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ORIGINAL ARTICLE

Influence of Lβ-, Dα- and Dβ-Asp isomers of the Asp-76 residue on the properties of αA-crystallin 70–88 peptide

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Abstract Proteins have been considered to consist exclusively of L-amino acids in living tissues. However, our previous studies showed that two specific aspartyl (Asp) residues in αA- and αB-crystallins from human eye lenses invert to the D-isomers to a high degree during aging. The reaction is also accompanied by isomerization into a form containing β-Asp (isoaspartate) residues. The appearance of D- and β-Asp in a protein potentially induces large changes to the higher order structure of the protein as well as to its function. However, it remains unclear whether the formation of the Asp isomer is the direct trigger of the change to the higher order structure and function. In this study, 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 αA-crystallin and its corresponding diastereoisomers in which Lα-Asp was replaced with Lβ-Asp, Dα-Asp, and Dβ-Asp at position 76 and compared their biochemical properties with that of normal peptide. The peptides containing abnormal isomers (Lβ-Asp, Dα-Asp, and Dβ-Asp residues, respectively) were more hydrophilic than the normal peptide (containing Lα-Asp), lost β-sheet structure and changed to random structures. The normal peptide promoted the aggregation of insulin while the other three isomers suppressed the aggregation of insulin. This is the first evidence that a single substitution of an Asp isomer in a peptide induces a large change to the properties of the peptide.

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Abbreviations
αAC αA-Crystallin
ThT Thioflavin T

Introduction

Protein α-amino acids contain one (or more) asymmetric tetrahedral carbon atoms. Therefore, the molecules can exist as two nonsuperimposable mirror images, that is, they can be right-handed (D-enantiomer) and left-handed (L-enantiomer) structures. However, only L-amino acids were selected in polymerization and formation of peptides and proteins after the emergence of life. Although the chemical and physical properties of L-amino acids and D-amino acids are extremely similar, with the exception of their optical character, the reasons for the elimination of D-amino acids and why all living organisms are now composed predominantly of L-amino acids are not clear. However, it is clear that it was necessary that one of the enantiomers was selected because polymers which consist of different amino acid diastereomers would not be able to fold into correct structures in a manner similar to current proteins. Thus, homochirality is essential for the development and maintenance of life. Once the L-amino acid world was established, D-amino acids were excluded from living systems except for the D-amino acids in the cell wall of microorganisms. However, recently, D-amino acids have been found in various higher organisms in the form of free amino acids, peptides, and proteins (Konno et al. 2007).

Thus, free D-serine was found in the mammalian brain and can function as an endogenous neurotransmitter interacting with the glycine-binding site associated with the NMDA-receptor complex (Hashimoto et al. 1992). Free D-aspartic acid (D-Asp) has been observed in mammalian endocrine organs at specific stages of development. Many researchers suggest that D-Asp has a role as a messenger in the maturation and differentiation of tissues in mammalian organs (Furuchi and Homma 2005).

Small peptides containing one D-amino acid have been found in both vertebrates and invertebrates. Dermorphin, from the skin of a frog, is the first example of a D-amino acid-containing peptide and is an opioid peptide with the sequence Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (Montecucchi et al. 1981). Its biological activity is about 1,000 times greater than that of morphine (Broccardo et al. 1981). Substitution of L-Ala for D-Ala causes a loss of this activity. Therefore, D-Ala is essential for its activity.

