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Simultaneous Stereoinversion and Isomerization at the Asp-4 Residue in βB2-Crystallin from the Aged Human Eye Lenses

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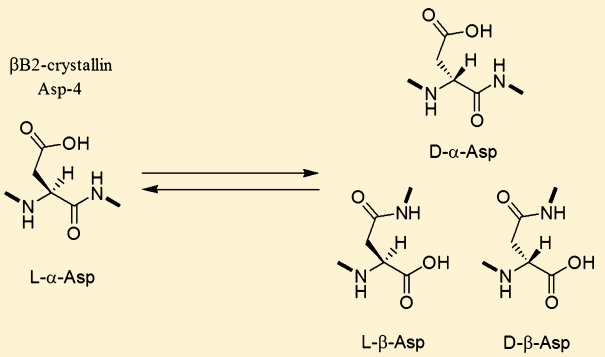
ABSTRACT: The lens proteins are composed of α-, β-, and γ-crystallins that interact with each other to maintain the transparency and refractive power of the lens. Because the lens crystallins are long-lived proteins, they undergo various post-translational modifications including racemization, isomerization, deamidation, oxidation, glycation, and truncation. In βB2-crystallin, which is the most abundant β-crystallin, the deamidation of asparagine and glutamine residues has been reported. Here, we found that the aspartyl (Asp) residue at position 4 of βB2-crystallin in the lenses of elderly human individuals undergoes a significant degree of inversion and isomerization to the biologically uncommon residue D-β-Asp. Surprisingly, the D/L ratio of β-Asp at position 4 in βB2-crystallin from elderly donors (67−77 year old) was 0.88−3.21. A D/L ratio of amino acids greater than 1.0 is defined as an inversion of configuration from the L- to D-form, rather than a racemization. These extremely high D/L ratios are equivalent to those of Asp-58 and Asp-151 (D/L ratio: 3.1 for Asp-58 and 5.7 for Asp-151) in αA-crystallin from elderly donors (~80 year old) as reported previously. Initially, we identified specific Asp residues in the β-crystallin family of proteins that undergo a high degree of inversion. These results show that the isomerization and inversion of Asp residues occurs both in the α- and β-crystallins of the lens. Inversion of these Asp residues directly affects the higher order structure of the protein. Hence, this modification may change crystallin−crystallin interactions and disrupt the function of crystallins in the lens.



The homochirality of proteins composed of L-amino acids was believed to be maintained throughout the entire lifespan of an organism. Over the past few decades, however, D-aspartic acid (D-Asp) residues have been detected in various tissues from elderly individuals such as tooth,[1,2] bone,[3] aorta,[4] brain,[5−7] eye lens,[8,9] retina,[10] conjunctivae[11] and cornea,[12] skin,[13,14] ligament[15] and cardiac muscle,[16] blood vessels of the lung,[16] chief cells of the stomach,[16] and longitudinal and circular muscles of the stomach, small intestine, and large intestine.[16] Importantly, the proteins containing D-amino acids are derived from tissues that are metabolically inert. Thus, D-amino acid residues arise due to racemization of amino acids in the protein during the lifespan of the individual. Of all the naturally occurring amino acids, aspartic acid (Asp) is the most susceptible to racemization. The emergence of D-Asp isomers in a protein can cause major structural changes because different side chain orientations can induce an abnormal peptide backbone. In addition to D-Asp formation, the β-linkage of Asp may affect the quaternary structure because the main chain of the protein is likely to be elongated. Therefore, the presence of the isomers could trigger abnormal aggregation and thereby induce partial unfolding of the protein, which may in turn lead to a disease state. Indeed, lens crystallin obtained from cataract donors that contain large amounts of D-β-Asp undergo abnormal aggregation to form massive and heterogeneous aggregates, leading to a loss of chaperone activity.[17]

A cataract, which is the most common age-related disease, is caused by clouding of the eye lens that may lead to a partial or total loss of vision. The mechanism of cataract development is not well understood. However, it is thought that eye lens proteins of a cataract are abnormally aggregated, resulting in clumping that scatters the light and interferes with focusing on the retina. Human lens proteins are mainly composed from the α-, β-, and γ-crystallin superfamily of proteins. The overall structure, stability, and short-range interactions of these proteins are thought to contribute to the transparent properties of the lens. α-Crystallin is a large molecule with a molecular mass of ~800 kDa and is comprised of two kinds of polypeptides: αA and αB. Given that each αA- or αB-crystallin monomer has a mass of ~20 kDa, the α-crystallin molecule is an aggregate containing approximately 40−50 subunits. The β/γ-crystallin superfamily comprises oligomeric β-crystallin and monomeric γ-crystallin. The β-crystallin is classified into four acidic (βA1, βA2, βA3, and βA4) and three basic (βB1, βB2, and βB3) polypeptide subunits, which associate as either homo- or hetero-oligomers with sizes ranging from 50 to 200 kDa in the lenses.[18,19] All β-crystallins have structures consisting of two

