RACEMIZATION OF ASPARTIC ACIDS AT SPECIFIC SITES IN α A-CRYSTALLIN FROM AGED HUMAN LENS

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ABSTRACT

D-Aspartic acid (D-Asp) was found in a high content in α -crystallin isolated from the water-soluble fraction of aged human lens. α -Crystallin was separated to α A- and α B-crystallins by reverse-phase chromatography. Racemization of aspartic acid proceeded to a larger extent in α A-crystallin than in α B-crystallin. Purified α A-crystallin was treated with trypsin to yield peptides that were then separated by HPLC using a C18 column. Tryptic peptides of α A-crystallin were separated and analyzed for amino acid composition and racemization of aspartic acid. Each aspartyl and asparaginyl residue in the protein was racemized to a different extent. Asp⁵⁸ (D/L ratio of Asp, 1.41) and Asp¹⁵¹ (3.04) were highly racemized. The D/L ratio of other aspartyl residues ranged from 0.1 to 0.2.

The biologically uncommon D-aspartyl residues have been found in long-lived proteins, such as tooth (9) and eye lens (4-6, 13-15, 18) of aged mammals. Aspartic acid (Asp) is the most easily racemizable amino acid (1), and it has been considered that D-aspartic acid (D-Asp) may be formed by racemization in the metabolically inactive tissues during the natural aging process. In the lens protein, D-Asp has been found only in the water-insoluble fraction of aged lens (4-6, 13-15). We have isolated a D-Asp-containing protein from the water-insoluble fraction of bovine adult lens (4) and confirmed that the origin of this protein was α A-crystallin from the water-soluble fraction (15). It was considered that the water-soluble α A-crystallin had been converted to a water-insoluble fraction with aggregation and racemization during aging. α A-Crystallin is the major component of lens protein and its function is related to maintenance of transparency of the lens (2). If the racemization of Asp occurred in α A-crystallin, the higher structure and the function of the protein would be affected. Therefore, as the next step of our studies, we have directed our attention to the racemization of Asp in α A-crystallin in the water-soluble fraction from

aged human lens. Recently, Oetelaar *et al.* (18) reported the D/L ratio of Asp in the water-soluble and water-insoluble proteins of the aged human lenses. The water-soluble proteins contain, α , β , γ -crystallins (2). Oetelaar *et al.* (18) did not indicate which crystallin was racemized.

As an extension of our previous study (15), we have clarified in the present experiment that racemization occurs predominantly in the water-soluble α A-crystallin and that the extent of racemization of Asp at two specific sites of α A-crystallin is extremely high.

MATERIALS AND METHODS

Purification of α A-crystallin from Human Lens

Forty aged human (about 80 years old) lenses were homogenized in 2 mM 2-mercaptoethanol/35 mM Tris-HCl buffer (pH 7.4). The proteins were fractionated into water-soluble and water-insoluble fractions by centrifugation at 20,000 g for 60 min at 4°C. The water-soluble fraction was dialyzed against 0.1 M Tris-HCl buffer (pH 7.4) and was fractionated by DEAE-Toyopearl column equilibrated with the same buffer. The elution was car-

ried out stepwise with 0, 0.15 and 2 M NaCl in the same buffer. Effluent fractions at each step were combined, dialyzed against distilled water and lyophilized. The 2 M NaCl fraction obtained by the DEAE-Toyopearl column was subjected to reverse-phase HPLC (RP-HPLC) using a COSMOSIL 5C18-AR column (4.6×150 mm). Elution was carried out with a linear gradient of solvent A (0.1% trifluoroacetic acid and 2 mM 2-mercaptoethanol in water), and solvent B (0.1% trifluoroacetic acid and 2 mM 2-mercaptoethanol in 2-propanol:acetonitrile, 7:3, v/v).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The identification of each crystallin fraction from the DEAE column and RP-HPLC was based on the subunit analysis by SDS-PAGE (2). SDS-PAGE was carried out in 12% polyacrylamide gels, at pH 8.8 according to the method of Laemmli (12). Gels were stained with Coomassie brilliant blue. Standard proteins used as molecular weight markers were phosphorylase *b* (94 K), bovine serum albumin (66 K), ovalbumin (45 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (20 K) and α -lactalbumin (14 K).

