparison of the two populations directly did not reveal statistical significance at the $P < 0.05$ level ($P = 0.20$), a significant difference was observed when the genotype frequencies in the old population were compared with the population frequencies expected by Hardy–Weinberg equilibrium based on the allele frequencies observed in the younger population ($P = 0.049$). This result suggests the possibility of a heterozygote advantage and that a selection for having both forms of the gene may occur for individuals that have "successfully aged," but further studies will be needed to validate this hypothesis. This putative hybrid vigor may offer a protective advantage for the $Ile_{119}/Val_{119}$ genotype in the repair of protein damage associated with aging.

Table 1
Is there an advantage in successful aging to having the valine or isoleucine polymorphic form of the repair methyltransferase?

| | Healthy young Ashkenazi Jewish (ages 21–74 years, average 38.2 years) | | | Healthy old Ashkenazi Jewish (ages 75–104 years; average 87.2 years) | | |
|---|---|---|---|---|---|---|
| | Ile/Ile | Ile/Val | Val/Val | Ile/Ile | Ile/Val | Val/Val |
| Individuals | 9 | 17 | 11 | 6 | 26 | 8 |
| Allele frequency | 0.473 | | 0.527 | 0.475 | | 0.525 |
| Expected distribution | 0.224 | 0.499 | 0.277 | 0.226 | 0.499 | 0.275 |
| Actual distribution | 0.243 | 0.459* | 0.257 | 0.150 | 0.650* | 0.200 |

Comparison of gene frequencies in a "successfully aged" population with that of an ethnically-matched "young" population (data from DeVry and Clarke, 1999).

* $P < 0.05$ comparing the fraction of heterozygotes in the young and old populations.

## 8. Species differences in methylation and repair pathways in nature

Genes encoding the L-isoaspartyl methyltransferase have been found in almost all organisms studied (Kagan et al., 1997a; Ichikawa and Clarke, 1998). Apparent orthologs of this enzyme have been found to be encoded in the genomes of all of the Gram-negative bacteria in the γ-subdivision with the exception of *Haemophilus influenzae*. However, no corresponding sequences are found in the Gram-positive bacteria *Mycoplasma genitalium*, *Mycoplasma pneumoniae* or *Bacillus subtilis* (Clarke, 1999). 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. Interestingly, the gene for the methyltransferase is also not found in the genome of the yeast *Saccharomyces cerevisiae*. 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 (Mudgett et al., 1997). Overall, the L-isoaspartyl methyltransferase represents an extremely well-conserved protein in the eubacteria, archaea, and eukaryotes (Kagan et al., 1997a).

The number of amino acids in the encoded gene products (minus the initiator methionine which appears to be generally removed) varies from 207 in *E. coli* to 225 in *Drosophila melanogaster* and *Caenorhabditis elegans* to 226 and 228 in humans to 229 in wheat and *Arabidopsis thaliana* (Kagan et al., 1997a). Most of the orthologs of the enzyme from the recently completed eubacterial and archael genome projects have coding regions of similar size to the *E. coli* enzyme (Ichikawa and Clarke, 1998). However, the enzyme from the hyperthermophilic eubacterium *T. maritima* is 316 residues with the additional amino acids in a C-terminal domain of about 100 residues with no similarity to other protein sequences in nature (Skinner et al., 2000; Ichikawa and Clarke, 1998). 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 (Ichikawa and Clarke, 1998; Griffith et al., 2001).

While it appears that bacterial and animal methyltransferase 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 (Mudgett et al., 1997; Thapar et al., 2001).

## 9. What can we learn about the role of protein methylation from the analysis of knockout and overexpressing animals?

Several organisms have now been genetically altered to disrupt the single methyltransferase gene, including the eubacterium *E. coli* (Visick et al., 1998a), the nematode worm *C. elegans* (Kagan et al., 1997b), and the mouse (Kim et al., 1997, 1999; Yamamoto et al., 1998). In each case, disruption of the gene resulted in the complete absence of enzyme activity, suggesting that multiple genes for this enzyme are not present. The natural absence of the methyltransferase in the yeast *S. cerevisiae* may also make this organism a “knockout”

on its own. The methyltransferase has also been overexpressed in the fly *D. melanogaster* (Chavous et al., 2001).

