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Comparison of post-translational modifications of alpha A-crystallin from normal and hereditary cataract rats

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Summary. In order to investigate the relationship between lens opacities and the various modifications of lens proteins, we analyzed and compared the properties of lens proteins of 85-day old normal Wistar rats and the hereditary cataract model, ICR/f rats. The present study identified many differences between normal and mutant lens proteins. In the ICR/f mutant rats, the relative amounts of gamma-crystallin decreased and high molecular weight (HMW) protein increased. Racemization and isomerization of Asp-151 of alpha A-crystallin was observed in the mutant ICR/f rats, and Met-1 of alpha A-crystallin was oxidized to methionine sulfoxide. These modifications were not found in the age-matched normal rats. These tendencies are consistent with aged and cataractous human lenses.

Keywords: Aging – Alpha A-crystallin – Cataract – Isomerization – Oxidation – Racemization

Introduction

The ICR/f rat strain develops hereditary cataracts, which appear around 75 days of age. It has been reported that the cataracts are inherited through an autosomal recessive gene. Biochemical studies on ICR/f rat lenses showed that calcium content in the cataractous lenses was about 10-fold higher than in normal lenses (Takeuchi et al., 2000). In addition, glutathione content and water-soluble protein in mutant lenses clearly decreased after cataract formation.

We reported that the mutant rats differed from normal rats in water–soluble lens protein composition. The major water–soluble lens proteins are composed of alpha-, beta-, and gamma-crystallins. However, gamma, betaB1- and betaA3-crysltallin markedly decreased in the lenses obtained from the ICR/f rat strain (Takeuchi et al., 2000). Furthermore we found that m-calpain and transglutaminase were activated by calcium, with the consequences that crystallin proteins were partially degraded and cross-linked beta-crystallins were formed in the cataractous lenses (Takeuchi et al., 2001).

Alpha-crystallin, a major protein of the lens, is thought to play a role in maintaining lens transparency. In its native state, alpha-crystallin is a large, water–soluble aggregate with an average molecular mass of approximately 800 kDa. Alpha-crystallin is comprised two polypeptides, alpha A- and alpha B-crystallin, which contain 173 and 175 amino acid residues, respectively. Since alpha-crystallins have very long half-lives, they undergo numerous post-translational modifications, such as deamidation (Miesbauer et al., 1994), racemization and isomerization (Fujii et al., 1994a, 1994b), truncation (Miesbauer et al., 1994; Fujii et al., 1997a; Emmons et al., 1992; Takemoto et al., 1991; Kamei et al., 1997), phosphorylation (Takemoto, 1996), and oxidation (Takemoto et al., 1992). These modifications may induce a change of the higher order structure of alpha-crystallin, and could be related to opacification of lenses.

In previous studies, we have reported two biologically uncommon D-beta-aspartyl residues (Asp-58 and Asp-151) of alpha A-crystallin from the lenses of various year ranges of human donors (Fujii et al., 1999; 2001a). The formation of D-aspartic acid at Asp-151 of human alpha A-crystallin started shortly after birth, and approximately half of the normal amount of L-alpha-Asp-151 was lost by 30 years when it changed

to other isomers such as D-beta, D-alpha, and L-beta isomers (Fujii et al., 1999).

Racemization of the Asp-151 residue has been observed in aged cattle (Groenen et al., 1990) and mice (Momose et al., 1998), despite differences in amino acid sequences. Furthermore, UVB-irradiation of young rat lenses induced racemization of only the Asp-151 residue in alpha A-crystallin (Fujii et al., 1997b). These results indicate that the configuration of Asp-151 of alpha A-crystallin is stereochemically labile, allowing the conversion of L-Asp to D-Asp. We also found that oxidation of Met-1 proceeds during aging or stress.

In this study, we measured the racemization and isomerization of the Asp151 residue and the oxidation of Met-1 of the alpha A-crystallins obtained from normal and mutant ICR/f rats.

Materials and methods

Rats and lenses

Lenses were removed from 85-day-old Wistar rats (normal) and ICR/f rats (mutant) immediately after death under chloroform anesthesia, and stored at −80°C if necessary.

Determination of protein contents

Protein contents were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standards.

