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# Isoaspartyl Protein Damage and Repair in Mouse Retina

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**PURPOSE.** To determine the propensity of retinal proteins for spontaneous damage via formation of isoaspartyl sites, a common type of protein damage that could contribute to retinal disease.

**METHODS.** Tissue extracts were obtained from retinas and brains of control mice and from mice in which the gene for protein L-isoaspartate O-methyltransferase (PIMT; an enzyme that repairs isoaspartyl protein damage) was knocked out. PIMT expression in these extracts was measured by Western blot, and its specific activity was assayed by monitoring the rate of [<sup>3</sup>H]methyl transfer from S-adenosyl-[methyl-<sup>3</sup>H]L-methionine to  $\gamma$ -globulin. Isoaspartate levels in extracts were measured by their capacity to accept [<sup>3</sup>H]methyl groups via the PIMT-catalyzed methylation reaction. To compare molecular weight distributions of isoaspartyl-rich proteins in retina versus brain, proteins from PIMT knockout (KO) and control mice were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). Isoaspartyl proteins were <sup>3</sup>H-labeled on-blot using a PIMT overlay and imaged by autoradiography.

**RESULTS.** When normalized to the  $\beta$ -actin content of each tissue, retina was found to be nearly identical to brain with regard to expression and activity of PIMT and its propensity to accumulate isoaspartyl sites when PIMT is absent. The two tissues show distinct differences in the molecular weight distribution of isoaspartyl proteins.

**CONCLUSIONS.** The retina is rich in PIMT activity and contains a wide range of proteins that are highly susceptible to this type of protein damage. Recoverin may be one such protein. Isoaspartate formation, along with oxidation, should be considered as a potential source of protein dysfunction and autoimmunity in retinal disease.

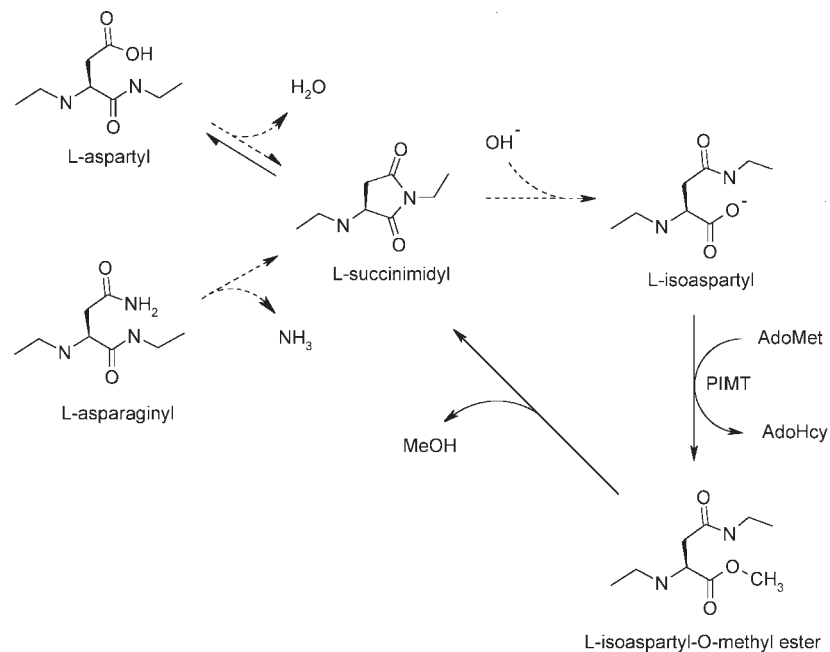
Keywords: protein damage, protein repair, isoaspartate, retina, brain

Isoaspartate (isoAsp) formation, through deamidation of asparaginyl residues or isomerization of aspartyl residues, constitutes a large proportion of spontaneous protein damage observed both in vitro and in vivo.<sup>1-6</sup> Generation of isoAsp sites is initiated by nucleophilic attack on the side-chain carbonyl of aspartate (Asp) or asparagine (Asn) by the C-flanking amide bond nitrogen, resulting in an intermediate succinimide (Fig. 1). Protein L-isoaspartate O-methyltransferase (PIMT; EC 2.1.1.77) selectively methylates the  $\alpha$ -carboxyl group of L-isoaspartyl residues, and the isoAsp methyl ester formed spontaneously demethylates to reform a succinimide that can restore a normal  $\alpha$ -linked Asp-Xaa bond.<sup>7-11</sup> Continuing cycles of PIMT action efficiently repair L-isoAsp sites, as has been demonstrated in vitro with a number of peptides and proteins.<sup>12-15</sup> A repair function for PIMT in vivo is supported by the observation that reduction of PIMT activity in cultured cells or PIMT knockout (KO) mice dramatically increases the level of isoAsp-containing proteins.<sup>16-19</sup> A critical need for PIMT action in the brain is evident by its high specific activity in this tissue<sup>20,21</sup> as well as the overt neurological phenotype of PIMT KO mice: increased brain size, abnormal neuroanatomical and electrophysiological properties of hippocampal cells, atypical open-field behavior, and fatal epileptic seizures beginning at 4 weeks of age.<sup>17,18,22-24</sup> Perhaps because of its small size, the retina of the PIMT KO mouse has apparently received no attention with regard to alterations in its morphology, physiology, biochemistry, or function. Remarkably,

no published studies to date mention any evidence for a vision deficit in the PIMT KO mouse. Such a deficit could be masked by (or consistent with) their atypical behavioral characteristics and the fact that olfactory and tactile functions serve them well in a caged environment.

