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Changes of Secondary Structure Detected by Laser Raman Spectroscopy in Model Peptides of Human αA-Crystallin Due to Substitution of D-Aspartyl Residues for L-Aspartyl Residues

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Purpose: We have found that two aspartyl (Asp-151 and Asp-58) residues of αA-crystallin are inverted and isomerized to the biologically uncommon D-β-Asp residues during aging. In order to elucidate the correlation between the formation of the D-β-Asp isomer and the environment surrounding the Asp in the protein, we performed a Raman spectroscopic study using two synthetic peptides: T6 peptide containing Asp-58, and T18 peptide, containing Asp-151, which correspond to the tryptic peptides of human αA-crystallin.

Methods: Both T6 (Thr-Val-Leu-Asp[58]-Ser-Gly-Ile-Ser-Glu-Val-Arg) and T18 (Ile-Gln-Thr-Gly-Leu-Asp[151]-ala-thr-his-ala-Glu-Arg) peptides were synthesized with four optical isomers which have L-α-, D-α, L-β and D-β-aspartyl residues. These peptides were subjected to Raman measurement.

Results: The Raman spectrum of the L-α-Asp T18 peptide measured as dry powder revealed that the secondary structure of this peptide is mainly anti-parallel β-sheet. The main structure of the D-β-Asp T18 peptide when in dry powder form was altered to an α-helix and/or random structure. The main structure of L-α-Asp T18 peptide when measured in aqueous solution also converted to an α-helix and/or random structure. The conversion of L-α-to D-β-Asp in T6 peptides when in dry powder form revealed no alteration of secondary β-sheeted structure.

Conclusion: Raman spectroscopy clearly revealed a large conformational change in the secondary structure of T18 peptide caused by substitution of normal L-α-Asp to biologically uncommon Asp-isomers. This result indicates that the inversion of an amino acid in a protein greatly affects the secondary structure of the protein. **Jpn J Ophthalmol 2000;44:354–359** © 2000 Japanese Ophthalmological Society.

Key Words: αA-crystallin, D-aspartic acid, model peptide, racemization, Raman study.

Introduction

Biologically uncommon D-aspartyl (Asp) residues have been reported in proteins of the tooth,[1] eye lens,[2] and aorta[3] in elderly humans. Aspartic acid is the most easily racemizable amino acid[4] and D-Asp may be formed by racemization in metabolically inactive tissues during the natural aging process. However, past studies have only observed D-Asp among all the proteins of whole tissues, and the localization of D-Asp was not known. In previous studies, we found D-Asp residues in specific sites of αA- and αB-crystallin, major components of human eye lens proteins. In aged αB-crystallin, the Asp-36 and Asp-62 residues are highly racemized.[5] A more interesting result is that the stereoconfiguration of the Asp-

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58 and Asp-151 residues is inverted to the D-isomer (D/L ratio of Asp > 1.0) in aged αA-crystallin.[6] Since racemization is defined as a reversible first-order reaction, when the D/L ratio reaches 1.0, the racemization is in equilibrium. Thus, D/L ratios >1.0 would not be defined as racemization, but as the inversion of configuration of L-amino acid to its D-isomer. This was the first observation of the inversion of the configuration of the amino acids in proteins in vivo during the natural aging process. Site-specific racemization of Asp-151 residue of αA-crystallin has also been observed in cow,[7] horse (unpublished data), and rat,[8] despite the difference in amino acid sequences.

These results suggest that the configuration of Asp-151 residue in αA-crystallin is stereochemically labile. On the other hand, these racemized/inverted Asp residues are simultaneously isomerized to form β-Asp residues in both αA- and αB-crystallin.[5,6] This result indicates that the normal L-α-Asp residues at specific sites of the protein are inverted to D-α, D-β-, L-β-Asp isomers, via a succinimide intermediate. Recently, we reported that the secondary structure in the region of Ala-155 to the C-terminus in αA-crystallin may constitute a field that causes the inversion of the Asp-151 to the D-isomer form.[9]

In order to elucidate the relationship between the four different isomers of Asp residues, L-α-, D-α, L-β, and D-β-Asp, in the peptides and their secondary structure, we performed a Raman spectroscopic study using a T6 peptide (Thr-Val-Leu-Asp[58]-Ser-Gly-Ile-Ser-Glu-Val-Arg) and a T18 (ile-Gln-thr-Gly-Leu-Asp[151]-Ala-Thr-His-Ala-Glu-Arg) peptide which both have L-α-, D-α, L-β, and D-β-aspartyl residues.