What are the phenotypes of organisms with disruptions in the L-isoaspartyl methyltransferase? For *E. coli* cells, the absence of the *pcm* gene encoding this enzyme results in decreased survival during stationary phase (where damaged proteins would be expected to accumulate), but only under specific environmental stress conditions (Visick et al., 1998a). These stresses include incubation in high salt, low concentrations of methanol, paraquat, or exposure to multiple cycles of heat shock. All of these treatments can result in protein unfolding and the subsequent more rapid generation of damaged aspartyl species (see above). The *pcm* gene is co-transcribed in an operon with the *surE* gene, which thus may have a related function. However, mutants in *surE* survive as well as wild type cells and actually suppress the stress-survival phenotype seen in the *pcm* mutant cells (Visick et al., 1998b). Interestingly, the absence of either functional gene did not lead to the accumulation of damaged protein substrates for the L-isoaspartyl methyltransferase. But a double *pcm–surE*-knockout did give a significant accumulation of altered proteins (Visick et al., 1998b). These results suggest that the methyltransferase may work in concert with the *surE* product to metabolize altered proteins containing L-isoaspartyl residues.

The short-lived soil nematode worm *C. elegans*, where powerful genetic approaches can be used, has provided a good model for aging studies (Johnson et al., 1993). Knockout worms deficient in the L-isoaspartyl methyltransferase display apparently normal morphology and a normal life span (Kagan et al., 1997b). Mutant worms, however, did not survive as well in the dauer stage, a developmental form exhibited by worms when starved for nutrients. The reproductive fitness of methyltransferase-deficient animals was also decreased (Kagan et al., 1997b). Only a relatively small accumulation of altered aspartyl residues was found in the proteins of mutant worms (Niewmierzycka and Clarke, 1999). It is possible that a parallel system, perhaps similar to that described above for bacterial cells, exists that can keep damaged protein levels low even in the absence of the repair methyltransferase.

Probably the best clues to the functioning of the repair methyltransferase in mammals have come from a number of recent studies on mice with a disrupted *pcmt1* gene for this enzyme on chromosome 10. Here, mice were found to be very short-lived, dying of strong seizure activity at an average age of about 42 days (Kim et al., 1997, 1999; Yamamoto et al., 1998). No major pathology has been observed in these young mice. However, abnormal proteins were found to accumulate to relatively high levels in a number of tissues in young knockout mice (Kim et al., 1997, 1999; Yamamoto et al., 1998). 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 to remove damaged proteins containing L-isoaspartyl or D-aspartyl residues seen in worms or bacteria. However, the recent construction of a "rescue" strain where a cDNA for the L-isoaspartyl methyltransferase under the control of a neuron-specific enolase promoter was introduced as a transgene in the *pcmt1*-deficient mice has suggested that additional proteolytic systems do exist in mice (Lowenson et al., 2001). These mice have no expression of the methyltransferase outside the brain. Surprisingly, when the accumulation of damaged substrates was measured, the accumulation leveled off in mice between 50 and 100 days of age (Lowenson et al., 2001). How did these mice manage to stem the accumulation of damaged proteins? Examination of the urine suggested that proteolysis might be responsible since additional

methylatable proteins or peptides were found in the urine. The proteolytic system responsible has not been identified but may be only induced when damaged proteins reach a critical level.

The connection of damaged protein accumulation and age-dependent epilepsy appears to be complex. It appears that a focus of damage is the hippocampus, where neurons are involved in memory and learning (Ikegaya et al., 2001). In humans, there appears to be reduced expression of the L-isoaspartyl methyltransferase in mesial temporal lobe epilepsy, and an accumulation of altered proteins including tubulin (Lanthier et al., 2002). This latter result suggests that damaged protein accumulation may be a causative factor in epilepsy. However, the absence of the L-isoaspartyl methyltransferase in mouse brain can also lead to alterations in the concentrations of *S*-adenosylmethionine and *S*-adenosylhomocysteine (Farrar and Clarke, 2002). Significantly, this latter metabolite appears to have anti-epileptic activity and its lowered level in *pcmt1*-deficient mice may thus contribute directly to the epilepsy phenotype (Farrar and Clarke, 2002).

One of the most exciting recent results is that the overexpression of the L-isoaspartyl methyltransferase in *D. melanogaster* can lead to a marked increase in life span under certain conditions (Chavous et al., 2001). Here, a three- to seven-fold enhancement in activity leads to a dramatic 32–39% increase in life span at 29 °C, a mild heat-stress condition for these flies. The fact that there was no change in life span at 25 °C indicates that the methyltransferase may only be limiting under stressful conditions (Chavous et al., 2001). This situation is similar to that seen in bacterial cells, where methyltransferase-deficient *E. coli* cells are only survival impaired under stress (Visick et al., 1998a).