In mammalian tissues, the specific activity of PIMT varies over a 10-fold range, with highest levels to date found in the brain and testis.<sup>17,18,20</sup> Given that the retina is an extension of the central nervous system, one would expect that it might also exhibit relatively high PIMT activity and potential for isoaspartyl protein damage. Modest PIMT activity has been reported in homogenates of whole eye and lens<sup>18,25</sup>; but to our knowledge, PIMT activity in the isolated vertebrate retina has never been investigated. This is unfortunate because isoaspartyl damage not only has the capacity to disrupt protein function, but also can trigger an immune reaction to self-proteins.<sup>26,27</sup> Either of these effects could potentially play a role in the development or exacerbation of eye diseases such as macular degeneration, autoimmune retinopathies, and retinal uveitis.

Because of their high metabolic demands and photosensitivity, retinal cells are subject to a high burden of oxidative stress that promotes oxidative damage of proteins and lipids.<sup>28,29</sup> A number of recent studies suggest that protein oxidation and isoAsp formation interact synergistically. Oxidation of purified hemoglobin with acetylphenylhydrazine produces a rapid increase in its isoAsp content, and exposure of erythrocytes to hydrogen peroxide has been found to induce



**FIGURE 1.** Mechanism of isoaspartate formation and PIMT-catalyzed repair. Under physiological conditions, deamidation of asparaginyl residues or isomerization of aspartyl residues results in the formation of an intermediate succinimide. Upon spontaneous hydrolysis, a mixture of L-Asp (~15%–30%) and atypical L-isoAsp (~70%–85%) linkages is produced. Using AdoMet as a methyl donor, PIMT then selectively methylates the isoaspartyl  $\alpha$ -carboxyl group to form a labile methyl ester. Spontaneous demethylation occurs within minutes to reform the original succinimide, with release of methanol as a by-product. This succinimide is now the starting point for further cycles of repair, resulting in near complete conversion of the isoaspartyl  $\beta$ -linkages to normal aspartyl  $\alpha$ -linkages. *Broken lines* represent the degradative pathway, and *solid lines* represent the repair pathway.

isoAsp formation in membrane proteins.<sup>30,31</sup> Ultraviolet A radiation has been found to trigger isoAsp formation in melanoma cells, while natural antioxidants such as hydroxytyrosol (found in olive oil) protect melanoma cells against both oxidative damage and isoAsp accumulation.<sup>32</sup> Cellular levels of PIMT can greatly affect the susceptibility of cells to apoptosis induced by oxidative stress, as observed in cultured human endothelial cells and the nematode *C. elegans*.<sup>33,34</sup> This synergism between two common forms of protein damage creates a strong rationale for investigating the potential role of isoAsp formation as a contributor to retinal dysfunction.

We report here the use of PIMT +/+ (WT, wild type),  $\pm$  (HZ, heterozygote), and –/– (KO) mice to investigate the expression and activity of PIMT in isolated retina, as well as the propensity of the retina to accumulate isoAsp-damaged protein when PIMT activity is absent. Our results confirm the expectation that mouse retina exhibits both a robust PIMT activity and a propensity to accumulate isoaspartyl proteins that rivals that of brain. This suggests that isoaspartyl protein damage, like protein oxidation, should be considered as a possible factor in one or more retinal diseases. It also raises the possibility that diminished visual function may be an unrecognized facet of the PIMT KO mouse phenotype.

## METHODS

### Mice

The PIMT KO mice used here originated from a line developed and described by Kim et al.<sup>17</sup> To start our colony, male PIMT HZ mice were kindly provided by Mark Mamula (Section of Rheumatology, Yale University School of Medicine) and mated with female C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME). Genotyping was done by Transnetyx, Inc. (Cordova, TN) using real-time PCR analysis of tail DNA with probes for both the neo cassette and the

*Pcmt1* gene. All mice were housed in groups of mixed genotypes and had access to food and water ad libitum in a colony room kept at 19°C to 22°C and 40% to 60% humidity, under a 12:12-hour light/dark cycle. Procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by University Laboratory Animal Resources of the University of California, Irvine. The mouse procedures were also in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Preparation of Tissue Extracts

