Size of Human Lens β-Crystallin Aggregates Are Distinguished by N-terminal Truncation of βB1*

(Received for publication, January 10, 1997, and in revised form, February 10, 1997)

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The aggregates formed by the interactions of the human lens β-crystallins have been particularly difficult to characterize because the β-crystallins comprise several proteins of similar structure and molecular weight and because their sequences were not known until recently. Previously, it could not be ascertained whether the species of various acidities were different proteins or modifications of the same proteins. The recent determination of the sequences permits calculation of molecular weights and unambiguous identification of the various β-crystallins and their modified forms by mass spectrometry. In this investigation, the components of the three sizes of β-crystallin aggregates, β1 (150,000), β2 (92,000), and β3 (46,000), were determined. The principal differences among the different β-crystallin aggregates was the presence of βA1 in β1 and β2, but not β3, and the length of the N-terminal extension of βB1. The size of the β-crystallin aggregate correlated with the length of the N-terminal extension of βB1, indicating that the flexible N terminus of βB1 is critical to the formation of higher molecular weight aggregates of β-crystallins. Separation of the components by ion exchange under non-denaturing conditions showed that βB2 occurs as homo-dimers and homo-tetramers as well as contributing to hetero-oligomers. Other β-crystallins were present only as hetero-oligomers.

The lens is a transparent avascular tissue with a high concentration of proteins closely packed to give a refractive index that will focus light on the retina (1). The lens proteins, which have monomeric molecular weights of approximately 20,000–30,000, form aggregates with molecular weights up to 1 million. It is believed that the clarity of the lens depends on the proper assembly of these aggregates (2). In the mammalian lens there are three primary groups of crystallins, α-, β-, and γ-crystallins, each composed of proteins homologous within the group but differing from the proteins in other groups. The recent identification of the same proteins. The recent determination of the sequences permits calculation of molecular weights and unambiguous identification of the various β-crystallins and their modified forms by mass spectrometry. In this investigation, the components of the three sizes of β-crystallin aggregates, β1 (150,000), β2 (92,000), and β3 (46,000), were determined. The principal differences among the different β-crystallin aggregates was the presence of βA1 in β1 and β2, but not β3, and the length of the N-terminal extension of βB1. The size of the β-crystallin aggregate correlated with the length of the N-terminal extension of βB1, indicating that the flexible N terminus of βB1 is critical to the formation of higher molecular weight aggregates of β-crystallins. Separation of the components by ion exchange under non-denaturing conditions showed that βB2 occurs as homo-dimers and homo-tetramers as well as contributing to hetero-oligomers. Other β-crystallins were present only as hetero-oligomers.

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and 70 years old. All lenses were clear; the donors had no diseases known to affect lens opacity. Each lens, analyzed individually, was homogenized in 2.5 ml of a buffer (0.05 M NaHSO₃, 0.05 M Tris, 0.02 M EDTA, 0.02% Na₂EDTA, pH 7.4). The water-soluble crystallins, removed as the supernatant after centrifugation at 27,000 × g for 15 min, were fractionated into α-, β₁-, β₂-, and γ-crystallins by size exclusion chromatography (70 × 2 cm Sephadex S-300HR column) using the homogenizing buffer as the mobile phase at a flow rate of 15 ml/min. The column was calibrated using thyroglobulin (M₉, 669,000), alcohol dehydrogenase (Mg, 150,000), bovine serum albumin (M₉, 66,000), and ovalbumin (Mg, 45,000). The absorbance of the eluate was monitored at 280 nm. Not all molecular weight determinations were performed on all lenses, but the chromatograms for the size exclusion, ion exchange, and reversed phase chromatograms, were approximately 3:1 in mass (Fig. 2) and reversed phase HPLC (Fig. 3).

Because there was some overlap between the peaks for B₁ and C, whether from the chromatograms for the size exclusion, non-denaturing anion exchange but changes the molecular weight by only 1 Da for a protein with a molecular mass of 20 KDa.

RESULTS

The data presented were obtained with the 56-year-old lens. Not all molecular weight determinations were performed on all lenses, but the chromatograms for the size exclusion, ion exchange, and reversed phase fractionation for the different lenses were similar, suggesting that there were only minor age-related differences in the water-soluble β-crystallins from ages 16 to 70. For the size exclusion chromatographic conditions used in this study, β-crystallins gave three peaks with aggregates of approximate molecular weights of 150,000 (β₁), 92,000 (β₂), and 46,000 (β₃) (Fig. 1). By employing several chromatographic techniques, size exclusion, non-denaturing anion exchange HPLC, and reversed phase HPLC, along with mass spectrometric determination of molecular weights of the fractionated proteins, it was possible to unambiguously determine the components of the β-crystallin aggregates (Table I). Each β-crystallin was identified by matching its molecular weight as determined by mass spectrometry with a molecular weight calculated from the known sequences of the β-crystallins. For all identifications, the agreement between the experimentally determined and calculated molecular weights was within 2 atomic mass units.

