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Accelerated aging of Asp 58 in αA crystallin and human cataract formation

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ABSTRACT

Racemisation of amino acids is one of the most abundant modifications in long-lived proteins. In this study racemisation of Asp 58 in the small heat shock protein, αA crystallin, was investigated. In normal human lenses, levels of L-isoAsp, D-isoAsp and D-Asp increased with age, such that by age 70 they accounted for approximately half of the total Asp at this site. Levels of D-isoAsp were significantly higher in all cataract lenses than age-matched normal lenses. The introduction of D-isoAsp in αA crystallin could therefore be associated with the development of cataract. Its more rapid formation in cataract lenses may represent an example of accelerated protein aging leading to a human age-related disease.

1. Introduction

Aggregation and the accumulation of insoluble protein are characteristic features associated with aging (Alavez and Lithgow, 2012) in a diverse range of organisms from short-lived animals such as *Caenorhabditis elegans* (David et al., 2010; Reis-Rodrigues et al., 2012) to humans (Heys et al., 2007). The reason for this is not well understood but it likely reflects denaturation/modification of susceptible polypeptides that renders them unable to be degraded effectively by cell re-cycling systems such as the proteosome (Snyder and Wolozin, 2004). Although knowledge of the basis for the denaturation of proteins *in vivo* is incomplete, some features have been elucidated. Particular amino acid residues are less stable to prolonged exposure under physiological conditions and are prone to decompose over time.

Foremost amongst these amino acids are asparagine and aspartic acid. Both of these residues can undergo spontaneous cyclisation reactions leading to the formation of a succinimide (Geiger and Clarke, 1987). Once formed, the succinimide intermediate can be hydrolysed yielding 4 possible structures: D-Asp, D-isoAsp, L-Asp and L-isoAsp. It is likely that if this process is extensive, it will lead to significant protein unfolding, since even the replacement of one Asn by an L-Asp can lead to measurable changes in physical properties (Takata et al., 2008).

As an indicator of the importance of this particular reaction, a ubiquitous enzyme, protein isoaspartate methyl transferase (PIMT) is present in cells and its function is to reverse such changes by methylating D-Asp and L-isoAsp and shunting the reaction back towards L-Asp. Although PIMT is not active on one of the isomers, D-isoAsp, and it also cannot reverse the deamidation associated with cyclisation of Asn, it is nonetheless important for survival since PIMT-knockout mice suffer seizures and have reduced life expectancy (Kim et al., 1997).

Due simply to the time of exposure, long-lived proteins are likely to undergo more degradative reactions than polypeptides whose lifetimes are measured in days. Knowledge of the lifetimes of individual human proteins is typically unavailable, however it has been established that elastin in the heart and lung (Shapiro et al., 1991) does not turnover and the most abundant protein in the body, collagen, has a half-life of decades (Cloos and Fledelius, 2000; Gineyts et al., 2000; Sivan et al., 2008; Thorpe et al., 2010). Recently it has been found that structural proteins that form part of the nuclear pore of neurons also do not turn over and they degrade over time. As a result of this degradation, the nuclei of older neurons become “leaky” and the cytosolic protein tubulin appears in the nucleus of neurons from 2 to 3 year old rats (D'Angelo et al., 2009).

Since the accumulation of insoluble protein with age appears to be universal (Jung et al., 2007), it could be hypothesized that the overall rate of protein denaturation might be important for the viability of older animals. If this were true, any mechanisms that promote, or ameliorate, these processes may well influence health

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and fitness in old age. One way to examine this is to modulate the levels of chaperone proteins (Sőti and Csermely, 2000). Molecular chaperones and the aging process, since these agents act to bind denaturing proteins in the cell (Muchowski et al., 1999).

In humans such experiments are clearly not possible, however some tissues are accessible and can be used to investigate the role of protein denaturation in age-related conditions and disease. The lens is one such tissue since it contains proteins that do not turn over and these undergo several PTMs that appear to be responsible for presbyopia (Truscott, 2009) where the lens becomes so stiff by middle age that it can no longer change shape to enable focussing on close objects.

Cataract is another condition whose incidence is markedly age-related (Taylor, 2003) and is associated with markedly increased levels of insoluble protein (Truscott and Augusteyn, 1977). If indeed protein denaturation were responsible for age-related cataract, some hypotheses can be tested. For example, if particular sites in proteins were crucial, one would predict that there should be a consistently greater degree of modification at these sites in cataract lenses than in age-matched normals.

This manuscript reports the outcome of a proteomic study to investigate the hypothesis that an accelerated rate of modification of crystallins at some sites, could be responsible for human age-related cataract. For example, Asn 76 in γS crystallin in cataract lenses has recently been shown to be more highly deamidated than in age-matched normal lenses (Hooi et al., 2012).

In the present study, the most abundant structural protein in the human lens, αA crystallin, was studied as it is known to undergo modifications with age and it is also a chaperone whose role is to bind to other proteins in the lens as they denature (Horwitz, 1992).