Mice were anesthetized with Euthasol (Virbac Corporation, Fort Worth, TX) and euthanized by decapitation at an age of 4 weeks. Brains were rapidly removed and homogenized as described below. Eyes were removed and retinas freed from surrounding ocular tissues under a dissecting microscope. Because of their small size, retinas (six total from three mice of each genotype) were pooled prior to homogenization. Brains were also pooled (three brains per genotype, but pooling was done after each individual brain was homogenized). Tissues were homogenized in a buffer composed of 5 mM K-HEPES pH 7.6, 0.5 mM EDTA, 10% sucrose, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM dithiothreitol (all from Fisher Scientific, Waltham, MA), and 1% mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged at 800g for 30 minutes at 4°C. The supernatants (hereafter “extracts”) were collected, and protein concentration was determined with a bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA). Samples were stored in aliquots at –80°C.

### Western Blot Analysis

Mouse brain and retina extracts were subjected to SDS-PAGE on a NuPAGE 10% Bis-Tris gel (Life Technologies, Carlsbad,

CA). After semidry transfer to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA), the membrane was blocked in 5% nonfat milk and probed simultaneously with primary antibodies against PIMT (1:3000; custom polyclonal [produced in house] against bovine brain PIMT) and  $\beta$ -actin (1:20,000, Cat. No. 4970S; Cell Signaling Technologies, Danvers, MA). After incubation with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (GE Healthcare, Pittsburgh, PA), detection was performed with enhanced chemiluminescence (ECL) Plus reagents (Thermo Scientific) using a Nikon D700 camera with subsaturating exposure times.<sup>35</sup> Band densities were determined with ImageJ (version 1.41 for Mac OSX; National Institutes of Health, Bethesda, MD) and corrected for background. Immunoblotting for  $\beta$ -actin was carried out because it is a very common loading control for these types of studies. As shown in our results, this proved to be especially important in comparing extracts of brain and retina.

### PIMT Assays, IsoAsp Assays, and On-Blot [<sup>3</sup>H]Methylation of IsoAsp Proteins

The assays for PIMT activity and isoAsp content in mouse tissue extracts were carried out as previously described for human brain extracts with minor modifications.<sup>36</sup> The PIMT assays measure the initial rate at which [<sup>3</sup>H]methyl groups are transferred from S-adenosyl-[methyl-<sup>3</sup>H]L-methionine ([<sup>3</sup>H]AdoMet) to  $\gamma$ -globulin, a known substrate for PIMT. Each 50- $\mu$ L reaction contained 10  $\mu$ g pooled mouse tissue extract protein, and reactions were carried out in triplicate.

For isoAsp assays, tissues extracts were incubated with [<sup>3</sup>H]AdoMet and sufficient recombinant PIMT to completely methylate all the available endogenous isoAsp sites. Each 18- $\mu$ L reaction contained 15  $\mu$ g pooled mouse tissue extract and 5  $\mu$ M PIMT, and reactions were carried out in triplicate.

Detection of isoAsp-containing proteins separated by SDS-PAGE was performed by on-blot PIMT-catalyzed [<sup>3</sup>H]methylation as previously described.<sup>37,38</sup> A Typhoon Trio+ variable mode imager (GE Healthcare) was used to capture the image from a tritium-sensitive phosphorimager screen.

We note that the all the assays mentioned above were carried out in buffers of low pH (6.0–6.2) to minimize loss of the <sup>3</sup>H-label via spontaneous demethylation that occurs rapidly at neutral and alkaline pH.<sup>38,39</sup>

