Journal of Chromatography B, 879 (2011) 3141–3147

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

# D-Amino acids in aged proteins: Analysis and biological relevance☆

Noriko Fujii[a,*], Yuichi Kaji[b], Norihiko Fujii[a]

[a] Research Reactor Institute, Kyoto University, Kumatori, Sennan, Osaka 590-0494, Japan
[b] Department of Ophthalmology, University of Tsukuba Institute of Clinical Medicine, Tsukuba, Ibaraki 305-8575, Japan

ARTICLE INFO

*Article history:*
Available online 12 June 2011

*Keywords:*
D-Amino acid
D-Aspartate
Aging
Lens
Crystallin
Skin
Oxidative stress
CML
Racemization
Isomerization
UV B irradiation

ABSTRACT

Homochirality is essential for life. L-Amino acids are exclusively used as substrates for the polymerization and formation of peptides and proteins in living systems. However, D-amino acids, which are enantiomers of L-amino acids, were recently detected in various living organisms in the form of free D-amino acids and D-amino acid residues in peptides and proteins. In particular, D-aspartyl (Asp) residues have been detected in various proteins from diverse tissues of elderly individuals. Here, we describe three important aspects of our research: (i) a method for detecting D-β-Asp at specific sites in particular proteins, (ii) a likely spontaneous mechanism by which Asp residues in proteins invert and isomerize to the D-β-form with age under physiological conditions, (iii) a discussion of factors that favor such a reaction.
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## 1. Introduction

During the emergence of life on the primitive earth only L-amino acids were selected for the formation of proteins. L- and D-amino acids are optical isomers of each other, representing right-handed (D-enantiomer) and left-handed (L-enantiomer) structures. The chemical and physical properties of L- and D-amino acids are extremely similar except for their optical characteristics. It is not known why L-amino acids were selected in preference to D-amino acids during chemical evolution and at what stage of evolution this process occurred. Nonetheless, it is important that only one enantiomer was selected because polymers comprising many diastereoisomers of amino acids do not fold properly. Therefore, 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 [1–3], bone [4,5], aorta [6], brain [7–9], erythrocyte [10], eye lens [11–13], retina [14], conjunctivae [15] and cornea [16], skin [17,18], ligament [19] and lung [20], as well

☆ This paper is part of the special issue "Analysis and Biological Relevance of D-Amino Acids and Related Compounds", Kenji Hamase (Guest Editor).

* Corresponding author. 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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doi:10.1016/j.jchromb.2011.05.051

**Table 1**
D-Amino acid containing proteins are observed in various tissues of the living body.

| Tissue | Protein | Amino acid | Disease |
|---|---|---|---|
| Teeth | Phosphophoryn | D-Asp | ? |
| Bone | Osteocalcin | D-Asp | ? |
| Aorta | Elastin | D-Asp | Arteriosclerosis |
| Brain | β-Amyloid | D-Asp, D-Ser | Alzheimer |
| Brain | α-synuclein | D-Asp | Parkinson |
| Lens | αA, AB-crystallin | D-Asp | Cataract |
| Retina | α | D-Asp | AMD[a] |
| Conjutivae | ? | D-Asp | Pingueculae |
| Cornea | ? | D-Asp | CDK[b] |
| Skin | Elastin | D-Asp | Elastosis |
| | Keratin | D-Asp | Sun damage |
| Lung | Elastin | D-Asp | ? |
| Intestine | ? | D-Asp | ? |

[a] AMD: age-related macular degeneration.
[b] CDK: climatic droplet keratopathy.

as cardiac muscle [21], blood vessels of the lung [21], chief cells of the stomach [21], longitudinal and circular muscles of the stomach, small intestine and large intestine [21]. D-serine (D-Ser) was also found in β-amyloid protein of patients with Alzheimer's disease [22]. These findings are summarized in Table 1. 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 life span of the individual. Of all the naturally occurring amino acids, aspartic acid (Asp) is the most susceptible to racemization. The reason why Asp residues are particularly susceptible to racemization will be discussed in Section 3. Initial studies in this area simply reported the presence of D-Asp from whole tissue samples. The specific sites of Asp residue racemization to form D-Asp were only discovered recently. Intriguingly, however, Asp residues do not racemize uniformly. Presumably, specific Asp residues are more susceptible to racemization than others because of sequence context and/or structural considerations. Therefore, it is necessary to determine the nature of the aspartyl residues at specific sites within particular proteins. The specific sites of D-Asp in some proteins have been determined. These include αA- and αB-crystallins from lens [12,13], β-amyloid protein in brain [9], type I collagen telopeptide in urine [5] and histone of the canine brain [23]. Here, we describe a method to determine the specific D-Asp sites in a protein of interest. We also describe the likely mechanism for the formation of D-Asp at specific sites in α-crystallin as an example. Finally, we discuss the factors that favor the racemization reaction.