Anion exchange chromatography was performed without urea in the buffer to observe which components were associated

1 The abbreviations used are: HPLC, high performance liquid chromatography; ESIMS, electrospray ionization mass spectrometry; BB, Bull and Breeze hydrophilicity index.

and in the β-crystallin aggregates. Under these non-denaturing conditions, three peaks labeled A, B, and C (Fig. 2) were evident. The intensities of A, B, and C varied considerably among the different groups of aggregates. For example, peak A from B₁ was small (Fig. 2a), but it was approximately one-third of B₂ (Fig. 2b) and B₃ (Fig. 2c). Peak B was a relatively minor component of B₁ but a major component of B₂ and B₃. Peak C was a major component of all three size exclusion fractions. Although the chromatograms were generally similar for all lenses, the relative intensities of peaks B and C derived from B₂ and B₃ varied somewhat, from about 3:2 to 2:3, among the lenses.

The reversed phase chromatogram of proteins eluting in peak A had only one peak, with an elution time corresponding to B₂ (Fig. 3a) (7). ESIMS analysis of this peak showed the presence of one protein with a molecular weight (Mₗ, 23,291) (Fig. 4) that confirmed its identity as βB₂ (Mₗ, 23,291) (7). The peak labeled A’ (Fig. 2c) also had a molecular weight matching B₂. Since deamidation of a protein causes it to elute later on anion exchange but changes the molecular weight by only 1 mass unit and therefore cannot be distinguished by ESIMS determination of its molecular weight, it seemed likely that A’ is a deamidated form of B₂.

Reversed phase analysis of ion exchange peaks B and C gave similar chromatograms, each with two major peaks (Fig. 3, b and c). One peak had a retention time of B₂ (peak 1 of Fig. 3), and the other had a retention time corresponding to βB₁ (peak 3 of Fig. 3) (6) and βA₄ (5), which co-elute with these reversed phase conditions. Identification of βB₂, βB₁, and βA₄ was confirmed by ESIMS analysis (Table I). Finding the same masses for the proteins in B and C suggested that B and C contain the same proteins but perhaps with the proteins in C modified by deamidation. The βB₂-crystallins in peaks B and C, whether from β₁, β₂, or β₃, were all intact (Mₗ, 23,291) (Fig. 4). βA₄-crystallin, which also was present only as the intact protein (Mₗ, 22,282), was found in size exclusion fractions B₁ and B₂; a mass corresponding to βA₄ was not detected in B₃. In contrast, βB₁-crystallin was identified by ESIMS in several forms (Fig. 5) with molecular weights corresponding to βB₁ and its truncated products at the N terminus (6). The relative amounts of βB₂:βB₁:βA₄, based on their intensities in the reversed phase chromatograms, were approximately 3:1 in peak B and 1:1.5 in peak C. A minor component, labeled 2 in Fig. 3, present in both ion exchange peaks B and C from β₁ and β₂, was identified by its reversed phase elution time as βA₃/βA₁ (5).

The electrospray mass spectra for the proteins with reversed phase elution times appropriate for βB₁ were remarkably dif-
Truncated $\beta B1$ in Human Lens $\beta$-Crystallin Aggregates