Proteins containing D-Asp residues have been detected in various tissues such as tooth (Masters 1983), bone (Ritz et al. 1996), aorta (Powell et al. 1992), brain (Fisher et al. 1986; Roher et al. 1993), eye lens (Fujii et al. 1994a, b), and skin (Fujii et al. 2002) from elderly individuals. The presence of D-Asp in aged tissues of living organisms is thought to result from the racemization of aspartyl residues in the protein where the proteins in such tissues are metabolically inert. In previous studies, we reported that specific Asp residues in αA- (Fujii et al. 1994a) and αB-crystallin (Fujii et al. 1994b), which is the most abundant eye lens protein, were highly inverted from the L-isomer to the D-isomer and isomerized from the normal peptide-bond α-linkage to the β-linkage via a succinimide intermediate (Fig. 1). Therefore, four isomers, consisting of normal Lα-Asp and the biologically uncommon Lβ-Asp, Dα-Asp, and Dβ-Asp are formed in αA- and αB-crystallins (Fujii et al. 1994a, b, 1999). The appearance of D-Asp isomers in protein can cause major changes in structure, since 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 would be elongated. Therefore, the presence of the isomers may be one of the triggers of abnormal aggregation and can induce the partial unfolding of a protein which may lead to a disease state. In fact, αA-crystallin containing large amounts of Dβ-Asp obtained from elderly donors undergoes abnormal aggregation to form massive and heterogeneous aggregates, leading to loss of its chaperone activity (Fujii et al. 2007). Since the chaperone activity plays an important role in preventing the aggregation and insolublization of other lenticular proteins, the loss of the activity affects the maintenance of the transparency of the eye lens (Horwitz 1992). Ecroyd et al. described the interaction of αB-crystallin and the other small heat shock proteins with amyloid fibril-forming proteins in a detailed review (Ecroyd and Carver 2009). Many previous studies also suggested that the inversion of L- into the D-amino acid in a protein had the potential to induce abnormal folding of the protein and decrease its activity (Fisher et al. 1986; Fujii et al. 1994a, b, 2002; Powell et al. 1992; Roher et al. 1993).

In this study, in order to further clarify the effect of the inversion to D-isomers in a protein, we synthesized peptides composed of all L-amino acids and the corresponding diastereoisomers in which the Lα-Asp residue at position 76 was replaced with a Lβ-Asp, Dα-Asp, or Dβ-Asp residue and compared their biochemical properties. To our knowledge, no such study focusing on the difference in the properties of peptide isomers has yet been published. As a first approach, we selected the peptide corresponding to amino acids 70–88 (KFVIFLDVKHFSPEDLTVK) of human αA-crystallin. This choice was made based on the fact that

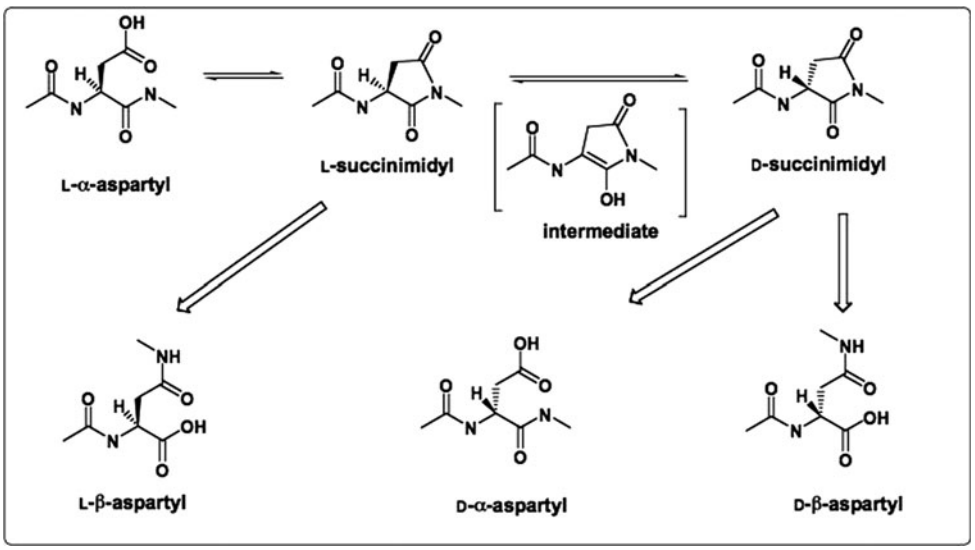

Fig. 1 Possible reaction pathways for spontaneous inversion and isomerization of aspartyl residues in protein

this peptide has been well characterized by Sharma et al. and corresponds to the β3 and β4 region present in the α-crystallin domain of sHSP16.5 and functions as the chaperone sites of αA-crystallin (Sharma et al. 2000). Tanaka et al. also showed that the same peptide accelerated the aggregation of insulin (Tanaka et al. 2008). In this study, we synthesized the same model peptide 70–88 (KFVIFLD76VKHFSPEDLTVK) and replaced Asp-76 with Dα-Asp, Lβ-Asp, or Dβ-Asp.

