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Determination of rate constants for β-linkage isomerization of three specific aspartyl residues in recombinant human αA-crystallin protein by reversed-phase HPLC☆

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ABSTRACT

The major soluble eye lens protein, αA-crystallin, has a very long half-life. Thus, many post-translational modifications, including stereoinversion, have been found in its constituent amino acids. We determine the rates of β-linkage isomerization, which is the main reaction through the formation of a succinimide intermediate, of specific Asp residues of recombinant human αA-crystallin protein by simple RP-HPLC method. Kinetic analyses of the β-linkage isomerization were performed on the three Asp residues of αA-crystallin, ^{58}Asp, ^{84}Asp, and ^{151}Asp, because the D/L ratios of both the ^{58}Asp and ^{151}Asp residues were higher than 1.0 in the αA-crystallin isolated from aged human eye lens. The β-linkage isomerizations of both the ^{58}Asp and ^{84}Asp residues were suppressed in the recombinant protein by approximately 0.4–0.5 times compared to those in the synthetic peptide below 50 °C, whereas the isomerization of the ^{151}Asp residue occurred at the same rate for the whole protein and synthetic fragmentary peptide. The suppression of ^{58}Asp isomerization in the recombinant protein relaxed to some extent when the αA-crystallin protein was incubated at a high temperature. The far-UV CD spectra showed that the secondary structure of the protein was partially disordered at temperatures greater than 60 °C in the recombinant αA-crystallin protein. These results suggest that the ^{58}Asp residue was restrained from forming the succinimide intermediate by the higher order structure of the αA-crystallin protein, and that the structural environment around the ^{151}Asp residue of the αA-crystallin was similar to that of the synthetic fragmentary peptide with respect to succinimide formation. The difference in the influence of the secondary structure of the αA-crystallin protein inverts the order of the succinimide formations of the ^{58}Asp and ^{151}Asp residues in the recombinant protein as compared with the order in the synthetic fragmentary peptides.

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1. Introduction

The main soluble protein in human lens, α-crystallin, is composed of αA- and αB-subunit polypeptides, which noncovalently form protein aggregates ranging from 500 to 800 kDa [1], and functions to maintain lens transparency. Because α-crystallin has a very long half-life, many post-translational modifications have been found in the amino acids of the protein such as oxidation, phosphorylation, and stereoinversion [2–4]. The stereoinversion of Asp residues was also found in the eye lens with cataract obtained from elderly donors [5,6] and the size distribution of αA-crystallin aggregates that contain isomerized Asp residues markedly increase [7]. These results suggest that the stereoinversion of Asp residues in αA-crystallin protein may cause damage to the transparent property of eye lens. Fujii et al. reported that the stereoinversion of amino acids occurred at specific L-aspartyl (Asp) residues, i.e., only the ^{58}Asp and ^{151}Asp residues among fifteen Asp residues of αA-crystallin [8], and found that the D/L ratios of both the Asp residues were higher than 1.0 in the αA-crystallin obtained from aged human eye lens [8]. These results suggest that the stereoinversions at specific Asp residues of the protein are enhanced by the unique conformation of aged human αA-crystallin protein, that is, a chiral reaction field that promotes the inversion of L-Asp to D-Asp residues exists in the αA-crystallin protein.

The stereoinversion of the Asp residue arises through intramolecular rearrangement, such as *via* a succinimide intermediate (Fig. 1) [9–11]. The L-succinimide intermediate, which has

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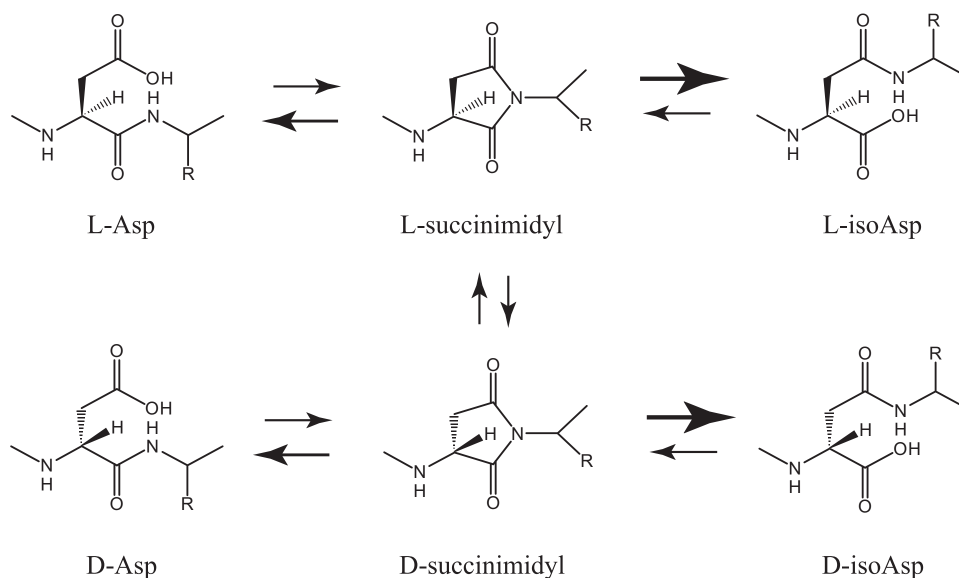

