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Alpha B- and βA3-crystallins containing D-Aspartic acids exist in a monomeric state

Hiroaki Sakaue [a], Takumi Takata [b], Norihiko Fujii [c], Hiroshi Sasaki [d], Noriko Fujii [a,b,*]

[a] Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
[b] Research Reactor Institute, Kyoto University, Kumatori-cho, Sennan-gun, Osaka 590-0494, Japan
[c] Radioisotope Research Center, Teikyo University, Kaga Itabashi-ku, Tokyo 173-8605, Japan
[d] Department of Ophthalmology, Kanazawa Medical University, Uchinada, Kahoku-gun, Ishikawa 920-0293, Japan

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ABSTRACT

Crystallin stability and subunit–subunit interaction are essential for eye lens transparency. There are three types of crystallins in lens, designated as α-, β-, and γ-crystallins. Alpha-crystallin is a hetero-polymer of about 800 kDa, consisting of 35–40 subunits of two different αA- and αB-subunits, each of 20 kDa. The β/γ-crystallin superfamily comprises oligomeric β-crystallin (2–6 subunits) and monomeric γ-crystallin. Since lens proteins have very long half-lives, they undergo numerous post-translational modifications including racemization, isomerization, deamidation, oxidation, glycation, and truncation, which may decrease crystallin solubility and ultimately cause cataract formation. Racemization and isomerization of aspartyl (Asp) residues have been detected only in polymeric α- and oligomeric β-crystallin, while the situation in monomeric γ-crystallin has not been studied. Here, we investigated the racemization and isomerization of Asp in the γ-crystallin fraction of elderly donors. The results show that Asp residues of γS-, γD- and γC-crystallins were not racemized and isomerized. However, strikingly, we found that a portion of αB-crystallin and βA3-crystallin moved to the lower molecular weight fraction which is the same size of γ-crystallin. In those fractions, Asp-96 of αB-crystallin and Asp-37 of βA3-crystallin were highly inverted, which do not occur in the native lens higher molecular weight fraction. Our results indicate the possibility that the inversion of Asp residues may induce dissociation of αB- and βA3-crystallins from the polymeric and oligomeric states. This is the first report that stereoinversion of amino acids disturbs lens protein assembly in aged human lens.

1. Introduction

Eye lens protein aggregates with aging finally resulting in loss of vision with the onset of formation of senile cataract. Although cataract is a major cause of blindness, there is no treatment except for surgery. The mechanism of cataract formation is obscure, but it is believed that damaged and modified eye lens proteins aggregate abnormally, resulting in clumping that scatters the light and interferes with focusing on the retina. Human eye lens proteins are mainly composed of α-, β- and γ-crystallins. Alpha-crystallin exists as a polydisperse hetero-polymer with a molecular mass of 800–1000 kDa, which is composed of αA- and αB-crystallins, each of which has a molecular mass of 20 kDa and has a chaperone function for protecting lens transparency. Beta-crystallin exists as a homo- or hetero-oligomer with a molecular mass of 50–200 kDa and is composed of seven subunits (βA1, βA2, βA3, βA4, βB1, βB2 and βB3) each with a molecular mass of 20–30 kDa. Monomeric γ-crystallin consists of seven subunits (γA, γB, γC, γD, γE, γF and γS) each with a molecular mass of 20 kDa [1]. All crystallin monomers have a similar molecular mass of approximately 20 kDa, but α-, β-, and γ-crystallins exist in a polymeric (35–40 subunits), oligomeric (2–6 subunits) and monomeric state, respectively in the lens (or native state). Therefore, α-, β- and γ-crystallins can be separated by size exclusion chromatography and each subunit of these crystallins can be separated by reversed phase high performance liquid chromatography (RP-HPLC).