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β-sheet domains, with each domain formed from two Greek key motifs and N- and C-terminal extensions of varying lengths. The crystal structure of βB2-crystallin, which is the most abundant constituent among the basic subunits, has been reported.[20−22] The βB2-crystallin has a two-domain β-structure, folded into four very similar Greek key motifs. βB2-Crystallin is thought to play a role in the stabilization of hetero-oligomer formation through interactions between the N-terminal arms and thereby help maintain their solubility in the lens.[22]

During the aging process crystallins undergo several post-translational modifications that lead to aggregation, cross-linking, and insolubilization, culminating in the development of a cataract. Examples of post-translational modification of α-crystallin that have been reported include racemization of aspartyl residues,[9,23] disulfide bonding,[24] deamidation[25] of asparagine or glutamine residues, methionine oxidation,[26] and backbone cleavage.[27] The post-translational modification of human β-crystallin was reported as backbone cleavage, deamidation, and oxidation.[28−31] Among β-crystallin subunits, βA3/A1, βA4, βB1, and βB2 crystallins undergo deamidation of asparagine (Asn) and glutamine (Gln) residues, oxidation of methionine and tryptophan residues, and truncation. Although all the β-crystallin subunits were deamidated, βB2 underwent the least amount of deamidation. Residues Gln-6, Gln-8, Gln-13, Gln-183, and Asn-16 were reported as sites of deamidation in βB2-crystallin.[28] Recently, Lampi et al.[29] suggested that Gln-71 and Gln-163 undergo deamidation and that this modification destabilizes the formation of βB2-crystallin dimers.

Many deamidation sites in all the β-crystallin subunits have been reported. Therefore, deamidation is thought to be the most prevalent post-translational modification in the lens. Deamidation, oxidation, and truncation of a protein results in a mass difference that can be detected by mass spectrometry. However, analysis of the racemization is not as straightforward to identify because there is no difference in mass between D- and L-amino acid-containing peptides. In order to determine the racemization site, the following steps are required. (i) The protein of interest is digested with a protease such as trypsin. (ii) The resulting peptides are separated by RP-HPLC. (iii) The peptides are identified by mass analysis and/or protein sequencing. (iv) The identified peptides are hydrolyzed with 6 N HCl and derivatized to the diastereoisomers. (v) The diastereoisomers are analyzed by RP-HPLC and the D/L ratio of amino acids determined by analysis of the respective peak areas. Hence, the analysis of the racemization of specific residues in a protein is a technically demanding process. With the exception of our previous reports describing the racemization of Asp in αA- and αB-crystallin, there are no other papers describing the racemization of residues in lens proteins. Here we demonstrate for the first time that the Asp-4 residue of βB2-crystallin undergoes inversion to the D-β-form in the aged lens. We also discuss the mechanism of the inversion reaction and its implications in terms of the higher order structure around the Asp-4 residue of βB2-crystallin.

EXPERIMENTAL PROCEDURES

Purification of βB2-Crystallin from Human Lens. Lens samples from elderly individuals (70−80 year old) were homogenized in 20 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid (EDTA) and then clarified by centrifugation at 12000*g* for 30 min at 4 °C. The supernatant was fractionated by size exclusion chromatography using a Sephacryl S-300 column (GE Healthcare, Piscataway, NJ) equilibrated in 20 mM Tris/HCl, pH 7.8, and 150 mM NaCl to obtain high molecular weight (HMW) α-, β-, and γ-crystallin. According to the method of Zhang et el.,[28] the β-crystallin was further fractionated into βB2-crystallin and other β-crystallins by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C4 column (VyDAC 4.6 × 250 mm, Pierce, Rockford, IL) with a linear gradient of 24−60% acetonitrile in the presence of 0.1% trifluoroacetic acid (TFA), at a flow rate of 0.8 mL/min.[28] The purity of the βB2-crystallin was checked by SDS-PAGE using 15% polyacrylamide gels according to the method of Laemmli.[32]

Enzymatic Digestions of βB2-Crystallin and Isolation of the Resulting Peptides. The βB2-crystallin was digested with trypsin for 17 h at 37 °C in 0.1 M Tris/HCl pH 7.6, 20 mM $CaCl_2$ buffer, at an enzyme-to-substrate ratio of 1:50. The digestion of the βB2-crystallin was expected to yield 24 tryptic peptides, based on the known sequence of the protein. The resulting peptides were separated by RP-HPLC 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.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOFMS). All spectra were obtained using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOFMS) (AXIMA-TOF^2; Shimadzu, Kyoto, Japan). The MALDI-TOFMS spectrometer was operated with a 337 nm nitrogen laser and an ion acceleration voltage of 20 kV. Data were collected in positive ion reflection mode. Apply 1 μL of 20 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in acetone onto a stainless MALDI plate and allow to air-dry before adding 1 μL of the peptide solution.