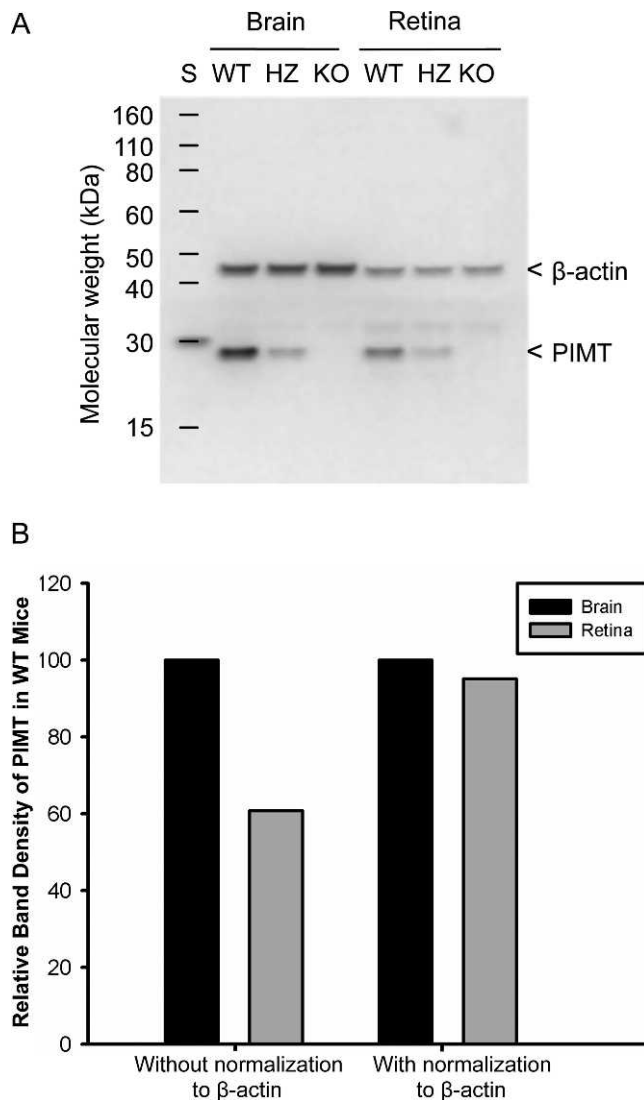
### Statistical Analysis

Comparisons were made with the two-tailed Student's *t*-test. Differences were considered significant when  $P < 0.05$ .

## RESULTS

### PIMT Expression in Retina Versus Brain of PIMT WT, HZ, and KO Mice

To explore PIMT expression levels, equal amounts of pooled extract protein from retina or brain of PIMT WT, HZ, and KO mice were examined by Western blot (Fig. 2A). The blot was developed with a mixture of two primary antibodies, one against PIMT and the other against  $\beta$ -actin. As expected for brain extracts, WT mice showed strong bands (lane 1) for PIMT (24.5 kDa) and  $\beta$ -actin (41.6 kDa), while extracts from KO mice (lane 3) showed only a band for  $\beta$ -actin. The PIMT band in brain extracts from the PIMT HZ mice had an intensity that was roughly 50% of that in the WT mice, in line with previous observations of both PIMT expression and activity in these mice. ECL signals for retina (lanes 4–6) paralleled those for the

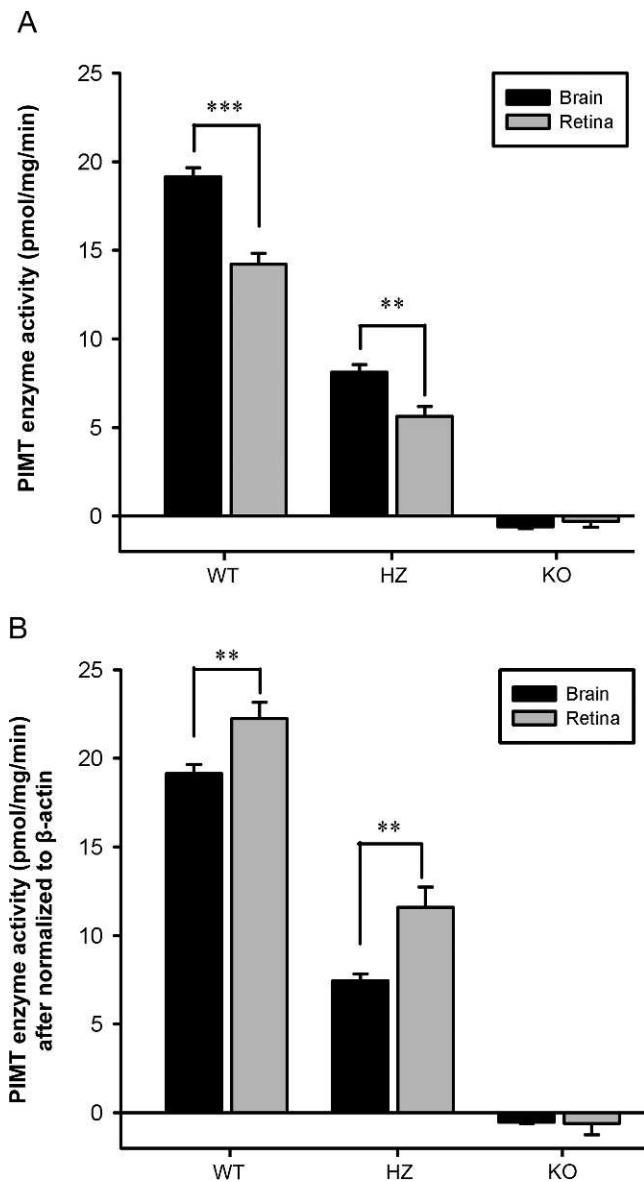


**FIGURE 2.** PIMT expression in brain and retina of WT, HZ, and KO mice. (A) Western blot showing expression of PIMT in brain and retina. Each lane represents 10  $\mu$ g extract from a pool of three brains, or a single pool of six retinas from three mice, of the indicated genotype. (B) Band densities of WT samples from (A) before (*left*) and after (*right*) normalization to the  $\beta$ -actin band in the same lane. The band density of the brain sample was set to 100 and the retina values were adjusted accordingly.

brain, except that band intensities for both PIMT and  $\beta$ -actin were reduced by approximately 40% (Fig. 2B, left half) even though equal amounts of brain and retina extract protein had been loaded onto the gel. When the Western signals for PIMT were normalized to  $\beta$ -actin, PIMT expression in retina was roughly equal to that in brain (Fig. 2B, right half).