Experimental procedures

Protein and reagents

Bovine insulin and Thioflavin T (ThT) were purchased from Sigma (St. Louis, MO, USA).

Peptide synthesis

The four isomers of the peptide KFVIFLDVKHFSPEDLTVK, corresponding to the amino acid sequence 70–88 of human αA-crystallin, were synthesized with the Lα-, Dα-, Lβ- , and Dβ-isomers of Asp-76 by Peptide 2.0 Inc. (Chantilly, VA, USA). In this study, the peptides contain Lα-, Dα-, Lβ- , and Dβ-isomers at Asp-76 were designated αAP-Lα76, αAP-Dα76, αAP-Lβ76, and αAP-Dβ76, respectively, as shown in Table 1. These peptides were synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid phase chemistry. The crude peptides were purified by reverse-phase HPLC (RP-HPLC) and the purity was confirmed to be more than 95% by analytical RP-HPLC. The optical purity of the Asp-76 residues of the peptides was checked before use according to the procedure described elsewhere (Fujii et al. 1994a). In brief, the peptides were hydrolyzed and derivatized with *o*-phthaldialdehyde (OPA) and *N-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The determination of the D/L ratio of amino acids was performed by RP-HPLC. The optical purity of the Asp-76 residues in the peptides was confirmed to be >99% (data not shown).

Table 1 Name and sequence of the peptides used in this study

Peptide name	Sequence	$M + H^+$ *m/z* (calc.)
αAP-Lα76	KFVIFLD(Lα)VKHFSPEDLTVK	2262.25
αAP-Dα76	KFVIFLD(Dα)VKHFSPEDLTVK	2262.25
αAP-Lβ76	KFVIFLD(Lβ)VKHFSPEDLTVK	2262.25
αAP-Dβ76	KFVIFLD(Dβ)VKHFSPEDLTVK	2262.25

The calculated mass-to charge ratios (*m/z*) of the protonated molecules ($M + H^+$) are indicated in the right column.

HPLC analyses

The crude peptide was injected onto a C18 column (Shiseido CAPCELL PAK UG80 3.0 × 250 mm) equilibrated with water containing 0.1% trifluoroacetic acid (solvent A). The bound peptide was eluted with a linear gradient (0–60% B) formed by mixing of solvent A and solvent B (100% acetonitrile containing 0.1% trifluoroacetic acid). A flow rate of 0.5 ml/min over 170 min was maintained. The elution was monitored at 215 nm for absorption.

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-TOF2, 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 reflection mode in positive ion mode. For the matrix, α-cyano-4-hydroxycinnamic acid (CHCA 10 mg) was dissolved in 1 mL of solution containing 0.1% trifluroacetic acid in water and acetonitrile at a ratio of 1:1. The sample peptide (0.5 µl) was added to an equal volume (0.5 µl) of the matrix solution on the plate and then dried. Each sample was present at a level of a few picomoles per spot.

Circular dichroism

The αAC AC70-88 peptide and its isomers (0.5 mg/ml each) were dissolved in 50 mM phosphate buffer (pH 7.5) with 100 mM NaCl. Circular dichroism (CD) spectra in the far-UV regions were obtained with a spectropolarimeter (Jasco J-720, Tokyo, Japan). A cylindrical quartz cell with a 0.2 mm path length was used. Far UV-CD spectra were measured at 25 and 60°C.

ThT fluorescence assays

The ThT fluorescence assays were performed according to the method reported by Tanaka et al. (2008). Alpha AC70-88 peptide and its isomers, insulin, and ThT were dissolved in 50 mM phosphate buffer (pH 7.5) with 100 mM NaCl. The insulin (2 mg/ml), the αAC peptide and its isomers (0.25 mg/ml each), and ThT (20 µM) were incubated in 96-microwell plates at 37°C with shaking at 900 rpm in a Deep Well MAXIMIZER (M13R Tytec, Japan). The fluorescence intensity of the excitation at 420 nm and emission at 485 nm were measured using a Wallac 1420 multi label counter.