Determination of the D/L Ratio of Amino Acids in βB2-Crystallin. 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, and briefly incubated with *o*-phthalaldehyde (OPA) and *N*-(*tert*-butyloxycarbonyl)-L-cysteine (Boc-L-cys) to form diastereoisomers. The D/L ratio of the amino acids was determined using RP-HPLC with a C18 column (Nova-Pak ODS, 3.9 × 300 mm; Waters) and fluorescence detection (344 nm excitation wavelength and 433 nm emission wavelength). Elution was carried out with a linear gradient of 7−47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate buffer, pH 6.0, in 120 min at flow rate of 0.8 mL/min at 30 °C.

Peptide Synthesis of T1 Peptide Containing Four Different Asp Isomers. T1 peptide containing four different Asp isomers were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid-phase chemistry using the automated solid-phase peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan). Fmoc-L-Asp(OtBu)-OH, Fmoc-D-Asp(OtBu)-OH, Fmoc-L-Asp-OtBu, and Fmoc-D-Asp-OtBu were used as building blocks to synthesize L-α-, D-α-, L-β-, and D-β-isomers, respectively. The coupling reaction was carried out using each Fmoc amino acid (10 equiv), PyBOP (10 equiv), 1-hydroxybenzotriazole (HOBt) (10 equiv), and *N*-methylmorpholine (7.5 equiv) in dimethylformamide (DMF). The N-terminal Fmoc group was deblocked with 20% piperidine in DMF. Simultaneous cleavage of the peptide from the resin and removal of the protective groups was achieved by treatment with a cocktail containing

90% TFA, 5% 1,2-ethanedithiol, and 5% thioanisole. The crude peptides were purified by RP-HPLC using a C18 column (Capcell pak C18 ACR, 30 × 250 mm; Shiseido) with a linear gradient of 0−50% acetonitrile in the presence of 0.1% TFA, at a flow rate of 3.0 mL/min, with monitoring at 215 nm. The purity of each peptide was confirmed to be >95% by analytical RP-HPLC and mass spectrometry.

RESULTS

Identification of the Fragments of the βB2-Crystallin from Elderly Donors. Figure 1 shows a typical RP-HPLC chromatogram of βB2-crystallin derived from the lens of a 72 year old individual. The βB2-crystallin was eluted as the first peak. This result is consistent with that described in a previous paper.[28] As shown in Figure 2, the βB2-crystallin is composed of 204 amino acids and contains 11 Asp and 8 Asn residues. In order to determine the D/L ratio of the respective Asp/Asn residues in the βB2-crystallin, the protein was subjected to tryptic digestion. Based on the known amino acid sequence of βB2-crystallin, this procedure was expected to yield 24 tryptic peptides (T1−T24 peptides) as shown in Figure 2. The resulting peptides were separated by RP-HPLC fitted with a fraction collector. A typical RP-HPLC chromatogram of the tryptic peptides of βB2-crystallin is shown in Figure 3a. The peptide corresponding to each peak was subsequently identified by MALDI-TOFMS analysis. The numbers above the respective peaks in Figure 3a correspond to the tryptic peptides shown in Figure 2. With the exception of T6 and T7, all the peptides include Asp or Asn residue(s). Figure 3b shows the enlarged chromatogram of RP-HPLC shown in Figure 3a corresponding to a retention time of between 25 and 35 min. As shown in Figure 4a, the MALDI-TOFMS analysis showed that the peptides at the retention time of 29.1 and 32.0 min in Figure 3b had a protonated molecular mass of 1084.9 and 1084.7, respectively. These results are consistent with the theoretical mass of T1 peptides ($[M + H]^+$ = 1084.5). We designated the peptides with a retention time of 29.1 and 32.0 min as T1(1) and T1(2), respectively. Furthermore, the T1(1) and T1(2) peptides were analyzed by MS/MS analysis (Figure 4b). MS/MS analysis clearly showed that both peptides (i.e., T1(1) and T1(2)) corresponded to T1. Our previous reports indicated that the separation of the same peptides by RP-HPLC was caused by a difference between the α and β linkage of the Asp residue in the peptide. Specifically, the peptide containing a β-Asp residue elutes earlier than that containing the α-Asp residue.[9,23] We synthesized four isomeric T1 peptides, acASDHQTQAGK where the Asp residue was L-α-Asp, L-β-Asp, D-α-Asp, or D-β-Asp. The retention time for each isomeric peptide on the RP-HPLC was then confirmed under identical conditions as described in Figure 3. As shown in Figure 5, the retention time of the D-β-Asp, L-β-Asp, L-α-Asp, and D-α-Asp containing peptides were 29.4, 29.4, 31.1, and 31.9 min, respectively. The β-Asp and the α-Asp containing peptides were readily separated; i.e., the former was eluted earlier than the latter on the RP-HPLC, which is consistent with our previous results.[9,23] From these results, the peaks with the retention times of 29.1 and 32.0 min in Figure 3b were identified as β-Asp containing T1 peptide and α-Asp containing T1 peptide, respectively. The previous data clearly indicated that β-Asp formation is accompanied by stereoinversion from the L- to D-form. Therefore, it was expected that Asp-4 may undergo stereoinversion from the L- to D-form. We hydrolyzed all the peptides containing Asp residues and determined the D/L ratios of Asp in βB2-crystallin by the method described in the Experimental Procedures. As shown in Table 1, the D/L ratios of β-Asp specifically at position 4 of βB2-crystallin were extremely high, i.e., 0.88, 3.21, and 2.4 for three elderly donors of 67, 72, and 77 years of age, respectively. In contrast, α-Asp-4 did not display a particularly high D/L ratio by comparison with that of β-Asp-4. We found that Asp-4 of the T1 peptide in βB2-crystallin showed an extremely high level of inversion from the L- to D-form and isomerized from a α- to β-linkage. The other Asp/Asn residues in the protein were not racemized to any great extent, with D/L ratios of less than 0.1 (Table 2).

