Characterization of Covalent Multimers of Crystallins in Aging Human Lenses* 

Received for publication, August 12, 2003, and in revised form, November 17, 2003
Published, JBC Papers in Press, November 17, 2003, DOI 10.1074/jbc.M308884200

Om P. Srivastava†‡, Marion C. Kirk†, and Kiran Srivastava§
From the †Department of Physiological Optics and the ‡Department of Pharmacology and Comprehensive Cancer Center, Mass Spectrometry Shared Facility, University of Alabama at Birmingham, Birmingham, Alabama 35294

The purpose of this study was to characterize covalent multimers with molecular mass of >90 kDa in the water-insoluble (WI) proteins of aging human lenses. The experimental approach was to first separate the multimers (molecular mass >90 kDa) as individual spots by two-dimensional gel electrophoresis and next analyze compositions of each multimers by matrix-assisted laser desorption ionization-time of flight and electrospray ionization-tandem mass spectrometric (ES-MS/MS) methods. The WI proteins from lenses of 25- and 41-year-old subjects showed distinct 5- and 16-multimer spots on two-dimensional gels, respectively, but the spots from 52- and 72-year-old lenses were non-descript and diffused. ES-MS/MS analyses showed two types of covalent multimers in 25- and 41-year-old lenses, i.e., the first type composed of fragments of eight different crystallins (i.e., aA, aB, aB1, aB2, aB3, aA4, aA4, and aA4), and the second type composed of aB1, aA-crystallins (possibly fragments) and two beaded filament proteins (phakinin and filensin). The most commonly identified species in the complexes of 41-year-old lenses were: aA-crystallin (C-terminally truncated, residues 1–157), aB-crystallin (residues 83–90), aB1-crystallin (residues 60–71), aB3-crystallin (residues 33–44), aA4-crystallin (residues 106–117), filensin (residues 78–90), and phakinin (residues 77–89). Three post-translational modifications (i.e., oxidation of Met and Trp, conversion of Ser to dehydroalanine, and formylation of His) were observed in aA-crystallin fragment, and the first two modifications could cross-link proteins. Together, the results suggested that covalent multimers appeared early in life (i.e., 25 years of age) and increased in number with aging, and the two beaded filament proteins form covalent complexes with crystallin fragments in vivo.

Mammalian lens contains three major structural proteins that are known as α-, β-, and γ-crystallins. Among these, the α- and β-crystallins exist as oligomers, whereas the γ-crystallin as a monomer. The αA- and αB-crystallins, the two subunits and primary gene products of α-crystallin, aggregate to form 800,000-Da oligomers, whereas β- and γ-crystallins originated via gene duplication and form a superfamily. These structural proteins, by virtue of their special structural interactions and high concentrations, contribute to the transparency of the lens and provide refractive index to focus light on to the retina. With aging, crystallins show aggregation, cross-linking, and water insolubilization. Together, these processes contribute to the development of age-related lens opacity. Presently, the sequence of events that lead to development of opacity is not well understood. However, evidence suggests that a variety of post-translational modifications in crystallins lead to their aggregation, cross-linking, and eventually water insolubilization. Because of several of the post-translational modifications simultaneously occur during aging in the native, aggregated, and water-insoluble crystallins, the identification of a single or combination of potential modifications as the initiating factor in the aggregation and insolubilization process of crystallins remains unclear. Consequently, it is now believed that lens opacity may involve a multifactorial mechanism. To understand the cataractogenic process, lens researchers have attempted to determine how these post-translational modifications lead to aggregation and water insolubilization of crystallins.

Recent studies of water-insoluble (WI)1 proteins from normal human lenses (1, 2) showed that the major in vivo modifications occurred in αA- and αB-crystallins, which included disulfide bonding, deamidation, oxidation, and backbone cleavage. Previous studies showed that the modifications of crystallins that may contribute to aggregation and cross-linking included disulfide bonding (3), glycation (4), oxidation of Trp and His residues (5, 6), deamidation (7), transglutaminase-mediated cross-linking (8), racemization (9), stereoinversion (10), and phosphorylation (11). The relative importance of the modifications and their specificity to either aging and/or cataractogenic processes is not well established. However, certain cataract-specific modifications (i.e., not observed or occurring at relatively lower levels during aging) have been identified, which included increased degradation of α-crystallin in diabetic cataracts (12), presence of abnormal αB species in the human nuclear cataracts (13), and increased deamidation of γS-crystallin (14).