Purification of alpha A-crystallin from normal and ICR/f rats (mutant)

Eight normal and nine mutant rat lenses were homogenized in 0.05 M phosphate (pH 7.0) buffer containing 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3 at 4°C. The homogenate was then centrifuged at 15,000 × g for 30 min to remove the water- insoluble material. Then 3 ml of the supernatant were applied to a Superose 12 column (1.6 × 50 cm, Pharmacia) and eluted with 0.05 M phosphate buffer at a flow rate of 0.5 ml/min. The eluate was monitored at 280 nm, using FPLC (Amersham Biosciences Corp, Piscataway, USA).

The first peak was pooled and concentrated by an ultrafiltration membrane (MW 10,000 cut-off, Millipore). The alpha-crystallin was further applied to a Superose 6 column (1.6 × 50 cm, Pharmacia) and eluted with the same buffer at a flow rate of 0.5 ml/min. The alpha-crystallin fractions were pooled and exchanged in 5 mM Tris-HCl buffer, pH 8.0, containing 6 M urea (starting buffer) using an ultrafiltration membrane. The alpha A-crystallin fraction was further fractionated by anion exchange chromatography using a Bio-Scale Q5 column (5 ml) (Bio-Rad Laboratories, Hercules, USA) connected to a FPLC (Pharmacia) at a flow rate of 1.0 ml/min. The crystallins were eluted with a linear gradient of 5 mM Tris-HCl buffer (pH 8.0) containing 6 M urea to 200 mM Tris-HCl buffer (pH 8.0) containing 6 M urea for 100 min. Elution of the crystallins was monitored by absorbance at 280 nm.

Enzymatic digestion and isolation of peptides

All preparations of alpha A-crystallin were digested with trypsin (Wako, Osaka, Japan), for 20 h at 37°C in 0.1 M Tris-HCl buffer (20 mM $CaCl_2$, pH 7.6) at an enzyme-to-substrate ratio of 1:50 (mol/mol). The resulting tryptic (T) peptides were separated by RP-HPLC (LC-10A, Shimadzu, Kyoto, Japan) using a C_{18} column (TSK gel-ODS-80 TM, 4.6 × 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. The fractions containing the desired peptides were collected into tubes using a fraction collector (FRC10, Shimadzu).

Amino acid sequence analysis

Amino acid sequences were determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (Applied Biosystems 476A/120A, Foster City, CA, U.S.A).

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, Kompact MALDI IV, Shimadzu). 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 as signals of positive ions. For the matrix, alpha-cyano-4-hydroxycinnamic acid (CHCA, 10 mg) was dissolved in 1 ml of solution containing a 2:1 ratio of 0.1% trifluroacetic acid in water to acetonitrile. The sample peptide (0.5 ml) was added to an equal volume (0.5 ml) of the matrix solution on the plate, and then dried. Each sample was present at a level of a few picomoles per spot.

Determination of D/L ratio of amino acids

All glassware was baked at 500°C for 3 h. Peptide samples were lyophilized in tubes and were hydrolyzed with gas-phase 6N HCl for 7 h at 108°C (PicoTag Work Stations, Waters, Tokyo). The hydrolysates were evaporated under reduced pressure. After hydrolysis, the samples were derivatized with o-phtalaldehyde (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 with a C_{18} column (Nova-Pak ODS, 3.9 × 300 mm, Waters) using fluorescence detection (344 nm excitation wavelength and 433 nm emission wavelength). Elution was carried out with a linear gradient of 5–47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate buffer (pH 6.0) for 120 min at a flow rate of 0.8 ml/min, at 30°C.

Results

Change of gel filtration and ion exchange chromatogram profiles of crystallins from normal and age-matched the mutant rats

Figure 1 shows gel filtration profiles of water–soluble proteins of normal rats (Fig. 1a) and mutant rats (Fig. 1b) using a Superose 12 column. There were many differences between normal rats and mutant rats. As shown in Fig. 1a and 1b, the relative amounts of gamma-crystallin of the mutant rats decreased more than that of normal rats. The height of the first peak from mutant rats is higher than that of normal rats. Since the first peaks of Fig. 1a and 1b contain alpha-crystallin and high molecular weight proteins (HMW), the first peak fractions were applied to a Superose 6 column in order to separate them into

