Protein L-Isoaspartyl Methyltransferase Catalyzes in Vivo Racemization of Aspartate-25 in Mammalian Histone H2B*

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Protein L-isoaspartyl methyltransferase (PIMT) has been implicated in the repair or metabolism of proteins containing atypical L-isoaspartyl peptide bonds. The repair hypothesis is supported by previous studies demonstrating in vitro repair of isoaspartyl peptides via formation of a succinimide intermediate. Utilization of this mechanism in vivo predicts that PIMT modification sites should exhibit significant racemization as a side reaction to the main repair pathway. We therefore studied the D/L ratio of aspartic acid at specific sites in histone H2B, a known target of PIMT in vivo. Using H2B from canine brain, we found that Asp25 (the major PIMT target site in H2B) was significantly racemized, exhibiting D/L ratios as high as 0.12, whereas Asp51, a comparison site, exhibited negligible racemization (D/L = 0.01). Racemization of Asp25 was independent of animal age over the range of 2–15 years. Using H2B from 2–3-week mouse brain, we found a similar D/L ratio (0.14) at Asp25 in wild type mice, but substantially less racemization (D/L = 0.035) at Asp25 in PIMT-deficient mice. These findings suggest that PIMT functions in the repair, rather than the metabolic turnover, of isoaspartyl proteins in vivo. Because PIMT has numerous substrates in cells, these findings also suggest that L-aspartate may be more common in cellular proteins than hitherto imagined and that its occurrence, in some proteins at least, is independent of animal age.

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The abbreviations used are: PIMT, protein L-isoaspartyl methyltransferase; isoAsp, isoaspartate/isoaspartyl; KO, knock-out; HPLC, high pressure liquid chromatography.

linkages alters neither the mass nor the charge (at neutral pH) of a polypeptide.

The formation of isoaspartate in proteins is generally viewed as a deleterious event associated with protein aging (10). It has been suggested that PIMT-dependent methylation of isoaspartyl sites serves to either repair the damaged sites or to tag the damaged proteins for degradation (11, 12). A repair function is supported by in vitro studies using PIMT and defined polypeptide substrates (13–15). These studies indicate the pathway shown in Fig. 2 whereby methylation serves to activate the atypical α-carboxyl of the isoaspartyl site. This activation promotes rapid, spontaneous demethylation via formation of the same succinimide intermediate that occurs during isoaspartate formation. Each cycle of methylation/demethylation converts ~15–30% of the isoaspartyl linkage to a normal aspartyl linkage; however, after multiple cycles of methylation/demethylation, a majority of isoaspartyl sites are converted to normal aspartyl sites. In a study using purified PIMT and three different synthetic L-isoaspartyl peptides, we observed a net repair (L-isoAsp to L-Asp) efficiency of 65–80% after 24–48 h of incubation in the presence of excess S-adenosyl-L-methionine (16). The overall efficiency of the repair was limited by racemization of the succinimide intermediate (see Fig. 2) to produce a mixture of D-isoAsp and L-Asp forms of the peptide, neither of which are efficiently methylated by PIMT. Similar results have been reported by other laboratories (14, 15).

Further evidence that PIMT serves to repair L-isoaspartyl sites in proteins comes from two lines of study in which PIMT activity was artificially reduced in living cells. One line of study utilized the methylation inhibitor adenosine dialdehyde to lower PIMT activity in cultured rat PC12 cells (17). As predicted by the repair hypothesis, inhibitor treatment led to a dramatic increase in isoaspartate levels. A second line of study subsequently demonstrated that proteins in tissue extracts of PIMT-deficient (knock-out) mice show dramatically higher levels of isoaspartate than do wild type mice (18, 19). Although both of these observations are consistent with a repair role for PIMT, both are also consistent with a possible role for PIMT in facilitating the degradation of isoaspartyl proteins.

To more precisely define the in vivo function of PIMT, we thought it would be useful to determine whether an unusual degree racemization can be detected at protein sites that are known targets of PIMT-dependent methylation. If the repair mechanism of Fig. 2 applies in vivo, one would expect significant and selective racemization at such sites because of their repeated transit through a succinimide intermediate during cycles of PIMT-catalyzed methylation. An important corollary prediction is that such site-specific racemization should not be observed (or be much lower) in proteins from a PIMT-deficient...
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Fig. 1. Mechanism by which L-isoaspartyl sites arise from L-aspartyl and L-asparaginyl sites in peptides and proteins. This spontaneous intramolecular rearrangement occurs most readily at Asn-Gly, Asn-Ser, and Asp-Gly sequences in flexible regions of polypeptides. The L-isoaspartyl form (lower right) typically accounts for 70–85% of the succinimide hydrolysis product.

