Limited Accumulation of Damaged Proteins in L-Isoaspartyl (d-Aspartyl) O-Methyltransferase-deficient Mice*

Jonathan D. Lowenson‡, Edward Kim§, Stephen G. Young¶, and Steven Clarke∥

From the ‡Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095-1569, the ¶Gladstone Institute of Cardiovascular Disease, San Francisco, California 94141-9100, and the ∥Department of Medicine and the Cardiovascular Research Institute, University of California, San Francisco, California 94143

L-Isoaspartyl (d-aspartyl) O-methyltransferase (PCMT1) can initiate the conversion of damaged aspartyl and asparaginyl residues to normal L-aspartyl residues. Mice lacking this enzyme (Pcmt1/−/− mice) have elevated levels of damaged residues and die at a mean age of 42 days from massive tonic-clonic seizures. To extend the lives of the knockout mice so that the long term effects of damaged residue accumulation could be investigated, we produced transgenic mice with a mouse Pcmt1 cDNA under the control of a neuron-specific promoter. Pcmt1 transgenic mice that were homozygous for the endogenous Pcmt1 knockout mutation (“transgenic Pcmt1/−/− mice”) had brain PCMT1 activity levels that were 6.5–13% of those of wild-type mice but had little or no activity in other tissues. The transgenic Pcmt1/−/− mice lived, on average, 5-fold longer than nontransgenic Pcmt1/−/− mice and accumulated only half as many damaged aspartyl residues in their brain proteins. The concentration of damaged residues in heart, testis, and brain proteins in transgenic Pcmt1/−/− mice initially increased with age but unexpectedly reached a plateau by 100 days of age. Urine from Pcmt1/−/− mice contained increased amounts of peptides with damaged aspartyl residues, apparently enough to account for proteins that were not repaired intracellularly. In the absence of PCMT1, proteolysis may limit the intracellular accumulation of damaged proteins but less efficiently than in wild-type mice having PCMT1-mediated repair.

The spontaneous chemical modification of proteins by reaction with oxygen, water, sugars, and other abundant metabolites is unavoidable. The accumulation of such nonenzymatically altered proteins is associated with normal aging as well as atherosclerosis, Alzheimer’s disease, and diabetes (1–3). Organisms have several strategies for dealing with damaged proteins, including intracellular proteolysis mediated by proteasome and lysosome action (4–6). Some types of covalent damage, however, are simple enough to recognize and repair directly (7). Enzymes such as prolyl cis-trans isomerase (8), methionine sulfoxide reductase (9), and disulfide isomerase (8) can restore activity to proteins that have been chemically altered.

We are interested in a common type of spontaneous protein damage in which l-aspartyl and l-asparaginyl residues undergo an intramolecular reaction that converts them to l-succinimidyld residues (10, 11). Nonenzymatic hydrolysis of the succinimide ring readily occurs at either carbonyl to generate both normal aspartyl residues and isoaspartyl residues, in which the peptide backbone proceeds through the β-carbonyl rather than the α-carbonyl moiety (12). The succinimide also racemizes more rapidly than do the open chain forms, and hydrolysis of the d-succinimide produces d-aspartyl and d-isoaspartyl residues (12). Local protein structure causes some l-aspartyl and l-asparaginyl residues to be especially prone to succinimide formation, and the presence of damaged aspartyl residues at these sites can significantly alter the structure, function, and immunogenicity of the protein (10, 13).

To minimize the accumulation of damaged aspartyl residues in cellular proteins, all mammalian tissues possess an L-isoaspartyl (d-aspartyl) O-methyltransferase (EC 2.1.1.77; designated PCMT1 in mice) (14). This enzyme uses S-adenosyl-L-methionine (AdoMet) to methylate l-isoaspartyl (and, less efficiently, d-aspartyl) residues but not normal l-aspartyl residues (15). Nonenzymatic deesterification of the methylated residues returns them to the succinimide form much more rapidly than occurs in the absence of methylation, resulting in the eventual conversion of most of the damaged residues to the “repaired” l-aspartyl form (16, 17).