Results

HPLC analysis of the peptides

The four purified peptides, αAP-Dβ76, αAP-Lα76, αAP-Dα76, αAP-Lβ76 were analyzed by analytical RP-HPLC. As shown in Fig. 2, the four peptides eluted at quite different times in the RP-HPLC. The elution times of αAP-Dα76, αAP-Lβ76, αAP-Dβ76 and αAP-Lα76 were 93.2 min, 93.75 min, 93.95 min, and 95.2 min, respectively. This result clearly indicates that the αAP-Dα76 peptide containing Dα-Asp is the most hydrophilic and that the hydrophobicity of the peptides increases in the order αAP-Lβ76, αAP-Dβ76 and αAP-Lα76. The peptide αAP-Lα76 peptide containing Lα-Asp was the most hydrophobic. This result clearly shows that replacement with isomers at Asp-76 dramatically decreases the hydrophobic properties of the peptide.

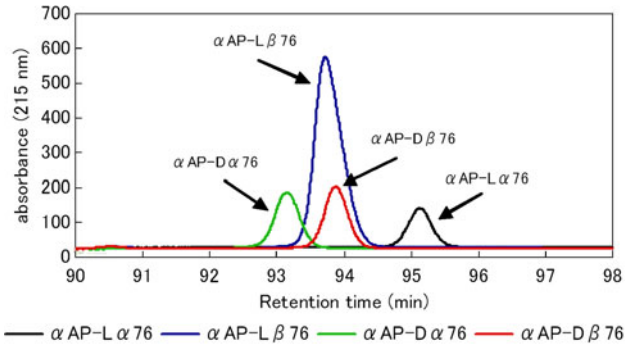

Fig. 2 Elution profile of the αA-crystallin 70–88 peptide isomers in which the normal Lα of Asp-76 was replaced with Dα, Lβ and Dβ isomers. Column; Shiseido UG80 3.0 × 250 mm. Eluent **a** 0.1% trifluoroacetic acid in water; **b** 0.1% trifluoroacetic acid in acetonitrile. Gradient 0–40% B in 170 min. Detection at 215 nm. Flow rate 0.5 ml/min

Mass analysis of the peptides

The purified peptides were analyzed by mass spectroscopy. As shown in Fig. 3a, the masses observed for the protonated precursor ions of the peptides were in the range 2262.3–2262.8, which is consistent with theoretical mass, $M + H^+$; 2262.25. Recently, we found that MS/MS analysis using post source decay (PSD) with a curved field reflectron could distinguish between the peptide containing β-Asp and the peptide containing α-Asp. As shown in Fig. 3b, the ratios of the y13/y12 ions from the peptides containing β-Asp (αAP-Lβ76 and αAP-Dβ76) were 1.13 and 1.06, respectively, while that from the peptides containing α-Asp (αAP-Lα76 and αAP-Dα76) were 0.38 and 0.36, respectively. The intensity of y13 ions from αAP-Lα76 and αAP-Dα76 was one-third of that from αAP-Lβ76 and αAP-Dβ76. The ratios of the y13/y12 ions are a useful indicator because y13 and y12 ions are a result of cleavage at the N- and C-terminus of aspartic acid in the peptide.

CD-analysis of the peptides

Figure 4a shows CD-spectra of the αAP-Lα76 peptide at 25°C and at 60°C. The secondary structure of this peptide is rich in β-sheet. This structure is very stable and retains the β-sheet structure when the peptide is incubated at a temperature of 60°C for 60 min (Fig. 4a). However, when Asp-76 was substituted with the other three isomers the secondary structure of the peptide isomers (αAP-Lβ76, αAP-Dβ76 and αAP-Dα76) dramatically changed to a random coiled structure at 25°C (Fig. 4b).