Enzymatic Digestions and Isolation of Peptides

α A-Crystallin was digested with trypsin for 4 h at 30°C in 0.1 M Tris-HCl buffer, pH 7.6, at the enzyme-to-substrate ratio of 1:100 (mol/mol). The digests were subjected to an HPLC column for separation of the peptides produced. Peptide mapping by HPLC was carried out using a COSMOSIL 5C18-AR column (4.6×150 mm) and a linear gradient of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in 40% acetonitrile).

Determination of the D/L Ratio of Amino Acids

All glasswares were baked at 500°C for 4 h in a furnace before use in order to avoid possible contamination of amino acids. The sample was hydrolyzed with 6 N HCl for 8 h in an evacuated sealed tube at 110°C. The hydrolyzate was evaporated to dryness under reduced pressure and the residual amino acids were converted to *N*-trifluoroacetyl amino acid isopropyl esters in the usual manner (10). The ratio of D- to L-amino acids was determined by gas-

liquid chromatography (Hitachi 163 Gas Chromatograph) using a chiral capillary column (Chirasil-Val 25 m×0.25 mm, Applied Science, U.S.A.).

Amino Acid Analysis

In order to assign the tryptic peptide of α A-crystallin, each peak obtained by HPLC-column was evaporated to dryness, and the residues were hydrolyzed with 6 N HCl in a sealed evacuated tube for 22 h at 110°C. The composition of amino acids was determined with an amino acid autoanalyzer, Dionex Model D-502.

RESULTS

The D/L ratio of Asp of the water-soluble fraction was 0.120. In order to isolate α - and β -crystallin, the water-soluble fraction was applied on a DEAE-Toyopearl column (Fig. 1). α -Crystallin is acidic compared with β -crystallin (2); therefore, β -crystallin was eluted faster than α -crystallin in DEAE-chromatography. It was confirmed by SDS-PAGE that β -crystallin and α -crystallin were obtained in the 0.15 M and 2 M NaCl fractions, respectively (Fig. 2). A higher D/L ratio of Asp was detected in the proteins eluted with 2 M NaCl (0.158) than with 0.15 M NaCl (0.079). This result shows that the extent of racemization of Asp in α -crystallin is higher than in β -crystallin. α -Crystallin is composed of 2 types of polypeptide chains, α A and α B, which show approximately 55% amino acid sequence homology (3, 11). Therefore, clear-cut separation of α A- and α B-crystallin had been difficult. We succeeded in separating α A- and α B-crystallins by reverse-phase chromatography (Fig. 3). As shown in SDS-PAGE (Fig. 4), α B-crystallin was eluted in peak 1 and α A-crystallin in peaks 2 and 3 in Fig. 3. After hydrolysis of these proteins, the D/L ratio of Asp was determined. The D/L ratio of Asp in peaks 1, 2 and 3 was 0.113, 0.158 and 0.191, respectively, indicating that α A-crystallin is racemized more intensively than α B-crystallin. In order to determine the D/L ratio of Asp of respective aspartyl residues in α A-crystallin, the peak 3 in Fig. 3 was subjected to tryptic digestion. A typical separation of the tryptic peptides of α A-crystallin is shown in Fig. 5. The symbols above the respective peaks in Fig. 5 correspond to the numbers of tryptic peptides of α A-crystallin shown in Fig. 6. The tryptic peptides containing aspartyl residues were outlined in circles. Determination of the D/L ratio

Fig. 1 DEAE-Toyopearl chromatography of water-soluble fraction of aged human lens. Elution was carried out stepwise with 0, 0.15 and 2 M NaCl in Tris-HCl buffer (pH 7.4). D/L ratio of Asp is 0.079 in peak 1, and 0.158 in peak 2.