The proportion of WI proteins increase in human lenses with aging and more so during cataract development (15), and the age-related water insolubilization of crystallins might be mediated via a precursor complex known as water-soluble, high molecular weight (HMW) proteins (16). One approach to deter-

† This work was supported by Grant EY-06400 from NEI, National Institutes of Health, by the National Center for Research Resources (Grants S10RR-11329 and S10RR-13795), by University of Alabama Health Services Foundation-General Endowment Funds, and by University of Alabama at Birmingham-Comprehensive Cancer Center Support Grant P30-CA13148. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Physiological Optics, University of Alabama at Birmingham, 924 S-18th St., Worrell Bldg., Birmingham, AL 35294. Tel.: 205-975-7630; Fax: 205-934-5725; E-mail: Srivasta@uab.edu.
§ The abbreviations used are: WI, water-insoluble; WS, water-soluble; HMW, high molecular weight; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; IEF, isoelectric focusing; HRTP, hydroxytryptophan; ES-MS/MS, electrospray ionization-tandem mass spectrometric; NFK, N-formylkynurenine.
mine the relative importance of different modifications in cross-linking of crystallins is to ascertain their presence in the species that form water-insoluble covalent multimers. The composition of the WI proteins has shown that several aggregated species increase in concentration and become water-insoluble with aging and cataractogenesis and show the modifications as described above (1, 2). The potential mechanisms that cause crystallin cross-linking during the cataractogenic process are identified, which include transglutaminase-mediated cross-linking (17), glycation-induced cross-linking (4), and disulfide bonding (3). Additionally, the cross-linked species might be represented by α-crystallin lens membrane (18–21), and α-crystallin intermediate filament proteins complexes (22). Together, these complexes may represent the supramolecular species in the lens, previously identified in vivo as 0.12- to 0.9-μm particles with an estimated molecular mass of 2 × 10⁶ Da by NMR spectroscopy (23) and by quasielastic light scattering (24).

As stated above, an intermediate state between WS proteins and WI proteins is believed to be the WS-HMW proteins, which is shown to exist in vivo by the light-scattering method (16). The constituents of the WS-HMW proteins have been reported (16, 25–27), but it provided little information regarding the mechanism of aggregation and cross-linking during the conversion of WS proteins to WS-HMW proteins. This was because the covalent multimers are difficult to isolate from the aging and cataractous lenses.

The purpose of this study was to identify covalent multimers with a molecular mass of >90 kDa in the WI proteins of aging human lenses. The experimental approach was to first separate the multimers as individual spots by two-dimensional gel electrophoresis (isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension) and then to analyze the single multimer spots by MALDI-TOF and ES-MS/MS mass spectrometric methods. Because our results showed that the covalent multimers are increasingly cross-linked, non-descript, and difficult to isolate as single species in the older human lenses (age > 40 years), the present study was focused on WI multimers from younger lenses of 25–40 years. The results presented show for the first time that two different types of covalent multimers exist in the aging human lenses (i.e., one composed of only the crystallin fragments and the other of crystallins and two headed filament proteins (filensin and phakinin)). Additionally, three specific post-translational modifications in the components of multimers (i.e., oxidation of Met and Trp, conversion of Ser to dehydroalanine, and formylation of His) were identified. These modifications might be causative factors for the crystallin cross-linking to form multimer species.

**EXPERIMENTAL PROCEDURES**

**Materials**—Normal human lenses with no apparent opacity were obtained from Dr. Robert Church of the Emory University or from the Shared Ocular Tissue Module at the University of Alabama at Birmingham. The lenses were retrieved within 48–72 h post-mortem, and stored in medium-199 without phenol red at −20°C until used. The pre-stained and unstained molecular weight protein markers were from Invitrogen and Amersham Biosciences, respectively. The chemicals for two-dimensional gel electrophoresis were from either Amersham Biosciences or Bio-Rad. Unless indicated otherwise, all other chemicals used in this study were purchased from Sigma (St. Louis, MO) or Fisher (Atlanta, GA) companies.

**Isolation of WS and WI Protein Fractions from Normal Human Lenses**

The two-dimensional gel electrophoretic analyses of WI protein fractions from lenses of 25-, 41-, 52-, and 72-year-old donors were shown in Fig. 1. The α-, β-, and γ-crystallins (molecular mass between 20–35 kDa) and their fragments (molecular mass < 20 kDa) were predominately present in the two-dimensional gel profiles of these lenses. Several minor spots with molecular mass >35 kDa were also seen in these lenses, which became increasingly more complex and non-descrip with aging. However, although some of these were separated as single spots in the WI protein fractions of 25- and 41-year-old lenses (shown with arrows in Fig. 1, A and B), they remained diffused and non-descrip in the lenses from 52- and 70-year-old donors (Fig. 1, C and D). Because the focus of this investigation was to characterize individual covalent multimers, the single spots with molecular mass >90 kDa from lenses of only 25- and 41-year-old donors were chosen for their compositional analysis by MALDI-TOF and ES-MS/MS methods.

As shown in Fig. 2A, five major spots (numbered as I to 5) were identified in the WI protein fraction of lenses from 25-year-old donors, whereas 16 spots were present in the 41-year-old lenses. The central region of each spot was excised and digested with trypsin as described under “Experimental Procedures.” Next, the tryptic fragments were analyzed by the MALDI-TOF method followed by their amino acid sequence analysis by ES-MS/MS method using Micromass-QTOF2. The amino acid sequences of the tryptic fragments from lenses of a 25-year-old donor revealed that the single multimer spots were...
of /H9251 arrows (identified by The spots with molecular mass increasingly complex and non-descript. with aging the proteins profiles became trumetric methods. MALDI-TOF and ES-MS/MS mass spec-

A (25-year-old) B (41-year-old) C (52-year-old) D (72-year-old)

Fig. 1. Two-dimensional gel electrophoretic protein profiles of WI proteins from lenses of donors with ages of 25 years (520 μg of protein applied, A), 41 years (720 μg of protein applied, B), 52 years (1.6 mg of protein applied, C), and 72 years (1.2 mg of protein applied, D). Note that with aging the proteins profiles became increasingly complex and non-descript. The spots with molecular mass >90 kDa (identified by arrows) from lenses of 25- and 41-year-old donors were analyzed by MALDI-TOF and ES-MS/MS mass spectrometric methods.