### PIMT Enzyme Activity in Retina Versus Brain

The same pooled extracts used for Figure 2 were used to determine PIMT enzyme activity under initial rate conditions using saturating levels of bovine  $\gamma$ -globulin as the methyl acceptor (Fig. 3). As expected, PIMT activity paralleled PIMT expression. When normalized to total protein, the specific activity of PIMT in retina was 70% of that seen in brain (Fig. 3A); but when corrected for relative expression of  $\beta$ -actin (using the  $\beta$ -actin band intensities in Fig. 2A), the specific

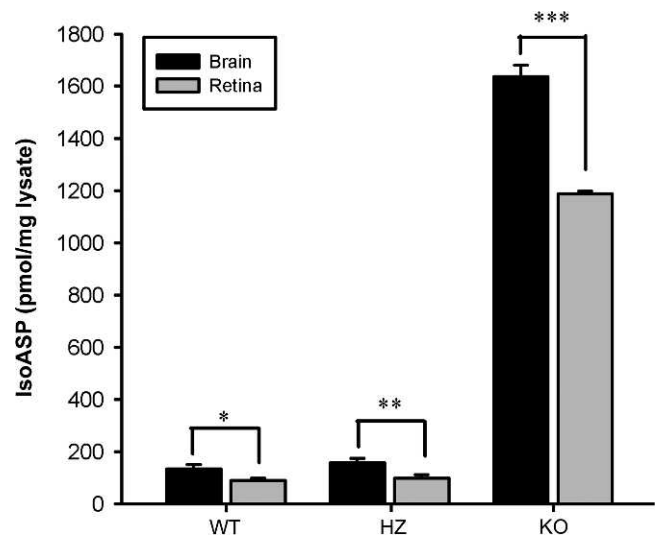


**FIGURE 3.** PIMT specific activity in brain and retina of WT, HZ, and KO mice. (A) Enzyme specific activity without normalization to relative  $\beta$ -actin levels. (B) Enzyme specific activity with normalization to relative  $\beta$ -actin levels. Enzyme assays for each tissue pool (as in Fig. 2) were run in triplicate. Error bars indicate the standard deviation of mean of the triplicate assays. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

activity of PIMT in retina of the WT mice actually exceeded that in brain by 16% ( $P = 0.05$ ) (Fig. 3B).

### Accumulation of IsoAsp in Retina Versus Brain of PIMT KO Mice

IsoAsp levels in brain extracts of 4- to 5-week-old PIMT KO mice are 5 to 12 times those found in WT mice due to lack of repair.<sup>17,18,40</sup> To measure isoAsp levels, tissue extracts were incubated with recombinant PIMT in the presence of [<sup>3</sup>H]AdoMet for 30 minutes in a pH 6.2 buffer. The protein methyl esters were isolated by precipitation with trichloroacetic acid and then hydrolyzed at alkaline pH. The resulting [<sup>3</sup>H]methanol was recovered by passive diffusion and quantitated by liquid scintillation counting. A comparison of methyl-accepting capacity for brain and retina extracts is shown in



**FIGURE 4.** Accumulation of L-isoaspartyl residues in extracts of brain and retina of PIMT WT, HZ, and KO mice. IsoAsp levels were determined by a methanol diffusion assay as described in Methods. Assays for each pool (as in Fig. 2) were run in triplicate.

Figure 4. The increased levels of isoAsp proteins in the retina and brain of KO mice are consistent with the results showing decreased PIMT activity in these tissues (Fig. 3B). For brain, PIMT deletion increased isoAsp to 12.5 times that in the WT mouse. A similar fold increase of 13.7 times is seen for retina. These data, which were not adjusted for  $\beta$ -actin levels, show that retinal proteins have a high propensity for isoAsp formation that equals, or slightly exceeds, that in brain.