The physiological importance of this pathway remained unclear until Pcmt1 knockout (Pcmt1/−/−) mice were created and found to display a distinctive phenotype (18, 19). Pcmt1/−/− mice have 2–6-fold higher levels of damaged aspartyl residues in their brain, heart, liver, and erythrocytes than are observed in wild-type tissues (18). Furthermore, Pcmt1/−/− mice are smaller than their Pcmt1+/− and Pcmt1+/+ littermates, undergo severe tonic-clonic seizures, and die at an average age of 42 days (18, 19). Electroencephalographic analysis shows that these mice suffer abnormal brain activity about 50% of the time, not just during the tonic-clonic seizures (20). Administration of the anti-seizure drug valproic acid enabled Pcmt1/−/− mice to attain the same size and weight as their wild-type littermates, suggesting that the absence of the methyltransferase did not interfere directly with food intake or metabolism (20). The combination of valproic acid and clonazepam prolonged mean survival but only by 36 days (20).

* This work was supported by National Institutes of Health Grants AG15451, HL41633 (to S. G. Y.), GM26029, and AG18000 (to S. C.), and by a grant from the University of California Tobacco-related Disease Research Program (to S. G. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

To whom correspondence should be addressed: 640 Paul D. Boyer Hall, 611 Charles E. Young Drive East, Los Angeles, CA 90095-1570.
Tel.: 310-825-8754, Fax.: 310-825-1968; E-mail: clarke@mbi.ucla.edu.

1 The abbreviations used are: PCMT1, L-isoaspartyl (d-aspartyl) O-methyltransferase; AdoMet, S-adenosyl-l-methionine; [14C]AdoMet, S-adenosyl[methyl-14C]-l-methionine; NSE, neuron-specific enolase; BisTris, 2,2-bis(hydroxymethyl)-2,2',2''-nitrotrilriethanol.
Experimental Procedures

Generation of Pcm1 Transgenic Mice—A rat neuron-specific enolase (NSE) promoter was used to direct the expression of mouse Pcm1 cDNA in the brains of transgenic mice. The methyltransferase coding sequence (including 119 base pairs of 5'–noncoding and 777 base pairs of 3'–noncoding sequence) was obtained from a 1580-base pair murine NSE-APP695 (22) after digestion with HindIII. After overhangs were filled with Klenow polymerase, the mouse Pcm1 cDNA and the rat NSE vector were ligated, and the NSE-Pcm1 transgenic construct was isolated by digestion with SstI. The transgene (2 ng/μl) was microinjected into F2 C57BL/6 × SJL fertilized mouse eggs by standard techniques (23). From 37 microinjected eggs, 33 pups were obtained, and 4 harbored the Pcm1 transgene. These transgenic founders were identified by polymerase chain reaction with primers corresponding to mouse Pcm1 exon 1 and 5′-CACCATTTCACACTGGATGTC-3′ from exon 1 and 5′-CACCATTTCACACTGGATGTC-3′ from exons 4 and 5). Southern blot analysis of tail DNA confirmed the integration of the transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation and hemizygous for the Pcmt1 transgene (Fig. 1).

Quantitation of Endogenously Methylated Damaged Aspartyl Residues—Damaged residues that are already methylated within cells by the endogenous methyltransferase and S-adenosyl-L-methionine are not measured in the assay described above but can be quantified after mild base treatment. Protein (8.3–9.6 mg) from homogenized frozen brains was incubated in 20 μl of 1 M potassium borate, pH 10.2. After times ranging from 5 to 360 min, 10 μl of 500 mM BisTris-HCl, pH 5.7, was added to lower the pH to about 6. Then, recombinant human methyltransferase (5 pmol/min) and S-adenosyl-L-methionine (10 μm final concentration) in 10 μl of 150 mM BisTris-HCl, pH 6.0, was added, and these reaction mixtures were incubated at 37 °C for 135 min. The reaction was stopped by freezing on dry ice, and the base-labile methyl esters were quantitated as described above.

Urine Collection and Analysis—Urine, freshly voided on Parafilm, was collected with a pipette and stored frozen until used. Creatinine in the urine was measured by a modified form of the procedure of Bosnes and Taussky (25). An aliquot of each urine sample (0.3–1 μl) or standard creatinine (0–25 μg) was diluted to 50 μl with water in duplicate tubes. Picric acid was added (25 μl of a 40 mM solution), and the tubes were capped and incubated in a boiling water bath for 45 min. After cooling to room temperature, 25 μl of 0.75 M NaOH was added. Within 15 min, 90 μl of each sample was transferred to a flat-welled microtiter plate (Costar), and absorbance was measured at 525 nm with a Beckman DU-660 plate reader. Damaged aspartyl residues in the urine were assayed with recombinant human methyltransferase as described above.