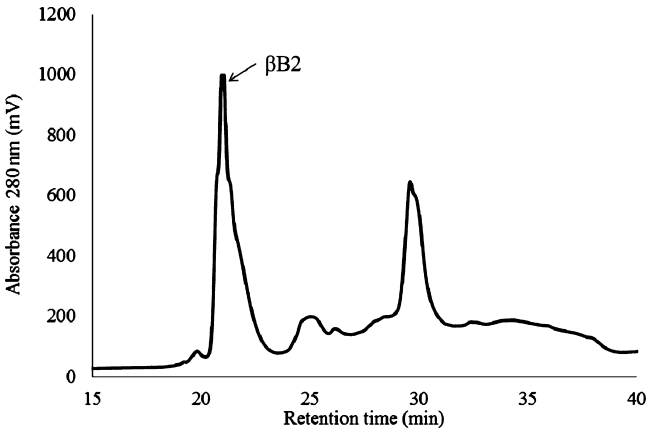

Figure 1. A typical RP-HPLC chromatogram of βB2-crystallin derived from a lens isolated from a 72 year old individual. The sample was applied to a C4 column (VyDAC 4.6 × 250 mm) and eluted using a linear gradient of 24−60% acetonitrile in the presence of 0.1% trifluoroacetic acid (TFA), at a flow rate of 0.8 mL/min.

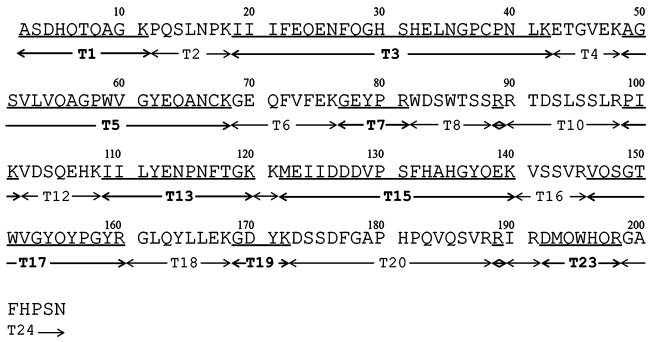

Figure 2. The primary structure of human βB2-crystallin. βB2-Crystallin was digested with trypsin (T).

DISCUSSION

β-Crystallin constitutes about half the soluble protein in the lens of elderly individuals and is composed of acidic βA1−A4 and basic βB1−B3 subunits. Previous studies concerning the post-translational modification of β-crystallin have been limited to analyzing the deamidation of asparagine (Asn) or glutamine (Gln) residues and oxidation of methionine and tryptophan residues. Here, we demonstrate for the first time stereoinversion and isomerization of Asp-4 in βB2-crystallin isolated from elderly individuals. As shown in Figure 3, the peak T1(2) includes L-β- and D-β-Asp-4 containing peptides while peak T1(1) includes L-α- and D-α-Asp-4 containing peptides. From the respective peak areas highlighted in Figure 3b, we calculated