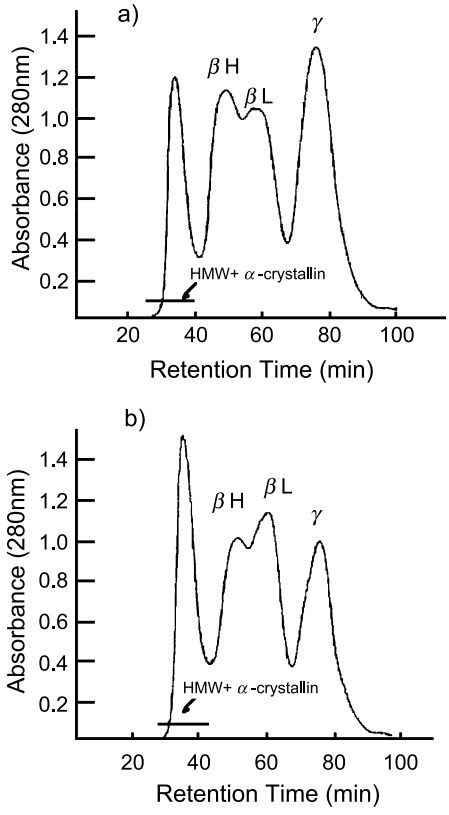

Fig. 1. Gel filtration profiles of lens proteins from normal and mutant rats using a Superose 12 column. **a** Normal rats, 85 days old; **b** mutant rats, 85 days old. Elution buffer: 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3. Flow rate: 0.5 ml/min

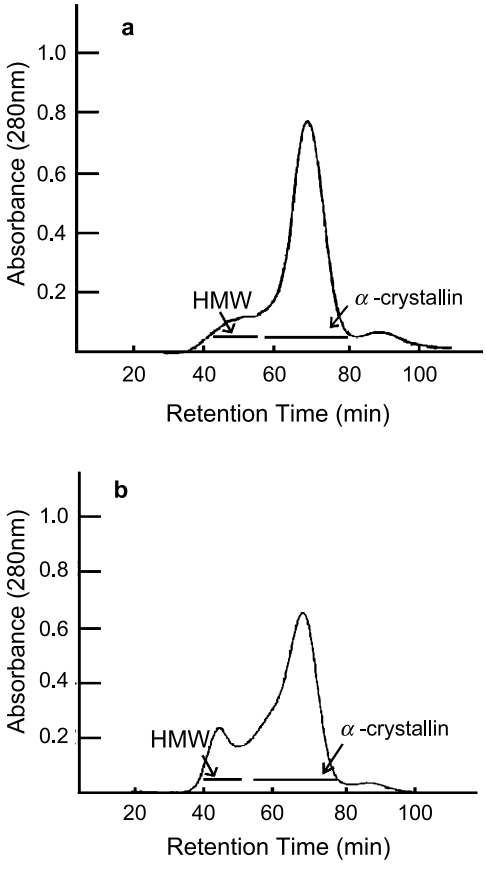

Fig. 2. Separation of HMW and alpha-crystallin using a Superose 6 column. Elution buffer: 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3. Flow rate: 0.5 ml/min. **a** Normal rats, 85 days old; **b** mutant rats, 85 days old

alpha-crystallin and HMW. As shown in Fig. 2a and 2b, the amount of HMW obtained from mutant rats is greater than that from normal rats. The alpha A-crystallin fractions were further fractionated from the alpha-crystallin fractions of Fig. 2a and 2b by anion exchange chromatography using a Bio-Scale Q5 column (Fig. 3a and 3b).

Comparison of aspartic acid isomers contents in alpha A-crystallin from normal and age-matched mutant rats

Alpha A-crystallins were digested with trypsin and the resulting peptides were separated by RP-HPLC. This procedure was expected to yield 20 peptides (T1–T20). The tryptic peptides were characterized on the basis of amino acid sequence analysis, and mass spectrometry. We identified the Asp-151 containing peptides, namely, T18 peptides (VQSGLD^{151}AGHSER) of the normal Wister and the mutant ICR/f rats alpha A-crystallin, and analyzed the stereoconfiguration of Asp-151 residues.