Amino Acid Analysis—Free amino acids and isosaportyl-containing dipeptides in urine were derivatized with o-phthalaldehyde and 2-mercaptoethanol, separated by reverse-phase high pressure liquid chromatography, and quantitated by fluorescence as described previously (26). A precipitate that formed upon mixing of the urine and derivatization reagent was removed by centrifugation at 20,800 × g for 3 min prior to injection. Urine that had been dried in 6 × 50-mm glass tubes was hydrolyzed in vaporized hydrochloric acid in vacuo at 108 °C for 3 h with a PicoTag Work Station (Waters); amino acids in the hydrolysates were quantitated as described above. The fluorescence color constants for these derivatives were determined with amino acid and dipeptide standards.
RESULTS

Expression of a Pcmt1 Transgene in Neurons Prolongs the Lives of Mice Lacking Endogenous Pcmt1—We generated two Pcmt1 transgenic mouse lines, lines 27 and 29, in which the murine Pcmt1 methyltransferase cDNA was placed under the control of a neuron-specific promoter. We then compared the survival of “transgenic Pcmt1+/− mice” with that of “nontransgenic Pcmt1+/− mice.” Whereas nontransgenic Pcmt1+/− mice died at a median age of 44 days (with only one mouse living beyond 150 days), the transgenic Pcmt1+/− mice lived much longer (Fig. 2). Of 11 line 27 transgenic Pcmt1+/− mice examined in this study, 6 died between 30 and 90 days of age, but 4 lived from 549 to 757 days. Line 29 transgenic Pcmt1+/− mice (n = 19) died at a median age of 213 days (Fig. 2), and 3 lived more than 400 days. The nontransgenic Pcmt1+/− mice began to die at about 21 days. In contrast, none of the line 29 transgenic Pcmt1+/− mice died at less than 52 days of age (Fig. 2).

Line 27 and most of the line 29 mice possessing one or two copies of the endogenous Pcmt1 gene were indistinguishable from comparable nontransgenic mice in size, weight, and behavior, although 15 of 94 line 29 mice ran rapidly in circles. Line 27 and line 29 mice appeared to have normal physiological functions and had unremarkable tissue histology. The transgenic Pcmt1+/− mice, however, differed from the nontransgenic Pcmt1+/− animals in several ways. First, although nontransgenic Pcmt1+/− mice weighed significantly less than age- and sex-matched Pcmt1+/− and Pcmt1+/+ littermates (18), the weights of transgenic Pcmt1+/− mice were identical to their Pcmt1+/− and Pcmt1+/+ littermates (data not shown). Second, due to their low grade seizure activity (e.g., facial grooming and myoclonic jerks), the nontransgenic Pcmt1+/− mice often could be distinguished by observation from wild-type and heterozygous littermates. In contrast, these abnormalities were not observed in the transgenic Pcmt1+/− mice. Finally, nontransgenic Pcmt1+/− mice of either sex never produced litters, even when housed with Pcmt1+/+ animals and given the anti-seizure drugs valproic acid and clonazepam, and only a single mating was observed (20). Two line 27 Pcmt1+/− animals, however, produced two small litters without the administration of any drug treatments, and 17 pairings involving male and/or female line 29 Pcmt1+/− mice have produced three litters (from two different Pcmt1+/− mothers).