Effects of four different isomers at Asp-76 of αA-peptide on the aggregation of insulin

Earlier studies indicated that the peptide KFVIFLDVK HFSPEDLTVK (residues 70–88 of αA-crystallin) is a functional element of α-crystallin (Sharma et al. 2000).

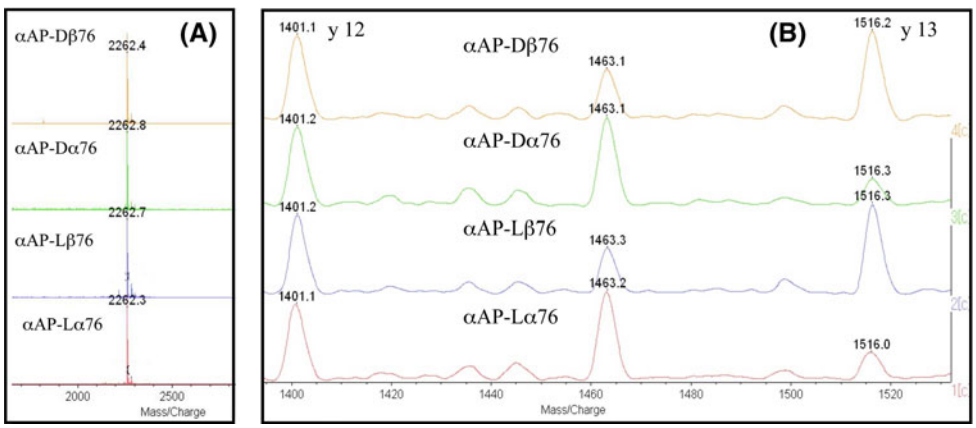

Fig. 3 Mass spectra of the protonated αA-crystallin 70–88 peptide isomers in which the normal Lα of Asp-76 was replaced with Dα, Lβ, and Dβ isomers. **a** Mass spectra of the precursor ion of the protonated peptides **b** Fragmentation spectrum of the protonated peptides

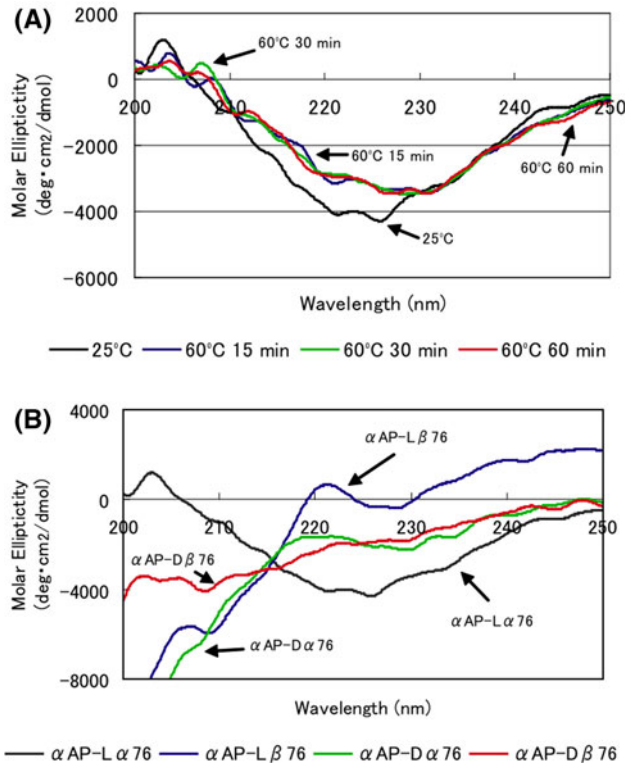

Fig. 4 **a** Far-UV CD spectrum of the αAP-Lα76 peptide at 25°C and 60°C. The αAP-Lα76 peptide retained β-sheet-rich conformation after heating for 60 min at 60°C **b** Far-UV CD spectrum of the αAP-Lβ76, αAP-Dβ76, and αAP-Dα76 peptides at 25°C

Tanaka et al. reported that αAC70-88 promoted the aggregation of insulin (Tanaka et al. 2008).