## 2. Analytical method for the determination of the specific sites of D-Asp residues in lens crystallins

Human lens is composed of three major structural proteins, α-, β- and γ-crystallins. α-Crystallin is a large molecule with a molecular mass of approximately 800 kDa and is comprised of two kinds of polypeptides, αA and αB-crystallins. Because αA- and αB-crystallin monomers have a molecular mass of approximately 20 kDa, the α-crystallin molecule must be made up of a complex of approximately 40–50 subunits. One human lens sample was homogenized and separated into water-soluble and water-insoluble fractions by centrifugation. The water-soluble proteins were then fractionated by size exclusion chromatography to obtain α-, β- and γ-crystallin. The α-crystallin fraction was subjected to reversed-phase high performance liquid chromatography (RP-HPLC) to obtain αA- and αB-crystallins (Fig. 1). Alpha A-crystallin has 15 Asp and 2 Asn residues, while αB-crystallin contains 11 Asp and 3 Asn residues. Digestion of either αA- or αB-crystallin with trypsin was expected to yield 20 tryptic (T1–T20) peptides. The resulting peptides contain only one or two Asp residues. Therefore, the D/L ratio of the

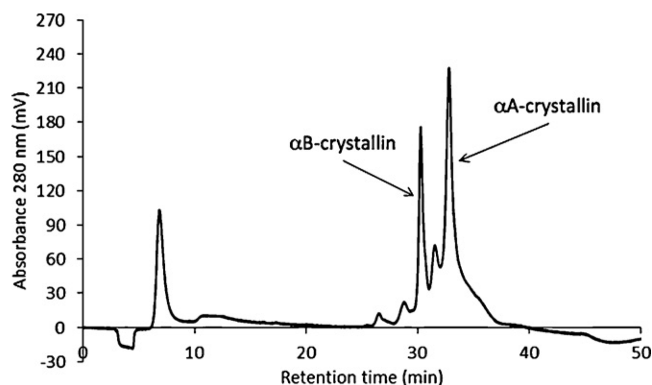



**Fig. 1.** Elution profile of the separation of αA-crystallin and αB-crystallin by reversed phase HPLC (RP-HPLC). Elution: solvent A: 0.1% trifluoroacetic acid/water. Solvent B: 0.1% trifluoroacetic acid/acetonitrile. Column: C4 column (VyDAC 4.6 mm × 250 mm, Pierce, Rockford, IL) Detection: absorbance at 280 nm. Gradient: 0–40% B in 60 min. Flow rate: 1.0 ml/min.

individual Asp residues in the protein can be determined by measuring the D/L ratio of the Asp residue derived from each tryptic peptide. Digestion with trypsin was performed at an enzyme-to-substrate ratio of 1:50 (mol/mol) in 0.1 M Tris–HCl buffer (pH 7.6) for 20 h at 37 °C. The resulting peptides were separated by RP-HPLC (LC-10A, Shimadzu, Kyoto, Japan) using a C18 column (TSK gel-ODS −80 TM, 4.6 mm × 250 mm, Tosoh, Tokyo, Japan) with a linear gradient of 0–40% acetonitrile in the presence of 0.1% trifluoroacetic acid, at a flow rate of 0.8 ml/min, with monitoring at 215 nm. Samples containing the desired peptides were collected into tubes using a fraction collector (FRC10 Shimadzu). Fig. 2 shows a typical RP-HPLC chromatogram of the tryptic peptides of αA-crystallin from an 80 year-old donor. The tryptic (T) peptides were identified by amino acid sequence analysis and mass spectrometry. We found that T6 and T18 peptides containing Asp-58 and Asp-151, respectively, are clearly separated into two peaks upon RP-HPLC (Fig. 2). The separation of these peptides is due to the difference between the α- and β-linkage of the Asp residues. The detection of a β-Asp residue in peptides is made possible by protein sequencing because the β-Asp containing peptides are resistant to Edman degradation. The β/α ratios of T6 peptides of elderly and young donors were 0.4 and 0.01, respectively. In contrast, the β/α ratios of T18 peptides of elderly and young donors were 1.0 and 0.05, respectively. The con-