Localization of Methyltransferase Activity in Tissues of Line 27 and Line 29 Transgenic Mice—The Pcmt1 transgene controlled by a neuron-specific promoter appeared to rescue, at least partially, the early death phenotype seen in mice lacking the endogenous methyltransferase. We next investigated where in the mouse, and at what level, the transgene was being expressed by assaying methyltransferase activity in various mouse tissues. As expected, transgenic mice possessing one or two copies of the endogenous Pcmt1 gene expressed the methyltransferase in the brain but not in the other tissues (Table I) (18). In contrast, transgenic Pcmt1+/− mice expressed methyltransferase activity in the brain but not in the other tissues (Table I), and Western blot analysis detected PCMT1 protein only in brain homogenates (data not shown), suggesting that the neuron-specific enolase promoter was properly directing expression to the brain but not to the other tissues. The amount of activity in brains from young (50 days) and older (370 days) transgenic Pcmt1+/− animals was not significantly different (data not shown). Since about half of the cells in the brain are not neurons (27), some reduction of methyltrans-
ferase activity was expected in these brains. The low activity observed here, however, suggested that even in neurons the expression of the transgene was weaker than that of endogenous Pcmt1 in wild-type neurons. Relatively low levels of neuronal expression from the neuron-specific enolase promoter have been reported by other investigators (28, 29).

Accumulation of Damaged Aspartyl Residues in Brain Tissue—The finding that transgenic mice expressing methyltransferase solely in neurons lived longer than nontransgenic knockout mice led us to compare the accumulation of damaged aspartyl residues in the brains of these animals. Recombinant human methyltransferase was used to label these residues in cytosolic/microsomal proteins with [14C]methyl groups from [14C]AdoMet in vitro. Examining 39 transgenic and 13 nontransgenic Pcmt1−/− mice, we found about 50% fewer damaged aspartyl residues when the transgene was present, indicating that the transgene-derived enzyme was repairing damaged neuronal proteins. However, the transgenic Pcmt1−/− brains still had about 4.5-fold more damaged residues than did Pcmt1+/+ and Pcmt1+/+/ brains (Fig. 3). These damaged residues could be accumulating both in neurons, due to the relatively low methyltransferase activity, and in glia, which should not express the transgene at all.

Examination of the levels of aspartyl damage in the brain with respect to age revealed several interesting points. First, these levels increased with age in young animals; 40-day-old and older mice had significantly more damaged residues per mg of protein than did the 13–21-day-old mice (p = 0.001 for Pcmt1−/− mice; p = 10−8 for Pcmt1+/− and Pcmt1+/+/ mice; Fig. 3). Second, the 13–14-day-old Pcmt1−/− animals already possessed about 8-fold more damaged aspartyl residues per mg protein than did age-matched Pcmt1+/− and Pcmt1+/+/ animals (Fig. 3). The difference in the amount of damage remained 8–11-fold as these mice aged to 91 days, demonstrating that both nursing and weaned Pcmt1−/− mice accumulate these residues. Finally, there was no significant increase in the level of damaged aspartyl residues in transgenic Pcmt1−/− mice after about 100 days of age (Fig. 3). In addition, although line 29 Pcmt1−/− mice averaged twice as much brain PCMT1 activity as did comparable line 27 mice, the plateau level of damaged aspartyl residues in these two lines was not significantly different. The quantity of damaged residues in Pcmt1+/− and Pcmt1+/+/ mice also attained an apparent steady state, although at a much lower level (Fig. 3). Because damaged residues were arising continuously in the cellular proteins, this stable level of damage probably represents a steady state between new damage, methyltransferase-linked repair, protein turnover, and perhaps unknown factors.

In control experiments, we asked whether the low level of damaged residues measured in the assays of Pcmt1+/− and Pcmt1+/+ mice resulted in part from the fact that some were already methylated by the endogenous enzyme. We therefore incubated brain cytosolic proteins under basic conditions where L-isoaspartyl α-methyl esters should hydrolyze within a few minutes to generate L-isoaspartyl residues with about an 80% yield (30, 31). Analysis of these samples with recombinant methyltransferase (data not shown) indicated that the true number of L-isoaspartyl residues in the Pcmt1+/− and Pcmt1+/+ proteins could be as much as 2.4-fold higher than the data shown in Fig. 3. Coupled with the 8–11-fold higher levels actually observed, this result indicates that the level of damaged aspartyl residues in Pcmt1−/− brain proteins was at least 4-fold higher than in heterozygous and wild-type brain proteins.

