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Differences in α-Crystallin isomerization reveal the activity of protein isoaspartyl methyltransferase (PIMT) in the nucleus and cortex of human lenses

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ABSTRACT

Although it is well-known that protein turnover essentially stops in mature lens fiber cells, mapping out the ensuing protein degradation and its effects on lens function over time remains challenging. In particular, isomerization is a common, spontaneous post-translational modification that occurs over long timescales and generates products invisible to most analytical methods. Nevertheless, isomerization can significantly impact protein structure, function, and solubility, which are all necessary to maintain clarity and proper refractive index within the lens. Herein, we examine the degree of isomerization occurring in crystallin proteins in the human eye lens as a function of both age and location within the lens. A novel mass spectrometric technique leveraging radical chemistry enables detailed characterization of proteins extracted from the cortex and nucleus of the lens. It is observed that the degree of isomerization increases significantly between the cortex and nucleus and between water-soluble and water-insoluble fractions. Interestingly, the abundance of L-isoAsp is low in the water-soluble cortex despite being the dominant product generated by isomerization of Asp *in vitro*, suggesting that Protein L-isoaspartyl methyltransferase (PIMT) is active in the cortex and suppresses the accumulation of L-isoAsp. The abundance of L-isoAsp increases dramatically in the nucleus, revealing that PIMT activity decreases over time in the center of the lens. In addition, the growth of L-isoAsp in the nuclear fraction suggests protein isomerization continues within the nucleus, despite the fact that most of the protein within the nucleus has become insoluble. Additionally, it is demonstrated that sequential Asp residues lead to isomerization hotspots in human crystallin proteins and that the isomerization profiles for αA and αB crystallin are notably different. Although αA is more prone to isomerization, αB loses solubility more rapidly upon modification. These differences are likely related to the distribution of Asp residues within αA and αB, which are in turn connected to refractive index. The high Asp content of αA is a hazard in terms of isomerization and aging, but it serves to enhance the refractive index of αA relative to αB, and may explain why αA is only found in the eye.

1. Introduction

Proteins within the human lens help provide the requisite refractive index needed for sight and inhibit molecular aggregation to particle sizes capable of scattering light (Horwitz et al., 1998; Rao et al., 1995). Given the lack of organelles within mature lens fiber cells, these functions must be performed in the absence of significant protein renewal or turnover (Samuel Zigler and Goosey, 1981). The proteins in the lens must therefore remain functional over significant lengths of time. The human lens also continues to grow throughout life, with newly synthesized cells continuously adding to the periphery (Augusteyn, 2007). Consequently, the approximate age of proteins within the lens is determined by their spatial location. The oldest proteins will be located in the central nucleus, while younger proteins will be found in the peripheral cortex. Crystallins constitute nearly 90% of the total water-soluble protein content in the lens fiber cells (Groenen et al., 1994). Protein degradation due to aging is thought to lead to loss of function in diseases such as cataracts and presbyopia, but complete characterization of all age-related protein post-translational modifications (PTMs) that might contribute to these maladies has proven elusive.

Among the major protein decay pathways, the mass-shifting PTMs in crystallins have been studied extensively, including disulfide bond formation, oxidation, phosphorylation, deamidation, and truncation (Ma et al., 1998). More subtle modifications such as epimerization and isomerization, which do not yield easily detectable mass-shifts, have received less attention. Epimerization is a specific type of isomerization

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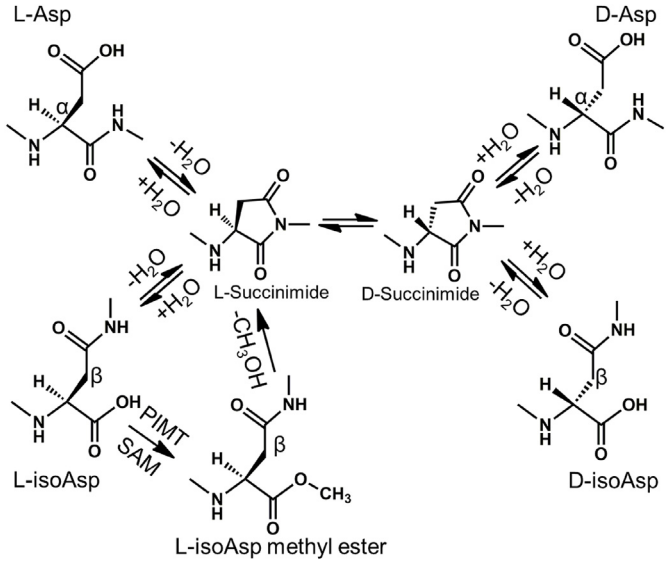

Fig. 1. Spontaneous aspartic acid isomerization via a succinimide intermediate. Alpha and beta carbons are labeled. PIMT conversion of L-isoAsp back to the L-succinimide intermediate via L-isoAsp methyl ester. (After last paragraph of introduction).

that occurs when any single residue in a peptide converts from the L to D configuration at the alpha carbon position. Isomerization can also occur without affecting chirality, which in peptides occurs primarily through the formation of iso-aspartic acid (isoAsp). Importantly, recent work has suggested that, collectively, isomerization is the most prevalent class of PTM in the lens (Truscott and Friedrich, 2016). Although difficult to detect, isomerization significantly impacts protein integrity, contributing to loss of solubility and function by altering structure (Fujii et al., 2012, 2016; Lund et al., 1996; Masters et al., 1978). A study of α-crystallins in sheep lenses revealed that isomerization was highest in the disordered termini, which serve as intermolecular bridges in the formation of highly dynamic oligomers (Tao and Julian, 2014). It has also been shown that isomerization in the structured crystallin domain leads to reduced solubility (Lyon et al., 2017).

