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Review

Deamidation and isoaspartate formation in proteins: unwanted alterations or surreptitious signals?

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Abstract. Formation of β-linked Asp-Xaa peptide bonds – isoaspartyl (isoAsp) sites – arise in proteins via succinimide-linked deamidation of asparagine or dehydration of aspartate, reactions which represent a major source of spontaneous protein damage under physiological conditions. Accumulation of atypical isoaspartyl sites is minimized in vivo by the activity of protein L-isoaspartyl *O*-methyltransferase (PIMT), which regenerates a normal peptide bond. Loss of PIMT has harmful consequences, especially in neurons; thus, formation of isoAsp sites and their subsequent correction by PIMT is widely believed to constitute an important pathway of protein damage and repair. Recent evidence is mounting, however, that deamidation and isoaspartate formation may, in some instances, constitute a novel mechanism for intentional modification of protein structure. Herein we describe the mechanism of Asx rearrangement, summarize the evidence that PIMT serves an important repair function, and then focus on emerging evidence that deamidation and isoAsp formation may sometimes have a useful function.

Key words. Isoaspartate; protein-L-isoaspartyl *O*-methyltransferase; *S*-adenosyl-L-methionine; deamidation; protein methylation; peptide bond; protein damage.

A brief history of isoaspartate formation and modification by PIMT

Our current appreciation for the prevalence and importance of isoaspartate formation grew out of studies originally aimed at understanding the specificity and function of a protein carboxyl methyltransferase found to be rich in the brain. This enzyme was first described by Axelrod and Daley in 1965 as a methanol-forming enzyme found in pituitary extracts [1]. In their study, [^{3}H]-methyl labeled *S*-adenosyl-L-methionine (AdoMet) was added to extracts, and the release of radioactive methanol was observed. Over the next 8 years, two other research groups independently discovered the same enzyme activity and demonstrated that methylation was occurring at carboxyl groups presumed to be those found on the side chains of Asp and/or Glu residues [2, 3]. A large number of papers published between 1975 and 1984 attempted to link this ubiquitous protein carboxyl methyltransferase (PCMT) enzyme to a variety of cellular functions, especially those involving stimulus-secretion coupling in neuronal and neuroendocrine cells (reviewed in [4]). The biological significance of this methylation reaction remained unclear, however, due to difficulties in establishing its natural substrates.

In 1984, two simultaneously published studies reported that the free α-carboxyl group at an isoaspartyl (isoAsp) linkage in deamidated adrenocorticotropin (ACTH) was an excellent methylation site for this enzyme, that we now call PIMT (protein L-isoaspartyl *O*-methyltransferase) [5, 6]. An isoaspartyl linkage can arise from a spontaneous intramolecular rearrangement of the peptide bond

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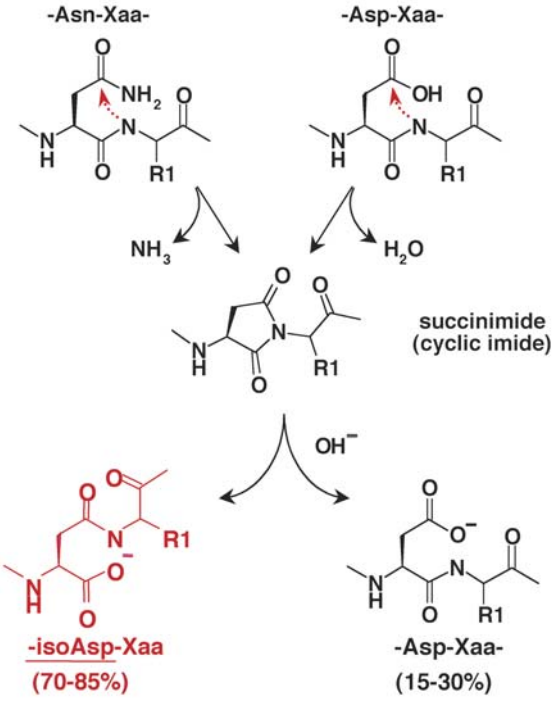

Figure 1. Formation of isoaspartate. Deamidation of asparagine or dehydration of aspartic acid occurs by nucleophilic attack of the α-amino group of the C-flanking amino acid. This leads to formation of a metastable succinimide (cyclic imide) intermediate, which hydrolyzes to a mixture of aspartyl and isoaspartyl linkages.

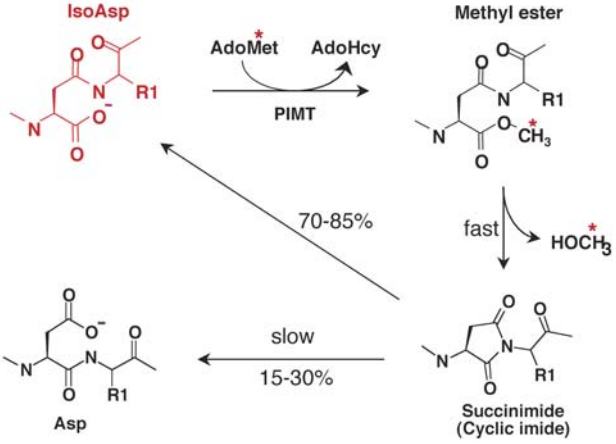

Figure 2. Methylation of isoAsp by PIMT. The free α-carboxyl group generated during isoAsp formation is methylated by PIMT with AdoMet serving as the methyl donor. Enzymatic methylation followed by spontaneous ester hydrolysis leads to reformation of the same cyclic imide intermediate shown in figure 1, which again hydrolyzes to a mixture of Asp and isoAsp. Due to the 70 : 30 isoAsp : Asp product formed by hydrolysis, each cycle of methylation by PIMT results in ~30% conversion of any remaining isoAsp to α-linked aspartic acid. The asterisk (*) indicates the fate of the radiolabel if methyl-labeled AdoMet is used as the methyl donor.

following Asn or Asp residues (fig. 1). When this occurs, the peptide bond becomes linked through the β-carboxyl group of the Asp- or Asn- side chain, leaving free the α-carboxyl group of the original peptide bond. The finding that the α-carboxyl group at an isoAsp site was the long-sought PIMT/PCMT target site helped to explain the instability and low stoichiometry of methylation observed in early research, as the methyl esters formed at isoAsp sites are highly susceptible to cyclization and hydrolysis (fig. 2).

The discovery that the β-linked form of a peptide was the methyltransferase substrate led to hypotheses that methylation might target an isoAsp-containing protein for repair or degradation, or alternatively that it might regulate protein activity [5, 6]. For the last 15 years, the damage/repair hypothesis has gained the most momentum [4, 7], although the degradation and regulation hypotheses have not been conclusively disproven. In fact, several recent studies discussed below support the idea that succinimide formation may represent a novel cellular mechanism for regulating protein localization and function. Further, the identification of significant isoAsp levels in proteins inaccessible to PIMT has raised the additional possibility that isoAsp content may provide a way to regulate protein function by a time-dependent molecular switch, in which the level of isoAsp reflects the time on a molecular clock. This review addresses key issues related to the biological significance of succinimide and isoAsp formation in proteins, as well as the biological function of PIMT.