Limited Accumulation of Damaged Aspartyl Residues in Heart, Testes, and Erythrocytes—The longer life span of the transgenic Pcmt1−/− mice enabled us to investigate whether accumulation of damaged aspartyl residues continued in tissues completely lacking methyltransferase-mediated repair as animals aged beyond the limit set by the early deaths of the nontransgenic knockout mice. In addition to heart (Fig. 4) and
Accumulation of damaged aspartyl residues in heart cytosolic/microsomal polypeptides. Levels of damage were assayed as described in Fig. 3. Open circles, Pcmt1−/− (n = 12); gray circles, transgenic line 27 Pcmt1−/− (n = 12); black circles, transgenic line 29 Pcmt1+/+ (n = 8); open crosses, Pcmt1+/+ (n = 6); gray cross, transgenic line 27 Pcmt1+/+ (n = 1); open triangles, Pcmt1+/+ (n = 9); and black triangle, transgenic line 29 Pcmt1+/+ (n = 1).

Levels of damaged aspartyl residues in tissues of adult mice over 100 days in age

Table II

Recombinant human l-isoaspartyl (D-aspartyl) O-methyltransferase was used to quantitate damaged aspartyl residues in polypeptides remaining in the 20,800 × g supernatant after centrifugation of whole tissue homogenates as described under “Experimental Procedures.” Values were first averaged from triplicate assays of individual tissue extracts and then averaged for the whole group of mice with standard deviation values. The number of animals used is given in parentheses. The error in each of the triplicate assays was generally very small (less than 5%).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue</th>
<th>Rate of accumulation (pmol methylatable sites/mg protein/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pcm1+/+ with transgene</td>
<td>Pcm1+/+ with or without transgene</td>
</tr>
<tr>
<td>Brain</td>
<td>829 ± 135 (21)</td>
<td>183 ± 29 (14)</td>
</tr>
<tr>
<td>Heart</td>
<td>356 ± 42 (11)</td>
<td>79 ± 14 (8)</td>
</tr>
<tr>
<td>Testis</td>
<td>500 ± 90 (13)</td>
<td>106 ± 56 (11)</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>77 ± 12 (20)</td>
<td>15 ± 2 (15)</td>
</tr>
</tbody>
</table>

Table III

Rates of accumulation of damaged aspartyl residues in Pcmt1−/− mice tissues

The rates at which damaged residues accumulated nontransgenic Pcmt1−/− mouse tissues over the first 21 days after birth were calculated from the data in Figs. 3–6. It was assumed for these calculations that there are no damaged residues in newborn mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rate of accumulation (pmol methylatable sites/mg protein/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>52</td>
</tr>
<tr>
<td>Testis</td>
<td>11.8</td>
</tr>
<tr>
<td>Heart</td>
<td>7.6</td>
</tr>
<tr>
<td>Liver</td>
<td>7.5</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>1.8</td>
</tr>
</tbody>
</table>

tissues, ranging from 51 pmol of methylatable residues/mg of protein/day in brains to 1.8 pmol of methylatable residues/mg of protein/day in erythrocytes (Table III). Because the rate at which aspartyl and asparaginyl residues arise in proteins should not decrease during the life of a mouse, we can use these rates to estimate the total number of damaged residues arising in adult mice. Assuming that a mouse is 15% protein by weight and that half of this protein is intracellular, a 20-g mouse would have 1.5 g of intracellular protein. If the average rate of damaged residue formation throughout the mouse is between 5 and 20 pmol/mg/day, as predicted from the values in Table III, there should be 7.5–30 nmol of newly damaged residues arising each day within the cells of an adult mouse.

Damaged Aspartyl Residues in Urine—The absence of increasing accumulation of damaged aspartyl residues in the cytosolic proteins of adult mice can result from repair of the damaged residues or from turnover of the proteins. Few peptidases cleave isoaspartyl bonds, but proteolysis of the surrounding residues creates isoaspartyl-containing dipeptides and tripeptides that can be excreted in the urine (33–36). Thus, if mice lacking endogenous PCMT1 do not have another repair pathway, they might excrete the damaged residues that are normally repaired in the cells of wild-type mice. To test this hypothesis, we examined urine from Pcmt1−/− and Pcmt1+/+ mice in several ways.

First, we directly quantitated urinary dipeptide levels after derivatization with o-phthalaldehyde and β-mercaptoethanol and separation by reverse-phase high pressure liquid chromatography. We did not detect significantly higher levels of isoaspartyl-containing dipeptides in urine from Pcmt1 knockout mice in several ways.