Fig. 1. Pathway for spontaneous β-linkage isomerization and stereoinversion of L-Asp residue *via* succinimide formation.

been generated from the native L-Asp residue in the protein, is quickly hydrolyzed and produces a mixture containing L-Asp and L-isoAsp residues in a ratio of 1:3.6. Some L-succinimide intermediate undergoes reversible stereoinversion, i.e., altering a D-succinimide intermediate, which also produces a mixture of D-Asp and D-isoAsp residues. The β-linkage isomerization and stereoinversion of Asp residues have been found in various proteins such as calmodulin [12,13] and amyloid β protein, which is related to Alzheimer's disease.[14], and occur spontaneously at a physiological temperature and pH. It has been reported that succinimide formation is affected by both the primary amino acid sequence and higher order structure of the protein. The amino acid residue on the carboxyl side of the Asp residue significantly affects the succinimide formation [11,15], which occurs rapidly when there are Gly, Ser, and Ala residues on the carboxyl side of the Asp residue. The secondary structure of the protein such as the α-helix and β-sheet tends to suppress the succinimide formation. Therefore, the formation rate drastically differs based on the location of the Asp residue of the protein [16–19].

In this study, we determined the rate constants for the β-linkage isomerization of three Asp residues, ^{58}Asp, ^{84}Asp, and ^{151}Asp, in recombinant human αA-crystallin because the configurations of both the ^{58}Asp and ^{151}Asp residues were biased toward D-isomers in the αA-crystallin isolated from aged human eye lens. We developed a simple method for quantifying the structural alterations of the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues in human αA-crystallin protein using RP-HPLC with a standard octadecylsilane (ODS) column by referring to our previous studies [20,21] because the traditional method for the quantification of β-linked Asp residues was time-consuming and not sensitive. Kinetic studies revealed that the β-linkage isomerization of the ^{58}Asp residue was suppressed in the recombinant protein (by approximately 0.4–0.5 times compared to Asp isomerization in the synthetic peptide), while the isomerization of the ^{151}Asp residue occurred at the same rate as that in the synthetic peptide. As a consequence, the isomerization of the ^{151}Asp residue at a lower temperature range of 37–50 °C was faster than that of the ^{58}Asp residue in the recombinant αA-crystallin protein, which corresponded to the D/L ratios of ^{58}Asp (D/L = 3.1) and ^{151}Asp (D/L = 5.7) even though the order of the β-linkage isomerization rates was ^{58}Asp > ^{151}Asp in the synthetic fragmentary peptides.

2. Experimental

2.1. Preparation of peptide and purification

Three peptide fragments of αA-crystallin, called T6 peptide (^{55}TVL^{58}DSGISER^{65}, 6th tryptic fragment from the N-terminus), T10 peptide (^{79}HFSPE^{84}DLTVK^{88}), and T18 (^{146}IQTGLD^{151}ATHAER^{157}) were prepared using standard Fmoc chemistry and a Shimadzu PSSM-8 solid-phase peptide synthesizer (Shimadzu, Japan). The Asp residues in the peptides were replaced with D-Asp, L-isoAsp, or D-isoAsp prepared using the reagents Fmoc-D-Asp(OtBu)-OH, Fmoc-L-Asp-OtBu, or Fmoc-D-Asp-OtBu (Watanabe Chemical Co. Ltd., Japan), respectively. All the peptides were purified by RP-HPLC under the isocratic condition and confirmed by mass spectrometry and analyses of their amino acid sequences.

2.2. Preparation of recombinant human αA-crystallin

Recombinant human αA-crystallin was prepared according to a previous report [21]. Briefly, the DNA fragment encoding αA-crystallin protein was inserted into a pET-3*d* expression vector and transformed into *Escherichia coli* BL21 (DE3) pLysS cells. The cells were grown at 37 °C in Lenox broth medium, and the expression of αA-crystallin was induced by the addition of isopropyl thio-β-D-galactoside to a final concentration of 0.3 mM. After culturing for 5 h at 37 °C, the collected cells were disrupted by a freezing and thawing cycle, and the supernatant was recovered by centrifugation at 20,000 × *g* for 10 min. The αA-crystallin was separated by gel filtration chromatography (Sepharose CL-6B, GE-Health Care Bioscience Co., Ltd.) and ion-exchange chromatography (DEAE TOYOPEARL, Tosoh Co., Ltd.) The specimen was concentrated and dialyzed against 50 mM phosphate buffer (pH 7.4).

2.3. Heat treatment of peptides and recombinant protein

The synthetic peptides were dissolved in 50 mM phosphate buffer (pH 7.4) to obtain a final concentration of 100 μM and placed in glass tubes (7 mm id × 80 mm length), which were sealed using a gas burner. Recombinant αA-crystallin was dissolved in 50 mM phosphate buffer (pH 7.4) to obtain a final concentration of 2 mg/ml

and then packed into glass tubes. The samples were incubated at 37 °C, 50 °C, and 90 °C using a bath heater and the reaction was stopped by freezing at −30 °C.