Materials and Methods

Synthesis of Peptides

We synthesized T6 and T18 peptides, which correspond to the tryptic peptides of human αA-crystallin. The sequences of the peptides are: Thr-Val-Leu-Asp[58]-Ser-Gly-Ile-Ser-Glu-Val-Arg: T6 and ile-Gln-thr-Gly-Leu-Asp151-Ala-Thr-His-Ala-Glu-Arg: T18 peptides. Both T6 and T18 peptides were synthesized with L-α-, D-α, L-β, and D-β-amino acids at Asp-58 or Asp-151 residues, respectively.

These peptides were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid phase chemistry in dimethylformamide.[10] Benzotriazole-1-yl-oxy-tris-pyrrolydino-phosphonium hexafluorophosphate and hydrobenzotriazole were used as the catalysts. The synthetic peptides were removed from formic groups with piperidine. The peptides were purified (>80%) by reverse-phase high pressure liquid chromotography (RP-HPLC) using a C18 column with an elution gradient of different mixtures of acetonitrile and 0.1% trifluoroacetic acid. All peptides synthesized were characterized by RP-HPLC, amino acid analysis and mass spectroscopy.

Raman Spectroscopic Study

Each peptide sample (about 1 mg) was transferred to a small depression (about 10 mL in volume) on an aluminum plate. With the incident laser beam illuminated from above the sample, each sample was first measured by Raman spectroscopy in the powder state. The sample was then dissolved in 10 μL of pure water and measured by Raman spectroscopy in the solution state.

An NRS-2000 Raman spectroscope (JASCO, Tokyo) with CCD detector (LNCCD 1100PB; Princeton Instruments, Cambridge, MA, USA) was used for this study. A wavelength of 514.5 nm was supplied from an ion laser (NEC, Kawasaki). The laser power was less than 10 mW at the sample position.

Results

The 200–1800 cm^{-1} region of the Raman spectra of T18 and T6 peptides was measured. In the present study, we particularly focused on the amide I and amide III regions.[11,12] The Raman spectrum of the L-α-Asp T18 peptide (L-α-Asp T18) in dry powder is shown in Figure 1. Several bands were assigned to be from the peptide backbone and residues, namely, amide III at 1238 cm^{-1}, amide I at 1671 cm^{-1}, CH_2 scissoring at 1437 cm^{-1}, and carbonyl bonds at 1735 cm^{-1}. A band from histidine was not detected. The main secondary structure is anti-parallel β-sheet because the frequency of amide III is at 1238 cm^{-1} and amide I is at 1671 cm^{-1}. In Figure 2, the spectrum of the D-β-Asp T18 peptide (D-β-Asp T18) measured in the powder state revealed that the secondary structure of this peptide most likely is mainly α-helix because of the shift of amide III (1238 cm^{-1}) to 1265 cm^{-1} and the appearance of another α-helical band at 937 cm^{-1}. The spectrum of the amide I band of D-β-Asp T18, which appeared at 1671 cm^{-1}, is smaller and wider than that of L-α-Asp T18, indicating a decrease in the amount of β-sheet structure in D-β-Asp T18 as compared to L-α-Asp T18 peptide.

Figures 3a–c show the spectra of T18 peptides with L-α-Asp as dry powder (a), D-α-Asp as dry powder (c), and L-α-Asp in aqueous solution (b). The spectrum of the D-α-Asp T18 peptide with only one inversion of L-α-Asp as dry powder revealed that the conformation of the T18 peptide had changed to an

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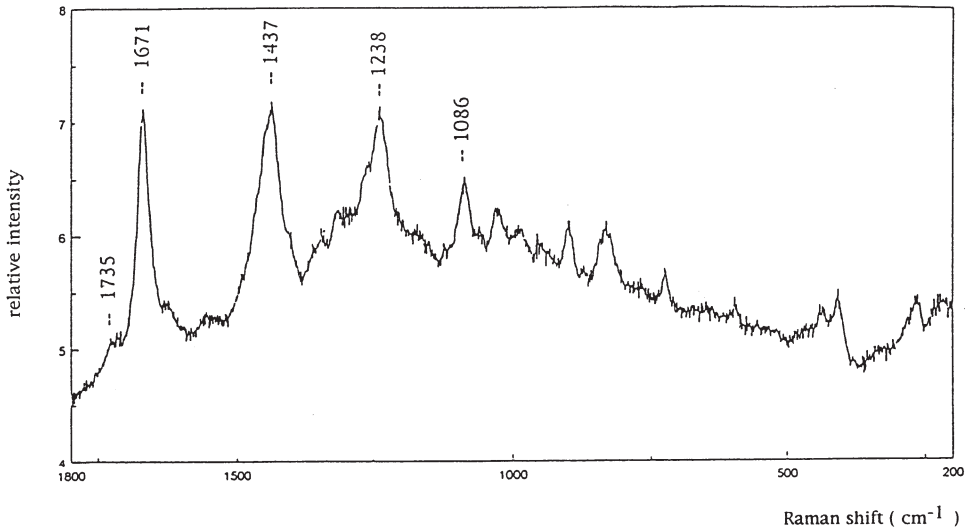