Testis (Fig. 6). Because erythrocytes are normally removed from the circulation after about 40 days (32), their average age in adult mice is constant, and older proteins cannot accumulate. Unexpectedly, the accumulation of damaged aspartyl residues in all of these tissues was quite similar. As in brain, the number of methylatable residues per mg protein increased only in animals younger than about 60 days and then leveled off as the mice got older (Figs. 4–6). The apparent plateau levels of damaged aspartyl residues in heart, testes, and erythrocytes from transgenic Pcmt1−/− mice averaged 4.5-, 4.7-, and 5.2-fold higher, respectively, than the average levels in tissues from mice expressing the endogenous methyltransferase, very similar to the 4.5-fold difference observed in brain. However, the absolute plateau level of damage in each tissue was significantly different, with brain having the highest and erythrocytes having the lowest levels in both the presence and in the absence of endogenous PCMT1 (Table II). As expected, the transgenic PCMT1 had no effect in tissues where it is not expressed.

The rates at which damaged residues in cytosolic proteins accumulated in young Pcmt1−/− mice have been calculated from the data in Figs. 3–6. These rates differ greatly between
mice (data not shown). For example, isoaspartylglycine ("β-
aspartylglycine"), the most abundant of these dipeptides in human urine (35), was present at 28.2 pmol/μg creatinine in urine from a 91-day-old Pcmt1−/− mouse and at 27.5 pmol/μg creatinine in urine from a 40-day-old wild-type mouse. These results suggest that much of this dipeptide might originate in dietary, extracellular, or unrepairable intracellular proteins rather than from the lack of repair in Pcmt1−/− cells.

Second, peptides with an N-terminal isoaspartyl residue might accumulate because the isopeptide bond is resistant to aminopeptidase activity. To determine whether more of these peptides accumulated in Pcmt1−/− than in Pcmt1+/+ mice, we compared the urinary levels of free amino acids and of peptides accumulated in Pcmt1−/− mice (data not shown). For example, isoaspartylglycine ("β-
aspartylglycine"), the most abundant of these dipeptides in human urine (35), was present at 28.2 pmol/μg creatinine in urine from a 91-day-old Pcmt1−/− mouse and at 27.5 pmol/μg creatinine in urine from a 40-day-old wild-type mouse. These results suggest that much of this dipeptide might originate in dietary, extracellular, or unrepairable intracellular proteins rather than from the lack of repair in Pcmt1−/− cells.

Second, peptides with an N-terminal isoaspartyl residue might accumulate because the isopeptide bond is resistant to aminopeptidase activity. To determine whether more of these peptides accumulated in Pcmt1−/− than in Pcmt1+/+ mice, we compared the urinary levels of free amino acids and of peptides accumulated in Pcmt1−/− mice (data not shown). For example, isoaspartylglycine ("β-
aspartylglycine"), the most abundant of these dipeptides in human urine (35), was present at 28.2 pmol/μg creatinine in urine from a 91-day-old Pcmt1−/− mouse and at 27.5 pmol/μg creatinine in urine from a 40-day-old wild-type mouse. These results suggest that much of this dipeptide might originate in dietary, extracellular, or unrepairable intracellular proteins rather than from the lack of repair in Pcmt1−/− cells.

Second, peptides with an N-terminal isoaspartyl residue might accumulate because the isopeptide bond is resistant to aminopeptidase activity. To determine whether more of these peptides accumulated in Pcmt1−/− than in Pcmt1+/+ mice, we compared the urinary levels of free amino acids and of peptides accumulated in Pcmt1−/− mice (data not shown). For example, isoaspartylglycine ("β-
aspartylglycine"), the most abundant of these dipeptides in human urine (35), was present at 28.2 pmol/μg creatinine in urine from a 91-day-old Pcmt1−/− mouse and at 27.5 pmol/μg creatinine in urine from a 40-day-old wild-type mouse. These results suggest that much of this dipeptide might originate in dietary, extracellular, or unrepairable intracellular proteins rather than from the lack of repair in Pcmt1−/− cells.