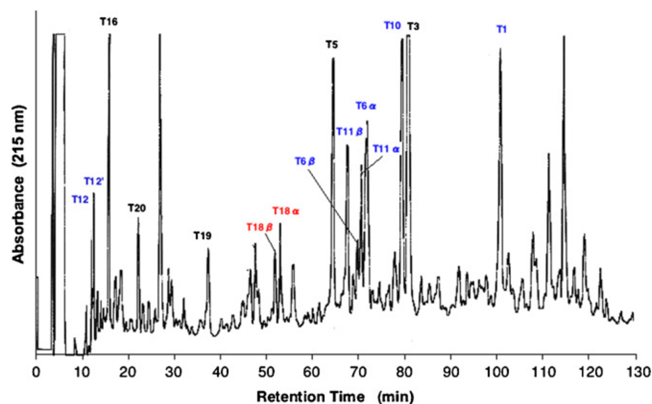



**Fig. 2.** Elution profile of tryptic peptides of aged human αA-crystallin. Elution: solvent A: 0.1% trifluoroacetic acid/water. Solvent B: 0.1% trifluoroacetic acid/acetonitrile. Column: TSK gel-ODS-80 TM, 4.6 mm × 250 mm, Tosoh, Tokyo, Japan. Detection: absorbance at 215 nm. Gradient: 0–40% B in 120 min. Flow rate: 0.8 ml/min. Peptides were detected by measuring their absorbance at 215 nm. αA-crystallin-derived peaks were identified on the basis of amino acid sequence analysis and MALDI-TOF-MS analysis.
N. Fujii et al. Biochem. Biophys. Res. Comm. 239, 918–923 (1997).