Figure 1. Raman spectrum of synthetic L-α-Asp T18 peptide corresponding to fragments of human αA-crystallin measured as dry powder.

α-helix and/or a random structure from the original β-sheet (Figures 3a,c). The spectrum of L-α-Asp T18 peptide in aqueous solution (Figure 3b) showed a conformational change from an anti-parallel β-sheet to an α-helix and/or a random structure. The amide III band of L-α-Asp at 1240 cm^{-1} in the powder state shifted to 1270 cm^{-1} with a shoulder at 1250 cm^{-1} when it was in aqueous solution. A small α-helical band at 935 cm^{-1} appeared in the spectrum of L-α-Asp measured in aqueous solution (Figures 3a,b).

The Raman spectrum of the D-β-Asp T18 peptide measured in aqueous solution (figure not shown) did not differ in conformation from that in the dry powder state (Figure 2). The secondary structure of the D-β-Asp T18 peptide remained an α-helix in both the powder state and aqueous solution.

The other two synthetic peptides of the T18 peptide, the L-β-Asp and D-α-Asp isomers, showed an α-helical conformation in both the dry powder state and aqueous solution because the band of amide III appears at 1265 cm^{-1} in these spectra of L-, β-, and D-α-Asp T18 peptide in both dry and aqueous states (figure not shown).

The only conformation of the L-α-Asp T18 pep-

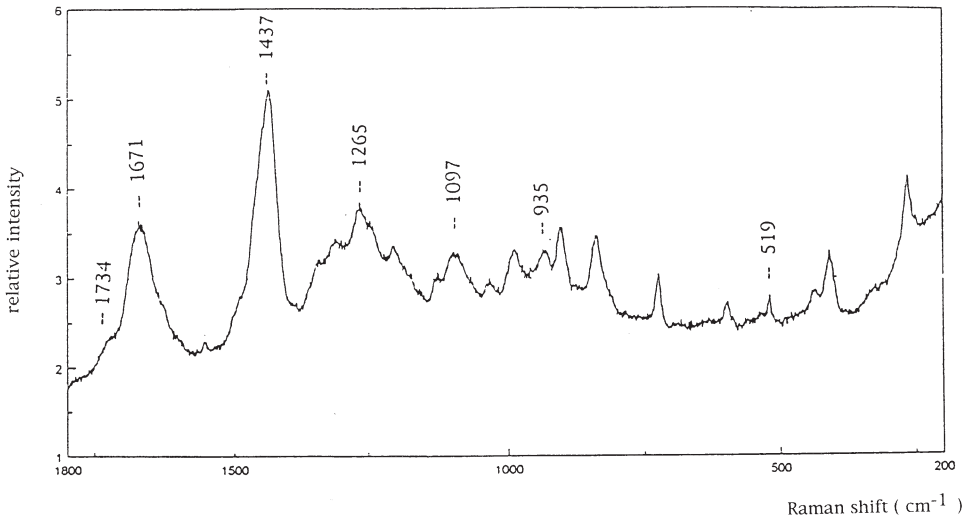

Figure 2. Raman spectrum of synthetic D-β-Asp T18 peptide corresponding to fragments of human αA-crystallin measured as dry powder.