Identification of individual spots on two-dimensional gels from WI proteins of lenses from donors of 25 years (A) and 41 years (B). A total of 5 and 16 spots (numbered in A and B) were identified in lenses of 25- and 41-year-old donors, respectively. The individual spots were analyzed by MALDI-TOF and ES-MS/MS methods.

Identification of composition of multimers in WI protein fraction of lenses from 25- and 41-year-old donors

| Spot number | Composition of multimers* |
|-------------|---------------------------|
| 1           | Filensin                  |
| 2           | αA and filensin           |
| 3           | αA, βA4, βA3, βB1, βB2, γS, and γD |
| 4           | αA and filensin           |
| 5           | αA, βB1, βA4, filensin, and phakinin |
| 6           | αA, βB1, βA4, and filensin |
| 7           | αA, βB1, γS, and phakinin |
| 8           | αA, βA4, βA3, filensin, and phakinin |
| 9           | αA, βB1, βB4, filensin, and phakinin |
| 10          | αA, βA4, and filensin     |
| 11          | αA, βA4, and filensin     |
| 12          | αA, βA4, and filensin     |
| 13          | αA, βA4, filensin, and phakinin |
| 14          | αA, βA4, βA3, βB1, and filensin |
| 15          | αA, βA4, and filensin     |
| 16          | αA and filensin           |

* The composition was based on sequence determination of tryptic fragments by ES-MS/MS using Micromass QTOF-2.

either made of several crystallin fragments or α-, β-, and γ-crystallins and filensin and/or phakinin (the two beaded fil-

TABLE I

ELS/MS Analysis of Components of Multimeric Complexes from Lenses of 25-Year-old Donors—The three tryptic peptide sequences of spot 1 on ES-MS/MS analysis showed amino acid sequences that matched with the sequence of filensin (Table II). The first peptide sequence of LINKEADEALLHNLR matched with the residues 144–157 of filensin, and the second and third peptides with sequences of SQLEEGREVLSHLAQQR and LGELAGPEDALAR matched with residues 223–239 and 78–90 of filensin, respectively. Human lens filensin has 665 amino acids with $M_r$ 74,496 (30). The fact that residues 79–239 of filensin were seen as a complex with molecular mass >90 kDa, suggested that the protein was truncated.

Spot 2 was a covalent complex of αA-crystallin and filensin, because ES-MS/MS analysis showed that it contained tryptic peptides with matching amino acid sequences of both proteins. The following tryptic peptide sequences of αA-crystallin were seen in spot 2: Ac-MDVTIQHPWF (residues 1–11, oxidized Met, and Trp with none, one or two oxygen), DVTIQHPWFKR (residues 2–12), TLGPFYFPSR (residues 13–22), TVLDSGIVSEVR (residues 55–65), SDRDKFVIFLDVK (residues 66–78), HFSPEDLTVK (residues 79–98), HFSPEDLTVKQDDFVE-
Covalent Multimers of Crystallins in Aging Human Lenses

### Table II

Amino acid sequences of components forming covalent multimers in lenses from a 25-year-old donor

| Spot number | α-Crystallin | β-Crystallins and γ | Filensin |
|-------------|--------------|---------------------|----------|
| 1 | A: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: DVTIQHPWFKR (#2–12) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: TLGPYPFSR (#13–22) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: TLGDGSISEVR (#55–65) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: SDRKDFV/FDLVK (#66–78) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: HFSPEELTVK (#79–88) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: HFSPEELTVK/QDDFVEIHGK (#79–99) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: VQDDFVEIHGK (#89–99) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: IQTGLDATHAER (#146–157) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: TGLDATHAER (#148–157) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
| 2 | A: TLGPYPFSR (#13–22) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: HFSPEELTVK (#79–88) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: APSWFDTGSLER (#37–69) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: APSWFDTGSLMNLK (#37–69) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: HFSPEELK (#83–90) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: DRFSVNLDVK (#73–82) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
|   | A: QDEHGFISLR (#108–116) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#223–239) |
|   | A: IPADVPDILTITSSLSDDGVLT(VNGPR (#124–149) | βA: ITIYDQENFQGKR (#33–45) | LGEAGLEDALAR (#78–90) |
| 3 | A: TLGPYPFSR (#15–21) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: TVLDSGISEVR (#55–65) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |
|   | A: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: DVTIQHPWFKR (#2–12) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |
|   | A: TLGPYPFSR (#13–22) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: TLGDGSISEVR (#55–65) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |
|   | A: SDRKDFV/FDLVK (#66–78) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: HFSPEELTVK (#79–88) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |
|   | A: HFSPEELTVK/QDDFVEIHGK (#79–99) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: VQDDFVEIHGK (#89–99) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |
|   | A: IQTGLDATHAER (#146–157) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
| 4 | A: TLGPYPFSR (#15–21) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#223–239) |
|   | A: TVLDSGISEVR (#55–65) | βA: VQDDFVEIHFGR (#106–117) | LGEAGLEDALAR (#78–90) |

* Residue numbers are shown in parentheses.