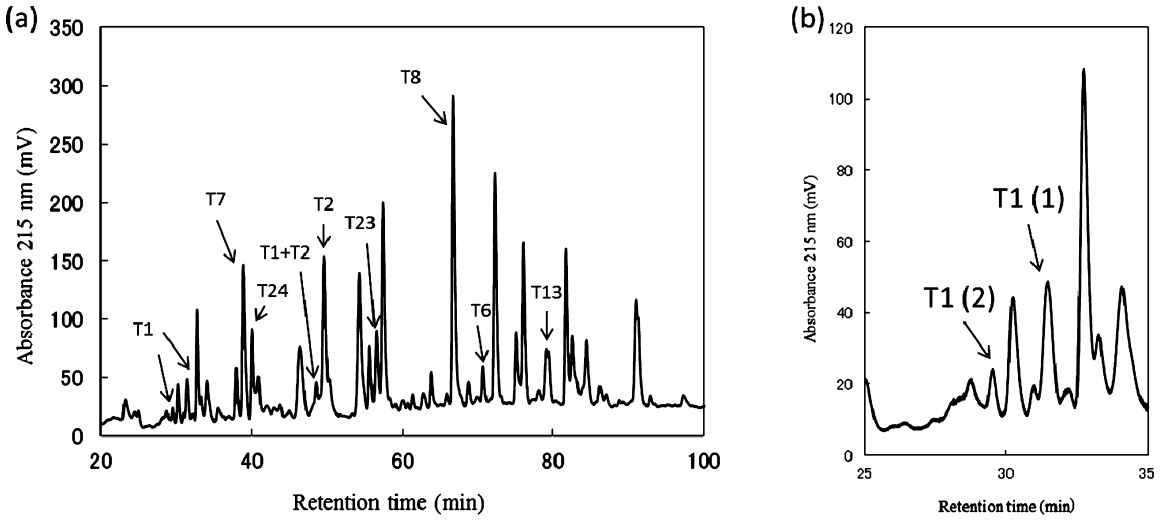

Figure 3. (a) Elution profiles of tryptic (T) peptides of human βB2-crystallin. Tryptic (T) peptides were separated by reverse-phase HPLC using a C18 column with a linear gradient of 0−40% acetonitrile in the presence of 0.1% trifluoroacetic acid. The peaks were identified by mass spectrometry. (b) Expanded view of part of the chromatogram shown in (a) with a retention time of between 25 and 35 min.

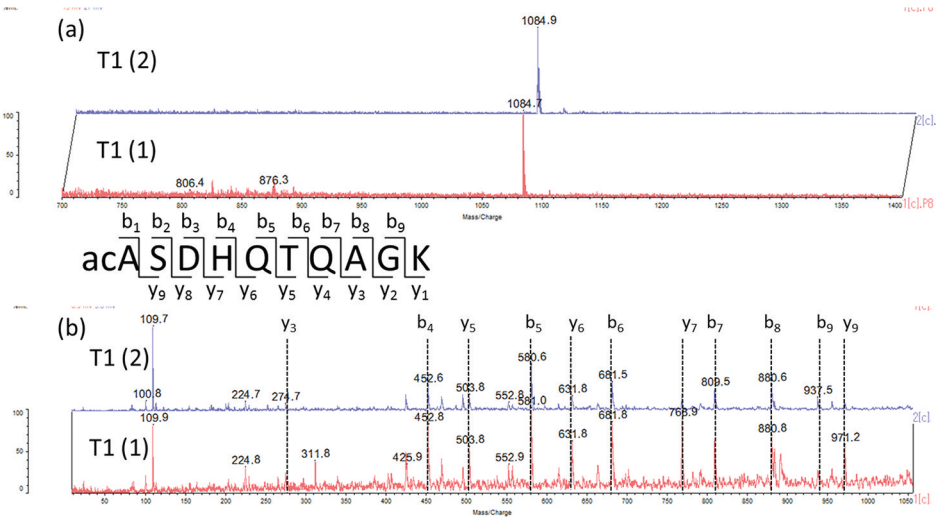

Figure 4. (a) Mass spectrum of a peak at retention time 29 and 32 min, shown in Figure 3b. (b) Fragmentation spectrum of a peak at retention time 29 and 32 min of Figure 3b.