2.4. HPLC analysis of isomerization of Asp residues in treated peptides

The isomerization ratios of the Asp residues in the T6, T10, and T18 peptides were determined by direct analysis using RP-HPLC. The incubated peptide samples were analyzed by HPLC using a JASCO HPLC system 880 (Nippon Bunko Co., Ltd); a C18 column, Develosil ODS-UG-5 (4.6 mm id × 150 mm length, Nomura Chemical Co., Ltd); or Capcell Pak C18 UG120 (4.6 mm id × 150 mm length, Shiseido Co., Ltd). The separation of T6 and T18 peptides was carried out according our previous study [20,21]. The separation of the T6, T10, and T18 peptides was carried out using the mobile phases of acetonitrile–sodium phosphate solutions (pH 5.1, 15 mM (15:85, v/v); pH 3.0, 15 mM (18:82, v/v); and pH 3.0, 15 mM (11:89, v/v); respectively).

2.5. Preparation of tryptic peptides from recombinant protein and HPLC analysis of isomerization of Asp residues

FPLC was applied to the incubated recombinant human αA-crystallin using a Superdex 75 10/300 GL column (10 mm id × 300 mm length, GE Health care Co., Ltd.), and the fraction eluted in the first peak was recovered. The following tryptic treatment and the separation of peptides were carried out referring to our previous study [21]. The specimen was digested with TPCK-treated trypsin (Sigma–Aldrich) for 1 h at 37 °C at an enzyme-to-substrate ratio of 1:10 (mol/mol) to prepare the T6, T10, and T18 peptides for determining the isomerization ratio of the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues in the protein. The tryptic hydrolysate was separated by linear gradient RP-HPLC with monitoring at 215 nm. Solvent A was water with 0.1% trifluoroacetic acid (TFA) and solvent B was an acetonitrile–water solution (40:60, v/v) with 0.08% TFA. The Capcell Pak C18 UG120 (4.6 mm id × 150 mm length) column was equilibrated at room temperature in solvent A. The peptide fragments were eluted at 1 mL/min by a liner gradient of 0–75% solvent B in 30 min. The peaks retained at 18.8–20.5 min and at 23–27.3 min were collected as a T18 peptide sample and a sample containing T6 and T10 peptides, respectively. The T18 peptide sample was analyzed using isocratic HPLC with the mobile phase of an acetonitrile-sodium phosphate solution (pH 3.0, 15 mM) (11:89, v/v). The T6 and T10 peptides were simultaneously analyzed using HPLC with the mobile phase of an acetonitrile–sodium phosphate solution (pH 4.5, 15 mM) (18:82, v/v).

2.6. Kinetic analyses

Kinetic studies of the β-linkage isomerization of Asp residue were carried out according to previous reports using αA-crystallin protein [22–24] and synthetic model peptides [10,25]. The isomerization of the Asp residue *via* succinimide formation has been reported to be a reversible first-order reaction [10,25]. Thus, it can be expressed as follows:

$$\frac{d[\text{isoASP}]}{dt} = k[\text{Asp}] - k'[\text{isoAsp}] \tag{1}$$

where [Asp] and [isoAsp] represent the concentrations of the α-linkage Asp (L-Asp and D-Asp) and β-linkage Asp (L-isoAsp and D-isoAsp) residues, respectively. Rate constants for the isomerization reaction (α-linkage to β-linkage) and for the reverse reaction (β-linkage to α-linkage) are represented by k and k', respectively. The integration of Eq. (1) gives

$$t = \frac{1}{k+k'} \ln |k([\text{Asp}] + [\text{isoAsp}]) - (k+k')[\text{isoAsp}]| + \text{constant} \tag{2}$$

At the initial condition, the [isoAsp] was estimated at 0 because the peptides containing isoAsp residue were not detected. Thus, Eq. (2) gives

$$-(k+k')t = \ln\left(1 - \frac{k+k'}{k}\frac{[isoAsp]}{[Asp]+[isoAsp]}\right) \tag{3}$$

The ratio between the rate constants k and k' is equal to that between the product concentrations [isoAsp] and [Asp] at equilibrium state. Because the isoAsp/Asp ratio in the T6 peptide [20] and model hexapeptide [10] was shown to reach 3.6 at equilibrium state, we assume k: k' = 3.6: 1. Thus, Eq. (3) gives

$$\frac{4.6}{3.6}kt = \ln\left(\frac{1+[isoAsp]/[Asp]}{1-(1/3.6)[isoAsp]/[Asp]}\right) \tag{4}$$

The rate constants for β-linkage isomerization of Asp residues were determined using Eq. (4).

2.7. Circular dichroism measurement

The CD spectra of the recombinant αA-crystallin were measured using a Jasco J-805 automatic recoding dichrograph with a temperature-control unit. The recombinant protein was dissolved in 50 mM phosphate buffer (pH 7.4) at 0.5 mg/mL. Far-UV CD spectra were measured three times with a 1.0 cm light path at 37, 47, 51, 55, 59, 64, and 70 °C. The symbol [θ] represents the mean residue ellipticity in degrees·cm^2/decimole based on the mean residue weight of 173 amino acids of human αA-crystallin (19909 Da).