**Note:** The sequences listed are those identified through MS/MS analysis, with oxidized Met and Trp indicated where applicable. Some sequences are truncated or fragmented, as indicated by the use of hyphens and parentheses. The table includes the identification of components forming covalent multimers in lenses from a 25-year-old donor, highlighting the complexity and diversity of the protein interactions in aging human lenses.
which show that a variety of fragments of α-, β-, and γ-crystallins and that of filensin and phakinin were present in the multimers. The components of the remaining spots could not be fully identified and, therefore, are not reported.

Spots 3 and 5 showed tryptic fragments with identical amino acid sequences suggesting their identical compositions except spot 5 lacked phakinin (Table III). Both spots were a complex of αA, βB1, βA4-crystallins, phakinin, and filensin. In both spots, αA-crystallin was apparently C-terminally truncated, and both Met and Trp residues at positions 1 and 9, respectively, were oxidized along with oxidized Met-113 residue in βB1-crystallin. The tryptic peptides present in the two multimers were as follows: αA: Ac-MDVTVIQHPWFK (#1–11, oxidized Met and Trp), TLGFPFYSR (#13–21), TVLDGSISEVR (#55–65), LDSGISEVR (#57–65), HFSPEDLTVK (#79–88) and VQDDFVEIHGG (#89–99); βB1: LVVFLEKENFQGR (#80–71), GEMFILEK (#110–117, oxidized Met), GYQYLLEPGDFR (#202–214); βA4: LTIFEQENFLGK (#107–118); filensin: LGELAGPEDALAR (#78–90), SQLEEGREVLSHLQAFR (#223–239), EVLSHLQAQR (#230–239), QLAVAQTLTK (#294–303); and phakinin: ALGISSVFQLGQR (#77–89), LMLQTPQIAGADDFFKR (#174–191), and YENEQPFR (#192–199).

Spot 6 multimer was a complex of αA, αB, βA3-crystallins, filensin, and phakinin. The tryptic peptide sequences observed in spot 6 were as follows: αA: Ac-MDVTVIQHPWFK (#1–11, oxidized Met and Trp), TLGFPFYSR (#13–21), TVLDGSISEVR (#55–65), LDSGISEVR (#57–65), HFSPEDLTVK (#79–88), VQDDFVEIHGG (#89–99), QDDHGYSIR (#104–112), IQTGLDATHAER (#146–157); αB: APSWFDTGLEM #(#57–69), HFSPEELK (#83–90); βA3: ITIYDQENFQGR (#83–94); filensin: LGELAPEDALAR (#78–90), QLAVAQTLTK (#294–303); and phakinin: ALGISSVFQLGQR (#77–89), LMLQTPQIAGADDFFKR (#174–191), and YENEQPFR (#192–199).

Identification of Post-translational Modifications Present in Components Forming the WI Multimers—As stated in the introduction, the formation of the cross-linked crystallin species during aging and cataractogenesis is believed to be primarily due to post-translational modifications in crystallins. The ES-MS/MS analyses of the complexes identified three major post-translational modifications in the αA-crystallin fragments. The three modifications were oxidation of Met-1 and Trp-9, conversion of Ser-59 to dehydroalanine and formylation of His-79. The oxidative modifications are listed in Tables II and III, and an example during ES-MS/MS analysis is shown in Fig. 3. Similarly, the conversion of Ser-59 to dehydroalanine in the αA-crystallin is shown in Fig. 4, and the formylation of His-79 in αA-crystallin is shown in Fig. 5. The significance of these modifications in the complex covalent formation is discussed below.

DISCUSSION

Because almost no information exists in the literature regarding the multimeric complexes of crystallins in human lenses, the purpose of this study was to identify crystallin multimers in the WI protein fraction of aging human lenses and then determine their composition by ES-MS/MS analyses. This aim was a part of the overall goal to identify species forming multimers and to elucidate the mechanism of their cross-linking and water insolubilization via modified amino acids. Although several modifications in specific amino acids in crystallins have been identified in past studies, their role in crystallin aggregation and cross-linking remains speculative (1, 2). An alternative approach involving the characterization of crystallin covalent multimers has been lacking in the literature, and it was adopted in the present study. This approach of identifications of crystallins multimers in human lenses has been precluded so far because of the difficulties in their isolation and availability of accurate methods for the compositional analysis.