Fig. 2. Mechanism for PIMT-dependent repair and racemization of L-isoaspartyl sites. PIMT catalyzes the methylation of L-isoaspartyl sites (top left) to form ω-aspartyl O-methyl esters (top right). At physiological pH and temperature, the methyl esters spontaneously demethylate with a half-life of ~5–15 min to form the more stable L-succinimide (L-imide, middle right), which has a half-life of several hours. Hydrolysis of the L-succinimide generates a mixture of L-aspartyl and L-isoaspartyl peptides, the former representing the completion of one repair cycle. Several additional cycles of methylation and demethylation convert nearly all of the original L-isoaspartyl sites to L-aspartyl sites. In the succinimide form, the acidity of the aspartate ω-carbon markedly increases, thereby promoting production of D-succinimide (bottom right) via spontaneous racemization. Both the L-succinimide and the D-succinimide hydrolyze to form the corresponding isoaspartyl and aspartyl peptides. Although succinimide racemization is slow compared with its hydrolysis, its relative stability, combined with its constant replenishment during repeated repair cycles, provides ample opportunity for accumulation of D-isoaspartyl and D-aspartyl sites, which are poor substrates for PIMT. AdoMet, S-adenosyl-L-methionine.

Tissue Sources—Frozen (−70 °C) coronal sections from the temporal lobe of male dog brains were obtained from the Institute for Brain Aging and Dementia Canine Brain Repository at the University of California, Irvine, CA. Frozen (−70 °C) brains from PIMT (+/+ or PIMT (−/−) mice, 2–3 weeks old, were obtained as described previously (20).

Histone Isolation—Isolation and purification of mammalian core histones was carried out according to Young et al. (20) with minor modifications. The starting material was canine brain (1.6–2.4 g), or pooled mouse brain (2.0–2.4 g). The precipitation of histones from the acid extract of the nuclear fraction was accomplished by addition of 25% (w/v) trichloroacetic acid. Individual histone peaks resolved by reversed-phase HPLC were identified by mass spectrometry and then dried in a vacuum centrifuge. Recombinant chicken H2B was expressed in Escherichia coli and purified to homogeneity as described elsewhere (21).

Mass Spectrometry—Individual histones or histone-derived peptide peaks from HPLC were identified by matrix-assisted laser desorption time-of-flight mass spectrometry on a Perseptive Biosystems Voyager-DE STR BioSpectrometry Workstation. Typically, 0.8 µl of histone or peptide (5–50 pmol) was mixed with 0.8 µl of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (histones) or α-cyano-4-hydroxycinnamic acid (peptides) in 50% (v/v) acetonitrile, 0.3% (w/v) trifluoroacetic acid, spotted onto the sample plate, and allowed to air dry. All spectra were acquired in the linear, positive ion mode.

Isolation of Individual Peptides from Histone H2B—Purified samples of H2B (60–120 µg) were digested for 6 h at 37 °C in reactions containing 0.31 mg/ml histone, 0.024 mg/ml endoproteinase Glu-C (Worthington), and 50 mM ammonium acetate, pH 4.0. After adding trifluoroacetic acid to a final concentration of 1% (w/v), peptides were separated by HPLC on an Aquapore RP-300 column (PerkinElmer Life Sciences/Brownlee) at 1.0 ml/min using a gradient of 3.5–49% (v/v) acetonitrile over 100 min in a base solvent of 0.1% (w/v) trifluoroacetic acid in water. Absorbance was monitored at 214 nm. Major peaks were collected, dried in a vacuum centrifuge, and identified by mass spectrometry. The quantity of peptide recovered in each peak was estimated from a peak area relative to an 18-mer peptide standard run under similar HPLC conditions. To analyze Asp33 from dog H2B, subdigestion of the pooled 36–68/71/76 Glu-C peptides (7–13 µg/sample) was carried out for 4 h at 37 °C in reactions containing 0.2 mg/ml peptide, 0.004 mg/ml trypsin (Sigma), and 96 mM ammonium bicarbonate, pH 7.8. Reactions were terminated by adding trifluoroacetic acid to a final concentration of 1% (w/v). Peptides were separated by HPLC and identified as described above for the Glu-C peptides.