The T18 peptides were separated into two peaks by RP-HPLC (data not shown) based on the difference between alpha- and beta-linkages of the Asp-151 residue as described in a previous paper (Fujii et al., 1994b). We designated the beta-Asp151 containing T18 peptide T18 beta, and the alpha-Asp151 containing peptide T18 alpha. Although the molecular masses of both T18 beta and T18 alpha peptides were equal to 1255.7, which is consistent with the theoretical mass of the peptide, we can distinguish T18 beta from T18 alpha by a protein sequencer. Sequence analysis of T18 alpha gave the expected sequence, from Val-146 to Arg-157, while T18 beta revealed only residues from Val-146 to Leu-150, because beta-linked-amino acid containing peptides are resistant to Edman degradation. The beta/alpha ratio of Asp-151 residues of alpha A-crystallin obtained from 85-day-old mutant rats is greater than that of the age-matched normal alpha A-crystallin (Table 1).

We analyzed the D/L ratio of the Asp residue in T18 beta and T18 alpha from normal and mutant lenses. The

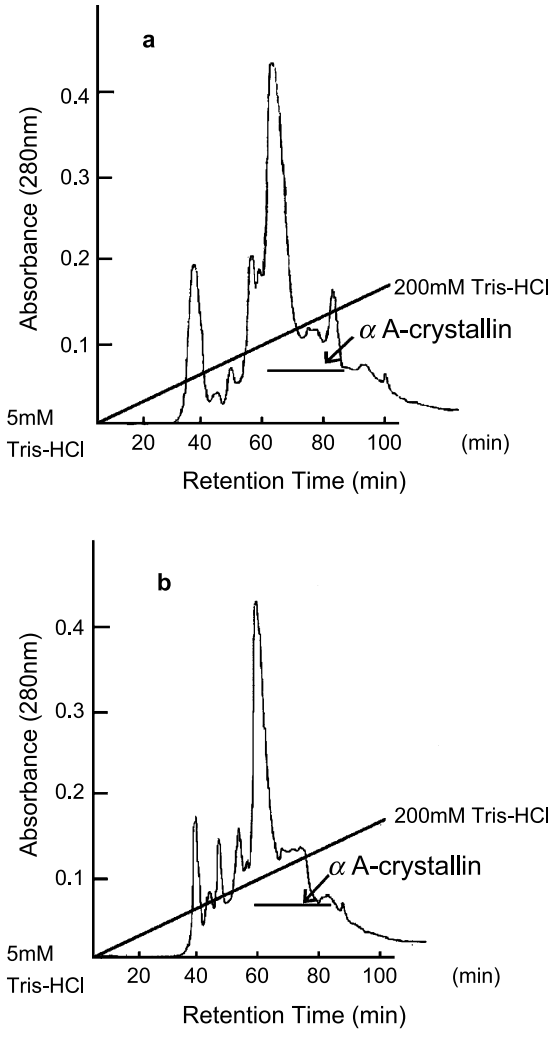

Fig. 3. Separation of alpha A and alpha B-crystallin using a Bio-Scale Q5 column. Flow rate: 1.0 ml/min. Gradient: 6 M urea/5 mM Tris-HCl (pH 8.0) to 6 M urea/200 mM Tris-HCl (pH 8.0) in 100 min. **a** Normal rats, 85 days old; **b** mutant rats, 85 days old

Table 1. Racemization, isomerization and oxidation of alpha A-crystallin from lenses of mutant (ICR/f, 85 days old) and normal (Wistar, 85 days old) rats

Modification	ICR/f (mutant)	Wistar (normal)
D/L of beta-Asp-151	0.14	0.04
D/L of alpha-Asp-151	0.04	0.04
beta/alpha of Asp-151	0.27	0.01
Ratio of oxidized Met-1	0.26	0.00

D/L ratio of beta-Asp151 residues of mutant alpha A-crystallin is 3 times as high as that of normal alpha A-crystallin. On the other hand, the D/L ratios of alpha-Asp-151 of normal and mutant alpha A-crystallins reflect low ratios of racemization (0.04), and there are no differences in the D/L value of either normal or mutant lenses (Table 1).

