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λ-Crystallin Related to Dehydroascorbate Reductase in the Rabbit Lens

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Purpose: To evaluate the relationship of λ-crystallin to reduced nicotinamide adenine dinucleotide (NADH)-dependent dehydroascorbate (DHA) reductase found specifically in the rabbit lens.

Methods: DHA reductase Fractions I–IV were separated from the λ/βL1-crystallin fraction of rabbit lens soluble protein by diethylaminoethyl (DEAE)-cellulose ion-exchange column chromatography, and then the enzyme was partially purified from Fraction II by rechromatography on the same ion-exchange column. The isolated DHA reductase fractions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, native isoelectric focusing and two-dimensional gel electrophoresis.

Results: Using Western blot and a probe of antiserum to recombinant λ-crystallin, the main 33-kDa protein band was strongly stained in all the rabbit lens DHA reductase fractions, and most of the additional protein bands of approximately 25–30 kDa were also detectable. In the partially purified enzyme, the 33-kDa subunit alone was identified as a distinct protein band by SDS-PAGE, and a main basic protein was found at pI 7.6 by native isoelectric focusing. In addition, many bands of more acidic proteins were separated from other enzyme fractions, and protein spots corresponding to the 33 and/or approximately 25–30-kDa subunits were detected in each of the more acidic proteins by two-dimensional gel electrophoresis.

Conclusion: These results suggest that λ-crystallin is closely related to the DHA reductase in the rabbit lens. The above heterogeneity of the enzyme-crystallin may arise from posttranslational modifications. **Jpn J Ophthalmol 2003;47:437–443**

Key Words: λ-Crystallin, dehydroascorbate reductase, enzyme-crystallin, heterogeneity, rabbit lens.

Introduction

The eye lens, a transparent organ, is characterized by the presence of very high concentrations of soluble structural proteins called crystallins.[1] They are classified into two groups, the ubiquitous crystallins (α, β, and γ) present in all vertebrates, and the taxon-specific, so-called enzyme-crystallins.[2] α-Crystallin is closely related to small heat-shock protein,[3] and functions as a molecular chaperone.[4] The taxon-specific enzyme-crystallins are either identical or related to metabolic enzymes. For example, ζ-crystallin discovered in the guinea pig lens is an active reduced nicotinamide adenine dinucleotide phosphate (NADPH)–quinone reductase.[5] And, ρ-crystallin identified in the lenses of amphibians belongs to an aldo–keto reductase (an osmotic stress protein) superfamily,[2] but the enzyme activity is lost.[6] It has been believed that crystallins have some connections with stress responses to such factors as heat, osmotic pressure, ultraviolet light, and oxidation.[2]

Oxidation of the lens constituents is an early event in the development of cataracts and, through this oxidation, lens insoluble protein aggregates are formed.[7] The lens is usually protected from oxidative stress by the antioxidation systems such as the ascorbate redox cycle[8] and the

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glutathione redox cycle.[9] The high ascorbate concentration may be beneficial in protecting the lens against oxidative and photoxidative damage if the high level is maintained.[10] However, the oxidation products of ascorbate are supposed to be cataractogenic, because it has been reported that these oxidation products lead to cross-linking of bovine lens protein,[11] and that opacity occurs in the rabbit lens exposed to a high concentration (1 mM) of dehydroascorbate (DHA), an oxidized form of ascorbate.[12] Therefore, lens ascorbate should be maintained in the reduced state. It has been known that ascorbate in the lens is predominantly regenerated from ascorbate free radical (AFR) by reduced nicotinamide adenine dinucleotide (NADH)-dependent AFR reductase,[8,13] and also from DHA by enzymatic[14] and/or nonenzymatic[15] reaction with glutathione.

Recently, in addition, we[16] found that NADH-dependent DHA reductase activity was detected only in the rabbit lens soluble fraction among several vertebrate lenses (bullfrog, Hartley guinea pig, Wistar rat, Japanese albino rabbit, swine, bovine, and human), and by gel filtration, eluted at the λ/βL1-crystallin fraction containing mainly λ-crystallin (a rabbit-specific enzyme-crystallin, 4–8% of total rabbit lens protein)[17,18] and oligomeric β-crystallin. Furthermore, we[16] reported that about 80% of the protein fractions separated from the λ/βL1-crystallin fraction by DEAE-cellulose ion-exchange column chromatography exhibited DHA reductase activity, and in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the separated enzyme fractions, protein subunits regarded as λ- and β-crystallin were commonly identified. These findings led to our interest in whether the DHA reductase is related to λ- and/or β-crystallin.