We investigated the effects of substitution of Lα at Asp-76 of αAC70-88 with Dα, Lβ, and Dβ isomers on the aggregation of insulin. The peptide αAC70-88 described in the paper of Tanaka et al. corresponds to αAP-Lα76 in this paper. The aggregation of insulin was monitored using the ThT fluorescence intensity at 415 nm. As shown in Fig. 5a–d, the aggregation of insulin (triangle in Fig. 5a–d, 2 mg/mL) increased during incubation at 37°C with shaking at 900 rpm for 0–8 h. When the 2 mg/mL insulin was incubated in the presence of 0.25 mg/mL αAP-Lα76 under the same conditions, the aggregation of insulin was rapidly promoted (circle in Fig. 5a). Self-aggregation of αAP-Lα76 was not observed upon incubation at 37°C (in Fig. 5a). These results are consistent with the report of Tanaka et al. (2008). However, when the 2 mg/mL insulin was incubated in the presence of 0.25 mg/mL αAP-Dα76 (circle in Fig. 5b), 0.25 mg/mL αAP-Lβ76 (circle in Fig. 5c), and 0.25 mg/mL αAP-Dβ76 (circle in Fig. 5d), under the same conditions, the aggregation of insulin was completely suppressed. As shown in Fig. 5b–d, the self-aggregation of all peptide isomers themselves, αAP-Dα76, αAP-Lβ76, and αAP-Dβ76 was not observed with incubation under the same conditions.

Discussion

Past studies have detected D-amino acid residues in various proteins from aged tissues and indicated a relationship between the presence of D-amino acids and age-related diseases. We have reported that a large amount of biologically uncommon Dα-, Lβ- , and Dβ-Asp is present at specific sites of αA- and αB-crystallins obtained from eye lenses of elderly people (Fujii et al. 1994a, b). As we have described in previous papers, Dβ-Asp is the most commonly found isomer of the four different possible Asp isomers (Fujii et al. 1999).

In mammalian lenses, αA- and αB-crystallin associate together non-covalently to form α-crystallin in the size range of approximately 700–1,000 kDa. Since the αA and αB monomers are approximately 20 kDa, the aggregate molecules contain between 35 and 50 subunits. Alpha-crystallin can function like a chaperone, binding to non-native or unfolded proteins and protecting them against aggregation induced by heat, reduction, and chemical modification (Bloemendal et al. 2004). However, modified α-crystallin composed of αA- and αB-crystallin contains a large amount of Dα-, Lβ- , and Dβ-Asp residues resulting in huge and heterogeneous aggregation and markedly decreased chaperone activity (Fujii et al. 2007). The results suggest that when normal Lα-Asp residues become inverted to uncommon Dα-, Lβ- , and Dβ-Asp residues in the protein, partial unfolding of protein can be induced which leads to a loss of chaperone function. However, there was no direct evidence linking the inversion of amino acids and the change in structure or function of protein. Therefore, in this study, we used αAC70-88 peptide which was previously well characterized as a functional peptide, and three different isomeric peptides which contained Asp-76 substituted with Dα, Lβ, and Dβ, respectively, and studied the effects of the isomers on the properties of the peptides.

The present study clearly indicates that the substitution of isomers of Asp (Dα-, Lβ- and Dβ-Asp) at position 76 induces a large change to various properties of the αAC 70-88 peptide (called αAP-Lα76 in this paper). When the Asp residue (Asp-76) was substituted with the other Asp isomers, the hydrophobicity of αAP-Lα76 dramatically decreased as shown in Fig. 2. There is a big difference in the hydrophobicity of peptides containing L-Asp and D-Asp in the α-linkage (αAP-Lα76 and αAP-Dα76), while the difference of hydrophobicity of peptides containing L- and D-Asp (αAP-Lβ76 and αAP-Dβ76) in the β-linkage was small. MS/MS indicated that the difference in the intensity of the y13 ion distinguished the β- and α-Asp containing peptides (Fig. 3b). This may depend on frequencies of cleavages at the N- and C-termini of the Asp and we will discuss this mechanism in another paper.