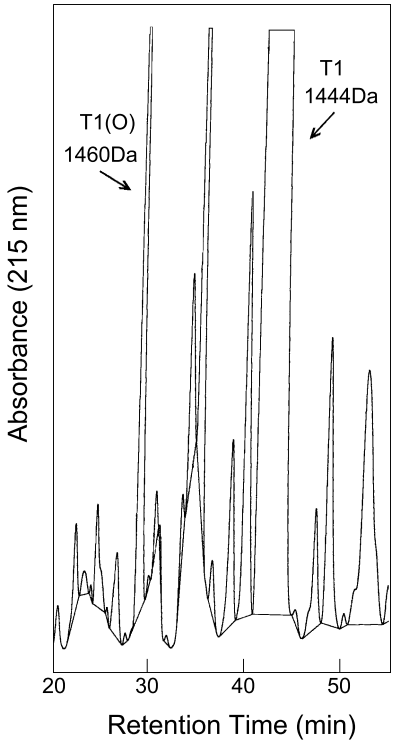

Fig. 4. Part of an RP-HPLC chromatogram of alpha A-crystallin obtained from lenses of ICR/f mutant rats. The oxidized T1 peptide (*T1(O) 1460 Da*) of alpha A-crystallin elutes earlier than the native T1 peptide (*1444 Da*)

Oxidation of T1 peptides of alpha A-crystallin

Figure 4 shows part of a RP-HPLC chromatogram of tryptic digest of alpha A-crystallin obtained from the lenses of the mutant rats. In addition to the T1 peptide of alpha A-crystallin (AcMDVTIQHPWFK: m/z 1444 Da), an oxidized T1 peptide (1460 Da) with a mass of 16 Da more than the native T1 peptide was observed in the alpha A-crystallin of the mutant rats. The relative amount of oxidized T1 was approximately 26% of the native T1 peptide. However, there was no oxidized T1 peptide in alpha A-cystallin from the normal Wister rats.

Discussion

In this study, we analyzed the properties of lens proteins of 85-day-old normal Wister and mutant ICR/f rats. The mutant rats had cataracts, while the lenses of the age-matched normal rats remained transparent. Previous studies have indicated that cross-linking of alpha A-crystallin in 85-day-old ICR/f rats increased more than that of

normal rats of the same age (Takeuchi et al., 2000). The present study shows the many differences between normal and mutant lens proteins. In the ICR/f mutant rats, the relative amounts of gamma-crystallin decreased (Fig. 1b) and HMW protein increased (Fig. 2b). As is well known, these changes are identical to those observed in aged and cataractous human lenses.

We observed that racemization and isomerization of Asp-151 of alpha A-crystallin from 85-day-old mutant rats are increased in comparison to normals. Although alpha A-crystallin has 14 Asp and 2 Asn residues, the racemization and isomerization were observed in only the Asp-151 residue but not in other Asp/Asn residues. This result indicates that the Asp-151 residue is stereochemically labile to allow the conversion of L-from to D-form in the protein. On the other hand, there was no racemization and isomerization in the age-matched normal rats. This tendency is consistent with changes observed in aged and cataractous lenses. In previous studies, we have shown that spontaneous racemization and isomerization occurs at the Asp-151 residue in alpha A-crystallin of aged or cataractous lenses (Fujii et al., 1999, 2000).

Generally, D-beta-Asp residues are derived from L-Asp/Asn residues, under physiological conditions, via a succinimide intermediate as shown in Fig. 5. This succinimide is readily formed by intramolecular cyclization, in which the peptide bond nitrogen of the residue following the Asp residue attacks the carbonyl group of the Asp residue side chain. Subsequently, the L-succinimide can be converted to D-succinimide, that is, racemization, and both succinimides can be hydrolyzed to D-beta-Asp, L-beta-Asp, D-alpha-Asp and L-alpha-Asp residues, respectively. It is known that the succinimide intermediate prefers to open to beta-Asp rather than to alpha-Asp (Geiger and Clarke, 1987). Therefore D-beta-Asp is a major isomer in mutant alpha A-crystallin. This reaction