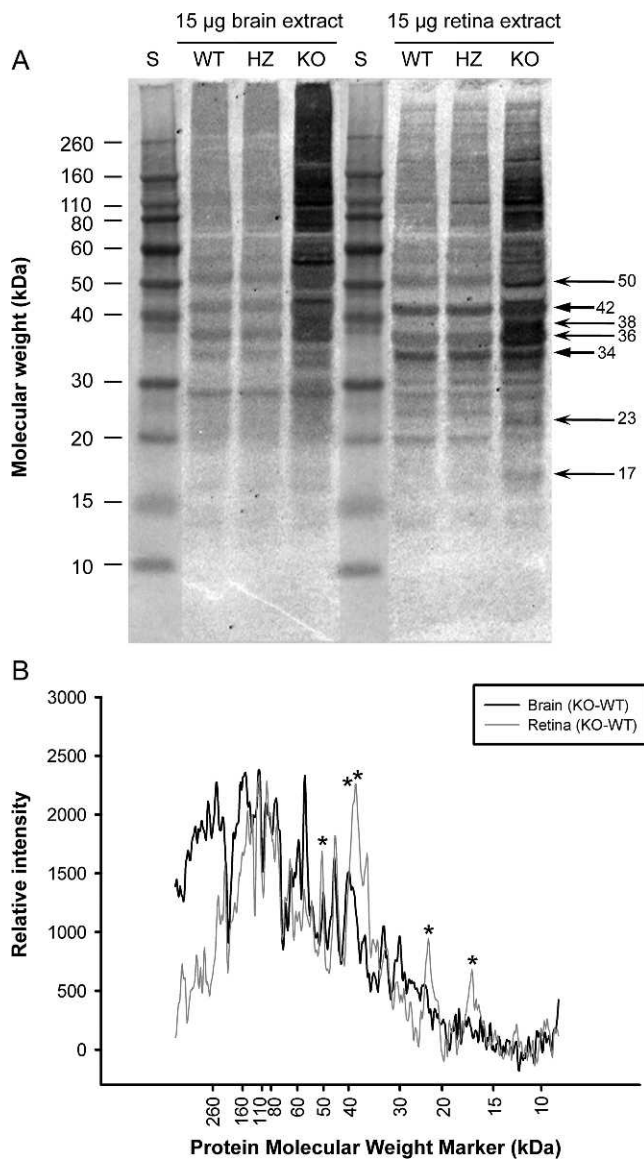
### Molecular Weight Distribution of Isoaspartyl Proteins in Retina Versus Brain

We compared patterns of isoaspartyl protein accumulation in retina versus brain by SDS-PAGE followed by on-blot [<sup>3</sup>H]methylation and autoradiography (Fig. 5A). In agreement with results shown in Figure 4, KO mice had significantly more isoaspartyl protein than WT or HZ mice in both retina and brain. Although overall levels of protein damage in retina and brain of the KO mice appear similar, the molecular weight profiles are clearly different. The molecular mass arrows on the right side of Figure 5A draw attention to seven bands in the KO-retina lane that appear to be more intensely labeled than the corresponding regions of the KO-brain lane. The five long arrows point to bands that appear to be endogenous substrates for PIMT because the labeling is more intense in the KO lane than in the WT or HZ lanes. The two short arrows point to bands where labeling is roughly equal in the retina lanes for all three genotypes. These latter two proteins probably exist in compartments such as the extracellular space or intracellular membrane-bounded compartments that do not contain any PIMT. Figure 5B shows superimposed scans of the KO-brain and KO-retina lanes from Figure 5A after subtraction of scans for the corresponding WT lanes. The five PIMT substrates indicated in Figure 5A are marked with an asterisk.

## DISCUSSION

### Retinal Substrates for PIMT

The long arrows in Figure 5A point to bands that appear to be endogenous PIMT substrates enriched in retina compared to



**FIGURE 5.** Comparison of major isoaspartyl proteins in pooled extracts of mouse brain and retina. (A) Extract samples were subjected to SDS-PAGE and transferred to a PVDF membrane. On-blot [ $^3\text{H}$ ]methylation by PIMT overlay was carried out as described in Methods. On the *right edge* are marked the positions of retinal protein bands that appear to be richer in retina than in brain. Here, the *longer arrows* indicate proteins whose isoAsp content increases significantly when PIMT is absent, while the *shorter arrows* indicate proteins whose isoAsp content seems to be relatively independent of PIMT status. (B) Densitometric difference scans (KO-WT) scans of brain and retina. Marked (*asterisk*) peaks indicate bands corresponding to the *long arrows* in (A), that is, retina-enriched proteins whose isoAsp content is sensitive to PIMT activity.

brain. A proteomic analysis of retinal PIMT substrates utilizing on-blot methylation on two-dimensional (2D) gels should allow identification of these and other isoAsp-prone proteins in the retina as has already been done for mouse brain.<sup>37</sup> A general proteomic analysis of normal rat retina employing 2D gels in combination with peptide mass fingerprinting has previously identified 145 retinal proteins, 6 of which were categorized as dedicated to “visual function.”<sup>41</sup> One of these latter proteins (recoverin) has a molecular weight (23.3 kDa) consistent with the band marked at 23 kDa in Figure 5A. As highlighted in the

sequence alignment of Figure 6, both mouse and human recoverin contain multiple hot spots (Asp or Asn linked to Gly or Ser) for isoAsp formation. Based on the crystal structure of recombinant human recoverin (Protein Data Base [PDB] ID: 2D8N; available in the public domain at <http://www.rcsb.org/pdb>), all of the highlighted residues in both mouse and human recoverin reside in relatively unstructured regions of the polypeptide chain (see also online, the Secondary Structure details for human recoverin at [expasy.org](http://expasy.org) [in the public domain], Accession No. P35243). This combination of sequence and presumed flexibility supports the idea that recoverin may be one of several major targets for PIMT in the retina. Moreover, the putative identity of recoverin in Figure 5 is consistent with its abundance in retina, as gleaned from the 2D gel study cited above in which recoverin was easily detected with Sypro Ruby dye, and exhibited an unusually strong sequence coverage (67%) in peptide mass fingerprinting.