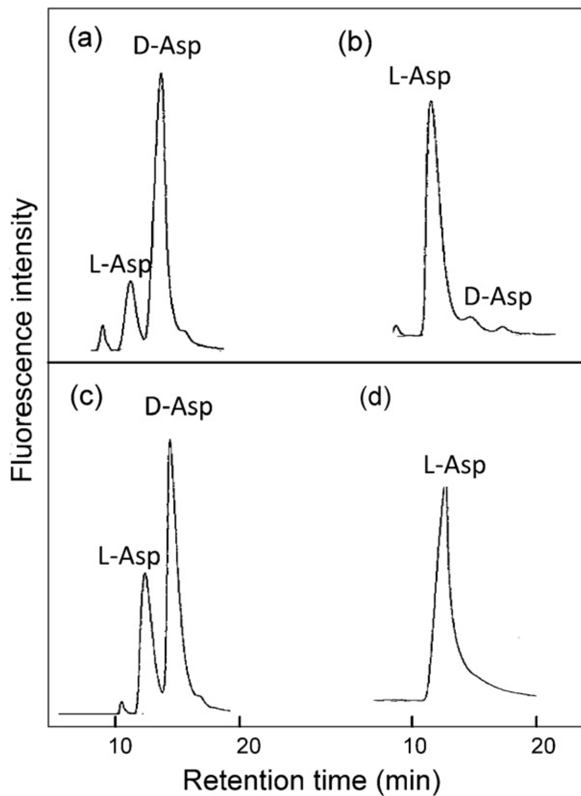



**Fig. 3.** Elution profile for the enantiomeric separation of Boc-L-Cys-/OPA aspartic acid derivatives from the hydrolysates of the T6 peptides of αA-crystallin obtained from elderly (80 year old) and young (11 month old) donors. (a) T6 peptide containing β-Asp 58 residue of αA-crystallin from elderly donor, (b) T6 peptide containing α-Asp 58 residue of αA-crystallin from elderly donor, (c) T6 peptide containing β-Asp 58 residue of αA-crystallin from young donor, (d) T6 peptide containing α-Asp 58 residue of αA-crystallin from young donor. Elution buffer: Solvent A: 5% acetonitrile, 3% tetrahydrofuran/0.1 M acetate buffer (pH 6.0), solvent B: 47% acetonitrile, 3% tetrahydrofuran/0.1 M acetate buffer (pH 6.0). Column: Nova-pak ODS (3.9 mm × 300 mm, Waters). Detection: Ex = 344 nm, Em = 433 nm. Gradient: solvent A–B in 120 min. Flow rate: 0.8 ml/min. Temperature: 30 °C.

tent of β-linked forms in the Asp-58 and Asp-151 residues of aged αA-crystallin was relatively high compared with samples from young individuals. The D/L ratios of individual Asp and asparagine (Asn) residues in the peptides were measured by the following method. The identified tryptic peptides were hydrolyzed by treatment with 6 M HCl for 7 h at 108 °C and derivatized using *o*-phthalaldehyde (OPA) and *N-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The D/L ratio caused by racemization during hydrolysis under these conditions was less than 0.02. The products of the reaction were then subjected to RP-HPLC and the D/L ratio of amino acids was determined by analyzing the ratio of the respective peak areas. Fig. 3 shows a typical RP-HPLC chromatogram of the T6 peptides of αA-crystallins from young and old donors. The D/L ratio of β-Asp-58 residue in the αA-crystallin of aged donors was extremely high (D/L ratio of Asp; 3.1, Fig. 3a), while the α-Asp-58 residue was scarcely racemized (Fig. 3b). Furthermore, although the amounts of β-Asp-58 residue in the young αA-crystallin was very low (about 1%), the D/L ratio was high (D/L ratio of Asp; 1.7, Fig. 3C). The α-linked Asp-58 residue in αA-crystallin from young donors was not racemized. In addition, at Asp151 residues in aged αA-crystallin, β-Asp was more highly inverted to D-isomers (D/L ratio of β-Asp-151; 5.7) than that of the Asp-58 residue (D/L ratio of β-Asp-58; 3.1). Moreover, α-linked Asp at 151 was also highly racemized (D/L ratio of α-Asp-151; 0.58), which was different from α-linked Asp at 58 (D/L ratio of α-Asp-58; 0.00) [13]. The Asp-36 and Asp-62 residues in αB-crystallin

**Table 2**
The D/L ratios of Asp residues in aA-and aB-crystallins from lenses of 80-year-old donors.

| Crystallin | Asp | | |
|---|---|---|---|
| | Site | D/L Ratio | Linkage |
| αA | Asp-2 | 0.01 | α |
| | Asp-58 | 3.10 | β |
| | Asp-67/69 | 0.07 | α |
| | Asp-76 | 0.11 | α |
| | Asp-84 | 0.12 | α |
| | Asp-91/92 | 0.03 | α |
| | Asn101 | 0.05 | α |
| | Asp105/106 | 0.13 | α |
| | Asp-151 | 5.70 | β |
| αB | Asp-2 | 0.00 | α |
| | Asp-25 | 0.01 | α |
| | Asp-36 | 0.92 | β |
| | Asp-62 | 0.57 | β |
| | Asp-73 | 0.00 | α |
| | Asp-78/80 | 0.00 | α |
| | Asp-96 | 0.00 | α |
| | Asp-109 | 0.07 | α |
| | Asp-127/129 | 0.01 | α |
| | Asp-140 | 0.01 | α |
| | Asp-146 | 0.02 | α |

from elderly donors were highly racemized (D/L ratio of Asp-36, 0.92; Asp-62, 0.57) [12] (Table 2). These Asp residues were isomerized to the β linked form. The D/L ratios of the other Asp residues in aged αA- and αB-crystallins were not as high (Table 2). This result clearly indicates that Asp residues do not invert uniformly and that particular Asp residues have a greater tendency to inversion than others within the crystallin molecule. Inversion to the D-Asp form is accompanied by isomerization to β-Asp in the protein. Therefore, analysis of the β-Asp in the peptide is very important. However, it is time consuming to detect β-Asp in the peptide using a protein sequencer. Recently, we found that MS/MS analysis using post source decay (PSD) with a curved field reflectron could distinguish between the β-Asp and α-Asp containing peptides. The relative content of β-Asp in a peptide was successfully estimated from a unique ratio, $y_n$:$y_{n+1}$, derived from tryptic peptides of a protein [24]. Fig. 4 shows a typical MS/MS analysis of T6 peptides, which contain four isomers of Asp 58. The y8 ion of the α-Asp containing peptide is weak in comparison to that of β-Asp containing pep-