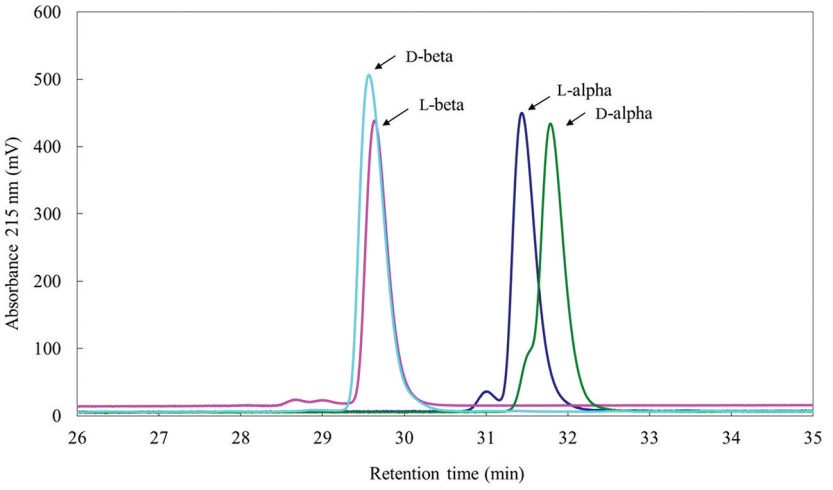

Figure 5. Elution profile of the βB2-crystallin T1 peptide isomers in which the normal L-α of Asp-4 was replaced with D-α, L-β, and D-β isomers.

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Table 1. The D/L Ratios and Linkage of Asp-4 Residue in the T1 Peptide of βB2-Crystallin from Elderly Donors

age (years)	linkage	D/L ratio of Asp-4
67	α	0.27
	β	0.88
72	α	0.09
	β	3.21
77	α	0.07
	β	2.4

Table 2. The D/L Ratios of Asp Residues in the Tryptic Peptides Obtained from Figure 3

retention time	mass (peptide name)	D/L ratio of Asp in the tryptic peptide
36.3–36.9	621.6 (T7)	0.04
46.4–48.0	783.8 (T2), 1849.7 (T1–T2)	0.09
53.1–53.8	1000.7 (T23)	0.03
64.0–65.4	1024.6 (T8)	0.04
68.0–68.9	983.6 (T6)	0.02
76.3–77.3	1410.0 (T13), 976.7	0.05

the amount of L-β- and D-β-Asp-4 to be ~20% that of L-α- and D-α-Asp-4. Previous studies have shown that the amount of D-α-Asp-containing peptide is likely to constitute less than half the amount of D-β-Asp-containing peptide.[33,34] Thus, we estimate that the amount of D-α-Asp-4 accounts for a further 5% of isomers. Hence, the total amount of L-β-Asp, D-β-Asp, and D-α-Asp-4 probably amounts to 25% that of L-α-Asp-4. Moreover, we identified an extremely high D/L ratio corresponding to a specific β-Asp residue in β-crystallin, i.e., D/L ratio for Asp-4: 0.88, 3.21, and 2.4 for a 67, 72, and 77 year old donor, respectively. Stereoinversion of specific Asp residues was not detected except in our earlier studies of D-Asp residues in αA- and αB-crystallin. The observed scatter in the D/L ratio between individual human donors arises because a cataract can develop from various causes, e.g., diabetes, exposure to cigarette smoke or UV B irradiation, short-sightedness, and certain allergies.

We previously reported the presence of D-isomers at Asp-58 and Asp-151 in αA-crystallin[23] and at Asp-36 and Asp-62 in αB-crystallin[9] from aged human lenses. D-Asp formation was accompanied by isomerization from the natural α-Asp to the abnormal β-Asp.[9,23,35] Asp is the most easily racemized amino acid to be found in proteins. Usually, this racemization proceeds with difficulty under physiological conditions. However, as shown in Figure 6, Asp residues in proteins are susceptible to racemization because D-Asp formation occurs via a succinimide intermediate as follows: (i) When the carbonyl group of the side chain of the L-α-Asp residue is attacked by the nitrogen of the amino acid residue following the Asp residue, L-succinimide is formed by intramolecular cyclization; (ii) L-succinimide may be converted to D-succinimide through an intermediate that has the prochiral α-carbon in the plane of the ring; (iii) D- and L-succinimide are hydrolyzed at either side of their two carbonyl groups, yielding both β- and α-Asp residues, respectively. The rate of succinimide formation is expected to depend on the neighboring residue of the Asp. When the neighboring amino acid of the Asp residue has a small side chain, such as glycine, alanine, or serine, formation of the succinimide intermediate occurs easily because there is no steric hindrance.[33] The Asp-58 and Asp-151 residues in αA-crystallin (D/L ratio of 3.0 and 5.7, respectively, from an 80 year old donor) are followed by Ser and Ala, which may favor formation of a succinimide intermediate. Therefore, the high level of stereoinversion may proceed depending on the neighboring amino acids.