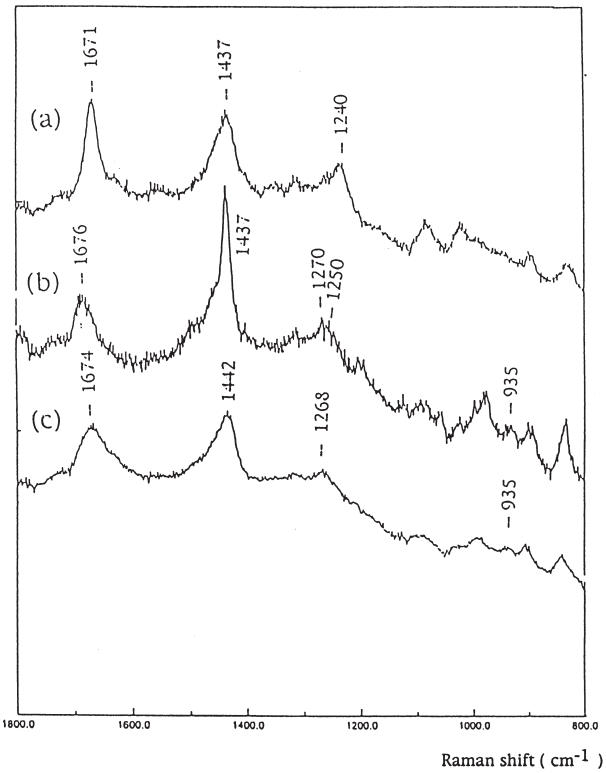

Figure 3. Raman spectra of synthetic peptides corresponding to fragments of human αA-crystallin. (**a**) L-α-Asp T18 as dry powder, (**b**) L-α-Asp T18 peptide in aqueous solution (after digital subtraction of water background), and (**c**) D-α-Asp T18 peptide as dry powder.

tide in the dry powder state was β-sheet. Inversion of the configuration of aspartic acid from the L-form to D-form, isomerization of aspartic acid from an α-bond to a β-bond and the hydrostatic change of the T18 peptide from a hydrophobic (in dry powder) to hydrophilic state (in aqueous solution) may cause a conformational change in T18 from a β-sheet structure to α-helix and/or random structure. These results are summarized in Table 1.

The spectrum of L-α-Asp T6 peptide in dry powder is shown in Figure 4a. The band of amide III is at 1235 cm^{-1}, which means that the secondary structure of the T6 peptide is mainly β-sheet. The spectrum of the D-β-Asp T6 peptide in dry powder (Figure 4b) also reveals that the conformation of the T6 peptide remained mainly β-sheet in inverted and isomerized forms of aspartic acid in the dry powder state.

Discussion

Raman spectroscopy and other methods[13] have shown that the conformation of lens proteins is mainly an anti-parallel β-sheet structure. The advantage of the β-sheet structure of lens proteins is that it maintains the transparency of the lens itself.[14,15]

There have been a few studies by Raman spectroscopy on the secondary structure of peptides,[16,17,18] but the conformation of the fragments of lens proteins has not been well investigated spectroscopically. T18 and T6, as model peptides corresponding to fragments of human αA-crystallin, contain one aspartic acid each. In the native, aged human αA-crystallin, Asp-151 in T18 peptide and Asp-58 in T6 peptide are susceptible to inversion from the L-form to D-form, and both Asp residues are isomerized from the α-bond to β-bond.[9,19,20] In the present study, the Raman spectra of T6 peptide in the solid state demonstrated that the conformation of T6 peptide retained β-sheet structure even if L-α-Asp was replaced with D-β-asp isomer (Figure 4).

However, the conformation of the T18 peptide changed under certain conditions. The only conformation of L-α-Asp T18 in the dry powder state was β-sheet, as shown in Figure 1. When the L-α-Asp residue in the T18 peptide was replaced by D-α-Asp, the main secondary structure of the β-sheet changed

Table 1. Secondary Structure of T18 and T6 Peptides Containing Optimers of Asp Residues

	T18			T6	
Asp Isomer	State	Secondary Structure	Asp Isomer	State	Secondary Structure
L-α	Solid	β-sheet	L-α	Solid	β-sheet
L-α	Aqueous	α-helix/random	L-α	Aqueous	nd
D-α	Solid	α-helix/random	D-α	Solid	nd
D-α	Aqueous	α-helix/random	L-α	Aqueous	nd
L-β	Solid	α-helix/random	L-β	Solid	nd
L-β	Aqueous	α-helix/random	L-β	Aqueous	nd
D-β	Solid	α-helix/random	D-β	Solid	β-sheet
D-β	Aqueous	α-helix/random	D-β	Aqueous	nd

nd: not detected due to interference of fluorescence.