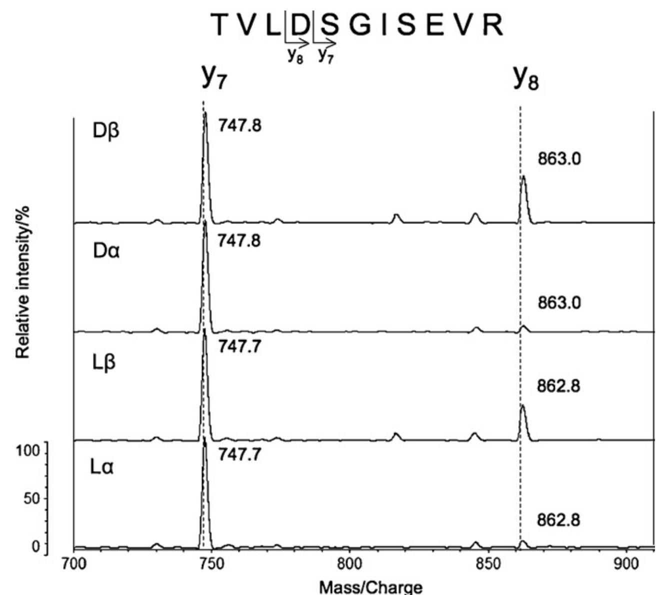
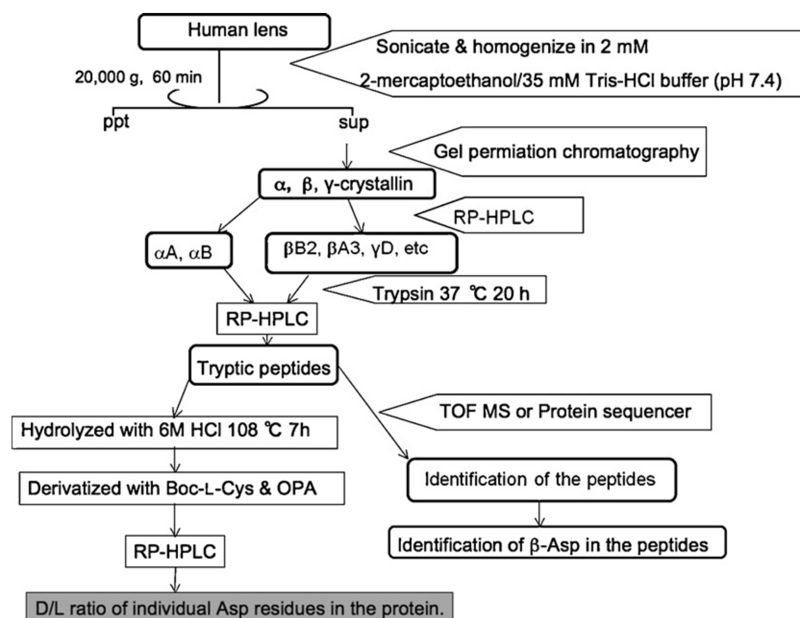



**Fig. 4.** Post source decay (PSD) spectra of T6 peptides obtained from αA-crystallin on a curved field reflectron MALDI-TOF MS (AXIMA TOF[2], Shimadzu).




**Scheme 1.**

tide. Finally, we found that D-Asp formation was accompanied by isomerization from the natural α-Asp to the abnormal β-Asp. The outline of the analytical method is shown in Scheme 1.