It has been reported that λ-crystallin shows 30% homology to L-3-hydroxyacyl-CoA dehydrogenase and contains a putative NADH-binding site.[17] However, its real enzyme activity is as yet unidentified. The present investigation demonstrates that in the rabbit lens, λ-crystallin is closely related to NADH-dependent DHA reductase based on our findings in partial enzyme purification and Western blot analysis. Heterogeneity of the enzyme-crystallin has also been studied.

Materials and Methods

Lenses and Preparation of the Lens Soluble Fraction

Rabbit lenses were obtained from freshly enucleated eyes of Japanese albino rabbits (about 6–20 months old) sacrificed with overdoses of anesthetics. Bullfrog, guinea pig (Hartley), and rat (Wistar) lenses were similarly obtained from euthanized laboratory animals, and swine and bovine lenses from those eyes obtained from a local slaughterhouse. The lenses were kept frozen at −80ºC until used. All animal procedures were in accordance with the ARVO resolution on animals and ophthalmic research.

Lenses from different species were homogenized separately in about 10 times their weight of 0.1 M KC1, 10 mM K-phosphate (pH 7.2) in a glass homogenizer in ice, and the soluble fraction was separated from the insoluble fraction by centrifugation at 15,000 ×*g* for 1 hour at 4ºC. Protein concentration was assayed by the bicinchoninic acid method[19] using bovine serum albumin as standard.

Isolation of DHA Reductase by Column Chromatography

All chromatography was carried out at 0º–4ºC. As reported previously,[16] four DHA reductase fractions, I–IV (shown in Figure 1) were separated from the λ/βL1-crystallin fraction in the rabbit lens soluble fraction by DEAE-cellulose (DE52) ion-exchange column chromatography. Fractions I–III were eluted from the column by 10 mM K-phosphate (pH 7.2), and Fraction IV done by 10–50 mM KC1 in the same buffer.

Fraction II, a main DHA reductase fraction was subsequently applied to rechromatography on the same DE52 ion-exchange column, as described previously.[20] The DHA reductase was adsorbed on the column equilibrated with 2 mM K-phosphate (pH 7.2), and re-eluted as a single peak (Fraction II-1) with a small tailing shoulder (Fraction II-2) by 10 mM phosphate buffer. This rechromatogram is superimposed in Figure 1.

DHA reductase activity was determined by spectrophotometric measurement of the oxidation rates of 100 μM NADH at 340 nm in the presence of 500 μM DHA, as described in our earlier report.[16]

Preparation of λ-Crystallin cDNA and Recombinant λ-Crystallin

Total RNA of the rabbit lens was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. The complete nucleotide sequences of cDNA of λ-crystallin have been reported by Mulders et al.[17] Based on the sequences, cDNA to λ-crystallin mRNA in the total RNA was produced and amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) (TaKaRa RNA PCR Kit (AMV) Ver. 2.1, Takara Shuzo, Otsu, Shiga) using a sense primer GGATCCGCTTCCCCGGCAGCCGGCG complementary to residues 64–82 of λ-crystallin cDNA and an antisense primer CTCGAGAAGTTTACTGGGGC complementary to residues 1014–1029 of λ-crystallin cDNA. These two primers were chosen so that the cDNA PCR product would