Fig. 2 SDS-PAGE of β - and α -crystallin separated by DEAE-chromatography. Lane 1, peak 1 in Fig. 1 (β -crystallin); Lane 2, peak 2 in Fig. 1 (α -crystallin); M, molecular weight markers. SDS-PAGE was performed in 12% polyacrylamide gel. Gels were stained with Coomassie brilliant blue. The identification of each crystallin fraction from the DEAE column was based on the subunit analysis by SDS-PAGE (2).

of respective aspartyl residues in αA -crystallin indicated that each aspartyl and asparaginyl residues was racemized to a different extent (Table 1). Two tryptic peptides, T6 (D/L ratio of Asp⁵⁸, 1.41) and T18 (D/L ratio of Asp¹⁵¹, 3.04) were extremely racemized. The D/L ratio of other aspartyl residues ranged from 0.1 to 0.2.

Four peptides (T10, T11, T12 and T18) were separated into 2 or 3 peaks by reverse-phase HPLC.

Fig. 3 Separation of αA and αB -crystallins by reverse-phase chromatography. Column: COSMOSIL 5C 18-AR (4.6 $\times$ 150 mm). Eluent: A, 0.1% trifluoroacetic acid and 2 mM 2-mercaptoethanol in water; B, 0.1% trifluoroacetic acid 2 mM and 2-mercaptoethanol in 2-propanol:acetonitrile (7:3, v/v). Gradient, 100% A in 10 min, and to 40% A in 70 min. Flow rate, 1 ml/min. Detection, at 280 nm. The D/L ratio of Asp is 0.113 in peak 1, 0.158 in peak 2, and 0.191 in peak 3.

These peptides were shown to possess different D-Asp contents and are diastereoisomers that are separable by column chromatography.

DISCUSSION

In this study, it was shown that the αA -crystallin

Fig. 4 SDS-PAGE of α A and α B-crystallins separated by reverse-phase chromatography. Lane 1: α B-crystallin, peak 1 in Fig. 3; Lane 2: α A-crystallin, peak 2 in Fig. 3; Lane 3: α A-crystallin, peak 3 in Fig. 3. SDS-PAGE was performed in a 12% polyacrylamide gel. Gel was stained with Coomassie brilliant blue. Molecular weight on the left

Fig. 5 An elution profile of tryptic peptides of aged human α A-crystallin. Column, COSMOSIL 5C 18-AR (4.6 $\times$ 150 mm). Eluent: A, 0.1% trifluoroacetic acid in water; B, 0.1% trifluoroacetic acid in acetonitrile. Gradient: 0% B (100% A) to 40% B. Flow rate, 1 ml/min. Detection at 230 nm. Each peak was identified by amino acid analysis as described in Materials and Methods.

Fig. 6 Primary structure of human α A-crystallin (3)

was one of the most easily racemizable components in the water-soluble fraction of aged human lens (Figs. 1 and 3). As shown in Table 1, the individual Asp/Asn residues in α A-crystallin do not racemize uniformly, but the extent of racemization is different. The outstanding finding is that the D/L ratio of Asp¹⁵¹ in T18 peptide is extremely high (3.04). This high D/L ratio may be explained by the coexistence of diastereomeric T18 peptide containing D- and L-Asp, and by an incomplete chromatographic separation of the diastereomeric peptides under the conditions used. The mechanism of formation of D-Asp from L-Asp in the peptide is not known. Geiger *et al.* (7) suggested that racemization of L-Asp/L-Asn could occur through a succinimide intermediate. Generally, Asn undergoes deamidation to form succinimide 10–100 times faster than Asp in a peptide, and therefore Asn residues can be racemized more easily. However, Asn¹⁰¹ in T12 peptide of aged human α A-crystallin is racemized to only a small extent (Table 1). The peptide bond cleavage at Asn¹⁰¹ in bovine α A-crys-

tallin was observed by Voorter *et al.* (20), who suggested that the cleavage occurred through succinimide ring formation leading to racemization. T12 peptide was not identified in the tryptic peptides of bovine α A-crystallin (8), and it is not clear whether or not Asn¹⁰¹ in bovine α A-crystallin is racemized. However, considering the low racemization extent of Asn¹⁰¹ in α A-crystallin from extremely aged human lens, it is unlikely that Asn¹⁰¹ was racemized significantly in bovine α A-crystallin. The racemization rate can also depend on the degree of the steric hindrance of the amino acids adjacent to Asp/Asn. Asn¹⁰¹ is followed by the bulky Glu, which may cause difficulty in forming a succinimide intermediate. In bovine (5 years old) α A-crystallin (8), D-Asp was found at only one site, i.e. at Asp¹⁵¹ (D/L ratio, 0.327) in T18 peptides. The amino acid sequence of α A-crystallin in vertebrates is relatively conserved. Human α A (3) and bovine α A chains (19) differ in 10 out of 173 amino acid residues. The 4 substitutions out of these 10 substitutions were concentrated in T18 peptides com-