Determination of Aspartate d/L Ratios—Acid hydrolysis of purified

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Aspartate 25 of Canine H2B Is Highly Racemized—As an initial investigation into the possible relationship between isoaspartate formation and racemization in vivo, we purified individual core histones from the post-mortem brains of 2-, 9- and 15-year-old dogs obtained through the Institute for Brain Aging and Dementia Canine Brain Repository at UC Irvine. (The sequences of the histones relevant to this study are shown in Fig. 3.) We first analyzed the D/L ratio of all Asx residues in acid hydrolysates of histones H2B and H3 (Fig. 4a). Histone H2B was chosen for reasons explained above. Histone H3 was chosen for comparison because it separates more cleanly from the other histones during HPLC purification (20) and because its overall aspartate and asparagine content is similar to that of H2B. As expected, the Asx D/L ratio for histone H3 was close to zero for all samples. In contrast, all of the H2B samples exhibited Asx D/L ratios significantly greater than zero, indicating that one (or more) Asx residues in H2B was at least partially racemized. We were somewhat surprised to see that the D/L ratios for H2B did not increase with animal age.

We next examined the site specificity of Asx racemization in canine H2B by comparing the D/L ratios of Asp25 and Asp51. As mentioned above, Asp25 is the major site of isoAsp formation and PIMT-dependent methylation in H2B. Asp51 was chosen as a comparison site because it was relatively easy to isolate from other Asx residues via proteolytic digestion. Fig. 4b shows that the D/L ratio of Asp25 (0.115–0.122) is considerably higher than the D/L ratio of the Asx-aggregate of whole histone H2B (0.049–0.070, Fig. 4a). In contrast, the D/L ratio of Asp51 in H2B (0.000–0.008, Fig. 4b) is in the same low range found for the aggregate of Asx residues in H3 (0.002–0.013, Fig. 4a). These findings confirmed our prediction that histone H2B would exhibit a higher content of D-aspartate than other histones and that the major contributor would be the succinimide-prone Asp25 residue. Examples of the original HPLC traces used to analyze the Asp25 D/L ratios in three dogs are presented in Fig. 4c. No data were obtainable from the 15.3-year-old dog because of poor recovery of the relevant peptide.

Racemization of Asp25 Requires PIMT Activity—To examine the possible role of PIMT in catalyzing the selective racemization of Asp25, we compared the D/L ratios at this residue in H2B isolated from wild type and PIMT knockout (KO) mice. PIMT-KO mice exhibit a nearly normal phenotype until one month after birth (18, 19). Between 30 and 60 days, most of the KO mice succumb to fatal epileptic seizures. Prior to death, all major tissues of the KO mice show dramatic elevation of protein isoaspartate levels compared with their wild type or heterozygote littermates. This is consistent with the presumed role of PIMT in catalyzing the repair of spontaneously arising isoaspartyl bonds via the mechanism shown in Fig. 2; it is also consistent with a role for PIMT in the metabolic turnover of isoAsp-bearing proteins. Unlike a turn-over role, the repair mechanism provides an opportunity for significant racemization because complete repair of each isoaspartyl site requires multiple cycles of methylation/demethylation, with each cycle resulting in formation of a metastable, racemization-prone succinimide.

Fig. 5a shows the Asx D/L ratios for all Asx-containing Glu-C peptides derived from wild type, PIMT-KO mice, and recombinant chicken H2B. The 1–35 peptide provides information on the racemization of Asp25, the only Asx residue in this peptide. The D/L ratio for wild type mouse was 0.141, similar to that found for Asp25 in canine H2B (Fig. 4b). This is 4.1 times the D/L ratio observed in the KO mice (0.034), indicating that PIMT makes a major contribution to the in vivo racemization of Asp25. The low but significant level of racemization of this same residue in the KO mouse may stem in part from the non-enzymatic succinimide formation that occurs during isoAsp formation (Fig. 1). We note, however, that the amount of racemization seen in the 1–35 peptide from the KO mice is only slightly greater than that seen in the 1–35 peptide from the recombinant chick H2B. We suspect that the 1–35 racemization observed for recombinant chick H2B (which is subject to minimal in vivo aging during its short expression period and no exposure to mammalian PIMT) occurs mainly from peptide...
handling during purification, protease digestion, and HPLC. If this is true, then this "in vitro" D/L contribution of 0.022 should be subtracted from the D/L ratio of Asp25 for both the wild type and KO mice. Doing this results in corrected D/L ratios of 0.119 and 0.012, respectively. Thus, PIMT is apparently responsible for 76% (uncorrected) to 90% (corrected) of the racemization observed at Asp25. The other two Glu-C peptides analyzed in Fig. 5 all showed AspxD/L ratios of 0.01 or less, regardless of the source. The 36–68 peptide contains two Asp and two Asn sites, whereas the 77–93 peptide contains a single Asn site. We conclude that Asp25 from wild type mice is the only Asx residue that exhibits significant racemization in vivo and that this racemization results from a combination of the inherent susceptibility of this site to isoaspartate formation coupled with the catalytic activity of PIMT.