Finally, we used the recombinant t-isoaspartyl/D-aspartyl methyltransferase as an analytical probe for t-isoaspartyl residues in peptides large enough to be methylated efficiently (tetrapeptides and larger) (15). Methylatable residues were found in all of the urine samples assayed, and the level of damage increased with the age of the mouse (Fig. 7). We observed no significant difference in the number of methylatable sites, relative to the amount of creatinine, in urine from Pcmt1−/− animals and from Pcmt1+/+ and Pcmt1+/+ controls in the 13–14- and 20–21-day-old groups (Fig. 7). However, urine from older Pcmt1−/− mice contained almost 2-fold more methylatable sites than urine from control animals (Fig. 7). Assuming that 435–783-day-old mice weighing 20 g excrete 0.67 mg of creatinine per day (37), then Pcmt1−/− mice excrete about 9.5 nmol and age-matched control animals about 5.1 nmol of methylatable t-isoaspartyl residues per day. Thus, at least 4.4 nmol of damaged residues that are repaired in wild-type mice are excreted each day by knockout mice that are not accumulating additional damaged residues within their cells. This daily excretion of damaged aspartyl residues in the urine represents 15–67% of the total daily production of intracellular aspartyl damage estimated above. This result indicates that proteolysis leading to urinary excretion is a significant factor in limiting the accumulation of damaged proteins in the absence, and perhaps as well in the presence, of the repair methyltransferase.
Fig. 7. Damaged aspartyl residues in mouse urine that are recognized in vitro by the l-isoaspartyl (o-aspartyl) O-methyltransferase. Each urine specimen was collected in a single voiding and was assayed without further treatment. Methylatable damaged residues were quantitated with recombinant human methyltransferase as described in Fig. 3. Open bars, urine from nontransgenic Pcmt1−/− mice; shaded bars, urine from nontransgenic Pcmt1+/− and Pcmt1+/+ mice. The difference between the values for these groups is statistically significant for the 43–103- and 435–783-day-old mice (p = 0.006 and 0.020, respectively) but not for the 13–14- or 20–21-day-old mice (p = 0.161 and 0.283, respectively).

DISCUSSION

We have been examining mice lacking PCMT1 to determine whether ineffective repair of damaged aspartyl and asparaginyl residues contributes to disease or to the deleterious effects of aging. We report here that damaged aspartyl residues in Pcmt1−/− brain proteins from 13 to 14-day-old mice were already eight times more abundant than those in aged-matched wild-type mice and accumulated at a rate of about 52 pmol/mg of protein/day until 20–21 days. Damaged residues continued to accumulate but at a lower rate, averaging 9 pmol/mg protein/day between days 21 and 55. The rate of accumulation appeared to decrease further in the brains of older Pcmt1−/− mice, but few of these mice live beyond this age. A similar pattern of damage accumulation has been recently observed by Shimizu et al. (38) in independently derived Pcmt1−/− mice. In contrast to the Pcmt1−/− mice, however, Pcmt1+/+ and Pcmt1+/− mice kept the level of damaged residues very low for at least 2 years. Although they had only about half of the methyltransferase activity, Pcmt1+/− mice survived as long as Pcmt1+/+ mice.

The high level of PCMT1 activity in wild-type brain, the fact that damaged residues rapidly accumulate in the brain in the absence of the methyltransferase, and the seizures in the Pcmt1−/− mice suggested that this enzyme plays a critical role in normal brain function. PCMT1, however, is expressed in all mammalian tissues and presumably is involved in repair throughout the body. We were therefore also interested in examining what would happen if tissues lacking this repair were allowed to accumulate higher levels of damage over several years. To answer these questions, we performed a “brain rescue” experiment by creating mice with a Pcmt1 transgene under the control of a neuron-specific promoter in a genetic background lacking the endogenous Pcmt1 gene. Here, we obtained mice expressing the methyltransferase only in the brain. Although the expression of the transgene-derived methyltransferase activity in the brains of these mice was only 6.5–13% of the level in wild-type mice, the transgenic mice lived much longer and accumulated only half the damaged residues found in nontransgenic knockout animals. The success of this rescue experiment further supported the importance of the methyltransferase in the brain.