The experimental approach in this study was to identify the single multimer spots in the WI protein fraction of aging human lenses following their separation by two-dimensional gel electrophoresis and then determine their composition and modified amino acids by the ES-MS/MS method. To ensure that the multimer spots were indeed non-disulfide-linked, the Immobi-
## Amino acid sequences of components forming covalent multimers in lenses from a 41-year-old donor

| Spot number | α-Crystallin | βCrystallin | γCrystallin | Filensin and/or phakinin |
|-------------|--------------|-------------|-------------|-------------------------|
| 3 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQGKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: SQLEEGRLSLQAQR (#223–239) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: EVLSHLAQQR (#230–239) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βA4: LTIFEQENFLGK (#106–117) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βA4: LTIFEQENFLGK (#106–117) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
|           | βA4: LTIFEQENFLGK (#106–117) | Phakinin: YEΝΕΚΩΨΦ (#192–199) |
| 5 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: SQLEEGRLSLQAQR (#223–239) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βB1: GYQYLFPGDFR (#202–213) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βA4: LTIFEQENFLGK (#106–117) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
|           | βA4: LTIFEQENFLGK (#106–117) | Phakinin: YEΝΕΚΩΨΦ (#192–199) |
| 6 αA: AC-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βB1: GYQYLFPGDFR (#202–213) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | γB: LFQYEDKNFQKR (#7–18) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
| 7 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βB1: GYQYLFPGDFR (#202–213) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | γB: LFQYEDKNFQKR (#7–18) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
| 8 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βB1: GYQYLFPGDFR (#202–213) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | γ: LFQYEDKNFQKR (#7–18) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
| 9 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: QLAVAQHTLKV (#294–303) |
|           | βB1: GYQYLFPGDFR (#202–213) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | γ: LFQYEDKNFQKR (#7–18) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
| 10 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Phakinin: ALGISSVFLQGLR (#77–89) |
|           | βA3: ITIYDQENFQKR (#33–44) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
|           | βA3: ITIYDQENFQKR (#33–44) | Phakinin: LMLQΤΕΤΗQAGΔΔΦΚΕ (#174–191) |
| 13 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: LGELAGPEDALAR (#78–90) |
|           | γ: LFQYEDKNFQKR (#7–18) | Filensin: LGELAGPEDALAR (#78–90) |
| 14 αA: Ac-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |
| 15 αA: AC-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |
| 16 αA: AC-MDVTIQHPWFK (#1–11, oxidized Met and Trp) | βB1: LVVFENFQKR (#60–71) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GEMFILEK (#110–117, Met oxidized) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βB1: GYQYLFPGDFR (#202–213) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA4: LTIFEQENFLGK (#106–117) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |
|           | βA3: ITIYDQENFQKR (#33–44) | Filensin: LGELAGPEDALAR (#78–90) |

*Residue numbers are shown in parentheses.*
Covalent Multimers of Crystallins in Aging Human Lenses

TABLE IV

| Amino acid sequences | Spot numbers |
|----------------------|-------------|
| αA (#1–11)           | 3,5,6,7,8,9,15,16 |
| αA (#13–21)          | 3,5,6,7,8,9,13,14,15,16 |
| αA (#55–65)          | 3,5,6,7,8,9,13,15,16 |
| αA (#79–88)          | 3,7,9,13 |
| αA (#89–99)          | 3,6,7,8,9,13,14 |
| αB (#57–69)          | 6,7,9 |
| αB (#83–90)          | 6,7,8,9,13,14 |
| βB1 (#60–71)         | 3,5,7,14 |
| βB1 (#110–117)       | 3 |
| βB1 (#202–213)       | 3,7 |
| βB2 (#108–119)       | 14 |
| βA3 (#33–44)         | 6,8,9,14 |
| βA4 (#106–117)       | 3,5,8,9,13,15 |
| γS (#7–18)           | 7 |
| Filensin (#78–90)    | 3,6,8,9,13,14,15,16 |
| Filensin (#99–106)   | 8 |
| Filensin (#223–239)  | 3,5 |
| Filensin (#240–254)  | 5,15 |
| Filensin (#284–303)  | 3,6 |
| Phakinin (#77–89)    | 3,6,7,8,9,13,15 |
| Phakinin (#174–191)  | 3,15 |
| Phakinin (#192–199)  | 3 |

line strips, after first dimension of IEF separation, were treated with 100 mM dithiothreitol and 300 mM iodoacetamide prior to the second dimension of SDS-PAGE analysis. Because the multimer components were identified based on their amino acid sequences, the identity of these species and the modified amino acids were definitive. Furthermore, because the water-insoluble multimeric species as opposed to water-soluble multimers were identified, the results were significant in providing information about the potential role of modified amino acids in cross-linking and water insolubilization.