Formation of the β-linkage

The possibility that certain Asx-Xaa sequences in peptides could form isoAsp was understood before this kind of linkage was known to be a biologically relevant modification and methyltransferase substrate. For example, the presence of a β-aspartyl peptide bond was found in the early 1960s to terminate sequencing by Edman degradation [8]. In addition, it was recognized that peptides containing Asn-Gly sequences can be particularly difficult to synthesize, due to the susceptibility of this site to deamidation and isomerization [9].

The mechanism for isoAsp formation is shown in figure 1. A metastable succinimide/cyclic imide (typical half-life of about 4 h at pH 7.4 and 37 °C) forms following nucleophilic attack of the peptide bond α-amino group on the side-chain carbonyl group, resulting in deamidation of asparagine or dehydration of aspartic acid. This intermediate then hydrolyzes to a mixture of Asp (~ 30%) and isoAsp (~ 70%) residues [10–12]. The isoAsp is typically the predominant product at this step due to the asymmetry of the succinimide structure [13].

The influence of local sequence and solution environment on the rates of succinimide formation has been extensively studied using synthetic peptides (reviewed in [13]). Isomerization associated with deamidation of asparagine typically occurs more rapidly than at aspartic acid residues

within the same sequence [10, 14]. Isoaspartate forms most readily at sequences in which the side chain of the C-flanking amino acid is relatively small and hydrophilic, and is less likely to be formed where bulky or hydrophobic residues are in this position. The most favorable C-flanking amino acids are Gly, Ser and His, leading to so-called isoAsp hot spots at Asn-Gly, Asn-Ser, Asn-His and Asp-Gly sequences. Recently, Robinson and Robinson [15] published a comprehensive study of the deamidation rates for 306 synthetic pentapeptides of the sequence Gly-X_{xx}-Asn-Y_{yy}-Gly. Their results confirmed and extended previous work (reviewed in [13]) showing that deamidation is relatively insensitive to the amino acid in the X position, but is highly sensitive to the amino acid in the Y position, with Gly, Ser and His being the most favorable.

In addition to local amino acid sequence, secondary and tertiary protein structure substantially affect the rate of isomerization (reviewed in [16]). IsoAsp linkages generally occur within flexible protein sequences and are less likely to be found within secondary structures such as α helices or β sheets [17]. In the absence of direct measurements of peptide chain flexibility such as thermal factors obtained by x-ray diffraction, flexibility plots [18] of a sequence may be used to estimate the propensity for a given sequence to generate an isoAsp site. Flexibility plots correctly predict the major sites of isoAsp formation seen, for example, during in vitro aging of calmodulin [19], human growth hormone [20] and tissue plasminogen activator [21].

Robinson and Robinson have recently designed a computational method by which to estimate the probability of deamidation within a folded protein [22]. This method combines the aforementioned data on deamidation rates of 306 synthetic pentapeptides with available three-dimensional (3D) structural information for 23 proteins previously reported to undergo deamidation, to determine a coefficient of deamidation (C_D) for a given Asn residue. After applying their prediction method to 13,335 proteins in the Brookhaven 3D protein database, they concluded that deamidation may be a biologically relevant event in the lifetime of many proteins [23].

IsoAsp accumulation in proteins aged in vitro

A useful way to empirically determine the susceptibility of a purified protein to form isoAsp sites is by in vitro aging at physiological pH and temperature, followed by measurement of isoAsp content [24]. In vitro aging under conditions of physiological pH and temperature has been used to study isoAsp accumulation in numerous proteins [24]. After aging at 37 °C for up to 4 weeks, isoAsp content ranges from approximately zero to nearly 1.5 mol of isoAsp per mole of protein. Some examples of proteins for which in vitro aging yields significant isoAsp include calmodulin [25], tissue plasminogen activor [21], tubulin [26], human growth hormone [20], serine hydroxymethyltransferase [27] and angiogenin [28].

Among all proteins studied to date by this method, the most rapid accumulation of isoAsp is found in synapsin I, a synaptic vesicle-associated protein that is believed to regulate the availability of synaptic vesicles [29]. When aged for 22 days in Hepes buffer at pH 7.4 and 37 °C, isoAsp linkages accumulated at an average rate of ~ 0.06 mol per mole of synapsin per day, reaching a stoichiometry of 1.3 mol per mole after 22 days [30].

Evidence that PIMT mediates protein repair in vitro and in vivo

Isoaspartyl methylation by PIMT is dependent upon donation of a methyl group by AdoMet, which generates *S*-adenosyl-L-homocysteine (AdoHcy) as a by-product (fig. 2). Methylation of isoAsp sites by PIMT facilitates reformation of the cyclic imide intermediate, which then hydrolyzes nonenzymatically to a mixture of Asp and isoAsp products in a ratio of about 30 : 70 [10, 14]. Because of this activity, PIMT has become widely considered to function as a protein repair enzyme, catalyzing conversion of β-linked isopeptide bonds back to the conventional α-linked form [7]. Although this repair is noticeably inefficient given the 30 : 70 mixed product from each cycle, the energy requirement for repair is far lower than for degradation and resynthesis of a new protein. The synthesis of a typical protein requires thousands of ATP molecules; in contrast, 10 cycles of isoAsp methylation and succinimide hydrolysis can result in nearly complete protein repair [31], requiring only 10 ATP equivalents in the form of AdoMet. Thus, only a small percentage of the ATP equivalents required to synthesize a new protein would be required for repair by PIMT.

Repair of isoAsp linkages in vitro has been demonstrated with synthetic isoAsp-containing peptides [31–33] as well as with aged proteins [33–35]. Repair of isoAsp-containing proteins in vitro can restore loss of activity associated with isoAsp formation. For example, repair of age-damaged calmodulin increased its ability to activate the calcium/calmodulin-dependent protein kinase from 18% of normal back to 68% [34]. Repair of deamidated HPr phosphocarrier protein to aspartic acid also led to a partial recovery of function, as assayed by its phosphohydrolysis activity [35].

In vivo studies using cultured cells, genetically altered *Drosophila* and knockout mice also support a physiological role for PIMT as a protein repair enzyme. In rat PC12 cells, the compound adenosine dialdehyde (AdOx), which indirectly inhibits cellular methyltransferase activity by raising levels of AdoHcy (a strong competitive inhibitor of many AdoMet-utilizing methyltransferases), was used to determine whether or not levels of isoAsp in proteins would change in response to PIMT inhibition. The level of

methyl-accepting proteins in PC12 cells was increased to 2.5 times normal following treatment for 3 days with 10 μM AdOx [36]. This effect could be completely reversed in less than a day following removal of AdOx from the culture medium [36]. In *Drosophila*, transgenic overexpression of PIMT led to a 3–7-fold increase in PIMT activity and 32–39% longer lifespan compared with control transgenic flies [37]. This extension was dependent on growth of flies at elevated temperatures, perhaps indicating a special need for PIMT in response to stressful conditions that may accelerate the formation of isoAsp sites.

The generation of PIMT-deficient knockout mice has provided valuable insight into the consequences of uncontrolled isoAsp accumulation in mammals [38]. Analysis of cytosolic proteins from various tissues of knockout mice indicate isoAsp levels 4–8-fold higher than from wild-type mice. A particularly dramatic accumulation of isoAsp-containing proteins was seen in the brain, where the absolute levels of isoAsp were found to be the highest of any tissue in the knockout mice. The major phenotype of these mice includes retarded growth and fatal epileptic seizures occurring at 4–6 weeks of age. Similar deficits have also been observed in independently engineered knockout mice [39].