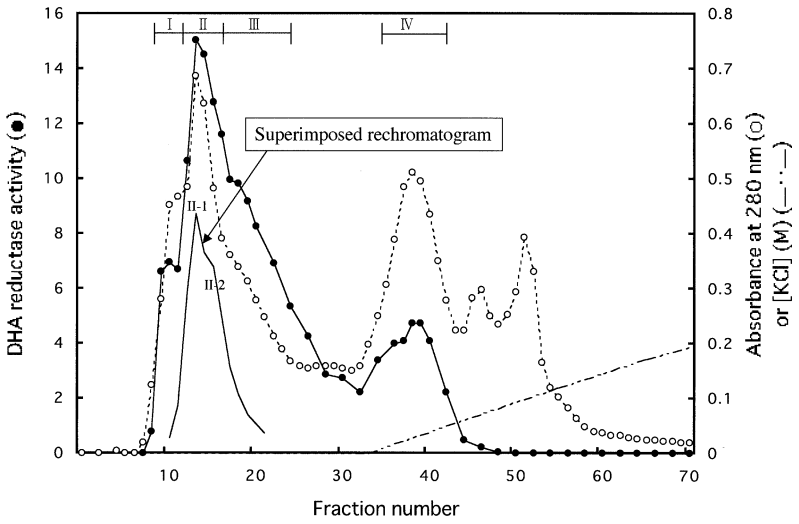

Figure 1. Separation of rabbit lens dehydroascorbate (DHA) reductase Fractions I–IV from λ/βL1-crystallin fraction by DE52 ion-exchange column chromatography, and superimposed rechromatogram of Fraction II. Modified from our previous papers.[16,20]

include a whole open reading frame (957 nucleotides) of λ-crystallin cDNA. To these sense and antisense primers were added the recognition sequences (GGATCC and CTCGAG) of restriction endonucleases *Bam*HI and *Xho*I on the 5′-ends for subsequent cloning of the PCR product, respectively. The PCR product of λ-crystallin cDNA was ligated into pCR 2.1 vector, and cloned by the TA Cloning Kit with INVα F′ *Escherichia coli* (Invitrogen). Clones with the correct insert were confirmed by the size determination and sequence analysis of the insert cDNA.

To generate glutathione-S-transferase (GST)-λ-crystallin fusion protein, plasmids from one of the clones with the correct insert were cleaved by restriction endonucleases *Bam*HI and *Xho*I (Takara Shuzo), and the λ-crystallin cDNA released was ligated into pGEX 6P-1 (Amersham Biosciences, Piscataway, NJ, USA) linearized with the same endonucleases, and then transformed into *E. coli* XL1-blue. Incorporation of λ-crystallin cDNA into pGEX 6P-1 was confirmed by DNA sequence analysis. Expression of the GST-λ-crystallin fusion protein was induced in the cell culture using 100 μM IPTG (isopropyl-β-D(−)-thiogalactopyranoside), and the GST fusion protein was purified by affinity chromatography on a Glutathione Sepharose 4B (Amersham Biosciences) column. Recombinant λ-crystallin was finally cleaved from GST of the fusion protein by PreScission Protease (Amersham Biosciences), and recovered as the nonadsorbed eluent without GST and the protease by the same affinity column chromatography. Purity and molecular weight of the recombinant crystallin were ascertained by SDS-PAGE.

Preparation of Antiserum Against Recombinant λ-Crystallin

Rabbits were immunized with 200 μg of recombinant λ-crystallin emulsified with Freund's complete adjuvant. Subsequently, booster injections with 100 μg of the antigen in Freund's incomplete adjuvant were administered three times every 2 weeks. The rabbits thus immunized were bled, and serum was collected and used for experiments as antiserum against the crystallin.

SDS-PAGE and Western Blotting

SDS-PAGE of the isolated DHA reductase fractions was performed on slab gels of 5–20% acrylamide, as described previously.[16] After the electrophoresis, the gels were stained for protein with Coomassie Brilliant Blue, or applied to Western blotting.

In Western blotting, proteins in the gels were transferred to nitrocellulose membranes. The resulting blots were used for immunostaining, or for protein detection with Coomassie Brilliant Blue. Immunoblots were probed with the following primary antibody, a 1/3000 dilution of rabbit polyclonal antibody (antiserum) against recombinant λ-crystallin (produced in this study) or a 1/2000 dilution of anti-β-crystallin mouse monoclonal antibody (raised against bovine βH-crystallin; StressGen Biotechnologies, Victoria, BC, Canada). As the secondary antibody, goat anti-rabbit or mouse IgG-horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) was used at a 1/3000 dilution. Protein bands reacted with the antibodies were visualized by the peroxidase reaction utilizing 3,3′-diaminobenzidine as a substrate.

Bovine lens βH-crystallin, an immunoblotting positive control was purchased from Sigma Chemical (St. Louis, MO, USA).