Table 1 *D/L Ratio of Aspartyl Residues in Tryptic Peptides of α A-Crystallin from Aged Human (80 Years Old) Lens*

	Tryptic peptides	D/L of Asp
T1:	ac-Met-Asp ² -Val-Thr-Ile-Gln-His-Pro-Trp-Phe-Lys	0.099
T6:	Thr-Val-Leu-Asp ⁵⁸ -Ser-Gly-Ile-Ser-Glu-Val-Arg	1.41
T7-8:	Ser-Asp ⁶⁷ -Arg-Asp ⁶⁹ -Lys	0.103
T9:	Phe-Val-Ile-Phe-Leu-Asp ⁷⁶ -Val-Lys	0.087
T10(1):	His-Phe-Ser-Pro-Glu-Asp ⁸⁴ -Leu-Thr-Val-Lys	0.071
T10(2):	His-Phe-Ser-Pro-Glu-Asp ⁸⁴ -Leu-Thr-Val-Lys	0.163
T10(3):	His-Phe-Ser-Pro-Glu-Asp ⁸⁴ -Leu-Thr-Val-Lys	0.186
T11(1):	Val-Gln-Asp ⁹² -Asp ⁹³ -Phe-Val-Glu-Ile-His-Gly-Lys	N.D
T11(2):	Val-Gln-Asp ⁹² -Asp ⁹³ -Phe-Val-Glu-Ile-His-Gly-Lys	0.086
T12(1):	His-Asn ¹⁰¹ -Glu-Arg	0.141
T12(2):	His-Asn ¹⁰¹ -Glu-Arg	0.075
T13:	Gln-Asp ¹⁰⁵ -Asp ¹⁰⁶ -His-Gly-Tyr-Ile-Ser-Arg	0.244
T18(1):	Ile-Gln-Thr-Gly-Leu-Asp ¹⁵¹ -Ala-His-Thr-Glu-Arg	3.04
T18(2):	Ile-Gln-Thr-Gly-Leu-Asp ¹⁵¹ -Ala-His-Thr-Glu-Arg	0.428

ND: D-Asp was not detected.

posed of 11 amino acids residues. The number of amino acids of human T18 peptide was 1 amino acid less than that of bovine. It is interesting that an extreme and site-specific racemization of Asp¹⁵¹ in T18 peptide proceeds in bovine and human α A-crystallin, in spite of the difference in amino acid sequence. The high site-specific racemization of Asp¹⁵¹ in α A-crystallin suggests that the specific steric area exists in conformationally flexible region of the protein.

The other peptide which contained a highly racemized Asp residue was T6 peptide. T6 peptide contains Asp-Ser sequence and has the chemical susceptibility to racemization of Asp⁵⁸ through imide ring formation. Human and bovine T6 peptide has a common amino acid sequence. D-Asp has not been found in bovine T6 peptide (8), in contrast to human α A-crystallin (Table 1). The high racemization rate of Asp of human T6 peptide could be due to a longer aging process of materials used in this study.

The racemization may be caused not only by the primary structure but also by the secondary structure of the protein. The secondary structure of α A-crystallin has been reported to consist of 0–9% α -helix and 51–68% β -sheet (17). T6 and T18 peptides have been predicted to exist in regions consisting of β -turn or β -sheet structure. Recently, Shapira *et al.* (16) found D-Asp-containing peptides in aged human (74 years old) myelin basic protein. They suggested that the most highly racemized aspartyl

residue (D-Asp contents, 10–17%) adjoined arginine residue, and that the racemized aspartic residue may exist in a β -turn region.

Further investigations concerning the secondary and higher structures of α A-crystallin are needed to elucidate the racemization mechanism of aspartic acid residues at the specific sites of the protein.

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