Treatment of the knockout mice with a combination of the antiepileptic drugs valproic acid and clonazepam delayed onset of seizures and increased average lifespan by 4–5 weeks [40]. Drug treatment with valproic acid alone increased the body weight of knockout but not wild-type animals, thereby equalizing them in size and suggesting that attenuated growth is secondary to a more fundamental neurological problem. Further analysis has indicated that the knockout mice exhibit abnormal electroencephalogram (EEG) activity well before the appearance of seizures.

Histological, behavioral and physiological studies have indicated additional, more subtle abnormalities. The brains of the knockout mice are ~15% larger than those of wild-type mice, despite the overall smaller size of the animal [39]. In addition, neurons develop abnormal dendritic morphology, in association with the formation of irregular microtubule bundles. Hippocampal slices in culture exhibit a lack of long-term potentiation or paired-pulse facilitation at mossy fiber-CA3 synapses, and the animals display impaired spatial memory as determined by a Morris water maze task [41].

The phenotype of the knockout mice suggests a unique vulnerability of the brain to the accumulation of isoAsp-containing proteins. In order to directly address the significance of PIMT activity in the brain, Lowenson et al. selectively inserted a PIMT transgene into neurons of the knockout mice under the control of a neuron-specific enolase promoter [42]. These mice had brain PIMT activity of 6.5–13% compared with wild-type mice, but were of similar size to wild-type and heterozygous mice and lived 5 times longer (median survival 213 days versus 44 days) than the nontransgenic knockout mice. Thus, even fractional enzyme activity in the brain restores a near normal phenotype and significantly delays epilepsy onset, despite dramatic and sustained isoAsp accumulation in other tissues, such as heart, liver and kidney.

The availability of PIMT knockout mice provides a useful means to identify proteins that form isoAsp in vivo. To date, two such proteins have been reported, histone H2B and synapsin I [43, 44]. Histone H2B was initially identified based on its selective accumulation of isoAsp in PC12 cells treated with adenosine dialdehyde [43]. This in vivo accumulation was then confirmed by comparison of isoAsp content in H2B purified from PIMT wild-type and knockout mice. H2B from the knockout mice contained about 80 times the isoAsp content of H2B from wild-type mice (8.1 vs. 0.09 mol percent). Interestingly, H2B is the only core histone in mice that accumulates isoAsp. The isoAsp site in in vitro aged H2B has been localized to an -Asp-Gly- sequence in the flexible N-terminal domain of the histone [W. Carter, K. Reissner and D. Aswad, unpublished observations].

It was mentioned earlier that purified synapsin I rapidly forms isoAsp sites during in vitro aging. Because of this and the fact that the PIMT knockout phenotype may be related to a deficit in synaptic transmission, we investigated the isoAsp levels in knockout and wild-type mice. Synapsin I partially purified from knockout mice was found to contain 0.9 mol of isoAsp per mole of protein, while isoAsp in wild-type synapsin I was below the level of detection. This stoichiometry is 5–7 times greater than the isoAsp content determined for the average protein in a crude homogenate, nuclear, cytosolic or synaptic vesicle preparation and is more than 10 times higher than H2B from the same knockout mice [44]. A similar isoAsp content in synapsin I from PIMT knockout mice has recently been reported by Shimizu et al. [45].

While other isoAsp-containing proteins from these animals remain to be characterized, these two provide a start for the identification of in vivo substrates for PIMT that may contribute to the phenotype of knockout mice. High levels of isoAsp in proteins such as these may contribute to other biological abnormalities not apparent in the knockout mice, due to their early deaths. For example, isoaspartyl peptides have been shown to trigger an autoimmune response [46], and it has been hypothesized that the presence of isoAsp in key proteins may contribute to autoimmune diseases such as lupus erythematosus [43]. In fact, both H2B and synapsin I have been identified as autoantigens in some forms of this disease [47, 48].

A potential role for deamidation and PIMT activity in apoptosis

A possible role for PIMT as a mediator of cell survival has been found in a screen for anti-apoptotic proteins [49].

Differential display reverse transcription-polymerase chain reaction (RT-PCR) was used to identify mammalian genes responsible for the anti-apoptotic effects of the drug CGP3466, a compound related to the anti-Parkinsonian drug deprenyl. Following the addition of CGP3466 to cells in culture, upregulated genes were isolated and sequenced. This approach indicated that PIMT messenger RNA (mRNA) was increased in drug-treated primary astroglial cells in culture by up to eightfold over mock-treated cells. Interestingly, mechanical injury in the absence of drug treatment led to a threefold increase in PIMT expression over noninjured controls, suggesting that independent stresses can lead to recruitment of PIMT transcripts in these cells. Transfection of the pro-apoptotic protein Bax into mouse primary cortical neurons resulted in only 10% cell survival [49]. However, cotransfection of PIMT and Bax into these cells led to a 56–92% protection from apoptosis. This result compared with 65 and 98% survival, respectively, when Bax was cotransfected with anti-apoptotic proteins Bcl-2 and Bcl-x_L. Similar results were observed in transfected COS cells.

The direct route of PIMT-mediated protection from Bax-induced apoptosis remains unclear. However, fluorescence microscopy of growth medium-deprived Chinese hamster ovary (CHO) cells cotransfected with PIMT (wild-type or mutant) and β-tubulin green fluorescent protein indicated that tubulin in cells containing mutant PIMT was relatively unpolymerized and aggregated, compared with tubulin in cells transfected with wild-type PIMT [49]. Tubulin was previously found to accumulate isoAsp both during in vitro aging and in adenosine dialdehyde-treated PC12 cells [26]. Concomitant with isoAsp accumulation, tubulin aged in vitro formed extensive intermolecular cross-links, some of which were disulfide bonds. It was suggested that the observed nonreversible aggregation might arise from lysinoalanine cross-links, or possibly by aspartyllysine cross-links between the ε-amino group of lysine side chains and succinimide intermediates generated during isoAsp formation. It would be interesting to know whether or not the molecular nature of the tubulin aggregation seen in the transfected CHO cells is similar to the aggregation of tubulin observed during in vitro aging.

Aritomi et al. [50] found that recombinant rat Bcl-x_L, an important anti-apoptotic protein, exists as a mixture of native Asn- and deamidated isoAsp-containing forms. Deamidation sites were localized to two candidate Asn-Gly sequences within a disordered region of the protein. These two forms migrate separately in high-percentage SDS-polycrylamide gel electrophoresis (PAGE) gels in a manner also observed on Western blots of Bcl-x_L from rat and human tissue [51, 52], suggesting that deamidation of Bcl-x_L pathway may also occur in vivo. The relative abundance of each band of the doublet observed on Western blots from tissue samples shifts during development [51, 52] and was postulated to be caused by an unidentified posttranslational modification [52]. More recently it has been demonstrated that Bcl-x_L does become deamidated via the isoAsp pathway in vivo, and that the relative levels of the native and deamidated forms are affected in hepatocellular carcinomas [53].