The major findings of this study were: 1) The covalent crystallin multimers in aging human lenses appeared early in life, i.e. 25 years of age, and became water-insoluble. These multimers were separable as individual spots by two-dimensional gel electrophoresis in lenses up to 40 years of age, but in the older lenses (e.g. 52 and 72 years old), the spots became diffused and non-descript, and were difficult to visualize as single spots. 2) The number of theses complexes increased with aging; i.e. a greater number of multimers existed in lenses of 41-year-old compared with 25-year-old subjects. 3) All the WI complexes in the 41-year-old human lenses were composed of α-, β-, and γ-crystallins and phakinin and/or filensin. The data suggested that the two beaded filaments proteins play a major role in the formation of covalent multimers with crystallin fragments. 4) Spot 3 in the 25-year-old lenses was composed of only the truncated species of eight crystallins (i.e. αA-, αB-, βA-, βA4-, βB1-, βB2-, γS-, and γD-crystallins). This suggested that the fragments of α-, β-, and γ-crystallins per se could form covalent multimers. 5) Although the crystallins forming the covalent complexes varied, αA-crystallin was invariably present in all the complexes in the 41-year-old lenses suggesting its major role during cross-linking. In each of the complexes, the αA-crystallin was C-terminally truncated and mostly contained residues 1–157, suggesting that a loss in the C-terminal region (residues 158–173) in αA-crystallin might induce the cross-linking process. 6) Only a few multimers contained the N- and C-terminally truncated αB-crystallin (regions with residues
57–69 and 83–90) in addition to the C-terminally truncated aA-crystallin, but the occurrence of the former was less frequent than the latter. 7) In the complexes with β-crystallin fragments, the most commonly occurring fragments were the two fragments from βB1-crystallin (residues 60–71 and 202–213), one from βA3 with residues 33–44, and one fragment of βA4 with residues 106–117. 8) The regions of filensin and phakinin present in all the complexes contained residues 78–90 and 77–89, respectively. 9) The γD- and γS-crystallin formed multimeric complexes with α- and β-crystallins in the young lenses 25 years of age, but their presence was minimal compared with α- and β-crystallin species in the complexes that existed with beaded filament proteins in older lenses. 10) Three major post-translational modifications were observed in aA-crystallin fragments, which included oxidation of Met-1 and Trp-9, formylation of His-79 and conversion of Ser-59 to dehydroalanine.

The results of this report provided information for the first time regarding the components of covalent complexes that existed in aging human lenses, and the important role that crystallin fragments and filensin and/or phakinin (two beaded filament proteins) might play in complex formation. Both phakinin (also known as CP1A9) and filensin (also known as CP115/CP95) are the major components of the backbone of the beaded filament in the lens (30, 31). The beaded filaments are composed of a thin backbone of 7–9 nm in diameter, which is decorated at regular intervals by globular beads of 12–15 nm in diameter, and are distinct from either actin-containing or vimentin-containing intermediate filaments (32). These beads are believed to be made of aA- and aB-crystallins (31). Both proteins are expressed only in the lens and in its differentiating fiber cells (34, 35). Furthermore, the cytoskeleton, beaded filaments, and α-crystallin exist together as a complex in the fiber cells (34). The potential role of phakinin in cataractogenesis was implicated, because mutation in the protein leads to inherited cataract (36, 37). In a recent report, the knockout of the intermediate filament protein phakinin (CP149) destabilized the cytoskeleton of fiber cells and decreased lens optical quality but did not induce cataract (38).

Results of this report also show two types of covalent complexes in the aging human lenses. The first complex was mainly composed of fragments of eight different crystallins (i.e. aA, aB, βA3, βA4, βB1, βB2, γS, and γD), and the second complex was made of α-, β-, and γ-crystallins and two beaded filament proteins (phakinin and filensin). In the first type complex, the crystallin fragments were covalently linked, because the observed molecular mass on SDS-PAGE was only ~100 kDa, whereas the total molecular mass of all the native crystallin combined would be 180,280 Da (i.e. aA: 19,909; aB: 20,159; βA3: 25,150; βA4: 22,243; βB1: 27,892; βB2: 23,249; γS: 20,875; and γD: 20,706). Furthermore, because the ES-MS/MS analyses identified only few trypic fragments of the crystallins, it suggested that the fragments of the crystallins formed the complex, but the presence of intact crystallins could not be ruled out.

The second type of the complex showed the presence of a variety of fragments of α-, β-, and γ-crystallins with fragments of filensin and phakinin. As stated above, in all the complexes, the C-terminally truncated aA-crystallin, and in few of them, both N- and C-terminally truncated aB-crystallin were present. The data suggested that a major role of α-crystallin in the formation of the multimer. Three major post-translational modifications were also identified in the αA-crystallin that formed the complexes; i.e. oxidation of Met-1 and Trp-9, conversion of Ser-59 to dehydroalanine, and formylation of His-79. An additional modification in βB1-crystallin involving oxidation Met-112 was also observed (spot 3, Table III). The Trp oxidation products of aA- and aB-crystallins have been identified in a previous study (39). Trp (M, 186), upon oxidation, acquires one oxygen and becomes hydroxyltryptophan (HRTP, M, 202) and, upon acquiring two oxygens, produces N-formylkynurenine (NFK, M, 218) (39). As shown under “Results,” the C-terminally truncated aA-crystallin species in the complexes contained modified Trp with either one or two oxygens, suggesting the conversion of the residue to HRTP and NFK. Because the oxidation is believed to play a major role in the development of senile cataract (40), both Trp oxidation products (i.e. HRTP and NFK) act as a photosensitizer, capable of producing reactive oxygen species (41); their presence in the in vitro produced multimers provides further evidence of the role of the oxidative process in the development of multimers. Because the metal-catalyzed oxidation is a primary cause of biomolecular oxidation, it may be responsible for the observed Trp oxidation. However, Trp has been shown to be oxidized by ionizing radiation, and it is not a site for metal binding (42), and, upon exposure of α-crystallin to iron, no Trp oxidation was reported (43).