## 3. Mechanism of D-Asp and β-Asp formation in protein

We also clarified the mechanism by which D-Asp residues are spontaneously formed in proteins. As shown in Fig. 5, the simultaneous formation of β- and D-Asp residues in the protein could be explained as follows: (i) when the carbonyl group of the side chain of the L-α-aspartyl 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 [I] that has the prochiral α-carbon in the plane of the ring. (iii) Protonation of the intermediate [I] may proceed from the upper or lower side of the plane in an

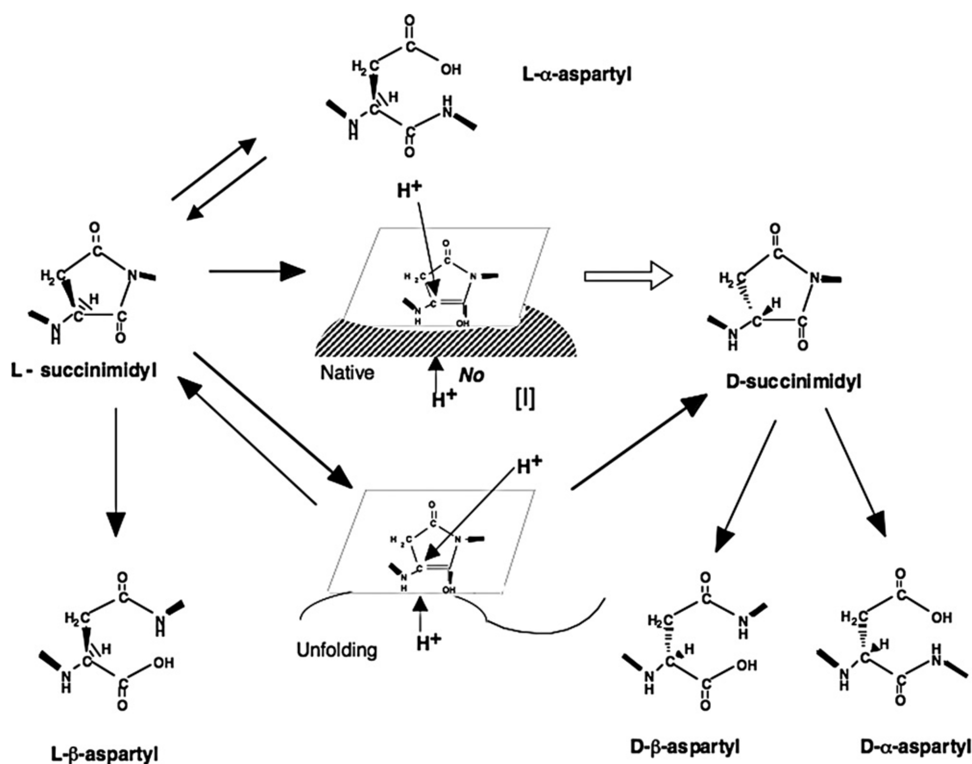



**Fig. 5.** Reaction pathways for spontaneous inversion and isomerization of aspartyl residues in proteins. The possible structural environment surrounding intermediate [I] that induces the inversion to the D-isomer. A local structure that hinders the protonation from the lower side may be present beneath the plane of intermediate [I] (shaded), resulting in the protonation of intermediate I from the upper side of the plane. The configuration is subsequently inverted to the D-form.
Fujii N. et .al. Biochim. Biophys. Acta 1549, 179–187 (2001).

**Table 3**
Sedimentation coefficient and chaperon activity of a-crystallin from elderly and young donors.

| | 80 year | 1 year |
|---|---|---|
| $S_{20,W}$ | 30–80 S | 17S |
| Chaperon Act | 40% | 100% |

$S_{20,W}$:sedimentation coefficient (20 °C in water).

ordinary peptide or protein. The area surrounding Asp-151 and Asp-58 residues in α A-crystallin is thought to form a chiral environment (Fig. 5, shaded parts) that promotes formation of D-succinimide over L-succinimide [25]. (iv) 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 residue. When the neighboring amino acid of the Asp residue has a small side chain, such as glycine, alanine or serine, the formation of succinimide occurs easily because there is no steric hindrance [26,27]. In the primary amino acid sequence of αA-crystallin, Asp-151 and Asp-58 are followed by alanine and serine, respectively. Therefore, formation of succinimide is anticipated to occur readily along with inversion.