With aging, metabolically inert lens crystallins undergo numerous post-translational modifications, such as deamidation, truncation, phosphorylation and oxidation [2,3]. These modifications have been described in many excellent reviews [3–5]. Of these modifications, deamidation has been reported as the most prevalent post-translational modification in aged lens [2]. Numerous studies of the effect of deamidation on crystallins have been performed [6–8]. As a result of deamidation, newly induced negative charge in lens crystallins may induce partial unfolding and incorrect subunit–subunit interactions between lens proteins, which may ultimately induce lens protein

* Corresponding author at: Research Reactor Institute, Kyoto University Kumatori-cho, Sennan-gun, Osaka 590-0494, Japan. Tel.: +81 72 451 2496; fax: +81 72 451 2630.
E-mail address: nfujii@rri.kyoto-u.ac.jp (N. Fujii).

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insolubilization. In addition to these modifications, racemization and isomerization of aspartyl residues to generate Dβ-, Lβ-, Dα-Asp residues in proteins have been proposed to be related to cataract formation [9–13].

These modifications may change higher order structure of crystallins, decrease crystallin solubility and be related to cataract formation. This is due to the fact that if D-Asp is formed in a protein, the configuration of the Asp residue would be opposite to the native configuration resulting in a possible change in the higher order structure of the protein. Consequently, as well as deamidation, the subtle effects of inversion may effect higher order structure of crystallins and alter the crystallin properties such as solubility, stability and crystallin–crystallin interaction. Furthermore, D-Asp formation is accompanied by the isomerization of the peptide bond from the normal α-linkage to the uncommon β-linkage, which may affect the quaternary structure of the crystallin because the main chain of the protein would be elongated. Therefore, the presence of the Asp isomers may be one of the triggers of aggregation and induce partial protein unfolding. It is
therefore noteworthy that D-Asp has been detected in aged proteins involved in aggregation disease, such as age-related eye diseases [14], photo-aging of skin [15], and Alzheimer disease [16].

Under physiological condition, Asp residues are easily inverted from the L-form to the D-form compared with other amino acid residues because the stereoinversion occurs via a succinimidyl intermediate [17,18]. Previously, we reported the presence of D-isomers at Asp-58 and Asp-151 of αA-crystallin [9], Asp-36 and Asp-62 of αB-crystallin in the high molecular weight α-crystallin fraction [10] and Asp-4 of βB2-crystallin in the β-crystallin oligomeric fraction [11] from human cataractous lenses of elderly donors. The study of stereoinversion of Asp residues in crystallin has been limited to α- and β-crystallins. In the present study, we investigated for the first time the specific D-Asp sites in γ-crystallin fractions obtained from the lower molecular weight (LMW) fraction of aged-related cataractous lenses.

2. Material and methods

2.1. Materials

2-({2-[Bis (carboxymethyl) amino] ethyl}(carboxymethyl) amino) acetic acid (EDTA), NaCl, Tris (hydroxymethyl) aminomethane and HPLC grade acetonitrile were purchased from Sigma-Aldrich Japan. Phenylmethylsulfonyl fluorides (PMSFs), trifluoroacetic acid (TFA) were obtained from WAKO pure chemicals. Sequencing grade-modified trypsin was purchased from Promega. All other chemicals were purchased from Sigma Chemical Co.

2.2. Isolation of water soluble γ-crystallin subunits from human cataract lenses

A total of 6 cataract lenses from subjects aged 48, 50, 67, 77, 86 and 87 years, were used in this study. Each lens was homogenized using an ultrasonic generator in 5 ml of a sonication buffer (20 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA) on ice. The homogenate was centrifuged at 16,000 *g* for 20 min at 4 °C. The supernatant was separated into α-, β- and γ-crystallin fractions by size exclusion chromatography on a Sephacryl S-300 column (GE Healthcare, Piscataway, NJ) equilibrated in 20 mM Tris/HCl, pH 7.8 and 150 mM NaCl at a flow rate of 1 ml/min using an AKTA prime (GE Healthcare, Piscataway, NJ). According to the method of Hanson et al. [19], the γ-crystallin was further separated into γC-, γD- and γS-crystallins by reversed phase HPLC (RP-HPLC) using a C4 column (VyDAC 4.6 × 250 mm, Pierce, Rockford, IL) with a linear gradient of 0–65% acetonitrile in the presence of 0.1% TFA at a flow rate of 0.8 ml/min. The elution of the γ-crystallin subunit was monitored at 280 nm and the proteins were collected manually.

