Post-translational Modification of α B-Crystallin of Normal Human Lens

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The current study reports several post-translational modifications of α B-crystallin in normal human lenses. The isoforms of post-translational modified α B-crystallin were isolated from the normal human lenses of >70-age group by ion exchange chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoform modifications were determined in detail by fast atom bombardment mass spectroscopy and amino acid sequence analysis. As the criterion of non-modified α B-crystallin, α B-crystallin from 1-d-old infant lenses were used. The modifications found in this study involve oxidation of the N-terminal methionine-1 residue, phosphorylation of the serine-59 residue, and truncation of four amino acids from the C-terminal position of the crystallin. The oxidation of methionine-1 was found in the early stage of human life in 1-d-old lens, although other modification of α B-crystallin were usually only found in old lenses (>70-age group).

Key words human; normal crystallin lens; α -crystallin; α B-crystallin; post-translational modification

α -Crystallin is one of the major structural proteins of the mammalian crystalline lens, and is mainly composed of two types of proteins, α A- and α B-crystallins.¹⁾ It is well known in ophthalmology that soluble high molecular weight protein in the human lens increases during aging and that such a phenomenon may be due to selective aggregation of α -crystallin. It is suspected that this observation may be related to the initial events in senile cataract formation, a major cause of blindness of aged persons.²⁾ Although numerous studies have been carried out to attempt to understand the mechanism of α -crystallin aggregation, the precise events are as yet unknown.

Recent studies on the mechanism of cataractogenesis suggest that post-translational modification may play an important role in the process of α -crystallin aggregation. Up to the present time, such investigations have been focused only on α A-crystallin, and several modifications have been discovered. These early studies detected racemization,³⁾ phosphorylation,⁴⁾ oxidation of methionine and/or tyrosine residues,⁵⁾ and deamidation.⁶⁾ Furthermore, a recent study revealed cleavage of amino acid residues from the C-terminus of α A-crystallin in aged lenses.⁷⁾ Although post-translational modification of α A-crystallin has been investigated in detail as described above, α B-crystallin has not been investigated yet in spite of being a major component of α -crystallin. Observed post-translational modifications of α B-crystallin from human lens were limited to phosphorylation of the serine-45 residue,^{4b)} cleavage of amino acid residues from the N-terminus,⁸⁾ and oxidation of methionine-68.^{3a)}

Recently, several investigators reported that the chaperone-like activity of α -crystallin may play an important role in preventing aggregation and insolubilization of the lens proteins, α -, β -, and γ -crystallin.⁹⁾ Furthermore, they suggested that post-translational modifications of α -crystallin might decrease its molecular chaperone-like activity and they emphasized the need to investigate the relationship between post-translational modification and the chaperone property of α -crystallin. Thus, it is clearly very important to precisely analyze post-translational modifications of α B-crystallin in order to understand the effects of the changes on total prop-

erties of α -crystallin.

The results from the current study demonstrate oxidation of the methionine-1 residue, truncation of four amino acid residues from the C-terminal site, and phosphorylation of serine-59 in the α B-crystallin of normal human lenses. These modifications have been identified for the first time.

MATERIALS AND METHODS

Lenses Used Human normal lenses were obtained from the Eye Bank of the Jules Stein Eye Institute, University of California, Los Angeles. These lenses were autopsy eyes that were removed 4 to 8 h after death. Upon removal, the eyes were stored at -160°C until use. The lenses used in this experiment derived from a 1-d-old infant and from the >70-age group.