Whether or not deamidation of Bcl-x_L is related to the anti-apoptotic activity of PIMT remains to be determined. Very recently, Deverman et al. reported that deamidation of Bcl-x_L is a determinant for the apoptotic effects of DNA-damaging agents in tumor cells [54]. In their study, treatment of a $p53^-$, Rb null osteosarcoma cell line (SAOS-2) with cisplatin led to a change in the migration pattern of Bcl-x_L similar to that previously mentioned. This altered form was correlated with cell death and could be blocked by induction of the antiapoptotic protein Rb in these cells. This group went on to show that the observed shift in migration was due to deamidation at asparagines 52 and 66, both sites found within the disordered region implicated by Aritomi et al. Transfection of SAOS-2 cells with deamidated constructs blocked the anti-apoptotic effects of Bcl-x_L. Further, fibroblasts devoid of p53 (which consequently cannot activate Rb) were vulnerable to cisplatin by deamidation of Bcl-x_L, as observed in the SAOS-2 cells. These findings indicate that suppression of deamidation may be important in the anti-apoptotic effects of Rb in both tumor cells and fibroblasts.

Caveats to the damage/repair hypothesis

The preceding sections of this review summarize literature that primarily assumes that deamidation and isoasp formation are undesirable changes in protein structure, and that PIMT serves to repair the isoAsp sites in an attempt to restore function. Although there are many data to support this hypothesis, there is one issue that, on the surface at least, does not fit well. This concern centers on the fact that PIMT converts isoAsp sites to Asp sites, regardless of whether they arise via deamidation/isomerization of an Asn or via dehydration/isomerization of an Asp. In the case of Asn deamidation, PIMT restores the normal peptide backbone but not the normal Asn side chain, so the 'repair' of Asn is technically incomplete. This is an important issue because it is widely believed that half or more of the isoAsp sites generated in vivo arise via deamidation of asparaginyl sites. Figure 3 addresses this concern by illustrating three possible outcomes wherein the action of PIMT on isoAsp sites following deamidation might provide a benefit to the cell even though the 'repair' is not always complete.

In this diagram, the isoAsp product of Asx degradation is drawn in brackets to emphasize that the steady-state level of isoAsp-bearing proteins is negligible in cells, except when PIMT activity is inhibited (as in AdOx treatment) or eliminated (as in the knockout mice). Three arrows indi-

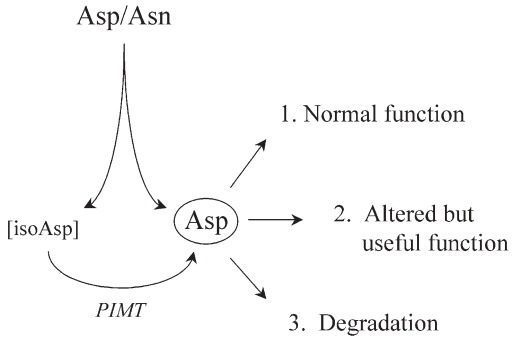

Figure 3. Metabolic fates of labile Asn and Asp sites. This figure combines the information shown in the previous two figures and goes further to indicate three possible ways in which the PIMT-catalyzed conversion of isoAsp to Asp can benefit the cell. Further discussion of this model is provided in the text.

cate three different interpretations of how this conversion might be useful to a cell. The top arrow symbolizes that some isoAsp-to-Asp conversion most likely does constitute complete repair. Certainly this is the case when the isoAsp site arises from an Asp site. It may also apply to some cases of Asn deamidation in which the side-chain alteration in going from Asn to Asp makes little or no difference in the function of the protein.

The second arrow in figure 3 specifically addresses the fate of Asn deamidation. This pathway proposes that an Asn-to-Asp conversion may result in a significant alteration of protein activity or other property, but one that is both useful and intentional. Deamidation of Asn via the succinimide route generates both isoAsp and Asp forms (fig. 1), but the action of PIMT assures that nearly all of the product ends up as Asp, i.e. a deamidation free of other complications. The idea that deamidation might be an intentional and useful event in the life of some proteins originated with the proposal of Robinson and Rudd, who first suggested that deamidation might signal the degradation of aged proteins [55]. More recently, the partial conversion of Asn to Asp at the N-terminus of the catalytic subunit of protein kinase A has been proposed as a mechanism of introducing additional heterogeneity into the protein that influences its subcelluar distribution [56]. This and other proposals regarding intentional deamidation are discussed in greater detail in the second half of this review.

The third arrow in figure 3 suggests that PIMT might sometimes function to assure that proteases can efficiently degrade deamidated proteins destined for disposal. IsoAsp sites are known to interfere with protease recognition [31, 57], and there is no known protease in mammalian cells that specifically degrade isoAsp-Xaa peptide bonds. Indeed, the absence (or low activity) of such an enzyme in mammalian cells is consistent with the observation that isoAsp-bearing proteins accumulate to high levels in PIMT knockout mice [38, 39]. The fact that this accumulation levels off after several months suggests that proteolysis does ultimately limit isoAsp levels. This may explain why organs other than the brain seem to be relatively unaffected by isoAsp accumulation in transgenic mice that express PIMT only in neurons. Incomplete degradation of isoAsp proteins by lysozomal and proteosomal enzymes might explain why isoAsp-Xaa dipeptides have been found in urine [40]. It should also be noted that some carboxypeptidases will cleave on the N-terminal side of isoAsp residues because isoAsp residues contain a free α-carboxyl group, a group normally found only on the C-terminal amino acid of a polypeptide [58].

The remainder of this review explores further the ideas and emerging evidence that spontaneous modification of Asx-Xaa sequences through the succinimide pathway may sometimes provide a subtle but useful mechanism for programming changes into a protein structure.

PIMT-dependent methylation in the regulation of cellular activity: beyond damage and repair

While the hyperaccumulation of isoAsp-containing proteins in PIMT knockout mice leads to abnormal development and premature death, the possibility remains that deamidation and isoAsp formation may play useful and intentional roles in some proteins. Coincident with the discovery that isoAsp sites are enzymatically methylated, it was suggested that interconversion between α and β configurations of the peptide bond might represent a molecular switch for the regulation of protein activity [5, 6]. Although evidence for this type of regulation has yet to be found, there is evidence that passage through the succinimide pathway is a significant event in the normal function of proteins as diverse as the cyclic AMP (cAMP)-dependent protein kinase and proteoglycans in the extracelular matrix of brain.

Protein Kinase A

The catalytic subunit of cAMP-dependent protein kinase (PKA-C) exists as three isozymes (Cα, Cβ and Cγ) which are further diversified by alternative splicing and post-translational modifications. Two of these isozymes, Cα and Cβ, are myristoylated at the amino terminus and also become >25% deamidated at Asn-2 in vivo, within the sequence Gly-Asn-Ala … [59, 60]. Deamidation occurs via the succinimide pathway, rendering a mixture of Asp and isoAsp products [60]. As N-terminal myristoylation is known to be inhibited by the presence of a negatively charged amino acid in the N+2 position, deamidation of Asn-2 following myristyolation of Gly-1 is the only apparent way to generate *myr*-Gly-Asp … in vivo [56].

An interesting and important question is whether or not the generation of these structural subforms has any func-

tional significance. Deamidation of PKA-C at Asn-2 has no apparent effect on enzyme activity nor association with the regulatory subunit or protein kinase inhibitor [56, 59]. However, the native form becomes localized in nuclei at concentrations 1.5–2.3 times higher than the deamidated form. The increased nuclear localization of the Asn-2 form over the Asp-2 form is reflected in an increased phosphorylation of the transcription factor cAMP-responsive element binding protein (CREB) associated with the native form [56] (fig. 4).