Aspartic acid is most susceptible to isomerization among the canonical amino acids, and asparagine can produce the same products via deamidation. The vulnerability of these residues is attributed to the susceptibility of the side-chain γ-carbonyl carbon to nucleophilic attack from the backbone nitrogen of the C-terminal amino acid (Fig. 1). The metastable L-succinimide ring thus formed can be hydrolyzed at one of two positions to form L-Asp or L-isoAsp. The increased acidity of the alpha-H in the succinimide ring also facilitates racemization to form D-succinimide and subsequent hydrolysis yields either D-Asp or D-isoAsp. Over time, sequences containing L-Asp will spontaneously convert into four isomeric forms. Both of the isoAsp products reroute the protein backbone through the sidechain, which induces major perturbations in protein structure (Noguchi, 2010). Protein L-isoaspartyl methyltransferase (PIMT) is the only known repair enzyme for age-related Asp isomerization. PIMT is an *S*-adenosyl-L-methionine (SAM)- dependent enzyme that methylates L-isoAsp residues and D-Asp (700-10,000-fold lower affinity), allowing partial reformation of the L-Asp form (Lowenson and Clarke, 1992; McFadden and Clarke, 1987). Gene knock-out studies have shown that PIMT-deficient mice exhibit a nine-fold increase of L-isoAsp in the brain and die from epileptic seizures between 4 and 12 weeks (Qin et al., 2015; Yamamoto et al., 1998), while overexpression of PIMT in *Drosophila* increased lifespan by nearly 30% (Chavous et al., 2001).

Isomerization is an inherently difficult PTM to detect, as isomers have similar chemical properties and identical mass. Nevertheless, new gas-phase techniques involving both ion mobility spectrometry (IMS) and tandem mass spectrometry have been developed to tackle this problem. For example, D-amino acid containing peptides (DAACP) can be site-specifically detected by changes in drift time using ion mobility spectrometry coupled to mass spectrometry IMS-MS (Jia et al., 2014). Ultrahigh resolution IMS-MS can separate amyloid beta peptides containing all four aspartyl isomers (Zheng et al., 2017). Mass spectrometry-based techniques focus on discerning isomers by creating diagnostic fragments or comparing changes in fragmentation intensities. Electron capture dissociation (ECD) and electron transfer dissociation (ETD) can distinguish Asp from isoAsp through observation of unique mass c and z-type ions (O'Connor et al., 2006). In particular, radical-directed dissociation (RDD) has proven to be well-suited for isomer detection because it can be implemented in the analysis of semi-complex samples, such as the protein complement found in the lens. In RDD experiments, a radical is created site-specifically and is then activated to cause dissociation. The radical will migrate away from the initial site by pathways dictated by the three-dimensional peptide structure. Various side-chain and backbone fragments will be generated with isomer specific intensities, allowing for isomer identification in a high-throughput fashion (Tao et al., 2012). Thus, RDD enables full characterization of isomers and epimers, which are typically invisible to traditional proteomics.

Herein, the differences in isomerization from the cortex and nucleus of aged, human lenses are detailed. Prior to examination, the two regions of each lens were separated, and then further divided based on solubility. Enzymatic digestion was performed on each of the fractions and followed by tandem LC-MS using both collision-induced dissociation (CID) and RDD to distinguish isomers. Crystallin isomerization was studied as a function of age, and the results are analyzed in terms of location within the lens, sequence, tertiary structure, and PIMT activity. Additional experiments were carried out *in vitro* to establish the intrinsic isomerization propensities and influence of PIMT for comparison with the results obtained from the lens. The most important factors influencing age-induced isomerization are revealed and discussed in relation to maintenance of lens function.

2. Methods

2.1. Protein extraction and digestion

Human lenses were acquired from the National Disease Research Interchange (NDRI) (Philadelphia, Pennsylvania). Each lens was snap frozen and sent on dry ice overnight. Upon arrival, the samples were stored at −20 °C until lens extraction was performed. The nuclei and cortices of the thawed lenses were separated using a 4 mm trephine. The endcaps of each nucleus were removed by gently scraping off the outer tissue until only the dense, nuclear portion remained. The nucleus and cortex were then homogenized in 50 mM Tris-HCl pH = 7.8. The lens fractions were then centrifuged at 15,100 g for 20 min at 4 °C to separate the supernatant from the precipitate. The supernatant (water-soluble) was purified by dialysis against water. The precipitate (water-insoluble) was solubilized in 6 M urea and purified by dialysis against 6 M urea. For water-soluble digestion, 50 μg of protein was dissolved in 50 mM NH_4HCO_3 buffer, pH = 7.8, disulfide bonds were then reduced with 1.5 μL of 100 mM DTT at 70 °C for 10 min. After returning to room temperature, reduced cysteines were capped using 3 μL of 100 mM iodoacetamide in the dark for 20 min. Finally, the proteins were digested with trypsin for 12 h at 37 °C using a 50:1 protein to enzyme ratio. For the water-insoluble digestion, 100 μg of protein was dissolved using 6 M urea in 50 mM Tris-HCl, pH = 8.0. Disulfide bonds were reduced using 5 μL of 200 mM DTT in Tris-HCl, pH = 8.0 at 37 °C for 20 min. Following this, 20 μL of 200 mM iodoacetamide in Tris-HCl, pH = 8.0 was added, and the mixture was incubated in the dark for 1 h. To consume unreacted iodoacetamide, 20 μL of 200 mM DTT was added and incubated for 1 h in the dark. Next, the urea concentration was diluted to < 0.6 M using 50 mM Tris-HCl, 1 mM $CaCl_2$, pH = 7.6. The