2. Materials and methods

2.1. Extraction and tryptic digestion of lens proteins

Normal human lenses were obtained from the Sydney Eye Bank, with ethical approval from the University of Sydney and fetal lenses from the Endocrinology Department, Prince of Wales Hospital, Randwick, NSW, Australia. Cataract lenses were obtained from the K.T. Seth Eye Hospital, Rajkot, Gujarat, India. For all lenses, the nucleus was separated from the cortex by coring through the visual axis with a 4.5 mm diameter trephine, which was pre-cooled at −20 °C. The de-capsulated lenses were placed in a pre-cooled Teflon holder with a diameter of 6 mm. A cold scalpel was used to remove ~0.5 mm of newly synthesized lens material from each end of the core. Only the nuclear regions of adult human lenses were used for analysis (Heys et al., 2007) and fetal lenses were used without dissection. Lens proteins were extracted using a previous method (Hooi and Truscott, 2011) and digested with trypsin as described previously (Hooi et al., 2012).

2.1.1. Peptide standards

The four isoforms of aspartate in TVLDSGISEVR, which correspond to tryptic peptide 55–65 of human αA crystallin, were synthesized by Peptide 2.0 (Chantilly, USA) with L-isoAsp/D-Asp/D-isoAsp at position 58. Each peptide was dissolved with FA:HFBA:H_2O (1: 0.05: 98.85, v/v) and analysed by analytical HPLC and LC-MS/MS. Optimized HPLC conditions that allowed separation of the four isoforms, were used to run human lens digests. Analytical HPLC separation was achieved using a 5 μm, C18 (4.6 × 250 mm) column (Phenomenex, Torrance, USA) at a flow rate of 1 mL/min. Samples (20 μL) were injected and a linear gradient used (10% B to 30% B in 115 min), where buffer B is acetonitrile: TFA (99.9:0.1, v/v). Confirmation of the separation of the αA crystallin peptides in lens digests was carried out by spiking in 1 pmol of the standard peptides.

2.1.2. Liquid chromatography mass spectrometry (LC/MS)

Tandem mass spectra were acquired using a Waters/Micromass quadruple time-of-flight (QTOF) Ultima mass spectrometer with a nanospray source (Manchester, UK). Peptides were separated by 1D LC using a nanoCap-LC auto-sampler system (Waters, Milford MA) as described (Hooi et al., 2012). Complete peaks collected from analytical HPLC were dried and examined by capillary LC. Capillary separation was performed on a ~20 cm fritless Magic C18 reversed phase column with 3 μm packing material (Michrom Bioresources, Auburn, CA) particle size 200 Å.

2.1.3. Data analysis

The triply charged ions for αA crystallin-derived tryptic peptides were extracted and the intensities from the extracted ion chromatogram (XIC) determined using MassLynx. Peak areas of specific peptides were calculated using a mean smoothing method (Number of Smooths: 2, window size (scans): ±3). The MS/MS spectrum of each peptide was matched to the XIC, ensuring the peak area used corresponded to that of the matched peptide and not an isobaric peptide. For αA crystallin, all forms of the peptide (L-Asp, L-isoAsp, D-Asp and D-isoAsp) were summed and modification for each one was expressed as a % of the total peak area. Simple linear regression analysis and Mann–Whitney *U* tests were performed using a previous method (Hooi and Truscott, 2011).

3. Results

3.1. Identification of Asp isomers in αA crystallin

Whilst investigating the LC/MS profiles of tryptic digests of human lens proteins, multiple peaks with almost the same MS/MS spectra were observed for a peptide derived from αA crystallin, corresponding to residues 55–65. Capillary LC conditions were developed that enabled good separation of the four Asp isoforms (Fig. 1), however LC/MS analysis of lens digests revealed that some of the peptide standards co-eluted with other lens peptides. Of particular note were versions of the αA crystallin tryptic peptide

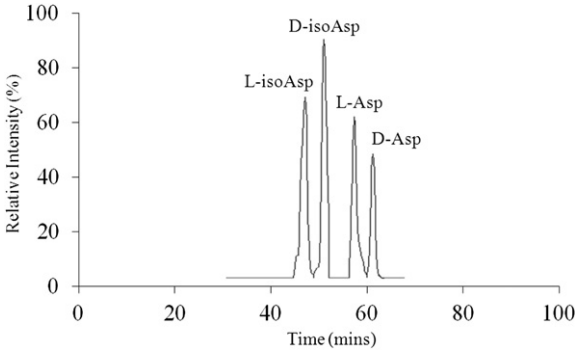

Fig. 1. Representative capillary LC/MS trace showing the separation of the four Asp isoforms of the αA crystallin tryptic peptide (55–65) TVLDSGISEVR. Peptides were synthesized containing D-Asp, D-isoAsp, L-Asp or L-isoAsp at position 58. The L-isoAsp (58) peak for TVLDSGISEVR was found to co-elute with L-Ser (59)/D-Ser (62) of TVLDSGISEVR; the D-isoAsp (58) peak with D-Ser (59)/D-Ser (62) of TVLDSGISEVR and D-Asp (58) peak with D-Ser (59)/L-Ser (62) of TVLDSGISEVR. Each of these Ser and Asp peptide isoforms has essentially the same MS/MS spectrum meaning that capillary LC/MS alone could not provide quantification of the Asp isomers. Therefore to enable accurate Asp quantification, an analytical HPLC step, as shown in Fig. 2, was added prior to capillary LC/MS.