The composition of deamidated PKA-C was further characterized by a combination of high-performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry. PKA-C was found to contain four separate species in the relative amounts L-Asp > D-Asp > D-isoAsp > L-isoAsp [61]. The relative amounts of each of these is shown in table 1. Since racemization is greatly accelerated at the succinimide stage, it is not surprising to find D-Asp and D-isoAsp present. Neither D-isoAsp nor D-Asp are effective substrates for PIMT, so they could be expected to accumulate, albeit slowly, as dead-end final products (fig. 5). The discovery of relatively high levels of racemized Asp in a protein of average-to-short half-life presents a compelling argument that this residue was subjected to numerous cycles of PIMT-catalyzed methylation and consequent succinimide formation in vivo.

Table 1. Configuration of Asp-2 in deamidated Cα and Cβ subunits of the cAMP-dependent protein kinase.

Asp-2 form	Percentage of deamidated Cα or Cβ	
	Cα	Cβ
Isoaspartyl	2.9	2.3
L-isoAsp	0.6	
D-isoAsp	2.3	
Aspartyl	97.1	97.7
L-Asp	86.5	
D-Asp	10.6	
Total L forms	87.1	
Total D forms	12.9	

Data are taken with permission from table 3 of Kinzel et al. [61]. The Cα and Cβ isozymes comprise 85 and 15%, respectively, of the C subunit. The N-terminus of Cα is coded as GNAAAAK- and Cβ is coded as GNAATAK-. The enantiomeric subforms of Cβ were not determined.

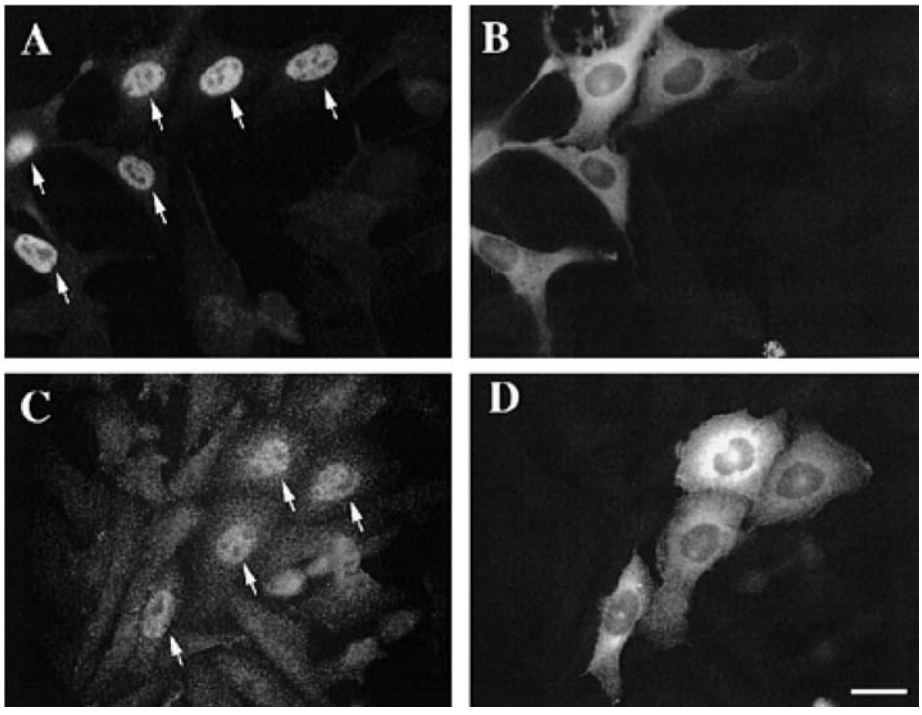

Figure 4. Evidence that deamidation of the catalytic subunit of PKA alters its intracellular distribution in a functionally important way. Native (Asn-2, panels *A* and *B*) and deamidated (mostly Asp-2, panels *C* and *D*) forms of bovine heart PKA-C were microinjected into NIH 3T3 cells along with purified mouse immunoglobulin (Ig)G. After 25 min at 37 °C, the microinjected cells were fixed and immunostained with primary antibodies directed against the phosphorylated form of CREB (cAMP response element binding protein, a nuclear substrate for PKA) or against mouse IgG. Staining for phospho-CREB is shown in *A* and *C* while the staining for IgG (used as a control to identify the injected cells) is shown in *B* and *D*. A comparison of *A* and *C* indicates that the native form of PKA-C is more effective at phosphorylating CREB than is the deamidated form. Separate experiments suggest this is because the native form concentrates more heavily in the nucleus than does the deamidated form. Reproduced from [56] by copyright permission of the Rockefeller University Press.

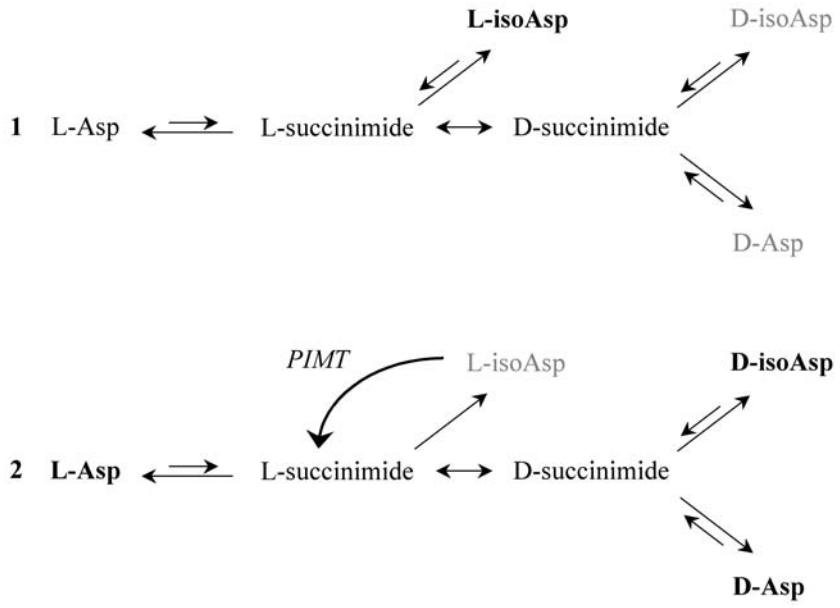

Figure 5. Effect of PIMT on the accumulation of altered forms of aspartate in proteins. Scheme 1 illustrates that succinimide formation from L-Asp (or L-Asn, not shown) generates L-isoAsp sites at a much greater rate than it generates D-Asp or D-isoAsp sites. This is because racemization of the succinimide is very slow compared with its rate of breakdown into the L-isoAsp and L-Asp forms. Scheme 2 illustrates that PIMT should markedly increase the rate of D-Asp and D-isoAsp accumulation because it greatly increases the steady-state concentration of the racemization-prone succinimide at the expense of the L-isoAsp form.

Histones

Histone H1° is a linker histone believed to be important in the packaging of chromatin and in the regulation of cellular proliferation and differentiation [62]. Two main subforms of H1° have been identified in human placenta (H1°a and H1°b), representing N-terminally acetylated and nonacetylated forms, respectively [63]. Each of these two becomes modified by deamidation at Asn3-Ser4, resulting in four chromatographically distinct forms of the same protein. The relative amount of acetylated isoforms and of deamidated isoforms increases steadily with age. Most notably, the deamidated form of H1° increases in both rat liver and brain by 3 and 7.5 times between 10 and 450 days, respectively. Similar increases were also found in murine liver and brain, as well as in the kidney of both species [64]. While the deamidation of H1° has not yet been directly demonstrated to occur through the succinimide pathway, this is believed to be the case [65]. As both acetylation and deamidation reduce the positive charge at the amino terminus, it has been postulated that these developmental changes influence chromatin structure and/or stability based on histone/DNA interactions, and that these changes may in part explain changes in chromatin structure associated with senescence during aging [64, 65].