Table I

Components of $\beta$-crystallin aggregates

| $\beta$-Crystallin | $\beta_1$-Aggregates ($M_r$ ~ 150,000) | $\beta_2$-Aggregates ($M_r$ ~ 92,000) | $\beta_3$-Aggregates ($M_r$ ~ 46,000) |
|-------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| $\beta A3/\beta A1$ | $\beta A3$-Aggregates ($M_r$ ~ 150,000) | $\beta A3$-Aggregates ($M_r$ ~ 92,000) | $\beta A3$-Aggregates ($M_r$ ~ 46,000) |
| $\beta A4$ | $\beta A4$-Aggregates ($M_r$ ~ 150,000) | $\beta A4$-Aggregates ($M_r$ ~ 92,000) | $\beta A4$-Aggregates ($M_r$ ~ 46,000) |
| $\beta B1$ | $\beta B1$-Aggregates ($M_r$ ~ 150,000) | $\beta B1$-Aggregates ($M_r$ ~ 92,000) | $\beta B1$-Aggregates ($M_r$ ~ 46,000) |
| $\beta B2$ | $\beta B2$-Aggregates ($M_r$ ~ 150,000) | $\beta B2$-Aggregates ($M_r$ ~ 92,000) | $\beta B2$-Aggregates ($M_r$ ~ 46,000) |
| $\beta B3$ | $\beta B3$-Aggregates ($M_r$ ~ 150,000) | $\beta B3$-Aggregates ($M_r$ ~ 92,000) | $\beta B3$-Aggregates ($M_r$ ~ 46,000) |

* ND, not detected.

Different for $\beta_1$, $\beta_2$, and $\beta_3$ (Fig. 5, a, b, and c, respectively). The $\beta B1$-crystallins showed progressive N-terminal truncation from $\beta_1$ to $\beta_2$ to $\beta_3$. For all the lenses, intact $\beta B1$ ($M_r$ 27,933) was a major component only of $\beta_1$, which also contained $\beta B1$ missing the first 15 residues ($M_r$ 26,535) and sometimes minor amounts with as many as the first 39 residues (Fig. 5a). In contrast, all the $\beta B1$ in $\beta_2$ was truncated at the N terminus with 15–41 residues missing (Fig. 5b), and most of $\beta B1$ in $\beta_3$ was even further truncated at the N terminus, with at least 34–41 residues missing (Fig. 5c).

Since non-denaturing conditions were used in the anion exchange chromatography, the proteins isolated in this separation could be expected to retain their native associations. Finding only $\beta B2$ in peak A of the ion exchange chromatograms of
Truncated βB1 in Human Lens β-Crystallin Aggregates

In previous investigations the components of the various aggregates of human β-crystallins were identified primarily by SDS-polyacrylamide gel electrophoresis (15, 16). Because the sequences of the human β-crystallins were not known and because there appeared to be several proteins with similar molecular weights, the exact composition of each of the aggregates could not be determined. Data from fetal lenses showed the presence of a 29-kDa protein, later identified as βB1 in both β1 and β2 (16). Whereas data from lenses more than 5 years old indicated the presence of βB1 only in β1, 15, 16, 16. From the SDS-polyacrylamide gel electrophoresis data presented in those studies, it appeared that βB1 was not a component of the lowest molecular weight aggregates. A protein of 24–26 kDa, first called βBp then renamed βB2, was a major component of β3, β2, and β1 from lenses of all ages (15, 16, 22). The other β-crystallins were not identified.

Results from our mass spectrometric investigation of β-crystallins, separated by ion exchange and reversed phase HPLC, have led to unambiguous identification of the components of each of the subgroups of β-crystallins (Table I). The data show that βB1 in a variety of forms truncated at the N terminus is present in the aggregates of β1, β2, and β3 and that the size of the aggregates correlates with the length of the N terminus of βB1. The largest aggregates, β3, are composed primarily of βB1 (both intact and with the first 15 residues missing), intact βB2, and intact βA4. The principal forms of βB1 present in β1, βB1(1–251) and βB1(16–251), correspond to bovine proteins previously identified as βB1a and βB1b (23). These two forms of βB1 are the major βB1-crystallins found in newborn human lenses (6). The ion exchange chromatography performed under non-denaturing conditions indicated that the oligomers of β1 are hetero-oligomers of βB1, βA4, and βB2. Because βB1 and βA4 co-elute on reversed phase HPLC, the relative amounts of each component were not ascertained. A very minor component in β3, had the correct retention time for βA3. Intact βA3 has not previously been detected only in fetal lenses; in adult lenses it was found missing the first 22 residues of the N terminus. An insufficient amount of this minor component was isolated from the 56-year-old lens for molecular weight determination, but it was presumed to be βA3 truncated at the N terminus based on its reversed phase HPLC elution time. A molecular weight corresponding to βA3(23–215), which is the same as βA1(5–197), was determined for this protein isolated from other adult lenses.

The tetrameric aggregates of the β-crystallins, βB1, included the same proteins as βB2, except that βB1 was found with further N-terminal truncation. Although no intact βB1 was present in β2, the following forms, truncated at the N terminus, were found: βB1(16–251), βB1(35–251), βB1(40–251), and βB1(41–251). As in β1, βB2 and βA4 were found only as intact proteins. The non-denaturing ion exchange of the aggregates in β2 indicated that the tetramers were homo-oligomers of βB2 and hetero-oligomers of βB1, βB2, and βA4.