3. Results

3.1. Determination of conditions for analyzing isomerization of ^{58}Asp, ^{84}Asp, and ^{151}Asp residues in incubated recombinant αA-crystallin

Recombinant human αA-crystallin was expressed in *E. coli* cells and purified by subsequent column operations, including gel filtration and ion-exchange chromatography. The purified protein formed a large aggregate with a molecular mass of 600–800 kDa, and the purity of the protein was found to be over 90% based on an SDS–PAGE analysis (data not shown). The recombinant αA-crystallin was incubated for 180 days at 37 °C and used as a model protein to determine the analytical conditions of RP-HPLC for the isomerization of the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues of αA-crystallin. The incubated αA-crystallin was digested by TPCK-treated trypsin, and the digested peptide fragments were separated using RP-HPLC with a linear gradient of 0–30% acetonitrile containing 0.1% TFA to fractionate the T6, T10, and T18 peptide isomers, which included the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues of αA-crystallin, respectively (Fig. 2a). To determine the fractionation times, the T6, T10, and T18 peptide isomers, which were contained in the L-Asp, D-Asp, L-isoAsp or D-isoAsp, residues of each isomer, were synthesized and then separated under the same conditions used for analyzing the digested peptides of αA-crystallin (Fig. 2b–d). The T6, T10, and T18 peptide isomers were eluted at 23.2–26.8 min, 24.0–27.0 min, and 19.0–20.3 min, respectively. Therefore, the digested peptides eluted at 18.8–20.5 min (indicated by the "T18" bar in Fig. 2a) were fractionated as a sample containing the T18 peptide isomers of αA-crystallin. The digested peptides eluted at 23.0–27.3 min (indicated by the "T6–T10" bar) were fractionated as a sample of mixed T6 and T10 peptide isomers because the synthetic T6 and T10 peptide isomers were eluted at almost

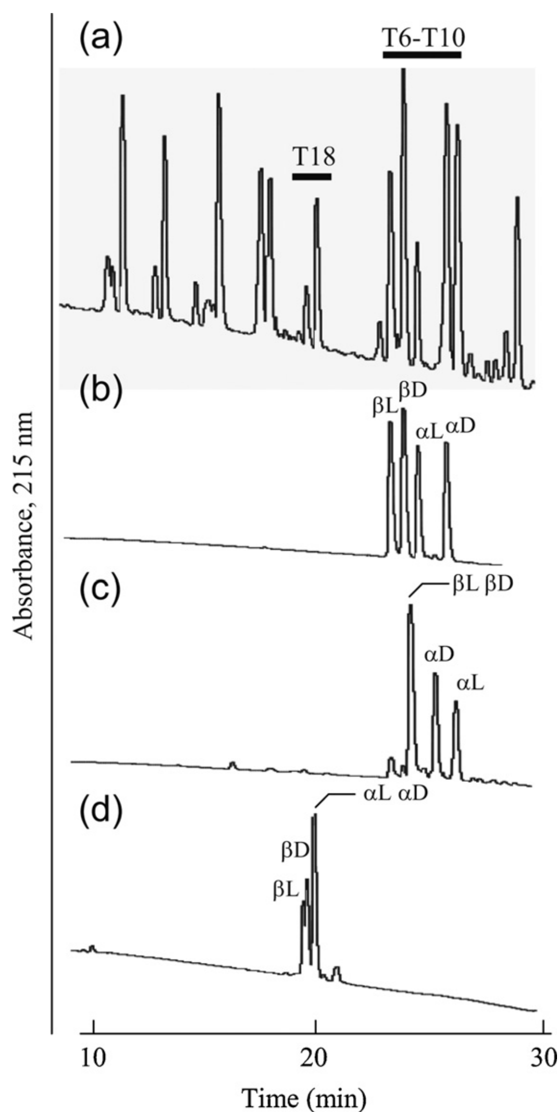

Fig. 2. Preparation of T6, T10, and T18 peptide isomers from tryptic hydrolysate of recombinant αA-crystallin incubated for 180 days at 37 °C. (a) The digested peptides were separated by RP-HPLC with a linear gradient of 0–30% acetonitrile in the presence of 0.1% TFA. The bars indicated by T18 and T6–T10 show the recovery regions of the digested peptides. Separations of the T6, T10, and T18 peptide isomers by RP-HPLC using the same condition as above are shown in (b), (c), and (d), respectively.

the same time (Fig. 2b and d). We further determined the isocratic RP-HPLC conditions for the simultaneous analysis of the T6 and T10 peptide isomers (Fig. 3). The analytical conditions were examined using various conditions for the acetonitrile–sodium phosphate solution, and we decided to use an acetonitrile–sodium phosphate solution (pH 4.5, 15 mM) (18:82, v/v) for a mobile phase to separate the T6–T10 sample. Under these conditions, to avoid the overlap of the T6 peptides containing L-isoAsp residue, T6 peptides containing L-Asp and D-Asp residues were not separated from each other. However, this was not a serious problem for determining the isoAsp/Asp ratio of the ^{58}Asp residue of αA-crystallin.