**DISCUSSION**

It is interesting that the extent of racemization of Asp25 in H2B is virtually the same in 2–3-week old mice (Fig. 5a), a 2-year-old dog (Fig. 4b), and a 15-year-old dog (Fig. 4b). Using rat PC12 cells, we previously estimated that isoaspartyl sites are generated in roughly 1% of H2B molecules/day in vivo (20). This supplies a steady stream of substrate for PIMT, which rapidly converts the isoaspartyl sites to racemization-prone succinimides. The incubation of a synthetic L-isoaspartyl peptide with PIMT and S-adenosyl-L-methionine resulted in the production of two major end products over a period of 24 h: 80% L-Asp peptide and 13% D-isoAsp/D-Asp peptide (16). Combining the rates of L-isoAsp generation in vivo with the capacity of PIMT to generate significant D-isoAsp and D-Asp from L-isoAsp provides a logical and compelling explanation for the extent of Asp25 racemization we see in 2–3-week-old wild type mice. This conclusion is supported by the recent studies of Kinzel et al. (23), who found significant levels of D-isoAsp and D-Asp at the Asn2 site in a deamidated subform of the catalytic subunit of protein kinase A and suggested a catalytic role for PIMT in the racemization. This conclusion is also corroborated by the earlier work of Perna et al. (24) who observed decreased levels of D-Asp/D-isoAsp in proteins from erythrocytes of uremic patients that have reduced activity of erythrocyte PIMT.

The reason why racemization does not continue beyond a D/L ratio of 0.12 or so may be because of a metabolic turnover of the D-isoAsp-containing histones. A similar explanation has been

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**FIG. 4. Analysis of aspartate D/L ratios in acid hydrolysates of histones from dog brain.** a, age dependence of D/L ratios for whole histone H2B (black) and whole histone H3 (gray). b, comparison of D/L ratios for Asp25 (black) and Asp51 (gray) in histone H2B from two dogs, ages 2.1 and 15.2 years. Note the larger vertical scale in this panel compared with the previous panel. c, examples of three individual aspartate D/L analyses of the H2B 1–35 Glu-C peptide (containing Asp25 as the only Asx residue) from dogs of ages 2.1, 9.4, and 15.2 years. The L-Asp peaks were all normalized for easy comparison of the D/L ratios. The retention time variation is because of the sensitivity of this isocratic separation to small differences in column equilibration between runs. The D/L ratios evident in this panel are higher than those shown in b, because the latter data have been corrected for acid hydrolysis-induced racemization as described under "Materials and Methods." Error bars in a and b represent the S.D. around the mean of triplicate D/L-Asp determinations from a given acid hydrolysate. The negative value of the Asp51 data for the 15.1-year-old dog reflects the fact that the uncorrected D/L ratio observed for this sample was slightly below that for the control peptide.
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are the most frequently encountered sites of rapid succinimide formation when proteins are aged in vitro at physiological pH and temperature (7). The fact that this racemization-prone site is conserved in mammals (Fig. 3) and that it occurs in a histone domain that interacts with DNA and chromatin remodeling proteins raises the possibility that racemization of Asp25 might be a functional modification rather than a purely deleterious side reaction. It will be of interest to determine whether the D-Asp25 form of H2B is differentially distributed between open and condensed regions of chromatin and whether it is relatively enriched or depleted in nucleosomes associated with active or repressed genes. Coincidentally, histone H2B is a major target of autoimmune responses in systemic lupus erythematosus (26). The formation of l-isoaspartyl sites, as well as other types of posttranslational modification of self-proteins, are believed to be key factors in eliciting autoimmunity in a number of diseases (27, 28).

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