Preparation of α B-Crystallin and Its Post-translational Modified Substances Each human lens was homogenized in 1.0 ml of 0.05 M phosphate buffer (0.15 M NaCl, 0.02% NaN_3 , 0.01 M EDTA, pH 7.2) at 4°C . After the homogenate was centrifuged at $15000\times g$, and 4°C for 50 min, the supernatant was fractionated by Superose12 and then Superose6 (1.6 $\times$ 50 cm column) gel filtration chromatography at 4°C , and eluted with the same buffer at a flow rate of 0.5 ml/min. The UV absorbance at 280 nm was measured using a Single Path Monitor UV-1 (Pharmacia Biotech). Fractions containing α -crystallin were pooled. To isolate α A- and α B-crystallins and modified isoforms, the α -crystallin fraction was fractionated by ion exchange chromatography on a Bioscale Q5 column (5 ml) (Bio Rad Laboratory) using a linear gradient elution of 50 mM Tris-HCl to 200 mM Tris-HCl in 6 M urea (pH 8.0) with a gradient HPLC system (Ternary Gradient Unit LG-980-02, Jasco Corporation, Japan). The UV absorbance was monitored at 280 nm with an Intelligent UV/VIS Detector UV-970 (Jasco Corporation, Japan). Each fraction was pooled and dialyzed against 0.1 M Tris-HCl, pH 7.6, using a dialysis membrane (Spectro/Pro, Cellulose ester membrane, molecular weight cut off=1000) for 24 h. After that, the inner solution was lyophilized. Then, the α B-crystallin species in respective fractions were determined by im-

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muno-dot blot analysis, as described below.

Enzyme Digestion Each lyophilized fraction containing approximately 10 nmol protein was digested with trypsin (sequence grade, Boehringer Mannheim) at 1:50=enzyme:protein in 100 μ l of 0.1 M Tris-HCl, pH 7.6, for 24 h at 37 °C. The digests were chromatographed on a Wakosil 5C18 column (0.46 $\times$ 25 cm, 5 μ m) using a gradient of 0–50% acetonitrile in 1% of trifluoroacetic acid over 150 min. The UV absorbance was monitored at 230 nm.

Dephosphorylation from phosphorylated peptide was performed using protein Phosphatase 1 (0.25 units/ μ l) (Promega) dissolved in 25 mM imidazole-HCl containing 2.5 mM EDTA, 25 mM dithiothreitol (DTT) and 50% glycerol, pH 7.5. Approximately 5 μ g of the peptide was mixed with 11.4 μ l of the enzyme solution and then incubated at 36 °C for 6 h. After desalting with a small sized column of Sephadex G10 by a conventional method, the peptide fraction was lyophilized and then analyzed by mass spectrometry.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) Mass spectral analyses of peptides in 50% acetonitrile containing 0.1% of trifluoroacetic acid were performed on a JEOL HX-110 mass spectrometer using a conventional probe or by direct injection through an HPLC-FAB-MS interface. A Magic Bullet (mixture of dithiothreitol:dithioerythritol=3:1, Tokyo Kasei Organic Chemical, Japan) was used as the FAB-MS matrix.

Analysis of Amino Acid Sequence Edman degradation of purified peptides was carried out using an Applied Biosystem Model 477/120A sequencer.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Two dimensional electrophoresis was carried out by both tube gel electrophoresis and SDS-PAGE according to the method of Henzel *et al.* Acrylamide gel, 12.5%, of 1 mm thickness was used for SDS-PAGE.¹⁰⁾ A mixture of Bio-Lyte 3/10:Bio-Lyte 7/9=1:4 was used as the tube gel. Conditions of tube gel electrophoresis were 20-min pre-scanning at 500 V and subsequently 180-min scanning at 1500 V.

Immuno-dot Blot Assay An immuno-dot blot assay was performed using a Bio-Rad-Immuno-Blot (Gar-HPR) assay system. Antibody against α B-crystallin was donated by Dr. Horwitz, Jules Stein Eye Institute, University of California, Los Angeles, California.