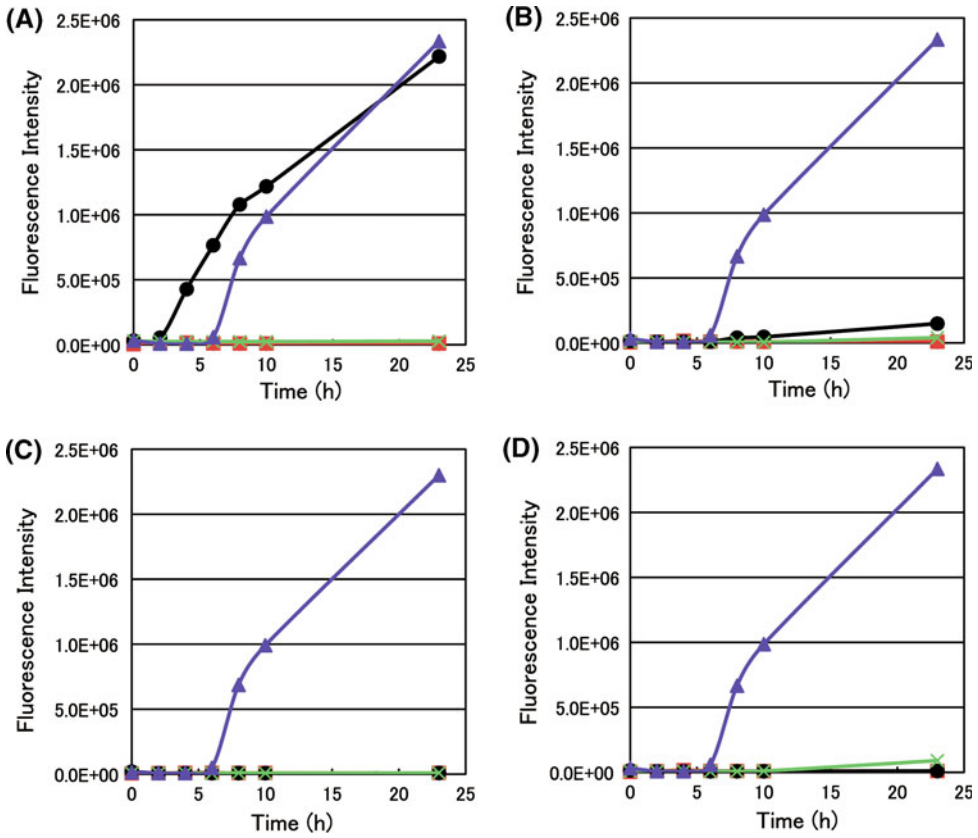

Fig. 5 ThT fluorescence assay for the effects of the peptide isomers (0.25 mg/mL) on the aggregation of insulin (2 mg/mL insulin). The time trace of the ThT fluorescence intensity of the peptide solutions incubated at 37°C with 900 rpm was monitored. **a** Insulin and ThT (*filled triangles*), insulin and ThT in the presence of αAP-Lα76 (*filled circles*), αAP-Lα76 alone (*times symbols*), Insulin alone (*filled diamonds*), ThT alone (*filled squares*) **b** Insulin and ThT (*filled triangles*), insulin and ThT in the presence of αAP-Dα76 (*filled circles*), αAP-Dα76 alone (*times symbols*), Insulin alone (*filled diamonds*), ThT alone (*filled squares*) **c** Insulin and ThT (*filled triangles*), insulin and ThT in the presence of αAP-Lβ76 (*filled circles*), αAP- Lβ76 alone (*times symbols*), Insulin alone (*filled diamonds*), ThT alone (*filled squares*) **d** Insulin and ThT (*filled triangles*), insulin and ThT in the presence of αAP-Dβ76 (*filled circles*), αAP-Dβ76 alone (*times symbols*), Insulin alone (*filled diamonds*), ThT alone (*filled squares*)

The secondary structure of αAP-Lα76 peptide clearly showed a β-sheet rich conformation at 25°C and this conformation was stable upon incubation at 60°C for 60 min (Fig. 4a), which is consistent with a previous result (Tanaka et al. 2008). However, the other three isomers, αAP-Dα76, αAP-Lβ76, and αAP-Dβ76 showed random secondary structure at 25°C (Fig. 4b) and the structure did not change at 60°C (data not shown). The reversed orientation of the side chain induced by the D-amino acid and elongation of the main chain induced by the β-amino acid may affect the hydrophobicity and the secondary structure of the peptides.