## 10. What is the fate of damaged proteins in the extracellular space?

The cytosolic localization of the L-isoaspartyl methyltransferase suggests that repair might only occur here and in the nucleus, where the enzyme can enter through the nuclear pores (Clarke, 1999). Thus, proteins containing damaged aspartyl residues 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 accumulation of isomerized and racemized species in intracellular cytosolic proteins, but not in extracellular plasma proteins, in methyltransferase-deficient mice (Kim et al., 1997).

However, methyltransferase would have access to the proteins in the extracellular environment when cells are lysed. This has been shown to occur where trapped methyltransferase is found in the extracellular space when rat blood vessels are injured (Weber and McFadden, 1997a). Furthermore, this enzyme can catalyze the methylation of aging collagen proteins (Weber and McFadden, 1997b). The function of the repair enzyme in the extracellular space would also be expected to require the methyl-donating cofactor *S*-adenosylmethionine. This cofactor is only known to be produced intracellularly, and extracellular levels are generally much lower than the $K_m$ for the L-isoaspartyl methyltransferase. Effective repair of extracellular proteins may thus be dependent upon the local concentration of *S*-adenosylmethionine. It is thus of considerable interest that oral or intravenous injection of *S*-adenosylmethionine

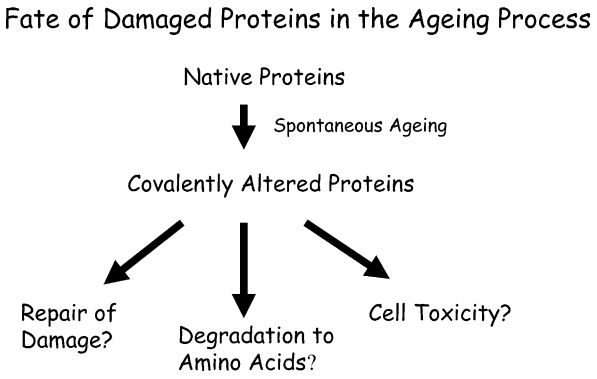


Fig. 7. Interaction of pathways for protein covalent damage, proteolytic degradation, and repair. Cellular failure to repair or degrade altered proteins to free amino acids can result in the age-dependent accumulation of these potentially toxic species.

in humans has a number of beneficial effects, even though it does not appear to enter most cells (Bottiglieri, 2002). These effects include an anti-depressant activity and amelioration of liver disease and osteoarthritis (Bottiglieri, 2002). This latter effect is very interesting because it suggests the possibility that raised *S*-adenosylmethionine levels in the extracellular environment may allow trapped L-isoaspartyl methyltransferase to repair proteins of the collagen/extracellular matrix complex whose covalent damage at aspartyl residues may contribute to osteoarthritis. It is clear that proteins of this complex, including collagen (Brady et al., 1999) and chondroitin sulfate (David et al., 1998), can have high levels of isoaspartyl residues and may represent some of the reason for the age-dependent loss of function of connective tissue in arthritis and other diseases. At a site of tissue injury, methyltransferase may be available but repair may be limited by the lack of *S*-adenosylmethionine. Its supplementation in the diet may allow such repair to occur.

## 11. Conclusion

The protein L-isoaspartyl (D-aspartyl) methyltransferase has been shown to have an important role in limiting the accumulation of damaged proteins that may accelerate the decline of organismal function in the aging process. It now appears that protein repair can be linked to proteolysis; both types of reactions can limit the functional impairment of cells by potentially toxic species (Fig. 7). This methyltransferase thus appears to function as one part of an overall metabolic strategy of organisms to limit the effects of the "bad chemistry" that spontaneously generates side products of the "good biochemistry" that makes life possible. Significantly, recent results have provided evidence that the full expression of the methyltransferase and the full activity of the repair process may be enhanced with a diet that maintains low plasma homocysteine levels. The possibility also exists that nutritional supplementation with *S*-adenosylmethionine may enhance extracellular repair, although there is presently no direct evidence that this is the case.

## Acknowledgements

I am grateful for research support for my laboratory from the National Institutes of Health, including National Institute of Aging Grant AG18000 and National Institute of General Medical Sciences Grant GM26020.

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