RESULTS

Preparation of α B-Crystallin and Its Post-translational Modified Substances For preparation of α B-crystallin and its post-translational modified substances, the α -crystallin fraction obtained by gel filtration chromatography (data not shown) was submitted to ion exchange chromatography on a Bioscale Q5 column. The separation was similar to that reported by another investigator.^{4b)} Along with the two major peaks for α A- and α B-crystallins, we found several minor peaks in the lenses of the >70-age group as shown in Fig. 1. The assignment of these peak peptides to α B-crystallin was carried out by immuno-dot blot analysis using antibody against α B-crystallin and by two-dimensional electrophoreses (data not shown). As a result, the minor peaks between $t_R=30$ min and $t_R=45$ min were tentatively identified as modified α B-crystallin crystallins. Therefore, these minor peaks

Fig. 1. Elution Patterns of α -Crystallin of Human Lenses of 1-d-Old (A) and of >70-Age Group (B) on a Column of Bioscale Q5 by Linear Gradient Elution Using 5 to 200 mM Tris-HCl in 6 M Urea (pH 8.0)

The respective peaks were collected and dialyzed against 0.1 M Tris-HCl (pH 7.6), and were identified by immuno-blot analysis, by SDS-PAGE and by sequence analysis.

were successively named α B1-, α B2- and α B3-crystallins. They were pooled and stored -80°C until analysis.

Enzyme Digestion with Trypsin One aliquot of purified α B fraction and its modified substances was digested with trypsin. The tryptic peptides generated were fractionated by reverse-phase HPLC on a Wakosil 5C18 column. Figure 2A shows the elution profiles of tryptic peptides of the α B fraction of 1-d-old lens. The tryptic peptides were (T1-T20) obtained from α B-crystallin were completely assigned and reported by Dubin *et al.*^{1a)} and Fujii *et al.*^{3a)} Figure 3 shows a part of the amino acid sequence of α B-crystallin. Next, the α B3 fraction from the lens of >70-age group was digested with trypsin and fractionated by reversed-phase HPLC. Six peaks (P1–P6) appeared including those from α B fraction of 1-d-old lens which had changed peak height or shape. These peptides were designated as candidates of post-translational modification and analyzed in detail by amino acid sequence analysis and FAB-MS. In this experiment, the α B3 fraction was mainly analyzed, since the amount of α B1 fraction was too small and the α B2 fraction showed a more complicated elution profile of tryptic peptides, making it difficult to fractionate each peak. As a result, three new post-translational modifications in α B-crystallin were found in addition to several modifications reported previously.^{3a,4b,8)}

1) Cleavage of Amino Acid Residues from the C-Terminal Region: From FAB-MS and sequence analysis, peak P1 ($t_R=36.2$ min) was a peptide with a molecular weight of m/z 844.4 ($M+H$)⁺ (Fig. 4A) and with the sequence EEKPAVTA by Edman degradation analysis. The calculated molecular weight of this peptide is 844.4 ($M+H$), consistent with the

Fig. 2. Elution Profiles of Tryptic Peptides Obtained from α B-Crystallin of 1-d-Old (A) and α B3-Crystallin of >70-Age Group (B) on a Column of Wakosil 5C18

After all peaks were collected, sequences were determined by sequence analysis and FAB-MS. As a result, new peaks that appeared in addition to those of α B-crystallin of 1-d-old lens, were identified and named as P1 to P6, corresponding to peptide modified post-translationally. The peaks P1 to P6 were designated as candidates of post-translational modification and analyzed in detail.

Fig. 3. A Part of Amino Acid Sequence of α B-Crystallin

The numbers (e.g. 18) described under tryptic peptides (e.g. T19) represent the peak numbers on the Wakosil 5C18 reversed chromatogram (Fig. 2). Twenty tryptic peptides (T1—T20) obtained from α B-crystallin were already assigned and reported.^{1a,3d} a) the region of residues 160—175 (T19), which involve the C-terminal region; b) the region of residues 1—13 (T1), which involve the N-terminal region; c) the region of residues 55—72 (T4); d) the region of residues 23—56 (T3).

molecular weight by FAB-MS. Therefore, the P1 peptide represents a peptide that has lost 3 amino acids from the C-terminal region of tryptic peptide T19 (Fig. 3A). The P1 peptide was tentatively identified as residues 164—171 of α B-crystallin, indicating the cleavage of 4 amino acids from the C-terminal region of α B-crystallin as a post-translational modification.