3.2. Kinetic studies of isomerization of ^{58}Asp, ^{84}Asp, and ^{151}Asp residues in both recombinant αA-crystallin and synthetic peptides

Three synthetic peptides, T6, T10, and T18, containing L-Asp residue were dissolved in 50 mM phosphate buffer (pH 7.4) and incubated at 37, 50, and 90 °C to analyze the β-linkage isomerization of the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues in the peptide fragments. The incubated T6, T10, and T18 peptides were analyzed using RP-HPLC with acetonitrile–sodium phosphate solutions (pH 5.1, 15 mM (15:85, v/v); pH 3.0, 15 mM (18:82, v/v); and pH 3.0, 15 mM (15:85, v/v); respectively) [20,21], and the rates of the β-linkage isomerization of the ^{58}Asp, ^{84}Asp, and ^{151}Asp residues were determined by comparing the peak areas of the isomerized peptides. The β-linkage isomerization was observed in all three Asp residues, ^{58}Asp, ^{84}Asp, and ^{151}Asp, and occurred in the manner of a first order reaction (Fig. 4, solid circles). In the examined temperature range of 37 to 90 °C, the order of the β-linkage isomerization rates was ^{58}Asp in T6 > ^{151}Asp in T18 > ^{84}Asp in T10.

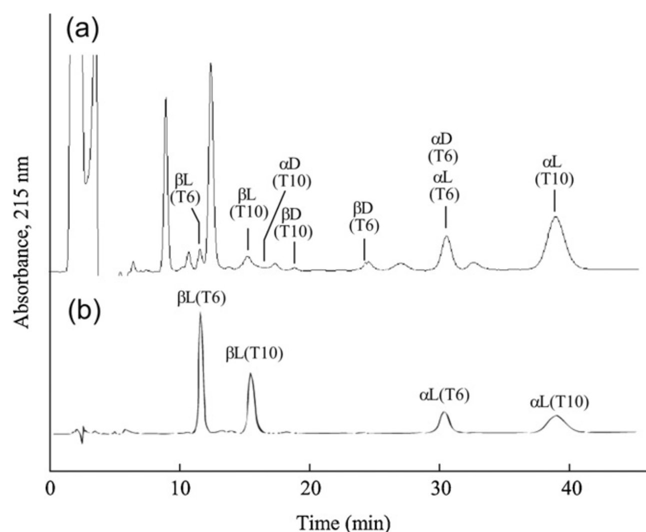

Fig. 3. Simultaneous analysis of T6 and T10 peptide isomers obtained from recombinant αA-crystallin incubated for 180 days at 37 °C. (a) The T6–T10 sample recovered in Fig. 2a was separated by RP-HPLC with an acetonitrile–sodium phosphate solution (pH 4.5, 15 mM) (18:82, v/v). The isomers are indicated at the tops of the peaks. (b) The mixture of T6 and T10 isomers, which contained L-Asp and L-isoAsp residues in each peptide, was separated by RP-HPLC using the same condition mentioned above.

To analyze the kinetics of the β-linkage isomerization of the Asp residues in the recombinant αA-crystallin protein, the purified protein was dissolved in 50 mM phosphate buffer (pH 7.4) and incubated at 37, 50, and 90 °C. After heat treatment, the recombinant protein that maintained the ability to aggregate was separated by gel filtration chromatography, and then digested with trypsin. Both the T18 and T6–T10 samples were fractionated using linear gradient RP-HPLC under the conditions described in Fig. 2. The β-linkage isomerizations of the ^{58}Asp and ^{84}Asp residues were determined by an RP-HPLC analysis of the T6–T10 sample using the mobile phase of an acetonitrile–sodium phosphate solution (pH 4.5, 15 mM) (18:82, v/v), as described in Fig. 3. The β-linkage isomerization of the ^{151}Asp residue was determined by an analysis of the T18 sample using isocratic RP-HPLC with an acetonitrile–sodium phosphate solution (pH 3.0, 15 mM) (11:89, v/v) [21]. In the recombinant αA-crystallin protein, the β-linkage isomerization was also observed in all three Asp residues, ^{58}Asp, ^{84}Asp, and ^{151}Asp, and occurred in the manner of a first order reaction (Fig. 4, open circles).

The β-linkage isomerizations of both the ^{58}Asp and ^{84}Asp residues of the αA-crystallin protein were significantly suppressed in comparison with those in the synthetic fragmentary peptides (Fig. 4). The rate constants of both Asp residues of the protein were approximately 0.4–0.5 times that in the synthetic peptides below 50 °C (Table 1). However, the difference in the rate constants of the ^{58}Asp residue between the protein and synthetic peptide became small at 90 °C (approximately 0.8 times), whereas the magnitudes of the rate constants of the ^{84}Asp residue were not altered at 90 °C.