However, Asp-36 and Asp-62 of αB-crystallin are followed by bulky amino acids (i.e., Leu and Thr, respectively) and are still subject to high levels of racemization. The presence of these bulky groups presumably inhibits formation of a succinimide intermediate. Moreover, even though αB-crystallin contains Asp-140-glycine and Asn-146-glycine, which are the most suitable for succinimide formation, racemization at these sites was not observed.[9] These findings clearly suggest that succinimide formation in a protein depends on higher order structures around the relevant Asp residue in addition to the effect of neighboring amino acid residues.

In our previous work, it was unclear why the D/L ratios of Asp-58 and Asp-151 in aged human αA-crystallin exceeded 1.0. Racemization is defined as a reversible first-order reaction. Hence, when the D/L ratio equals 1.0, the racemization reaction has reached equilibrium. Thus, the D/L ratios of greater than 1.0 should be defined as stereoinversion of L-Asp to its D-isomer, rather than racemization. Here, we show the reason why the D/L ratio of Asp-58 and Asp-151 from native aged αA-crystallin was greater than 1.0 in our previous study.[35] The stereoinversion caused by protonation of intermediate [I] does not occur with equal probability from above and below the plane shown in Figure 6. Namely, a sterically hindered arrangement composed of a native higher order structure of αA-crystallin may be present on the lower side of intermediate [I]. Such a mechanism would result in protonation of intermediate [I] from the upper side of the plane. This in turn causes the configuration to be inverted to the D-form. When the native higher order structure is destroyed by the unfolding of αA-crystallin, protonation to intermediate [I] proceeds with equal probability from both sides of the plane, resulting in an increase in L-Asp.[35] Our findings indicate that structural considerations determine whether the Asp-151 residue undergoes the transformation to D-β-Asp. Hence, the higher order conformation of αA-crystallin is critical to this reaction.

However, it is not possible to discuss the relationship between likely racemization sites of Asp residues in αA-crystallin and the higher order structure of the protein because such structural data are unavailable at the present time. We are interested in determining why racemization/inversion occur at specific sites in proteins. The formation of D-Asp depends on two conditions: (i) the level of steric hindrance from the amino acid next to the Asp residue; (ii) whether the area surrounding the Asp residue forms a chiral environment that promotes inversion of the L- to D-form. More recently, Laganowsky et al. reported the X-ray structure of truncated bovine αA-crystallin (59–163 residues) and truncated human αB-crystallin (68–162 residues).[36] The polypeptide chain between residues 147 and 155 of bovine αA-crystallin was shown to form a flexible hinge loop. However, the primary amino acid sequence corresponding to this region of αA-crystallin is different between the bovine and human forms of the protein (i.e., bovine: PSGVDAGHS; human: QTGLDATHA). Therefore, it is unclear whether or not Asp-151 of human αA-crystallin is located in a hinge loop region. Nonetheless, Asp-151 in bovine αA-crystallin was also subject to specific racemization. Therefore, these areas may have a common flexible structure that favors the racemization and isomerization of Asp.

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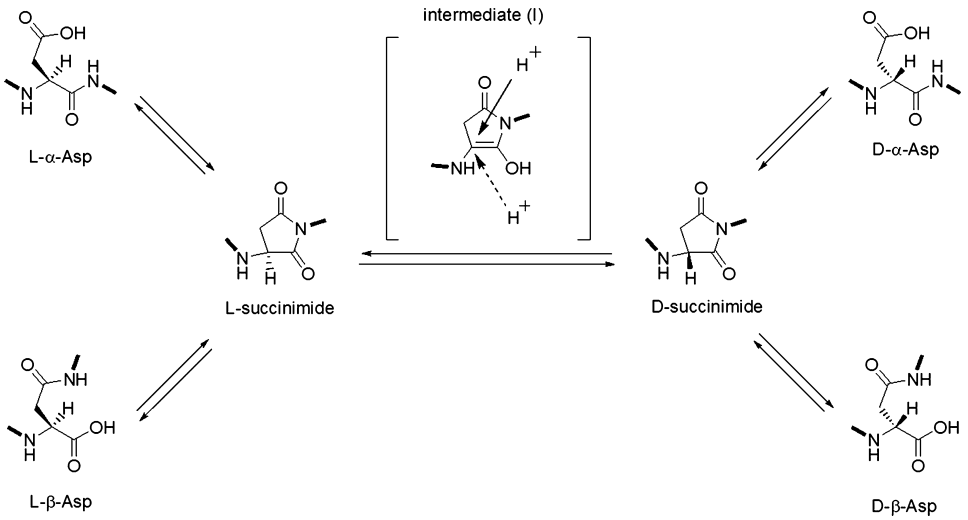

Figure 6. Possible reaction pathways for the spontaneous racemization and isomerization of aspartyl residues in proteins.