As shown in Fig. 5, D-Asp formation is also accompanied by isomerization from the natural α-Asp to the biologically uncommon β-Asp (isoaspartate). Thus, four isomers, L-α-Asp, L-β-Asp, D-α-Asp and D-β-Asp, are simultaneously formed in the protein. The formation of these isomers at Asp-151 and Asp-58 of human αA-crystallin begins shortly after birth and thereafter gradually accumulate during the aging process. Indeed, the amount of D-β-Asp was greater than that of normal L-α-Asp in αA-crystallin derived from the human lens of elderly individuals (~80 years of age) [28].

## 4. Aggregation of protein and the presence of D-β-Asp

### *4.1. D-β-Asp containing protein form massive and heterogeneous aggregates, which are associated with a loss of biological function*

The appearance of D-Asp isomers in proteins can cause major changes in the corresponding 3-D structure. This is because different side chain orientations may 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 elongated. Therefore, the presence of these isomers may be one of the triggers for abnormal aggregation. Moreover, these processes can induce the partial unfolding of the corresponding proteins, leading to a disease state. In fact, αA-crystallin containing large amounts of D-β-Asp obtained from donors of ~80 years of age has been shown to undergo abnormal aggregation to form massive and heterogeneous aggregates [29]. Specifically, αA-crystallin from normal young individuals (2 year-old, non racemized samples) had an average sedimentation coefficient of 17 S at 37 °C compared with the same protein from elderly individuals with sedimentation coefficient of 30-80 S. Changes in the self-association of α-crystallin aggregates have also been correlated to changes in chaperone activity. Alpha-crystallin from young donors display chaperone activity, but this activity reduces by 60% in aged α-crystallin aggregates [29] (Table 3). This chaperone activity plays an important role in preventing aggregation and insolubilization of other lenticular proteins. Hence, the loss of activity adversely affects maintenance of the transparency of the eye lens [30].

### *4.2. Is the formation of the Asp isomer the direct trigger of the change to a higher order structure and function?*

As described in Section 4.1, the appearance of D- and β-Asp in a protein potentially induces large changes to its higher order structure as well as to its function. However, it remains unclear whether the formation of the Asp isomer is the direct trigger for such a change. In order to clarify the effect of the inversion to D-isomers in a protein, we synthesized peptides corresponding to the 70–88 (KFVIFLDVKHFSPEDLTVK) fragment of human α-A-crystallin, which is known to have chaperon function [31]. 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 the aggregation of insulin [32]. This result clearly indicates that a single substitution of an Asp isomer in a peptide induces a large change in the properties of the peptide (Table 4).

**Table 4**
Influence of L-β-, D-α-, and D-β-Asp isomers at Asp-76 residues on the properties of αA-crystallin 70–88 peptide (KFVIFL[76]DVKHFSPEDL7VK).

| | |
|---|---|
| hydrophobicity | Lα > Dα > Dβ > Lβ |
| secondary structure | Lα ⇨ β-sheet rich<br>Lβ, Dα, Dβ ⇨ random |
| Interaction with Other protein | Lα ⇨ promote the aggregation of insulin<br>Lβ, Dα, Dβ ⇨ Inhibit the aggregation of insulin |

## 5. What are the factors that promote the formation of D-β-Asp in proteins?

### *5.1. The effects of UVB irradiation on D-β-Asp formation in proteins*

The racemization of Asp residues was observed in lens crystallins from elderly donors as described above. Furthermore, UV B irradiation of young rat lens induced the racemization of the Asp-151 residues in αA-crystallin [33].

We also found a D-β-Asp-containing protein in sun-damaged dermis of the skin from elderly donors using an anti-peptide 3R antibody we prepared [34]. The abnormal protein was localized to elastic fiber-like structures of dermis samples from elderly donors with actinic elastosis. The immunoreactivity of the D-β-Asp-containing protein in sun-damaged dermis from aged skin against anti-elastin antibody suggests this protein may be elastin [17]. In contrast, there was no immunoreactivity in sun-exposed skin from young donors [17]. The results clearly indicate that the formation of D-β-isomers in protein is correlated with both aging and exposure to sunlight. Recently, we also detected D-β-Asp containing proteins in the epidermis of UVB-irradiated mouse skin. These proteins were identified by proteomic analysis as members of the keratin family of proteins, including keratin-1, keratin-6B, keratin-10, keratin-14 (Mori et al. unpublished data). Clearly, because skin and lens tissue

are exposed to sunlight they are particularly susceptible to UV B irradiation.