Another observed modification in aA-crystallin was the conversion Ser-59 to dehydroalanine, which has been here reported for the first time. This modification is of great significance, because covalent binding has been shown to occur between two proteins involving dehydroalanine of one protein and cysteine of the other protein to form lanthionine (44). Such covalently linked proteins were not dissociated with SDS, urea, or disulfide reducing agents (44). Therefore, these covalent complexes observed in the WI proteins of aging human lenses will also be stable during similar treatment in the resolubilization buffer prior to the two-dimensional gel electrophoresis. A previous report has demonstrated the presence of lanthionine in cataractous human lenses (45). Recently the presence of lanthionine in human cataractous lenses has been again identified. A treatment of polyhistidine or copper complex of His residue with N-acetyldehydroalanine resulted in the formation of a new amino acid histidinalanine (46), whose presence has been shown to be ~70 times greater in human cataractous lenses compared with normal lenses (47). A similar role of dehydroalanine in the complexes reported herein might occur. Therefore, the conversion Ser-59 to dehydroalanine might cause cross-linking via cysteine and/or His of another protein, leading to stable multimers in aging and cataractous human lenses. The formylation of the His residue may provide reactive carbonyl residue, which is shown to increase with aging in a variety of tissues (48).

The presence of the phakinin and filensin with crystallin fragments in the multimeric complexes has been reported for the first time. The fact, that only one such multimer complex existed in the 25-year-old lenses, whereas the majority of the complexes in the 41-year-old lenses contained phakinin and/or filensin and crystallins, suggests that the involvement of beaded filament proteins might be an age-related process. Additionally, because the complexes made of only crystallin fragments existed in the 25-year-old lenses but none in the 41-year-old lenses, the cross-linking of crystallin fragments might precede the involvement of beaded filament proteins.

One major question arose from this investigation: What is the mechanism of complex formation between fragments of α-, β-, and γ-crystallins and the two beaded filament proteins? Because aA-crystallin fragments with the three modified amino acids (i.e. oxidation of Met-1 and Trp-9, conversion of Ser-59 to dehydroalanine, and formylation of His-79) were present in the multimeric complexes, it probably plays a central role during cross-linking. As stated above, the oxidized Trp and
conversion of Ser to dehydroalanine would lead to cross-linking among proteins. It might be possible that the specific fragments of filensin and phakinin containing residues 77–89 that are found most commonly in the complexes could also play major roles in the cross-linking process. Alternatively, the phakinin and filensin in the beaded filaments may per se provide the nucleation site for the formation of multimeric complexes with the crystallin fragments. Presently, further investigation in our laboratory is focused to answer these questions.

Acknowledgment—The help of Martha Robbins in preparation of the manuscript is greatly appreciated.