2.3. Enzymatic digestions of γ-crystallin subunits and isolation of the digested peptides

To remove acetonitrile from the eluted samples, γ-crystallin subunits were dialyzed in water using a dialysis membrane (Spectra/Por 7, 1000 MWCO, SPECTRUM LABS). The γ-crystallin subunits were digested with trypsin for 20 h at 37 °C in 50 mM Tris–HCl (pH 7.6) and 1 mM $CaCl_2$ at an enzyme-to-substrate ratio of 1:50 (w/w). The resulting peptides were separated by RP-HPLC set up with UV-970, LG-980-2, PU-980, DG-980-50 (JASCO, Tokyo, Japan) using a C18 column (Capcell pak C18 UG 80, 3.0 × 250 mm; Shiseido, Tokyo, Japan) with a linear gradient of 0–60% acetonitrile in the presence of 0.1% TFA, at a flow rate of 0.5 ml/min with monitoring at 215 nm. The fractions containing desired peptides were collected using a fraction collector (FRC10, Shimadzu, Kyoto, Japan).

2.4. Identification of tryptic peptides of γ-crystallin

To identify the tryptic peptides, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was employed (MALDI-TOF MS, AXIMA-TOF2; Shimadzu, Kyoto, Japan) as previously described [11]. The MALDI-TOF MS was operated with a 337 nm nitrogen laser and an ion acceleration voltage of 20 kV. Data were collected in a positive ion reflection mode. 1 μl of 20 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in acetone was applied onto a stainless steel MALDI plate, then air-dried, followed by the addition of 1 μl of peptide solution.

2.5. Determination of the D/L ratio of aspartic acid in γ-crystallin-derived peptides

A small amount of the collected samples (~50 μl) was dried under reduced pressure before hydrolysis. The peptides were hydrolyzed with gas-phase 6 M HCl at 108 °C for 7 h (PicoTag Workstation; Waters, Tokyo, Japan). The hydrolyzed samples were dissolved in 0.1 M borate buffer, pH 10.4, then incubated with *o*-phthalaldehyde (OPA) and *N* (*tert*-butyloxycarbonyl)-L-cysteine (Boc-L-Cys) to form diastereoisomers. The D/L ratio of the aspartic acid was determined by RP-HPLC using a C18 column (Nova-Pak ODS, 3.9 × 300 mm; Waters) with a fluorescence detector (344 nm excitation wavelength and 433 nm emission wavelength, Shimadzu). Elution of aspartic acid was carried out with a linear gradient of 7–47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate buffer, pH 6.0 over 120 min at a flow rate of 0.8 ml/min at 30 °C.

3. Results

3.1. Separation of lens water soluble fraction

Fig. 1 shows the size exclusion chromatogram of the water soluble fraction of the cataract lens from 50 year-old donors. HMW (high molecular weight proteins), α, β and γ-crystallins were eluted in order of size as shown in the chromatogram. The amount of HMW, α, β and γ-crystallins was dependent on the lens sample, however, each lens sample shows similar peak patterns. Fractions with retention times between 222 and 252 min in Fig. 1 were collected as the γ-crystallin fraction and used in RP-HPLC in Section 3.2.

3.2. Separation of γ-crystallin subunits

The pooled γ-crystallin fraction obtained by size exclusion chromatography was separated by RP-HPLC using a C4 column as shown in Fig. 2. The γ-crystallin fraction was separated into three peaks. In order to identify the proteins obtained from peaks 1, 2, and 3 of Fig. 2, the proteins were subjected to dialysis and tryptic digestion. The resulting peptides were further separated by RP-HPLC using a C18 column.