As described above, histone H2B forms isoAsp linkages in vivo that become repaired by PIMT. The prominent occurrence of Asx modification in two different histones in vivo collectively suggest that the succinimide pathway may mediate fine-tuning of molecular function in the nucleus and provide yet another example of posttranslational histone modification in vivo. As histones are exceptionally long-lived proteins, the recurring process of isoAsp formation and repair via the succinimide route should lead to significant racemization (fig. 5), as found already with PKA. Experiments to test this prediction are currently in progress in our laboratory.

A special role for PIMT in the brain and testes?

PIMT is a widely expressed and highly conserved enzyme and has been identified in organisms ranging from bacteria to plants to humans [66–71]. In mammals, PIMT levels are notably higher in brain and testes than in other organs [72–74], suggesting a particular importance for these tissues. Within the testes, particularly high levels of PIMT have been identified within spermatogenic cells, with an increase in both expression and activity correlated with progressive stages of spermatid development [75, 76]. PIMT has been postulated to be important for repair of damaged proteins within mature spermatozoa, as these cells are translationally silent during epidymal storage [75, 77].

The evidence for a special role for PIMT in the brain stems from its high level of expression there, as well as from the marked accumulation of isoAsp-containing proteins in the brains of knockout mice. The therapeutic effects of epileptic drug treatment [39, 40] and the recovery of phenotype in the transgenic animals [42] further support this hypothesis. Ikegaya et al. [41] recently proposed

that interconversion between asparagine/aspartic acid and isoAsp in presynaptic proteins may regulate synaptic transmission. This idea is consistent with the finding that synapsin I accumulates isoAsp to near stoichiometric amounts in PIMT-knockout mice (fig. 6). The isoAsp form of synapsin I also shows an unusually high affinity for PIMT ($K_M \cong 0.1$ μM) and is rapidly methylated by PIMT in vitro [78]. Synapsin I is a widely studied neuronal protein of critical importance to neurotransmission and to the formation and maintenance of functional synapses in the brain [29, 79]. A functional defect in synapsin I caused by formation of isoAsp remains to be determined; nonetheless, it seems reasonable to consider the possibility that the formation and reversal of isoAsp linkages in synapsin I may serve a functional role in regulating synaptic transmission.

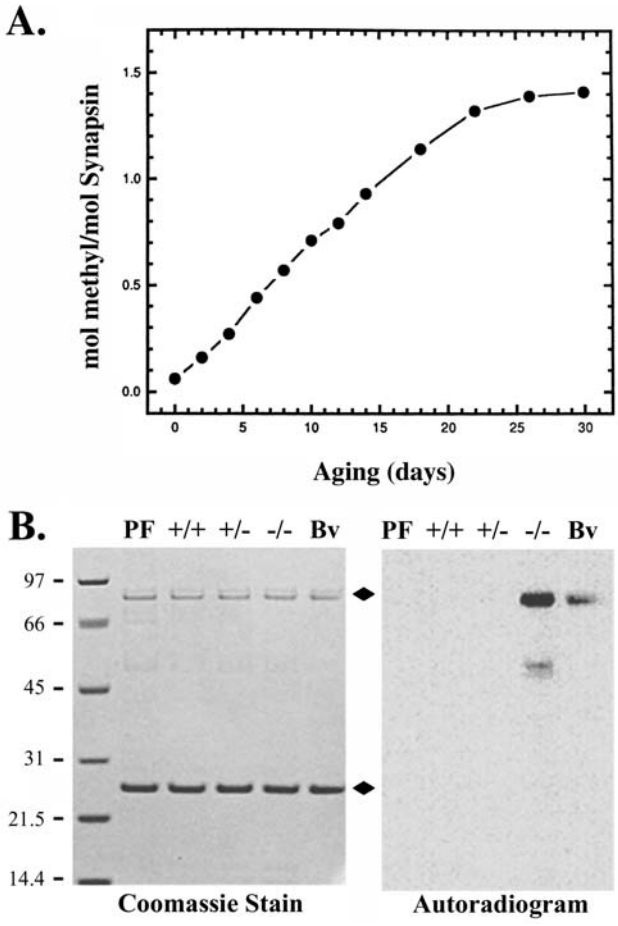

Figure 6. IsoAsp accumulation in synapsin I. (*A*) Time course for isoAsp accumulation during in vitro aging of purified bovine synapsin I, at pH 7.4, 30 °C. Taken with permission from [30]. (*B*) Methylation of synapsin I purified from PIMT knockout mice. Synapsin I samples were [^{3}H]-methylated for electrophoresis and autoradiography. PF indicates wild-type synapsin I purified from mouse brains purchased from Pel Freez Biologicals. Synapsin I purified from PIMT wild-type, heterozygous and knockout mice are indicated by +/+, +/– and –/–. Bv indicates in vitro aged bovine synapsin I containing 0.2 mol isoAsp per mole of protein. Diamonds indicate the presence of synapsin (82 kDa) and PIMT (25 kDa) used in the methylation reaction.

Given the studies suggesting particular relevance of PIMT to brain and testes, it is worthwhile to consider similarities between these tissues in the context of PIMT function. For example, both neurons and mature spermatozoa are terminally differentiated cells and may have special needs for repair of damaged proteins. In addition, both cell types rely on calcium-dependent release of contents within specialized vesicles. In the case of neurons, neurotransmitters are released into the synapse; in the case of spermatozoa, the acrosome reaction releases enzymes necessary for penetration of an egg. Both of these processes are characterized by vesicle docking and membrane fusion events mediated by some of the same players (e. g. NSF, Rab3 A [80], SNARE complex proteins [81, 82] and synaptotagmin isoforms [83]).

There is in fact a history of ideas regarding a role for PIMT (when it was known as PCMT or PCM) in the regulation of neurotransmission and neurotransmitter receptor activity, (reviewed in [84]). Diliberto et al. found in 1976 that carboxymethylation by this enzyme led to release of membrane proteins from chromaffin granules of the adrenal medulla and proposed that methylation was important for excitation-secretion coupling [85]. However, this hypothesis was later contested based on studies of protein secretion in the pancreas and parotid gland [86]. Studies performed in the early 1980s reported that pharmacologic activation of presynaptic dopamine autoreceptors was mediated by PIMT-dependent methylation in both synaptosomes and striatal slices [87, 88]. With the discovery in 1984 that PIMT selectively modifies L-isoAsp sites, the protein damage hypothesis became a major focus of the field.

Regulation of PIMT activity

The studies and speculation described so far in this review have dealt with the possible roles of isoaspartyl methylation in damage repair or regulation of protein activity. It is also worthwhile to consider if and how PIMT activity itself might be controlled. Analysis of the PIMT gene has provided interesting clues about regulation of the enzyme [69]. The 5′ untranslated region of the gene contains a CpG island but no TATA box, features consistent with a housekeeping gene. However, the gene also contains several motifs which indicate potential regulation by stress, including antioxidant, xenobiotic and estrogen response elements. As mentioned earlier, PIMT transcripts were found elevated following treatment of cells with CGP3466 [49], further indicating potential gene regulation in response to environmental factors. An increase in PIMT-dependent methylation was found in response to heat shock in HeLa cells [89]. However, this increase was not associated with increased transcription or increased enzyme-

specific activity. It was thus concluded that increased methylation resulted from greater substrate availability, i.e. formation of more isoAsp, and that PIMT itself is not regulated by heat shock.