Native Isoelectric Focusing and Two-dimensional Gel Electrophoresis

Native isoelectric focusing (without urea) and two-dimensional gel electrophoresis were carried out using

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Ampholine PAGplate pH 3.5–9.5 and ExcelGel SDS Gradient 8–18 (Amersham Biosciences), as reported previously by us.[21] For the two-dimensional gel electrophoresis, the native isoelectric focusing was run as the first dimension, and the second dimension was SDS-PAGE by the ExcelGel. Protein bands and spots were stained with Coomassie Brilliant Blue.

Results

Western Blotting of Rabbit Lens DHA Reductase

We[16,20] previously reported that rabbit lens DHA reductase was separated into four fractions, I–IV by DE52 ion-exchange column chromatography, and then, from Fraction II with the highest activity, the enzyme was partially purified as a single peak (Fraction II-1) with a tailing shoulder (Fraction II-2) by rechromatography on the same ion-exchange column (Figure 1).

By SDS-PAGE analysis, as described previously,[16,20] a 33-kDa protein and a few proteins with molecular weights of approximately 25–30 kDa were commonly detected in DHA reductase Fractions I, II-2, III and IV, and the 33-kDa subunit alone was identified as a distinct protein band in the partially purified enzyme, Fraction II-1 (Figure. 2A). In this study, furthermore, using Western blotting with antiserum to recombinant λ-crystallin, the 33-kDa protein was strongly stained in all the enzyme fractions, and most of the approximately 25–30-kDa protein bands were also detectable although immunoreactivity was weaker in those bands (Figure 2B).

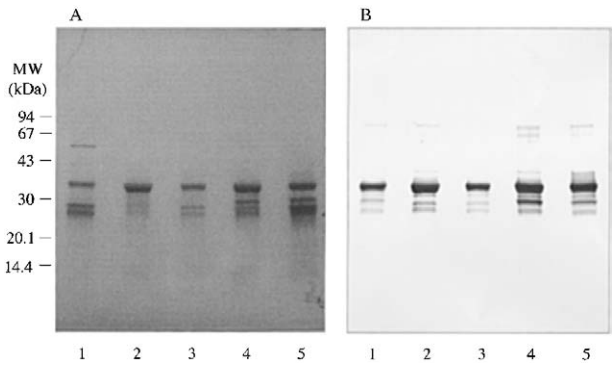

Figure 2. (**A**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rabbit lens dehydroascorbate reductase Fractions I, II-1, II-2, III, and IV. The gel was stained for protein with Coomassie Brilliant Blue. (**B**) Western blot of the same samples probed with antiserum to recombinant λ-crystallin. Lane 1: Fraction I (2.2 μg protein), lane 2: Fraction II-1 (1.7 μg protein), lane 3: Fraction II-2 (0.8 μg protein), lane 4: Fraction III (3.9 μg protein), lane 5: Fraction IV (3.5 μg protein). MW: molecular weight.

We[16] mentioned before that the approximately 25–30-kDa proteins might be β-crystallin subunits, considering the resemblance in molecular mass. However, a 25-kDa protein (βB2- or βA3-crystallin subunit)[22] was immunochemically detectable only in DHA reductase Fraction IV in a similar manner to bovine lens βH-crystallin control, but not detectable in Fractions I and III using Western blotting with anti-β-crystallin monoclonal antibody (Figure 3). Therefore, β-crystallin is unlikely to be associated with rabbit lens DHA reductase.

Immunoreactivity of Anti-Recombinant λ-Crystallin Antibody

To define the immunoreactivity of the antiserum to recombinant λ-crystallin, lens soluble proteins from bullfrog, guinea pig, rat, rabbit, swine, and bovine were separated by SDS-PAGE, and analyzed by Western blotting with antiserum to the recombinant crystallin (Figure 4). The antiserum was strongly and specifically reactive with the 33-kDa protein, and small amounts of approximately 25–30-kDa proteins and others were also detectable only in the rabbit lens (Figure 4B). Therefore, it is concluded that the antibody is practically specific to rabbit lens λ-crystallin.