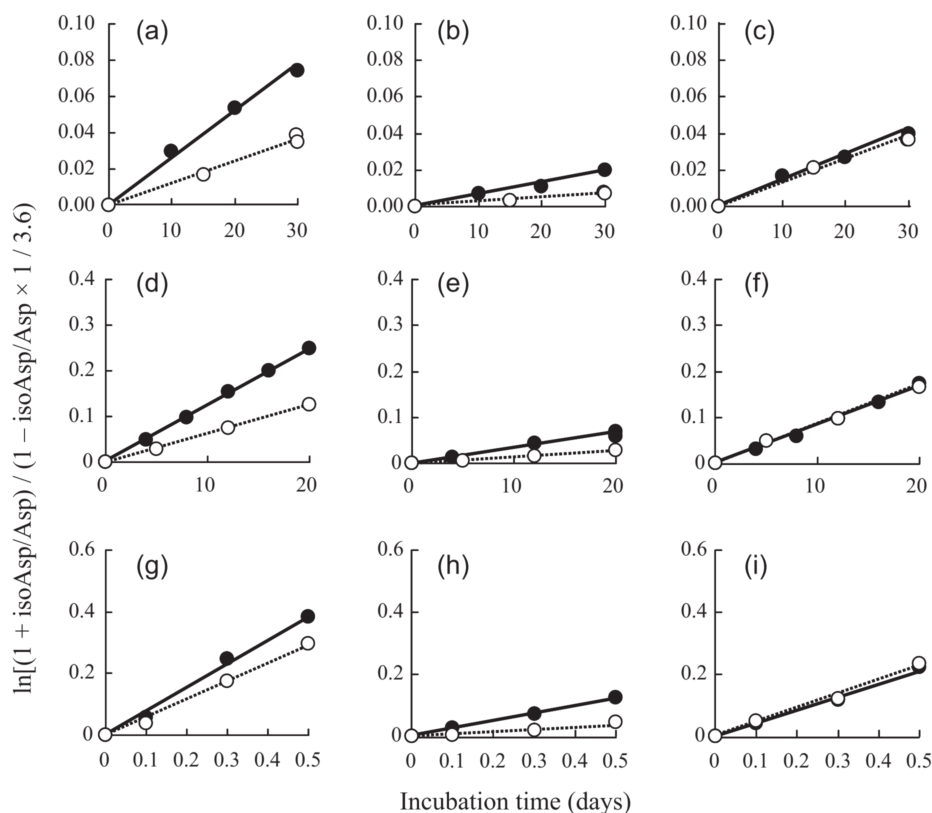

Fig. 4. β-Linkage isomerization of three Asp residues in synthetic fragmentary peptides (solid circles) and recombinant αA-crystallin protein (open circles) at various temperatures. The samples were incubated at 37 (a–c), 50 (d–f), and 90 °C (g–i), and the β-linkage isomerizations of the [58]Asp (a, d, g), [84]Asp (b, e, h), and [151]Asp (c, f, i) residues were quantified by RP-HPLC analyses. The rate constants of the β-linkage isomerization were determined from the slope of each line.

These results suggest that both the [58]Asp and [84]Asp residues of αA-crystallin are restrained from forming the succinimide intermediate by the higher order structure of the αA-crystallin protein. In contrast, the isomerization of the [151]Asp residue occurred at the same rate for the whole αA-crystallin protein and the synthetic fragmentary peptide even at 37 °C, which suggests that the structural condition for succinimide formation near the [151]Asp residue of αA-crystallin resembles that of the synthetic fragmentary peptide. As a consequence, the order of the β-linkage isomerization rates in the αA-crystallin protein was [151]Asp > [58]Asp > [84]Asp because of the suppression of the [58]Asp isomerization by the higher order structure of the protein below 50 °C.

3.3. Analysis of secondary structure of recombinant αA-crystallin at various incubating temperatures

To examine the effect of the incubating temperature on the secondary structure of the recombinant human αA-crystallin, the CD spectra were measured at various temperatures ranging from 37 to 70 °C (Fig. 5). The far-UV CD spectrum of the recombinant protein at 37 °C showed a minimum at 217 nm (Fig. 5a), the shape of which is similar to that of the spectrum measured with bovine native αA-crystallin (data not shown). The shape of the spectrum changed as the incubating temperature increased, and the minimum for the CD spectrum at 70 °C changed to 205 nm, which indicated that the structure of the αA-crystallin protein was partially disordered as the result of the high temperature. The measurement results for the ellipticity at 205 nm at various incubating temperatures showed that the major transition by a secondary structural change took place between 47 and 60 °C (Fig. 5b), which corresponded well with the alteration in the suppression of the [58]Asp residue by the higher order structure of the protein, which occurred between 50 and 90 °C (Fig. 4). This temperature agreement strongly suggests that the secondary structure of the αA-crystallin protein restricts the succinimide formation of the [58]Asp residue.

4. Discussion

The traditional methods for quantifying the β-linkage isomerization of amino acids are both time-consuming and quantitatively inadequate because the β-linkage of an amino acid is confirmed indirectly by an amino acid sequencer using the principle that β-linked amino acids are resistant to Edman degradation. In this study, we established a simple method for quantifying the struc-

Table 1
Rate constants of β-linkage isomerization in Asp residues.

Temperature (°C)	[58]Asp (/day)		[84]Asp (/day)		[151]Asp (/day)	
	In peptide	In protein	In peptide	In protein	In peptide	In protein
37	0.0020	0.0009	0.0005	0.0002	0.0011	0.0010
50	0.0098	0.0048	0.0026	0.0010	0.0066	0.0065
90	0.60	0.46	0.19	0.064	0.34	0.35

The rate constant k of each peptide was determined by the slope of the linear regression least squares line ($r^2 > 0.97$) of the first-order reaction in Fig. 4.