Taken together, the results suggest that the β-sheet rich conformation of the αAP-Lα76 peptide may be maintained by hydrophobic interaction and when the hydrophobicity of the peptides is decreased by substitution with the Asp isomers, the secondary structure of the isomers changes to a random structure.

Tanaka et al. (2008) characterized the peptides αAC70-88 (KFVIFLDVKHFSPEDLTVK), αAC71-88 (FVIFLD VKHFSPEDLTVK), and αAC70-88K70D (DFVIFLDV KHFSPEDLTVK). The secondary structure of αAC70-88 is a β-sheet-rich structure while those of αAC71-88 and αAC70-88K70D are random. αAC70-88 and αAC70-88K70D promoted aggregation of insulin while αAC71-88 suppressed aggregation of insulin. The self-aggregation of αAC71-88 and αAC70-88K70D was promoted, while self-aggregation of αAC70-88 did not occur at pH 7.5 with shaking at 900 rpm. In this study, αAP-Lα76 (which is the same as αAC70-88 in the paper of Tanaka et al.) promoted aggregation of insulin while the other three isomers suppressed aggregation. Self-aggregation of all peptides used in this study, αAP-Lα76, αAP-Dα76, αAP-Lβ76, and αAP-Dβ76 did not occur at 37°C, pH 7.5 upon shaking at 900 rpm. The previous studies suggested that the FVIFLD76 sequence of αAC70-88 is important for its self-amyloid fibril formation (Sharma et al. 2000; Tanaka et al. 2008). The substitution of the normal Lα form of Asp-76 with their biologically uncommon isomers dramatically

suppressed insulin aggregation. This result suggests that the chaperone-like activity of the αAC70-88 peptide was a result of substitution of the normal Lα form with their isomers, Dα, Lβ, and Dβ at Asp-76. The mechanism by which a peptide prevents or promotes the aggregation of other proteins is not fully understood. The interaction between αAP-Lα76 and insulin may be stronger than the insulin–insulin interaction. However, when the substitution of a single Asp isomer in a peptide changed the hydrophobicity and the secondary structure of the peptide changed from a beta-sheet-rich structure to a random structure, the alteration may induce a change in the location or number of the binding sites of αAC70-88 isomers for insulin, and thus the isomers may suppress insulin aggregation.

Generally, one point mutation in a protein seldom occurs within living tissues of mammals; however, the isomerization of Asp residues in protein or peptide occurs spontaneously and easily through a succinimide intermediate under physiological conditions. We previously measured the D/L ratios of individual Asp residues in αA-crystallin over a 80-year range. The D/L ratios of Asp-58 (D/L of Asp: 3.1) and Asp-151 (D/L of Asp: 5.8) residues were extremely high. In contrast, the Asp-2 residue (D/L of Asp: 0.01) which is located in the N terminal region was not racemized at all. The D/L ratios of the Asp-76 and Asp-84 residues in the aged αA-crystallin were 0.10 and 0.12, respectively. Unfortunately, since the tertiary structure of αA-crystallin is unknown, the means by which the Asp isomers affect the conformation of the regions surrounding the Asp-2, -58, and -151 residues in the protein is not understood. However, the properties of the αAC70-88 peptide used in this study are well known and thus, this peptide allows clarification of the relationship between the Asp isomer and properties of the peptide.

This study clearly indicates that the Asp isomers change the properties of the peptide with respect to hydrophobicity, secondary structure, and aggregation and interaction with other proteins (insulin in this study). This is the first study focusing on the influence of the peptides containing Asp isomers.

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