Heterogeneity of Rabbit Lens DHA Reductase

By native isoelectric focusing of the partially purified DHA reductase (Fraction II-1), a major protein band was identified at pI 7.6, and a minor protein band was also detected around pI 7.4 (Figure 5). However, proteins from

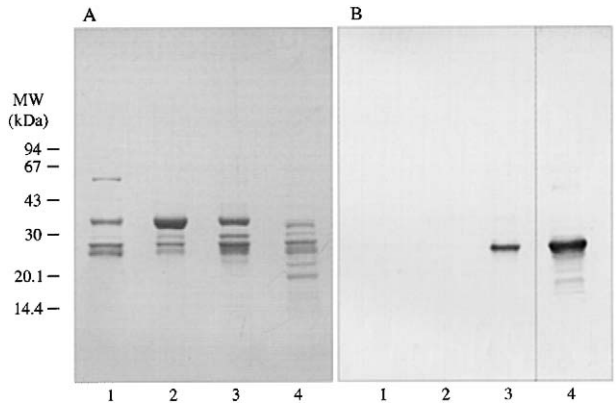

Figure 3. Western blot of rabbit lens dehydroascorbate reductase Fractions I, III and IV probed with anti-β-crystallin monoclonal antibody. (**A**) Blotting membrane stained for protein with Coomassie Brilliant Blue. (**B**) Immunostaining. Lane 1: Fraction I (2.2 μg protein), lane 2: Fraction III (3.9 μg protein), lane 3: Fraction IV (3.5 μg protein), lane 4: bovine βH-crystallin (2.8 μg protein). MW: molecular weight.

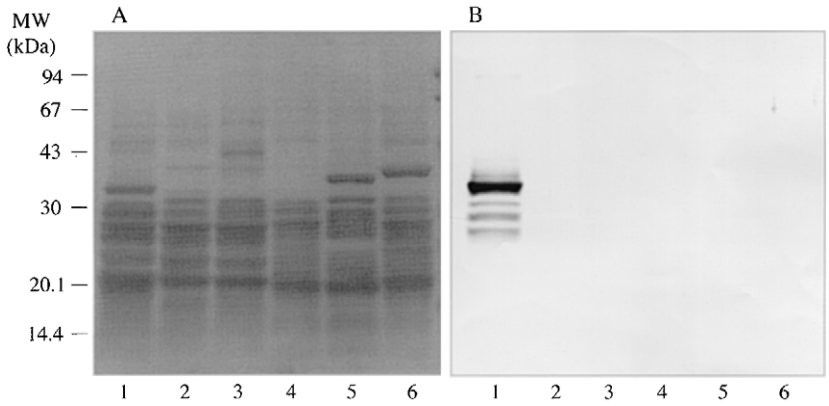

Figure 4. (**A**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lens soluble proteins from different species. The gel was stained for protein with Coomassie Brilliant Blue. (**B**) Western blot of the same samples probed with antiserum to recombinant λ-crystallin. Lane 1: rabbit (8.0 μg protein), lane 2: bovine (6.7 μg protein), lane 3: swine (6.8 μg protein), lane 4: rat (7.8 μg protein), lane 5: bullfrog (9.1 μg protein), lane 6: guinea pig (7.8 μg protein). MW: molecular weight.

DHA reductase Fractions I, III and IV were more heterogeneous in a wide range of pI 8.3 to 5.2, and most of the proteins were more acidic than pI 7.6 (Figure 5).

Two-dimensional gel electrophoresis of DHA reductase Fractions III and IV demonstrated that protein spots corresponding to the preceding 33 and/or approximately 25–30-kDa subunits were invariably found in each of the proteins with different pIs separated by isoelectric focusing (Figure 6).

These results suggest that the heterogeneity of rabbit lens DHA reductase may be the same as that of λ-crystallin.

Discussion

It has been reported that λ-crystallin, a rabbit-specific enzyme-crystallin, is dimers or tetramers composed of a subunit with a molecular weight of 33–35 kDa.[17,18] The present study (Figure 2) has shown that the 33-kDa protein is the main subunit of all the rabbit lens DHA reductase fractions, and is strongly immunoreactive with the antibody to recombinant λ-crystallin. The partially purified enzyme, Fraction II-1, is substantially composed of the 33-kDa protein alone (Figure 2). We[20] have recently