Fig. 4. FAB-MS of P1 Peptide (A) and P3 Peptide (B)

Details of the determination are described in Materials and Methods.

2) Oxidation of Methionine-1 Residue: The percentage of the P3 peak ($t_R=73.0$ min) in the total peak area was larger than that ($t_R=73.3$ min) from 1-d-old lens. This was unusual so the structure of the P3 peptide was examined. Edman degradation of the P3 peptide was examined, but it was not degraded. Therefore, this peptide was subdigested with the endopeptidase Asp-Nase and gave a small peptide with the sequence DIAIHHPWIR. This peptide was tentatively identified as residu 2—11 of the N-terminal region of α B-crystallin. The molecular weight of peak P3 peptide was 1446.6 by FAB-MS as shown in Fig. 4B. The difference between the observed molecular weight of the P3 peptide (1446.6) and the calculated molecular weight of the T1 peptide (ac-MDIAIHHPWIR; MW=1430.7) (Fig. 3B) is 15.9, suggesting that the P3 peptide represent an oxidized form of the unmodified T1 sequence. From these observations, it is suggested that the most probable site of post-translational modification would be formation of methionine sulfoxide at the N-terminus of the peptide. The peptide at $t_R=73.3$ min in the 1-d-old lens was assigned naturally. This peptide was also the T1 peptide in an oxidized form. Taken together, these results suggest that oxidation of methionine-1 at the N-terminus of α B-crystallin may occur before or immediately after birth. However, we can not easily eliminate spontaneous oxidation in the peptide preparation process.

We also found a second oxidation of methionine-68, as indicated by the P4 peptide ($t_R=77.0$ min), obtained from aged lenses from the >70-age group (data not shown). A peak with the same retention time as the P4 peptide in 1-d-old lens was also found. This peak was also investigated by Electrospray ionization mass spectrometry (ESI-MS) and Edman degradation analysis. However, this peak material gave no useful data, due to no mass peaks and no Edman degradation. We do not know whether this material is peptide or not. This second oxidation in aged lenses has already reported by

Fig. 5. FAB-MS of P5 Peptide (A) and Digest of P5 Peptide with Protein Phosphatase 1 (B)
Details of the determination are described in Materials and Methods.

Fujii, *et al.*,^{3a)} thus, oxidation of this peptide were not studied further.

3) Phosphorylation of Serine Residue: Two sites of phosphorylation in α B-crystallin were found in this experiment. The first is phosphorylation at the serine-59 residue. This was determined using the P5 peptide (t_R =83.2 min) from lens isolated from the >70-age group. This peptide was sequenced to 13 cycles and was found to be AP?WFDTGLSEMR, in which ? means unidentified amino acid residue. From sequence analysis, the P5 peptide was tentatively identified as residue 57–69 of α B-crystallin or as the tryptic T4 peptide (Fig. 3C). This strongly suggests that the unidentified amino acid residue must be a serine residue. The observed molecular weight of P5 peptide was m/z 1576.8 ($M+H$)⁺ by FAB-MS (Fig. 5A), even though the calculated molecular weight of the T4 peptide is 1496.7 ($M+H$). The difference between the observed molecular weight of the P5 peptide and the calculated molecular weight of the T4 peptide is 80.0. This suggested that the serine-59 residue in α B-crystallin may be phosphorylated. This assumption was confirmed indirectly by dephosphorylation from the peptide with protein Phosphatase 1. Figure 5B shows the FAB-MS spectrum of the peptide Phosphatase 1 digest of the P5 peptide. After digestion, material with m/z 1497.0 was observed. This indicates that a fragment with m/z 80 (observed value=79.9) was re-

moved from the P5 peptide by digestion with peptide Phosphatase 1. This indicates a phosphate group.