### IsoAsp Formation and Retinal Disease

Protein oxidation and immune dysregulation (autoimmunity and inflammation) have been strongly implicated in retinal diseases such as macular degeneration and autoimmune retinopathy. IsoAsp formation, like oxidation, is a common form of covalent protein damage, and may even exceed protein oxidation in its prevalence; yet, outside of the biopharmaceutical industry, isoAsp formation is often overlooked as a significant source of protein damage.<sup>6</sup> The low profile of isoAsp formation can be attributed to the subtle nature of this modification and the resulting technical challenges to its detection. Oxidation typically generates a polypeptide mass change (e.g., +16 for oxidation of methionine to its sulfoxide) that can be detected easily by even the simplest forms of mass spectrometry. Oxidized amino acids can be released and detected during Edman degradation and recognized by their unique retention times in chromatographic separations. Carbonylated proteins can be conveniently detected on immunoblots using kits that employ antibodies to their dinitrophenyl derivatives. IsoAsp proteins do not contain an atypical amino acid per se; rather they contain an unusual ( $\beta$ -linked) peptide bond between an Asp and its C-flanking neighbor (Fig. 1). IsoAsp formation gives rise to a mass change of only +1 (for Asn > isoAsp) or no change at all (for Asp > isoAsp). Edman degradation is of limited value because sequencing stops at the isopeptide bond.<sup>42</sup> Advanced applications of mass spectrometry have been used to locate isoAsp sites in polypeptides,<sup>39,43</sup> but these techniques are not widely accessible and are more qualitative than quantitative. A popular technique for detecting and quantitating isoAsp sites in proteins uses the PIMT enzyme to selectively tag the atypical  $\alpha$ -carboxyl with a [ $^3\text{H}$ ]methyl group. The methylation approach works well in several formats as long as one takes into account that the protein methyl ester formed at the most common hot spots are highly unstable, with half-lives as short as 7 minutes (at pH 7.4, 37°C) for the methyl ester of -isoAsp-Gly- sequence.<sup>38,39,44</sup>

IsoAsp formation can disrupt cell function by two different mechanisms: by directly affecting the biological activity of the protein, or by triggering an autoimmune reaction to the protein. Direct functional damage via isoAsp formation has been found in numerous proteins including calmodulin,<sup>45</sup> the bacterial phosphocarrier protein HPr,<sup>15</sup> ribonuclease A,<sup>14</sup> the antigen binding sites of various antibodies,<sup>46,47</sup> and recently 4EBP2,<sup>48</sup> a protein that regulates gene expression at the level of mRNA translation initiation. IsoAsp accumulation has also been associated with altered patterns of protein phosphorylation and acetylation in mouse brain and embryonic kidney

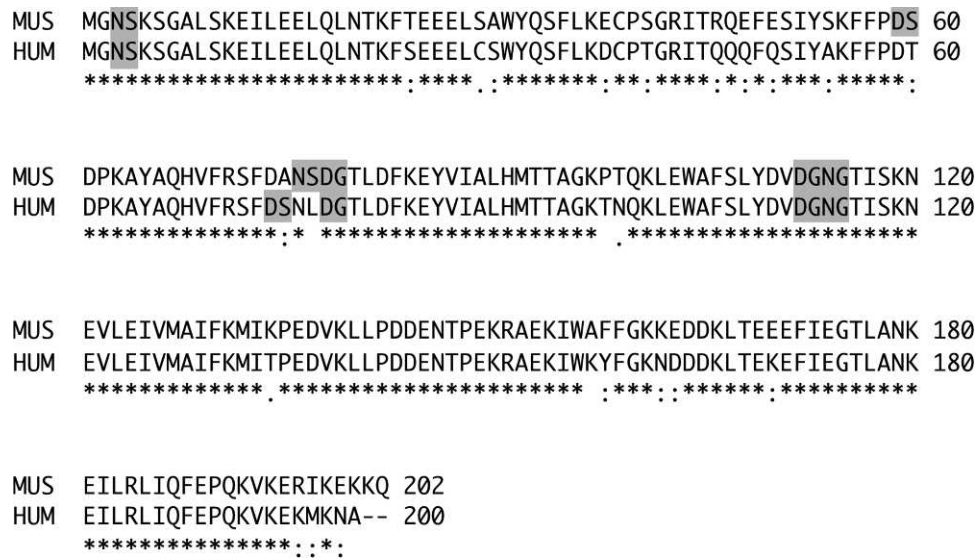


FIGURE 6. Sequence alignment of mouse (MUS: UniProtKB P34057; from the UniProt Knowledgebase available in the public domain at <http://www.uniprot.org>) and human (HUM: UniProtKB P35243) recoverins. IsoAsp “hot spot” sequences are *highlighted*.