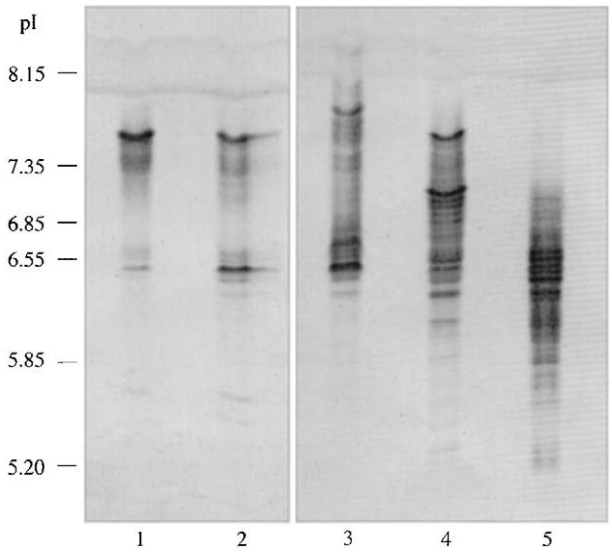

Figure 5. Native isoelectric focusing of rabbit lens dehydroascorbate reductase Fractions I, II-1, II-2, III, and IV. Protein bands were visualized with Coomassie Brilliant Blue. Lane 1: Fraction II-1 (13.3 μg protein), lane 2: Fraction II-2 (6.7 μg protein), lane 3: Fraction I (17.6 μg protein), lane 4: Fraction III (31.3 μg protein), lane 5: Fraction IV (28.1 μg protein).

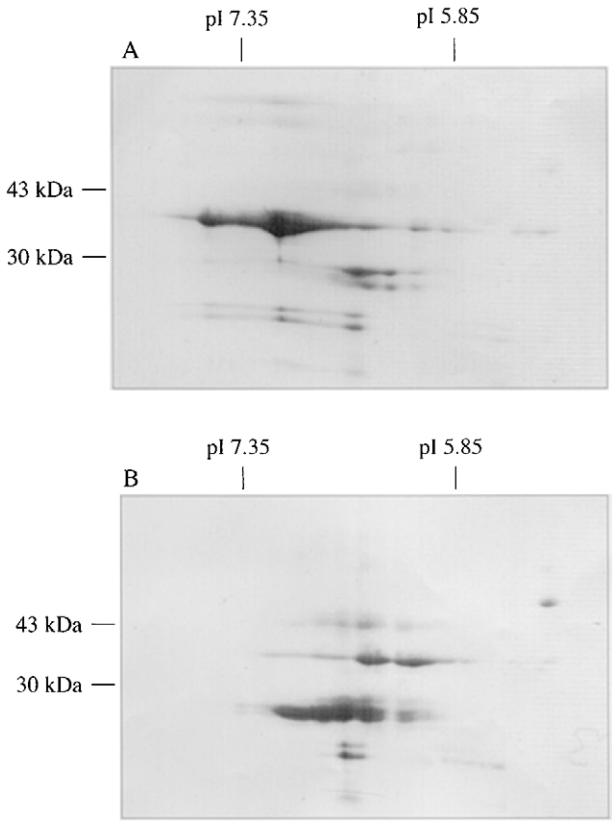

Figure 6. Two-dimensional gel electrophoresis of rabbit lens dehydroascorbate reductase Fractions III and IV. (**A**) Fraction III (31.3 μg protein), (**B**) Fraction IV (28.1 μg protein). Protein spots were visualized with Coomassie Brilliant Blue.

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reported amino acid sequences of two tryptic peptides from the partially purified enzyme. Figure 7 depicts the sequence alignment of the tryptic peptides and λ-crystallin. The sequences of the peptides are 100% identical to parts of the amino acid sequence[17] of λ-crystallin, as mentioned in that previous paper.[20] Thus, it is suggested that λ-crystallin is closely related to NADH-dependent DHA reductase in the rabbit lens.

Only one mRNA of 1.65 kilobases has been identified with λ-crystallin in the rabbit lens.[17] However, the present study has revealed that not only the 33-kDa protein, but the approximately 25–30-kDa protein subunits are also detected in the rabbit lens DHA reductase fractions (Figure 2A), and most of the additional subunits are significantly detectable by the antibody to recombinant λ-crystallin (Figure 2B). The lower molecular weight proteins may be posttranslationally generated by proteolytic and/or nonenzymatic cleavage of the 33-kDa subunit, because it has been reported that the specific cleavages of α- and β-crystallins occur during maturation of the bovine lens.[23,24] λ-Crystallin may be assembled with a combination of dimers or tetramers with the main 33-kDa subunit and the minor subunits of lower molecular weights.