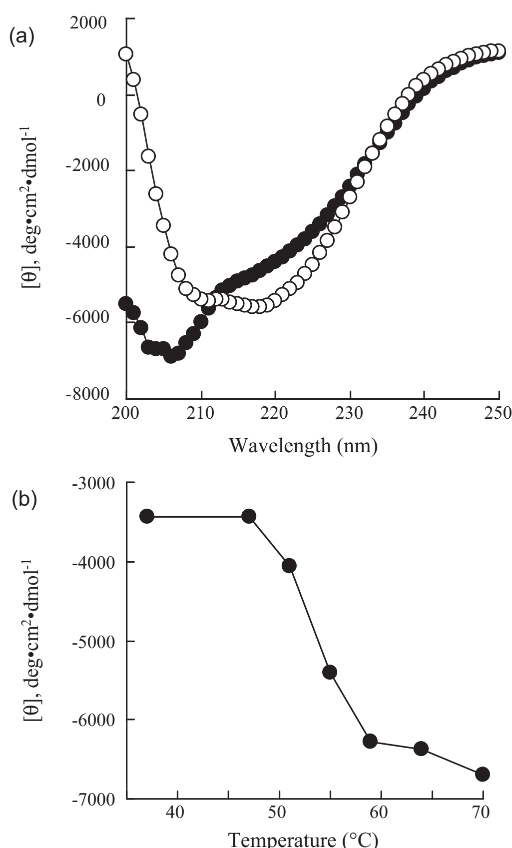

Fig. 5. CD spectra of recombinant human αA-crystallin incubated at various temperatures. (a) Far-UV CD spectra of αA-crystallin measured at 37 (open circles) and 70 °C (solid circles). (b) Change in ellipticity at 205 nm with respect to temperature.

tural alterations of specific Asp residues in the recombinant human αA-crystallin protein, and the development of this method made it possible to perform easy and rapid analyses of the β-linkage isomerizations of the three Asp residues at various temperatures. Almost all human proteins are initially made from L-amino acid in the manner of α-linkage ligation. Thus, if isomerization to the β-linkage or stereoinversion to the D-form occurs at a specific amino acid residue, the resultant protein becomes a diastereomer compared to the native one. Because an entire protein is not a suitable target for RP-HPLC separation, peptide fragments containing such structurally altered amino acid residues, which are also diastereomers of each other, should be prepared using an appropriate treatment of the specific protease. The standard RP-HPLC can separate and quantify the four diastereomers containing αL-, αD-, βL-, and βD-amino acids at a specific residue. Thus, we can easily quantify the structural alteration of Asp residues simultaneously without any extra operations. The key to our method is the determination of the RP-HPLC separation conditions for the four diastereomers. We established the HPLC conditions for analyzing structurally altered amino acid residues in the peptide fragments of human αA-crystallin: ^{55}TVLDSGISEVR^{65}, ^{79}HFSPEDLTVK^{88}, ^{146}IQTGLDATHAER^{157}, and the peptide fragment of the Prion protein, ^{106}KTNMKHMAGAAAAGAVVGGLG^{126} [20,21,26]. The structural alteration of the underlined Asp and Asn residues in these peptides can be quantified by our simple method using RP-HPLC. These successful separations suggest that our simple method using RP-HPLC can be used to easily and speedily analyze the structural alteration of specific Asp residues in a variety of proteins.

Kinetic studies using synthetic peptides with identical sequences of the three tryptic fragments of αA-crystallin (T6, T10, and T18 peptides) revealed that the order of the rate constants for β-linkage isomerization was ^{58}Asp in T6 > ^{151}Asp in T18 > ^{84}Asp in T10 peptides at temperatures ranging from 37 to 90 °C. The rate constants for the β-linkage isomerization of the ^{58}Asp and ^{151}Asp residues were 0.0098/day and 0.0066/day at 50 °C, respectively. The Ser residue, which is the amino acids at the carboxyl side of the ^{58}Asp residue in the T6 peptide, was reported to accelerate the succinimide formation by its hydroxyl catalysis [11]. Deprotonation from the amide group of the peptide chain may be assisted by the hydroxyl group of the serine residue, which results in an enhancement of the nucleophilicity of the nitrogen and accelerates the succinimide formation [27]. The ratio of the rate constants for the ^{58}Asp and ^{151}Asp residues was estimated to be approximately 1.5 using the data from the 50 °C incubation. This ratio well corresponds with that for succinimide formation reported previously using a model hexapeptide. The succinimide formation in Val-Tyr-Pro-Asp-Ser-Ala was 1.6 times faster than that of Val-Tyr-Pro-Asp-Ala-Ala when these peptides were incubated at 70 °C [11]. These two model peptides have the same amino acids on the carboxyl side of the ^{58}Asp and ^{151}Asp residues of αA-crystallin, i.e., Ser and Ala. These results show that the amino acid residue on the carboxyl side of the Asp residue is the main determinant of the β-linkage isomerization rates for the ^{58}Asp and ^{151}Asp residues in the synthetic peptides. Taken together, they show that the rate constant for the β-linkage isomerization at the initial phase represents that of succinimide formation.