REFERENCES

1. Lund, A. L., Smith, J. B., and Smith D. L. (1996) Exp. Eye Res. 63, 661–672
2. Hansen, S. R., Hasan, A., Smith, D. L., and Smith, J. B. (2000) Exp. Eye Res. 71, 195–207
3. Takemoto, L. (1996) Exp. Eye Res. 63, 585–590
4. Nagaraj, R. H., Sell, D. R., Prabhakaram, M., Ortwether, B. J., and Monnier, V. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10257–10261
5. McDermott, M., Chiesa, R., Roberts, J. E., and Dillon, J. (1991) Biochemistry 30, 8653–8660
6. Andley, U., and Clark, B. A. (1989) Invest. Ophthalm. Vis. Sci. 30, 706–713
7. Srivastava, O. P., and Srivastava, K. (2003) Mol. Vis. 9, 110–118
8. Lorand, L. (1998) in Advances in Post-translational Modifications of Proteins and Aging (Zappa, V., ed) pp. 79–94, Plenum Press, New York
9. Fujii, N., Ishibashi, Y., Satoh, K., Fujino, M., and Harada, K. (1994) Biochim. Biophys. Acta 1204, 157–163
10. Fujii, N., Matsumoto, S., Hiroki, K., and Takemoto, L. J. (2001) Biochem. Biophys. Acta 1549, 179–187
11. Takemoto, L. (1996) Exp. Eye Res. 62, 499–504
12. Jimenez-Asensio, J., Colvis, C. M., Kowalak, J. A., Duglas-Tabor, Y., Datiles, M. B., Meroni, M., Mura, U., Rao, C. M., Balsubraminian, D., Janjani, A., and Garland, D. (1999) J. Biol. Chem. 274, 32287–32294
13. Thampi, P., Hassan, A., Smith, J. B., and Abraham, E. C. (2002) Invest. Ophthalmol. Vis. Sci. 43, 3265–3272
14. Takemoto, L., and Boyle, D. (2000) Mol. Vis. 6, 164–168
15. Clark, R., Zigman, S., and Lehrman, S. (1969) Exp. Eye Res. 8, 172–182
16. Jedziniak, J. A., Nuoli, D. F., Baran, H., and Benedek, G. B. (1978) Invest. Ophthalmol. Vis. Sci. 17, 51–57
17. Shridas, P., Sharma, Y., and Balsubraminian, D. (2001) FEBS Lett. 499, 245–250
18. Takemoto, L., and Boyle D. (1998) Int. J. Biol. Macromol. 22, 331–337
19. Cobb, B. A., and Petrasj, J. M. (2000) J. Biol. Chem. 275, 6664–6672
20. Tang, D., Borchman, D., and Yappert, M. C. (1999) Ophthal. Res. 31, 452–462
21. Cenedella, R. J., and Fleischer, C. R. (1992) Curr. Eye Res. 11, 801–815
22. Carter, J. N., Hutcheson, A. M., and Quinlan, R. A. (1995) Exp. Eye Res. 60, 181–192
23. Morgan, C. F., Schleich, T., Caines, G., H., and Farnsworth, P. N. (1989) Biochemistry 28, 5064–5074
24. Benedek, G. B., Chylack, L. T., Liboni, T., and Pennett, M. (1987) Curr. Eye Res. 6, 1421–1432
25. Jedziniak, J., Kinoshita, J. H., Yates, E. M., and Benedek, G. B. (1975) Exp. Eye Res. 20, 367–369
26. Srivastava, O. P. (1988) Exp. Eye Res. 47, 525–543
27. Srivastava, O. P., Srivastava, K., and Silney, C. (1996) Curr. Eye Res. 15, 511–520
28. Herbert, B. (1999) Electrophoresis 20, 660–663
29. Lai, E.-K. (1995) Nature 326, 680–685
30. Hess, J. F., Casselman, J. T., Kong, A. P., and Fitzgerald, P. G. (1998) Exp. Eye Res. 66, 625–644
31. Goulasmos, G., Gounari, F., Remington, S., Palmer, S., Aebi, U., and Georgatos, S. D. (1996) J. Cell Biol. 132, 645–655
32. Quinlan, R. A., Carter, J. M., Sandilands, A., and Prescott, A. R. (1996) Trends Cell Biol. 6, 123–126
33. Hess, J. F., Casselman, J. T., and Fitzgerald, P. G. (1996) J. Biol. Chem. 271, 6729–6735
34. Fitzgerald, P., and Gottlieb, W. (1989) Curr. Eye Res. 8, 801–811
35. Masaki, S., and Watanabe, T. (1992) Biochem. Biophys. Res. Commun. 186, 190–198
36. Conley, Y. P., Erturk, D., Keeverline, A., Mah, T. S., Keravala, A., Barnes, L. R., Bruchas, A., Hess, J. F., Fitzgerald, P. G., Weeks, D. E., Ferrell, R. E., and Gorin, M. B. (2000) Am. J. Hum. Genet. 66, 1426–1431
37. Jakobs, P. M., Hess, J. F., Fitzgerald, P. G., Kramer, P., Weleber, R. G., and Litt, M. (2000) Am. J. Hum. Genet. 66, 1432–1436
38. Sandiland, A., Prescott, A. R., Wegener, A., Zoltoski, R. K., Hutcheson, A. M., Masaki, S., Kustak, J. R., and Quinlan, R. A. (2003) Exp. Eye Res. 76, 385–391
39. Finley, E. L., Dillon, J., Crouch, R. K., and Schey, K. L. (1998) Protein Sci. 7, 2391–2397
40. Spector, A. (1995) PASEB J. 9, 1173–1182
41. Beszka, K. J., Bliski, P., Chignell, C. F., and Dillon, J. (1996) Free Radic. Biol. Med. 20, 23–34
42. Stadtman, E. R. (1993) Annu. Rev. Biochem. 62, 797–821
43. Smith, J. B., Jiang, X., and Abraham, E. C. (1997) Free Radic. Res. 26, 103–111
44. Chiu, M. L., Folcher, M., Griffith, P., Hult, T., Klatt, T., and Thompson, C. J. (1986) Biochemistry 25, 2352–2361
45. Bessems, G. J., Rennen, H. J., and Hoenders, H. J. (1987) Exp. Eye Res. 44, 691–695
46. Kanayama, T., Miyanaga, Y., Horiuchi, K., and Fujimoto, D. (1987) J. Agric. Food Chem. 35, 163–168
47. Friedman, M. (1999) Trends Cardiovasc. Med. 9, 34–40
48. Berlett, B. S., and Stadtman, E. R. (1997) J. Biol. Chem. 272, 20313–20316
Characterization of Covalent Multimers of Crystallins in Aging Human Lenses
Om P. Srivastava, Marion C. Kirk and Kiran Srivastava

J. Biol. Chem. 2004, 279:10901-10909.
doi: 10.1074/jbc.M308884200 originally published online November 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308884200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 9 of which can be accessed free at
http://www.jbc.org/content/279/12/10901.full.html#ref-list-1