Moreover, the present experiments with two-dimensional gel electrophoresis (Figure 6) shows that the 33-kDa and lower molecular weight protein subunits are very heterogeneous with regard to charge characteristics. It is assumed that the original λ-crystallin has a basic isoelectric point of ≥7.6 (Figure 5). Other more acidic proteins are probably produced from the original one by deamidation and/or phosphorylation, although chemical analysis for the possible posttranslational modifications was not performed in the present study. Both the modifications responsible for increasing acidity are observed at specific sites of polypeptide chains in α-crystallin of the bovine lens,[23,25] and deamidation is also detected in β- and γ-crystallins as well as in α-crystallin in the human lens.[26]

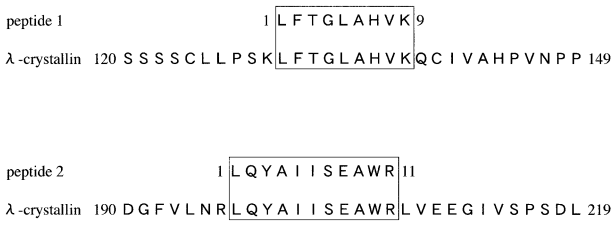

Figure 7. Sequence alignment of two tryptic peptides from the partially purified rabbit lens dehydroascorbate reductase and λ-crystallin. Amino acid sequences[20] of the two peptides (peptides 1 and 2) were 100% identical to partial sequences[17] of λ-crystallin, as shown by boxes.

We are interested in the function of λ-crystallin, related to DHA reductase, in the rabbit lens. Our unpublished observation demonstrated that NADH-dependent DHA reductase activity in the rabbit lens soluble fraction reached the maximum level at >5 mg soluble protein/mL, and the maximum activity level seemed to be equivalent to the nonenzymatic DHA reduction rate by about 1 mM glutathione (data not shown). However, the rabbit lens is known to contain glutathione at a high concentration, about 10 mM in the cortex and about 4 mM in the nucleus.[27] DHA must be predominantly reduced by the nonenzymatic reaction with glutathione in the rabbit lens. It has been reported that the nonenzymatic reduction of DHA is linked to the glutathione redox cycle and hexose monophosphate shunt.[12,28] The DHA reductase is not so effective for ascorbate regeneration in the lens unless glutathione decreases abnormally to <1 mM.

λ-Crystallin/DHA reductase may be involved in another peculiar function in the rabbit lens. As reported in our previous paper,[16] the rabbit lens DHA reductase was specific for NADH, and apparent K_m values of the crude enzyme were 4.0 μM for NADH and 5.7 mM for DHA. This result indicates that the DHA reductase may have a strong affinity for NADH. This is consistent with λ-crystallin having a putative NADH-binding fold.[17] The lenses of certain species such as bullfrog, guinea pig, and rabbit are known to contain high concentrations of reduced pyridine nucleotide, NADPH or NADH.[29] The high pyridine nucleotide levels in the lenses are presumably achieved by its binding to taxon-specific enzyme-crystallins, and are beneficial as near ultraviolet light filter.[29] In addition, the lenses with high pyridine nucleotide levels appear to be less susceptible to photo-oxidation.[30] Although ρ-crystallin in the bullfrog lens loses enzyme activity, this enzyme-crystallin retains NADPH-binding capacity.[6] ζ-Crystallin in the guinea pig lens possesses an active NADPH–quinone reductase activity,[5] and specifically binds NADPH as well.[31] It has been reported that a mutation of the ζ-crystallin gene is associated with an autosomal dominant congenital cataract in the guinea pig, and the mutant crystallin fails to bind NADPH and appears to lack NADPH–quinone reductase activity.[5,31] Therefore, NADH-binding ability and enzyme activity in λ-crystallin/DHA reductase may be responsible for the structural stability and function of the enzyme-crystallin in the rabbit lens. However, further investigation is needed to elucidate these possibilities. Effects of the heterogeneity of the rabbit lens enzyme-crystallin on its nucleotide binding capacity and enzyme activity also remain to be studied.

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