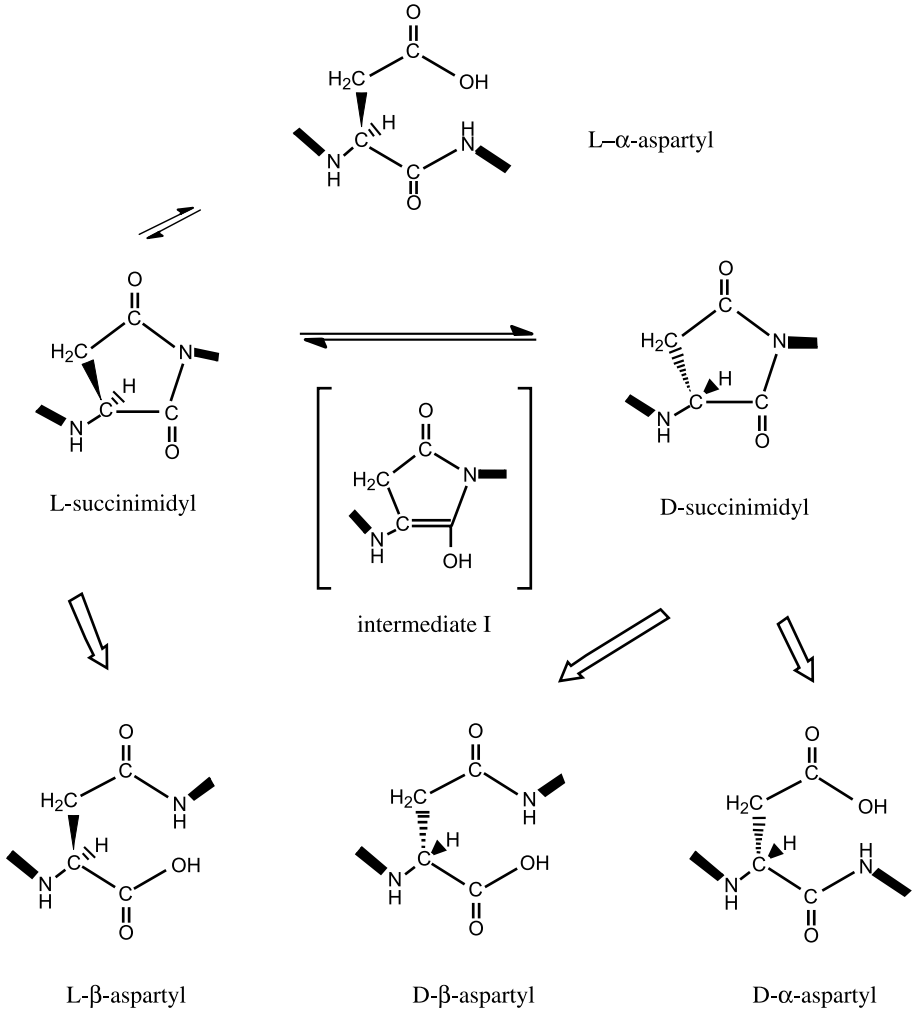

Fig. 5. Reaction pathways for the spontaneous racemization and isomerization of Asp residue in protein via a succinimide intermediate

depends on the rate of succinimide formation, which is believed to depend on the neighboring residue of the Asp residue. When the neighboring amino acid of the Asp residue has a small side chain, succinimide formation occurs easily because there is no steric hindrance. Since the Asp-151 residue in rat alpha A-crystallin is followed by alanine, succinimide is easily formed. So far, these abnormal isomers at the Asp-151 residue have been observed in alpha A-crystallin of long-lived animals such as cattle or humans. It is noteworthy that D-beta-Asp was formed in mutant alpha A-crystallin within a short time-period of 85 days.

Another modification, oxidation of T1 peptide, which resulted from the N-terminal Met of alpha A-crystallin, was observed in the mutant rat but not in the normal rat as shown in Fig. 4. The oxidation of lens proteins is believed to contribute to the formation of cataracts and the aging of the lens. However, oxidation of Met-1 was found even in fetal bovine lens (Kamei et al., 1997), showing that the modification occurred soon after synthesis of the protein. The previous study showed that Met-1 was oxidized to methionine sulfoxide by gamma-irradiation and the amount increased depending on radiation dose (Fujii et al., 2001b). The oxygen atom attack at the N-terminal of alpha A-crystallin occurred easily, showing that the N-terminus of the alpha A crystallin may be exposed to the outside.

The present study showed that various modifications, such as the increase of HMW protein, racemization and isomerization of Asp-151 of alpha A-crystallin, and oxidation of Met-1 of alpha A-crystallin occurred to a greater extent in the mutant rats than in age-matched normal rats. This tendency is consistent with aged and cataractous human lenses. Further biochemical studies, together with higher order structural studies of alpha A-crystallin, are needed to further investigate the possibility how post-translational modifications might affect the transparent properties of lenses. The present study showed that the ICR/f rats are suitable animal models for the studies of cataracts.

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