In the recombinant human αA-crystallin, the β-linkage isomerizations of the ^{58}Asp and ^{84}Asp residues were suppressed in comparison with those in the synthetic peptides, and the rate constants of both residues in the protein were approximately 0.4–0.5 times that in the synthetic peptides at a 37 °C incubation temperature. These results corresponded with the previous suggestion that the secondary structure of the protein restrains the Asn or Asp residue from forming the succinimide intermediate [17]. The nucleophile–electrophile distance should be shorter than 2 Å so that Asn residues in the protein undergo deamidation. However, the minimum critical distances in α-helix, β-sheet, and β-turn structures are estimated to be 2.5 Å, 3.5 Å, and 3.1–3.3 Å, respectively, which result in a slower deamidation of the Asn residues located in the secondary structures compared to that in unstructured regions [28]. The rate constant of the ^{58}Asp residue in the recombinant αA-crystallin protein came close to that in the synthetic T6 peptide at the higher temperature of 90 °C. This result corresponds to the partial disorder of the secondary structure of the recombinant αA-crystallin protein that occurs at temperatures over 60 °C, which agrees with the previous results using bovine αA-crystallin protein [29,30]. These results support the suggestion that the secondary structure of αA-crystallin suppresses the succinimide formation of the ^{58}Asp residue. The magnitude of the suppression of the β-linkage isomerization of the ^{84}Asp residue in the recombinant αA-crystallin protein did not alter for temperatures ranging from 37 to 90 °C. This result suggests that the secondary structure near the ^{84}Asp residue is resistant to high temperature. This suggestion is supported as follows: the αA-crystallin protein functions as a chaperon against heat denaturation and the secondary structure of the αA-crystallin shows thermal reversibility, which was confirmed by the result that the minimum of the far-UV CD spectra of the recombinant protein moved back to 217 nm after cooling to 37 °C (data not shown). The rate constant for the ^{151}Asp residue in the recombinant αA-crystallin protein was almost equivalent to that in the synthetic T18 peptide at temperatures ranging from 37 to 90 °C. This result suggests that the region near the ^{151}Asp residue of αA-crystallin was highly flexible. Recently, an X-ray structural analysis revealed the crystal structure of truncated bovine αA-crystallin (from 59 to 163 residues). In bovine αA-crystallin, the ^{84}Asp residue formed β-sheet or β-turn structures and the ^{151}Asp

residue was located in the unstructured region of the carboxyl terminus [31]. These results well correspond with our result obtained from analyses of the rate constants for β-linkage isomerization in the [84]Asp and [151]Asp residues. However, the present X-ray analysis does not give the structural information regarding [58]Asp residue, the β-linkage isomerization of which altered by the change of temperature. X-ray structural analysis of whole αA-crystallin protein is needed to discuss the relationship between heat-sensitive structure and β-linkage isomerization of Asp residue.

Fujii et al. suggested that αA-crystallin protein had a chiral reaction field that promotes the inversion of L-Asp to D-Asp residue because the D/L ratios of the [58]Asp and [151]Asp residues are higher than 1.0 [8,23,32–34]. However, the present study suggests that the [151]Asp residue of αA-crystallin is located in the flexible region of the protein, which was also shown by an X-ray structural analysis of truncated bovine αA-crystallin [31]. Because αA-crystallin has a long half-life, a large amount of isoAsp is accumulated spontaneously at the [151]Asp residue of αA-crystallin. There have been some reports that structurally altered Asp residues induce a drastic conformational change in protein. The substitution of isoAsp for the [7]Asp or [23]Asp residue increased the β-sheet content of the amyloid β protein$^{1-42}$ [35], and the substitution of D-Asp for the [23]Asp residue enhanced its fibril aggregation [36,37]. An experiment using five model synthetic peptides showed that the β-linkage isomerization of the Asp residue affected the major conformations of the synthetic peptides [38]. The weak denaturing condition was also reported to lead to the formation of amyloid fibril in an α-crystallin protein [39]. These results suggest that the conformational alteration of an α-crystallin protein may be induced by structural alteration of a specific Asp residue, which may create the chiral reaction field in the aged αA-crystallin protein. In the truncated αA-crystallin obtained from aged human lens, which had a carboxyl terminal region after the [155]Ala residue was deleted, the D/L ratios of the [151]Asp residue drastically decreased to 0.3 [32]. Therefore, the X-ray crystallography of the truncated αA-crystallin is incomplete for the elucidation of the structural environment near the [151]Asp residue. Further investigations are needed to obtain information related to the higher-order structure of the aged αA-crystallin protein, the specific Asp residues of which are replaced by the isoAsp or D-Asp residue.

5. Conclusions

We established a simple RP-HPLC method for the easy and rapid analysis of the structural alterations of Asp residues and used it to analyze the effect of a protein's higher-order structure on the succinimide formation in the recombinant αA-crystallin protein. Our present method was based on the separation of peptide diastereomers containing αL-, αD-, βL-, and βD-amino acids at specific residues, and succeeded in quantifying the β-linkage isomerization and stereoinversion of Asp residues on the peptides related to neuronal diseases such as Alzheimer's disease and Prion disease [26], as well as on the tryptic-digested peptide fragments of αA-crystallin protein. Because the spontaneous structural alterations of Asp and Asn residues cause biologically important changes in peptide and protein structures, and function as molecular clocks measuring the time of biological processes such as protein degradation, a simple quantification method using RP-HPLC should greatly facilitate the evaluation of the post-translational modification of a protein and reveal new biological roles for structural alterations at specific amino acids of the protein.

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