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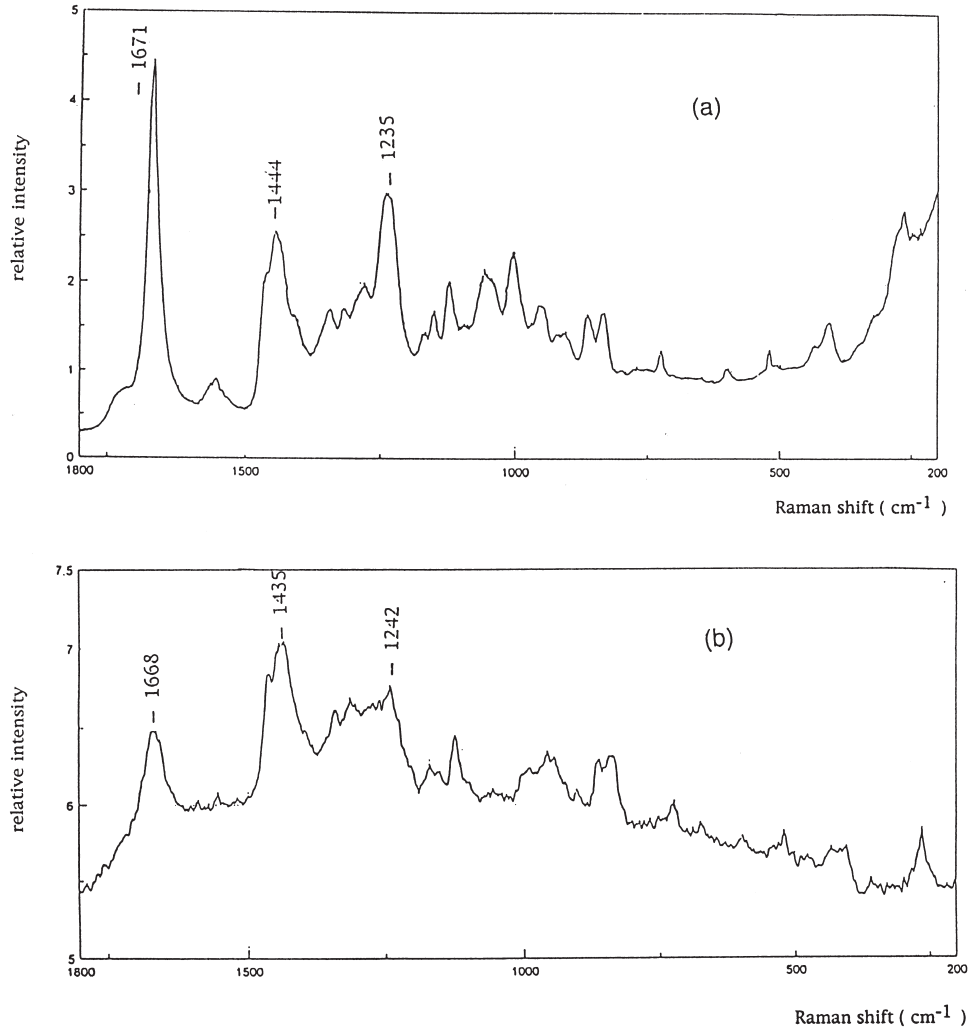

Figure 4. Raman spectra of synthetic peptides corresponding to fragments of human αA-crystallin (**a**) L-α-Asp T6 peptide and (**b**) D-β-Asp T6 peptide measured as dry powder.

to an α-helix or random structure on the basis of the Raman spectrum. This indicates the shift of amide III to 1265 cm^{-1} and the appearance of an α-helical band at 937cm^{-1} (Figure 2). The other isomers, L-β-Asp and D-β-Asp T18 peptides, have a random structure in the solid state (Table 1). In the aqueous state, all Asp isomers containing T18 peptides have an α-helix or random structure (Table 1). These observations indicate that the native L-α-Asp T18 in human αA-crystallin is originally in a hydrophobic state; however, when the T18 peptides are exposed to a hydrophilic condition the β-sheet structure is changed to an α-helix or random conformation. Our previous studies suggested that the secondary structure surrounding Asp-151 is closely related to the inversion of Asp-151 from L-α-form to D-β-form.[9,19,20] Assuming that the Asp-151 T18 peptide is synthesized in a hydrophobic condition buried in the αA-crystallin surface, the microenvironment of T18 might expose it to adjacent water during the aging process, causing it to change from a hydrophobic β-sheeted structure to a hydrophilic α-helix accompanied by Asp-151 inversion and isomerization. Exposure to water did cause the secondary structural change from β-sheeted to α-helix or random structure in this study (Figure 3). Thus, the D-β-form Asp in T18 peptide is gradually accumulated in αA-crystallin during the aging process and raises the D:L ratio in lens proteins of the elderly to more than 1.0. It was found, for example that the D:L ratio at 80 years of age was 5.7.[6,21] In the future, it will be necessary to study the precise stereoconfiguration of T18 in αA-crystallin and the possibility of microenvironmental

changes during aging, using other diagnostic methods such as NMR or x-ray diffraction.

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