The dimers of β-crystallin, βB1, included homo-oligomers of βB2 and hetero-oligomers of βB1 and βB2. The βB1 in β3 was degraded at the N terminus even further than in β2, with most of βB1 missing 34 or more residues from its N terminus. Finding βB1, although only in forms truncated at the N terminus, in β3, is in opposition to previous reports that βB1 exists only in larger aggregates (15, 17, 20, 21). These differing observations are easily explained by the fact that the βB1 products without the N-terminal 34–41 residues have molecular weights of 24,192–24,834, similar to the molecular weight of βB2 (23, 291) and may not have been recognized as βB1 by SDS-polyacrylamide gel electrophoresis analysis. Identification would have been further complicated by the fact that βB1 minus these residues has a pl (6.38) similar to the pl of βB2 (6.33). βB3, which has previously been isolated only from fetal or newborn lenses (5), was not detected in any of the subgroups of the β-crystallins from these adult lenses.

For bovine lens crystallins, it has been demonstrated that the various aggregates of β-crystallins are in a dynamic equilibrium with the size of aggregates affected by concentration, temperature, and ionic strength (24). Hydrophilic interactions appeared to be the main factor affecting the association-dissociation equilibrium (24). Even though these data may not be directly applicable to human lenses because the composition of bovine β-crystallins differs considerably from human β-crystallins, it is interesting to consider the effect hydrophilicity might have on the stability of the various human β-crystallin aggregates. The hydrophilicities of the β-crystallin or portions of them can be calculated from the Bull and Breese indices (25). The long N-terminal extension of βB1, which appears to play a unique role in the formation of large aggregates as demonstrated by the presence of intact βB1 only in β1-crystallins, has a Bull and Breese index (BB) of +365. The hydrophilicity indices for βB1 in the middle size aggregates of β3 and the dimers of β3, where 15–41 residues are missing from the N terminus, range from BB −20 to −57. (The more positive numbers indicate a more hydrophilic sequence). Further evidence of the likelihood that hydrophilicity is important in aggregate formation is demonstrated by the fact that βB2 forms both homo-dimers and tetramers, but βB1 and βA4 do not form homo-oligomers. Overall, βB2 is a more hydrophilic protein (BB +62) than either βA4 (BB +30) or βB1 (BB +5).

Comparison of the sequence of the first 34 residues of human βB1 with the N terminus of bovine βB1 shows that this region is not as rich in alanine and proline as bovine βB1 (23), but it does include 8 alanines, 5 prolines, and 4 glycines and no bulky amino acids, giving this region considerable flexibility. These characteristics, along with its hydrophilicity, may allow the N terminus to be flexible for easy interaction with charged portions of the other β-crystallins, stabilizing high molecular weight aggregates.
Previous investigations using mutant forms of βB2 have demonstrated that the linker portion (residues 80–88, linker sequence, are missing in N-terminal truncations, and that which is present lacks the first 22 residues of the N terminus formed smaller aggregates than A3 with only 6 residues missing (28). Such a role for A3 is present in adult lenses is truncated at the N A3 without the first 29 residues of the N A3 in human lenses is showed that, in addition to forming homo-dimers and hetero-dimers, βB2 could form homo-oligomers as well as hetero-oligomers with other acidic and basic β-crystallins. Our results for human lens β-crystallins showed that, in addition to forming homo-oligomers and hetero-oligomers, βB2 also formed homo- and hetero-tetramers. In bovine lenses, both B3 and A3 are major components, whereas in humans B3 is produced only in fetal and newborn lenses, and A3 is a very minor component. Furthermore, the A3 that is present in adult lenses is truncated at the N terminus (5). Slingsby and Bateman (27) concluded that, for bovine β-crystallins, oligomers larger than dimers required the presence of an acidic β-crystallin with a long N terminus, such as A3. This conclusion was supported by studies of mutant rat A3, showing that A3 without the first 29 residues of the N terminal formed smaller aggregates than A3 with only 6 residues missing (28). Such a role for A3 in human lenses is improbable because very little A3 is present in adult lenses and that which is present lacks the first 22 residues of the N terminus. It is much more likely that the long N terminus of B1 is the major determinant of the size of the β-crystallin aggregates in human lenses.

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Truncated βB1 in Human Lens β-Crystallin Aggregates

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