Recent studies have reported that isoaspartyl methylation of mammalian membrane proteins and kidney cytosolic extracts is enhanced by the presence of GTPγS [90, 91]. Follow-up studies have reported that GTPγS-dependent stimulation of isoaspartyl methylation of kidney cytosolic extracts can be inhibited be genistein, tyrphostin or vanadate, two tyrosine kinase inhibitors and a tyrosine phosphatase inhibitor, respectively [92]. These results suggest that the regulation of isoaspartyl methylation by guanine nucleotides may be mediated by tyrosine phosphorylation, and therefore that signal transduction pathways may modulate PIMT function. These studies collectively point to possible separate regulation of both gene expression and enzyme activity.

Proteins with naturally high levels of isoAsp

At least two proteins have been found to contain naturally high levels of isoAsp and do not appear to be in vivo substrates for PIMT-dependent methylation. The existence of such proteins suggests that formation of isoAsp in certain cases may have a unique and useful function and may not necessarily represent a state of damage. Deamidation has previously been suggested to serve as a molecular clock for regulating protein turnover, development, aging and homeostasis (discussed in [23]). In an extension of this idea, deamidation of asparagine or dehydration of aspartic acid leading to isoAsp may serve as a molecular timer for proteins that are not in vivo substrates for PIMT.

HMAP

The most striking example of stable isoAsp accumulation in vivo is found in the extracellular matrix of mammalian brain. The extracellular matrix contains a large protein complex (>500 kDa) which we call HMAP (high mass methyl-accepting protein) [93]. HMAP was first identified in soluble extracts from bovine brain on the basis of its extraordinarily high level of isoAsp as measured by PIMT-dependent methylation in vitro (fig. 7). Biochemical characterization of bovine HMAP indicated that it is a protein complex containing phosphacan, a brain-specific chondroitin sulfate proteoglycan, and tenascin-R, an extracellular glycoprotein [94]. Tenascin-R has been demonstrated to interact in vitro with phosphacan via the latter's core protein [95]. Rat brain HMAP is found in very low levels until ~10 days postnatal, at which time it increases linearly through at least 40 days postnatal (fig. 7B) [93]. This time course is very similar to that observed for rat brain phosphacan [96].

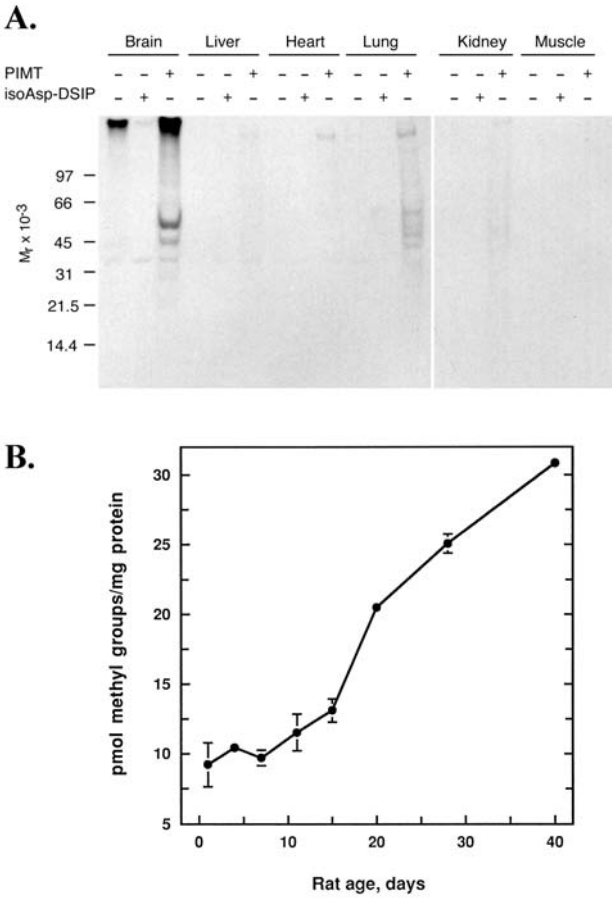

Figure 7. HMAP tissue distribution and developmental profile. (*A*) Soluble protein extracts were prepared from indicated rat tissues and methylated by PIMT and [^{3}H]-AdoMet for analysis by gel electrophoresis and autoradiography. An isoAsp containing low molecular weight peptide, δ-sleep-inducing peptide (DSIP), was included in some reactions as a competitive substrate of isoAsp-specific methylation. Figure taken with permission from [93]. (*B*) Soluble protein extracts from the brains of rats of the age indicated (abcissa) were analyzed as in *A* above. Following electrophoresis, bands containing HMAP were excised, and isoAsp content was determined by scintillation counting of eluted radioactivity. Taken with permission from [94].

Phosphacan represents >40% of the protein component of HMAP and is responsible for >90% of the isoAsp content. Using the calculated molecular weight for phosphacan, this corresponds to a stoichiometry of ~7 mol of isoAsp per mole of phosphacan [94]. This content is greatly in excess (on a mol/mol basis) of any protein reported in vitro or in vivo (synapsin I in both cases) and indicates the prevalence of isoaspartyl linkages in the extracellular matrix. Since phosphacan has been widely studied as an axon guidance molecule during brain development [97], one wonders if isoAsp sites might modulate phosphacan activity in this context. In such a case, isoAsp content could influence development as a time-dependent spontaneous change in protein structure. An attractive, albeit highly speculative idea, is that the isoAsp-prone se-

quences in phosphacan are programmed to undergo isomerization on a predictable time scale and that these structural changes alter the affinity with which phosphacan interacts with its physiological targets.

Ribosomal protein S11 of *Escherichia coli*

The ribosomal protein S11 found in the 30S ribosomal subunit of *E. coli* is another example of a protein which selectively accumulates significant levels of isoAsp in vivo [98]. This protein was also identified on the basis of its ability to serve as an exceptional methyl-accepting protein in cell extracts. The identity of this methyl-acceptor as a ribosomal protein was suspected based on its abundance during the growth phase, and was confirmed as S11 by Western blotting using a panel of antibodies specific for individual ribosomal proteins. The isoAsp content of S11 was estimated to be ~0.5 mol/mol. Levels of isoAsp-rich S11 were most pronounced during the logarithmic growth phase (when ribosomes are most abundant) and decreased during the stationary phase. Interestingly, no other major methyl-accepting proteins were observed during exponential growth, and only later during the stationary phase did other methyl acceptors begin to appear. These latter proteins likely reflect protein aging during this phase. The high content of isoAsp in S11 during rapid growth raises the possibility that isoAsp may play a unique role in ribosome function.