We reported previously (18) that mice lacking the repair methyltransferase are more sensitive to the seizure-inducing drug metrazol than are wild-type mice and that the anti-seizure drugs valproic acid and clonazepam prolonged the life span of these animals (20). It is likely that, by decreasing the accumulation of damaged aspartyl residues, the transgene-derived methyltransferase raised the seizure threshold of the rescued mice to an intermediate level between the thresholds of nontransgenic Pcmt1−/− and wild-type mice. Immunohistochemical studies revealed that the transgene-derived methyltransferase was expressed largely in neurons, in both line 27 and line 29. However, the pattern of neuronal expression was distinct in the two lines. These findings indicate that the expression of Pcmt1 in neurons (rather than glia) is paramount in the prevention of the fatal seizure disorder. At this point, however, we do not know whether the methyltransferase is critical for the function of all neurons or only for certain types of neurons.

Because many of the transgenic Pcmt1−/− mice lived for several hundred days, we were able to examine the long term accumulation of damaged residues in peripheral organs, where the methyltransferase is not expressed at all. We found no apparent defects in these mice, and initial pathological studies were unremarkable. We found that damaged aspartyl residues accumulated in an age-dependent manner only in relatively young mice and attained plateau levels by 100 days of age. At first approximation, the rate at which damaged residues arise in proteins remains constant with time; thus, the plateau levels of damage in tissues of older mice must result from processes of repair and/or turnover of these residues. We have been especially interested in the situation in mouse tissues that lack the repair methyltransferase, where the levels of damage are much greater but which still approach plateau values with age. We suggest that enhanced proteolytic degradation may limit the accumulation of proteins containing damaged aspartyl residues, especially in methyltransferase-deficient tissues. Clear evidence has been presented for the selective proteolytic degradation of spontaneously damaged calmodulin in both HeLa cells (6) and in Xenopus oocytes (39).

Isoaspartyl linkages themselves are generally not cleaved by mammalian proteases (33, 34). We have thus investigated the possibility that proteolysis of proteins containing damaged aspartyl residues in PCMT1-deficient mice may be reflected in the increased urinary output of peptides containing 1-isoaspartyl residues. In fact, damaged aspartyl residues in proteins fed

4 C. Farrar, E. Kim, S. Young, S. Clarke, and C. Houser, unpublished data.
to rats are excreted as isoaspartyl-containing dipeptides in the urine (40), and it has been proposed that at least some endogenous isoaspartyl residues are dealt with in a similar manner (41). Furthermore, an L-isoaspartyl residue that arises in collagen has been found in human urine as part of an eight-residue peptide (42). We found that urine from Pcm1−/− mice contains more peptides with damaged aspartyl residues than urine from Pcm1+/+ mouse urine. Because PCMT1 repairs intracellular proteins (14), our results provide the first evidence that damaged aspartyl residues that arise within cells can be excreted in the urine.

Is the elevated level of altered aspartyl residues in the urine from Pcm1−/− mice enough to account for the observed steady state level of damaged proteins in tissues despite continuing spontaneous generation of isoaspartyl residues? From the rates of damage accumulation measured in tissues of young Pcm1−/− mice, we estimated that 7.5–30 nmol of newly damaged aspartyl residues are generated each day. The daily excretion of damaged residues in the urine of Pcm1−/− mice was 4.4 nmol more than that in wild-type mice, demonstrating that a significant fraction of the damage can be metabolized by proteolysis. Additional isoaspartyl peptides may be present in the urine that are not readily recognized by the methyltransferase (15), although we did not detect increased levels of generally poorly recognized dipeptides and N-terminal L-isoaspartyl peptides.

Interestingly, the ability of the proteolytic pathway to stem the accumulation of damaged aspartyl residues is apparently insufficient to prevent seizures in animals lacking the repair methyltransferase. Thus, the repair methyltransferase is needed (at least in the brain) to lower the level of damaged residues beyond what the degradation mechanisms can accomplish. Furthermore, it is possible that proteolysis of proteins that contain covalent modifications important to learning and memory could have undesirable effects in that their replacement proteins would not be appropriately modified (43).

Unlike the brain, other tissues appear to be able to function relatively normally with higher levels of damaged aspartyl residues. The importance of the methyltransferase in maintaining a lower steady state level of damaged aspartyl residues in these tissues remains to be determined. It is certainly possible that mice reared under other conditions (e.g., outside of a vivarium) would be more susceptible to pathologies in the absence of the repair methyltransferase.

REFERENCES