In summary, this study clearly shows that the D/L ratio the Asp-4 residue of βB2-crystallin is much higher than 1.0, akin to that of Asp-58 and Asp-151 residues of αA-crystallin. The residue neighboring Asp-4 of βB2-crystallin is histidine, which has a bulky side chain, suggesting the unusual D/L ratio of Asp-4 is not influenced by the adjacent residue. Rather, inversion of Asp-4 in βB2-crystallin may proceed because of the chiral environment around this residue that promotes inversion of the L- to D-form.

The crystal structure of a human βB2-crystallin, which contains residues 15−195, was determined by Smith et al.[37] as shown in Figure 7. Human βB2-crystallin has four Greek key

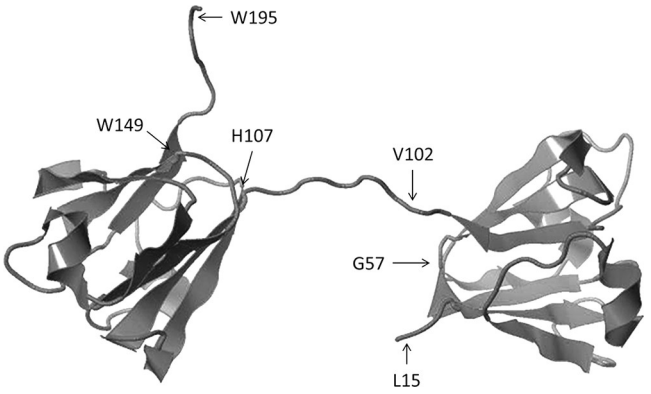

Figure 7. Crystal structure of human βB2-crystallin which contains residues 15 to 195.

motifs located between residues 17−56, 57−101, 107−148, and 149−191 as well as a connecting peptide from residue 102−106 and N- and C-terminal arms (residues 2−16 and 193−205, respectively). Importantly, however, residues 2−14 in the N-terminal arm and 196−205 in the C-terminal arm were not visible in the structure because of the inherent flexibility of these regions. Using ^{1}H NMR spectroscopy, Carver et al. also clearly showed that the N- and C-terminal extensions of bovine βB2-crystallin are largely unstructured. These extensions were shown to be accessible to solvent and possess significantly greater flexibility compared with the domain core of the protein.[38] Thus, the structural data support our hypothesis that the highly flexible region around Asp-4 could promote formation of the succinimide followed by inversion and isomerization. Furthermore, the N-terminal regions of βB2-crystallin are thought to be involved in intermolecular interactions. Trinkl et al. showed that the peptide linker in βB2-crystallin is necessary for dimerization, and the N- and C-terminal arms appear to be involved in preventing the formation of higher homo-oligomers.[39] Werten et al. reported that the N- and C-terminal extensions of βB2-crystallin are involved in protein−protein interactions in the βA3/βB2-crystallin heterotetramer.[40] The inversion and isomerization of Asp-4 could trigger major structural changes because different side chain orientations often induce an abnormal peptide backbone. We have already clarified the effect of the inversion to D-isomers in a protein. In a previous study, we synthesized peptides corresponding to the 70−88 (KFVIFLDVKHF-SPEDLTVK) fragment of human αA-crystallin, which is known to have chaperone function. The L-α-Asp, corresponding to position 76, was replaced by diastereoisomers L-β-Asp, D-α-Asp, and D-β-Asp, and the biochemical properties of the four different peptides were then compared. The peptides containing abnormal isomers (L-β-Asp, D-α-Asp, or D-β-Asp) were more hydrophilic than the normal peptide (containing L-α-Asp) and adopted a random coil structure, rather than the normal β-sheet motif. The normal peptide promoted the aggregation of insulin while the other three isomers suppressed its aggregation. A single substitution of an Asp isomer in a peptide induces a large change in the properties of the peptide.[41] Similarly, the properties of the T1 peptides of βB2-crystallin were altered by the introduction of isomers at Asp-4. As shown in Figure 5, the two isomeric T1 peptides of βB2-crystallin, containing the L-β- and D-β-form of Asp-4, were more hydrophilic than the normal peptide (containing L-α-Asp). Conversely, the other isomeric T1 peptide (containing D-α-Asp) was more hydrophobic than the normal peptide. These results clearly indicate that a single substitution of an Asp isomer in a peptide induces a large change in the properties of the peptide. The changes in hydrophilicity/hydrophobicity caused by the isomerization of Asp residues in βB2-crystallin may explain the observed biophysical properties of the aged protein. Therefore, these modifications can induce a partial unfolding of the protein that alters the intermolecular interactions, leading to a disease state.

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ABBREVIATIONS

MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; RP-HPLC, reverse-phase high-performance liquid chromatography; acA, acetylalanine.

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