Conclusions and Perspectives

With the exception of its role as an intermediate in protein self-splicing [99], succinimide formation in proteins has been regarded mainly as a source of protein damage and a consequence of 'aging', both in vivo and in vitro. Indeed, for many proteins this seems to be the case. To the extent that sequence plays a major role in determining the propensity of proteins to undergo this reaction, however, one can imagine that for certain proteins evolution may have chosen susceptible sequences as a means of producing an autocatalytic remodeling of protein structure that serves a useful function. The catalytic subunit of PKA is probably the best example to date of an intracellular protein in which succinimide-mediated 'degradation' of an Asn residue, working in conjunction with the isoaspartyl methyltransferase, may act by intentionally introducing a charge heterogeneity (Asn-to-Asp transition) that affects subcellular distribution of the subunit. This charge heterogeneity may also be in part responsible for the vulnerability of tumor cells to DNA-damaging antineoplastic agents [54], suggesting that inhibition of deamidation may be important to cellular activity and survival.

The 'reason' for succinimide formation at Asp-25 in histone H2B is less clear. Charge heterogeneity cannot explain why aspartate-25 in the N-terminal region of histone H2B constantly undergoes succinimide formation followed by PIMT-dependent conversion back to aspartate. It is tempting to assume that H2B exemplifies the widely subscribed damage/repair model and that the susceptible sequence could not easily mutate to a more stable sequence without compromising histone function. Because histones are especially long lived, it will be interesting to see whether, as predicted by the kinetics of succinimide formation and racemization, H2B selectively accumulates unusually high levels of D-Asp and/or D-isoAsp as an animal ages. If so, it would accordingly be interesting to investigate whether the rate of accumulation differs in regions of active chromatin versus silent chromatin. To the extent that succinimide formation is highly dependent on peptide flexibility and conformation around the susceptible residues, the rate and degree of racemization might be influenced by the presence or absence of specific histone-binding proteins associated with transcription and chromatin structure. Racemization could function, for example, to turn off genes over the long term and thereby play an active role in the general aging of an organism.

The phenotype of PIMT knockout mice is dominated by an apparent deficit in neuronal function, while the cells of tissues other than brain seem to tolerate well the limited accumulation of isoAsp sites. It may be that the complexity of processes such as synaptic transmission and the intricate balance of inhibitory and excitatory pathways in brain is such that there is little tolerance for this type of damage in neurons. A more speculative idea is that succinimide formation in proteins such as synapsin I serves a function that is also dependent on methylation of the resulting isoAsp sites. The role of succinimide formation and isoAsp methylation could be something simple like that found with the catalytic subunit of PKA, or something more complex such as a reaction that plays a direct role in synaptic transmission. Certainly more research on synapsin I and other neuronal substrates of PIMT is warranted in order to find out which sites in the protein are altered, and how the protein's function is affected, if at all.

IsoAsp accumulation in the extracellular matrix also challenges the idea that this process is simply a damage reaction. IsoAsp sites may be well tolerated and not alter protein function in the matrix, suggesting that evolution had no reason to select against susceptible sequences. A more intriguing, although currently unsupported, rationale is that isoAsp accumulation plays the role of a molecular clock that guides brain development through well-timed progressive changes in the affinity of protein-ligand interactions.

The ideas expressed here are summarized in figure 8 as four different scenarios that interrelate deamidation, isoAsp formation and PIMT function. Scheme 1 considers cases like histone H2B, where an -Asp-Xaa-linkage isomerizes in an intracellular protein that is accessible to

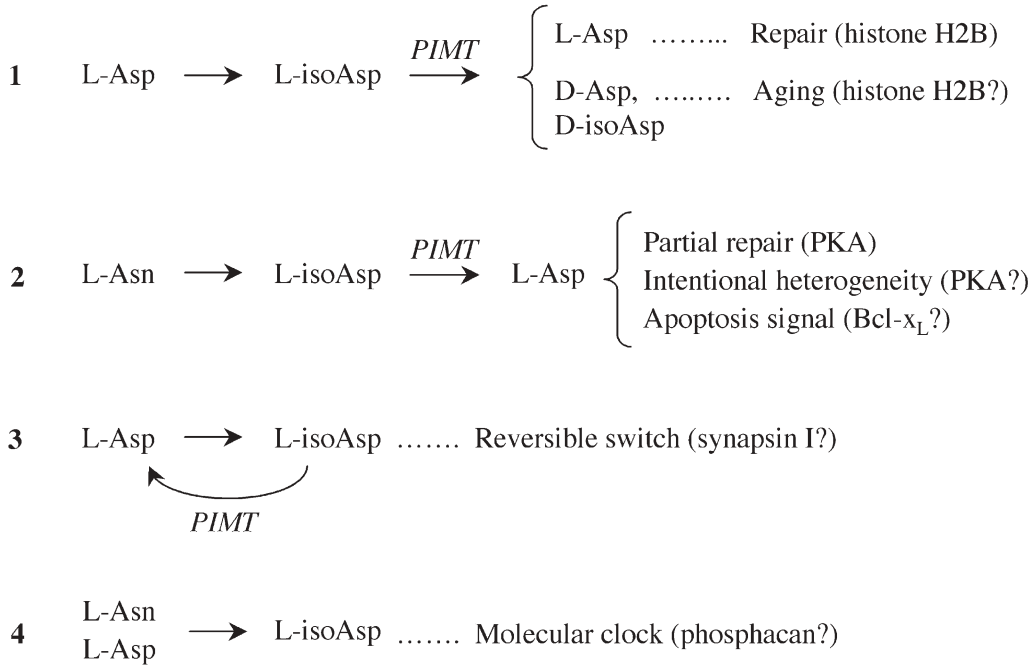

Figure 8. A summary of presumed and possible functions for deamidation and isoAsp formation in proteins (see text for details). Although not explicitly shown in these models, it is understood that a significant portion of the Asn sites that deamidate go directly to Asp without the assistance of PIMT (see fig. 1).

PIMT. The short-term consequence is repair of isomerized linkages, but a possible long-term effect is racemization of the L-Asp residue with marked accumulation over time of D-Asp and D-isoAsp forms.

Scheme 2 considers the deamidation of intracellular proteins such as PKA or Bcl-x$_L$ in which L-Asn residues deamidate to generate L-Asp residues with the help of PIMT. The increased negative charge associated with deamidation triggers a one-time alteration that may change the affinity of the protein with regard to its interaction with other macromolecules.

Scheme 3 considers the transition from Asp to isoAsp and back again as a reversible switch, analogous to phosphorylation/dephosphorylation, except that one half of the proposed switch (Asp→isoAsp) is autocatalytic. Although the autocatalytic transition from Asp to isoAsp occurs relatively slowly in model peptides, proper folding of protein domains in vivo could greatly accelerate this process and be regulated by interactions of a given domain with other proteins. The strong propensity for synapsin I to undergo isoAsp formation in vitro and in vivo, combined with the interesting phenotype of PIMT knockout mice, makes a strong argument for investigating the possibility that isoAsp formation in synapsin I (and other synaptic proteins) may play a regulatory role in synaptic transmission.

Scheme 4 considers the role of isoAsp in proteins such as phosphacan that are not accessible to PIMT. The progressive accumulation of isoAsp originating from Asn and/or Asp sites may alter the affinity of such proteins for one or more of their ligands. The rate of isoAsp accumulation could be fine tuned via amino acid sequence to generate a series of alterations that follow a chronological pattern important for the timing of critical developmental events. PKA, H2B, synapsin I and phosphacan represent several examples in which succinimide formation may modulate the function of proteins inside the cell and in the extracellular matrix. Hopefully, the next few years will provide important new information on the extent to which deamidation, succinimide formation, isoaspartyl sites and PIMT play roles that go beyond the damage-repair model.

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