The second phosphorylation was observed in the P6 peptide (t_R =118.2 min). The observed molecular weight of this peptide was m/z 4085.5 ($M+H$)⁺ by FAB-MS (data not shown). The sequence of 7 amino acids at the N-terminal region of this peptide was determined by Edman degradation, indicating that the sequence was LFDQFFG. From the results of this analysis, the P6 peptide was tentatively identified as the tryptic peptide T3 (Fig. 3D). However, the calculated molecular weight of the T3 peptide is 4005.5 ($M+H$). The difference in molecular weight between the P6 peptide and the T3 peptide is 80. This suggests that phosphorylation may occur in the T4 peptide, and reaction can occur on a serine residue in T3 peptide. However, in this experiment we could not determine the precise site of phosphorylation, because the P6 peptide was too large to sequence by Edman degradation.

DISCUSSION

The current study demonstrates three new types of post-translational modifications of α B-crystallin; oxidation of the N-terminus methionine-1 residue, cleavage of four amino acid residues from the C-terminal region and phosphoryla-

tion of the serine-59 residue.

Oxidation of a methionine residue was first discovered in bovine α A-crystallin by Takemoto *et al.*^{5a)} In addition, they reported that they could not find oxidation of methionine residues in human normal and cataractous lens proteins by FAB-MS analysis. However, they suggested the presence of methionine oxidation in human α A-crystallin by a sensitive radioimmunoassay.^{5a)} Recently, Fujii *et al.* reported the oxidation of methionine-68 in the α B-crystallin of aged human lens.^{3a)} In the current study, we could detect oxidation of the N-terminal methionine-1 by FAB-MS analysis and sequence analysis. This methionine-1 oxidation could be found in 1-d-old lens, indicating that oxidation occurred before birth or immediately after birth. We assume that Takemoto *et al.* did not find oxidation of methionine residues in human lens proteins, due to differences in the amount of oxidized methionine between human and bovine lenses.

Concerning truncation of amino acid residues from the C-terminal region, Takemoto *et al.* demonstrated the existence of bovine α A-crystallin devoid of the C-terminal serine residue.¹¹⁾ There is no reliable evidence regarding cleavage of amino acid residues from the C-terminal region of human α -crystallin until now, although Takemoto *et al.* suggested truncation of amino acid residues from the C-terminal region of human lens.^{6b)} As a result of detailed analysis, we found cleavage of four amino acid residues from the C-terminal region of human α B-crystallin, which was different from the case of bovine. Recent studies have revealed that the C-terminal region of α -crystallin might play an important role in its molecular chaperone properties, which protect the α molecule against protein denaturation.^{11a,b)} We postulate that senile cataractogenesis may result from the truncation of amino acid residues from the C-terminal region of α -crystallin.

Dubin *et al.* suggested that the phosphorylation sites of human α B-crystallin may be similar to those in bovine; that is Ser-19, Ser-45 and Ser-59.^{1a)} In the current experiment, we found phosphorylation of Ser-59 in human α B-crystallin, supporting the proposal of Dubin *et al.* On the other hand, Miesbauer *et al.* reported that Ser-45 is phosphorylated in human α B-crystallin.^{4b)} At the present time, phosphorylation of Ser-19 has not been found yet. The physiological function of phosphorylation of serine residues in the α B-crystallin of crystalline lenses is unknown yet, however, one proposal is that phosphorylated α -crystallin becomes more acidic, and thereby induces aggregation of crystallin from human lens. In the current study, we did not examine the P2 fraction, because this substance was analyzed in the previous study and we have previously found and reported truncation of amino acid residues from the N-terminus of α B-crystallin.⁸⁾

Taken together, we confirmed that post-translational modification may induce aggregation and insolubilization of lens

protein, and that this phenomenon may be a trigger of senile cataractogenesis. We feel that the effect of post-translational modification of lens proteins on their aggregation should be investigated further to clarify the mechanism of senile cataractogenesis.

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