### 5.2. *The effects of oxidative stress on* D-*β*-*Asp formation in proteins*

Our previous results strongly indicate that UV irradiation enhances D-β-Asp formation in protein with aging. In contrast, a previous study using an anti-carboxymethyl lysine (CML) antibody [35] reported that advanced glycation end products (AGEs) also increased in UV B irradiated skin. AGEs are formed by metal-catalyzed oxidation of glucose or Amadori products and are thought to be involved in aging or age-related diseases such as diabetes [36], atherosclerosis [37], Alzheimer's disease [38] and the formation of cataracts [39,40]. These findings are very similar to the detection of D-β-Asp in age-related disease. Recently, Kaji et al. reported that D-β-Asp and CML were commonly observed in the drusen between the retinal pigment epithelial cells and Bruch's membrane during the development of age-related macular degeneration (AMD) [41]. The AGEs are very complicated and include pyrraline [42], pentosidine [43] and carboxymethyl lysine (CML) [44]. CML is formed by oxidative cleavage of Amadori products or Schiff bases or by modification with glyoxal generated through auto-oxidation of glucose and/or lipid peroxidation [45,46]. CML has been proposed as a potential marker of oxidatively damaged tissues or proteins *in vivo*. Mizutari reported that CML modification occurred in radiation damaged areas of skin [35]. These results suggest that UV-induced oxidation accelerates the formation of CML in photo-aged skin. Moreover, although both D-β-Asp and CML formation have been observed in similar tissues with aging or upon UV B-irradiation, as yet, no clear correlation between these two processes has been identified. Recently, we used a double-labeling method with anti-peptide 3R and anti-CML antibody to show that the D-β-Asp and CML modified proteins co-localize to the same section of epidermis and dermis of sun-exposed skin. Furthermore, keratin family proteins were shown to undergo D-β-Asp and CML formation upon UV B irradiation by MALDI-TOF/MS/MS [47]. Moreover, Verzijl et al. recently used biochemical analysis to demonstrate that the AGE levels were linearly related to the degree of Asp racemization in both cartilage and skin collagen [48]. Taken together, these findings clearly indicate that the levels of D-β-Asp and AGE's both increase during aging and that these two components accumulate in metabolically inert proteins.

## 6. Prospects

Until recently, most researchers have held the view that L-amino acids in proteins can never change to D-isomers under physiological conditions. This general idea had no real basis in scientific fact but became established because D-amino acid residues could not be identified in nature. However, recent improvements in analytical techniques have facilitated the accurate analysis of amino acid enantiomers at the picomole level. Therefore, we were able to identify a very small quantity of D-aspartic acid residues at specific sites in lens proteins comprised almost entirely of L-amino acids. Intriguingly, the aspartic acid residues were not racemized uniformly. Instead, there appear to be 'hot spots' within specific proteins that are susceptible to racemization. We were also able to propose a mechanism for D- and β-aspartic acid formation in α-crystallin. Moreover, we have proposed a mechanism for D- and β-aspartic acid formation in α-crystallin. The formation of D-aspartic acids in proteins depends on the primary amino acid sequence and higher order structure surrounding the Asp residues. We propose that a chiral reaction field exists in the native higher order structure of human αA-crystallin that induces the inversion of L-Asp to D-Asp at Asp-151. D-amino acid formation with age proceeds, at least partially, in proteins that contain only one handed structures comprised of L-amino acids in a process opposite to the evolution of life.

D-Asp containing proteins can be identified by immunohistochemical analysis or 2D-PAGE and Western blotting as described in the previous section, even if there is no prior knowledge of the likely target protein. The identity of D-β-Asp containing proteins can subsequently be determined by MALDI-TOF-MS analysis. The specific sites of D-β-Asp residues in the identified protein can then be identified by biochemical analysis, assuming sufficient amounts of material can be isolated.

## Acknowledgements

This work was supported, in part, by a research grant from the Ministry of Education, Science, Sports and Culture of Japan.

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