cells.<sup>19,49,50</sup> In 2006 we identified 22 endogenous substrates for PIMT in the PIMT KO mouse brain.<sup>37</sup> Many of these proteins, such as synapsin 1 and creatine kinase B, are also found in the retina. If isoAsp formation in these proteins leads to brain dysfunction, it may lead also to retinal dysfunction.

Under what circumstances would isoAsp damage be relevant to retinal diseases? The answer is 2-fold: genetic mutations that lower PIMT activity, and advanced age. To date there are no reported cases of severe PIMT deficiency in humans; but if and when such individuals are discovered, we predict from the results presented here that they would have significant pathology in both the brain and the retina. In this regard it is interesting to point out that retinal function in the PIMT KO mouse has apparently never been assessed.

There are currently 11 known nonsynonymous single nucleotide polymorphisms (SNPs) in the PIMT of humans. One of these, corresponding to position 119 in the amino acid sequence, has two alleles (Val and Ile) that are both extremely common. Neither allele is firmly associated with any pathology, but the Clarke lab at UCLA has evidence that individuals who are heterozygous at position 119 may age more successfully

than either of the two homozygotes.<sup>51</sup> Our lab is planning to examine the PIMT gene status in children with a variety of neurodevelopmental disorders to see if a PIMT deficiency might play a role in a subset of these conditions. If such individuals are found, we would not be surprised to find visual acuity deficiencies as part of their phenotype.

With regard to aging, we have recently found interesting differences between PIMT WT and HZ mice. The latter mice have 50% to 55% of the PIMT activity found in the WT, indicating a simple gene dosage affect. Published reports to date have revealed little difference in the phenotype of the WT and HZ mice over their entire life span of 2 years. However, we have recently found that PIMT activity declines with age in the mouse brain and that the decline is somewhat greater in the HZ than the WT mice. Importantly, we also find that isoAsp levels increase six times faster with age in the brains of male HZ versus WT mice. Loss of cognitive ability and loss of retinal function are both hallmarks of advanced age in humans. Individual variation in PIMT levels was  $\pm 24\%$  of the mean in a study of 299 healthy individuals from Minnesota.<sup>52</sup> Individual variations over this range of PIMT levels, plus the tendency of

TABLE. Overlap of Human Retinal Autoantigens With Major PIMT Targets in Mouse Brain

Protein	Retinal Uveitis <sup>53</sup>	Autoimmune Retinopathy <sup>54</sup>	Age-Related Macular Degeneration <sup>55</sup>	PIMT Targets in Mouse Brain <sup>37</sup>
Actin-β	✓			✓
Aspartate aminotransferase	✓			✓
Creatine kinase B	✓			✓
Esterase D	✓			-
Retinol binding protein 3*	✓		✓	-
Tubulin-β	✓			✓
Voltage-dependent anion-selective channel	✓			✓
Carbonic anhydrase II		✓		✓
Enolase-α		✓		✓
HSC-70		✓		✓
Recoverin*		✓		-
Transducin-α*		✓		-
Aldolase C			✓	✓
Pyruvate kinase M			✓	✓

\* Proteins presumed to be highly enriched in retina compared to brain.

PIMT levels to decline with age, could make a significant contribution to the variability of age-related cognitive decline and retinal diseases in humans.

### IsoAsp and Autoimmunity

The pioneering work of Mamula and colleagues<sup>26,27</sup> has shown that the presence of isoAsp in a protein can greatly increase its ability to break tolerance in the innate immune system. In an early demonstration of this, the isoaspartyl form of a mouse cytochrome C-peptide was able to trigger an immune response directed at multiple epitopes on the mouse's own intact cytochrome C. Serum antibodies to retinal self-proteins have been documented in several eye diseases including uveitis, autoimmune retinopathy, and age-related macular degeneration. A compilation of retinal antigens reported in selected studies is presented in the Table. In the far right column of the Table we indicate which of these proteins were also found to be major targets of PIMT in our proteomics study of the PIMT KO mouse brain. Of the 14 retinal autoantigens listed, 11 are ubiquitous, while the 3 proteins marked with an asterisk (\*) are presumed to be highly enriched in retina relative to brain. Of the 11 ubiquitous autoantigens, all but one (esterase D) are among the 22 proteins identified as major PIMT targets in the KO mouse brain. This high degree of overlap is consistent with the idea that the antigenicity of autoimmune proteins in the retina (and perhaps other segments of the eye) arises in part because of their high propensity for isoAsp formation.

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