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Polydispersity of a mammalian chaperone: mass spectrometry reveals the population of oligomers in α B-crystallin

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Abstract

The quaternary structure of the polydisperse mammalian chaperone α B-crystallin, a member of the small heat shock protein family, has been investigated by using electrospray mass spectrometry. The intact assemblies give rise to mass spectra which are complicated by the overlapping of charge states from the different constituent oligomers. Therefore, to determine which oligomers are formed by this protein, tandem mass spectrometry experiments were performed. The spectra reveal a distribution, primarily of oligomers containing 24 to 33 subunits, the relative populations of which were quantified, to reveal a dominant species being composed of 28 subunits. Additionally, low levels of oligomers as small as 10mers, and as large as 40mers were observed. Interpretation of the tandem mass spectral data was confirmed by simulating and summing spectra arising from the major individual oligomers. The ability of mass spectrometry to quantify the relative populations of particular oligomeric states also revealed that, contrary to the dimeric associations observed in other small heat shock proteins, there is no evidence for any stable sub-structures of bovine α B-crystallin isolated from the lens.

Introduction

The small heat shock proteins (sHSPs) constitute a family of molecular chaperones involved in protein stabilization under conditions of stress (1). The mammalian sHSP α B-crystallin is systemically expressed (2) and, along with α A-crystallin, is also a major structural protein of the eye lens. α B-Crystallin has been demonstrated to display molecular chaperone activity by suppressing the aggregation of unfolded protein (3). Over-expression of α B-crystallin outside the lens is associated with a number of aberrant protein disease states, including Alzheimer's, Alexander's, Parkinson's and Creutzfeldt-Jakob disease (4-7). Despite increasing amounts of structural information for prokaryotic (8) and plant sHSPs (9), the structures of mammalian members of this family, including the α -crystallins, HSP27 and HSP20, have yet to be elucidated. These sHSPs are isolated from tissue as polydisperse multimers, and it is this heterogeneity which has confounded the efforts of structural biologists (10, 11).

α -Crystallin, in particular, has long been the subject of conflicting results regarding its molecular weight distribution (12), however it is accepted that from the lens, α -crystallin is isolated as a multimeric assembly ranging in molecular weight from 300 kDa to greater than 1 MDa (10). Recently, a combination of size exclusion chromatography and multi-angle laser light scattering was used to determine the mass range of human recombinant α B-crystallin as 530 to 684 kDa, with a population of 585 kDa at the peak of the elution profile (13). This represents the most accurate assessment of the polydispersity inherent in the α -crystallins to date, however it is confined to the major species in the elution profile, and provides little insight into the relative populations of oligomers within the distribution.

Electrospray ionization coupled to time-of-flight mass spectrometry (MS) provides a unique insight into defining the subunit composition of noncovalent complexes (14-16) including small heat shock proteins (17, 18) and other chaperones (19-21). It has not been possible, however, to interpret data from polydisperse proteins such as α B-crystallin. This is primarily due to their high molecular weight and the large number of oligomeric species involved, which give rise to overlapping peaks in the mass spectrum.

Tandem mass spectrometry (MS/MS), a method used routinely for peptide sequencing, involves selection of ions in a well-defined m/z range and subsequent collision-induced dissociation (CID) with an inert gas to produce fragment ions (22). This process can also be employed to dislodge individual subunits from noncovalent assemblies of proteins (14), resulting in a re-distribution of charge on the products. Here we show that by applying such an approach to α B-crystallin, we are able to define the range, and the relative populations of the different oligomers that constitute this polydisperse assembly.

Materials and Methods

Purification of Bovine α B-Crystallin.

α -crystallin was isolated from 2-4 week bovine calf lens extracts by gel filtration using a Sephacryl S300HR column (Amersham Pharmacia). Protein was equilibrated into buffer A (50 mM Tris-HCl, 2 mM DTT, 6 M urea, pH 8.5) and concentrated to 10 mgml⁻¹ in an ultra-filtration cell (Millipore). Samples were loaded onto a MonoQ ion-exchange column (Amersham Pharmacia) and eluted with buffer B (buffer A + 1 M NaCl) using a linear gradient of 0 to 14 %B, and peaks corresponding to the phosphorylated and unphosphorylated forms of the A and B subunits of α -crystallin were separated. The unphosphorylated α B-crystallin was concentrated to 30 mgml⁻¹ using a YM10 Centricon (Millipore). 70 μ l of the sample was loaded onto a Superdex 200HR10/30 gel filtration column (Amersham Pharmacia) and eluted at 0.3 mlmin⁻¹ with 200 mM ammonium acetate at 4 °C. Fractions of the refolded protein were collected and stored on ice to minimize quaternary structure rearrangement (25) and analyzed by MS within an hour of collection. Equal volumes of each of the collected fractions were pooled, resulting in a combined fractions protein concentration of 1.2 mgml⁻¹. An aliquot of this solution was acidified with AG 50W-X8 resin (BioRad) to obtain a denatured, monomeric sample, the mass of which (20079.2 ± 0.7 Da), agreed with the sequence mass of unphosphorylated bovine α B-crystallin of 20078.8 Da (spectrum not shown).

Mass Spectrometry.

Experiments were conducted using LCT (orthogonal time of flight) and modified Q-ToF 2 mass spectrometers (Micromass UK Ltd). Typically, 2 μ l of solution were electrosprayed from gold-coated glass capillaries prepared in-house as described previously (23). In order to preserve noncovalent interactions on the LCT, the following instrument parameters were used: capillary voltage 1.5 kV, cone gas 150 Lh⁻¹, sample cone 200 V, extractor cone 10 V, ion transfer stage pressure 9.0×10^{-3} mbar (1 mbar = 100 Pa), ToF analyzer pressure 3.0×10^{-6} mbar. Tandem MS experiments were performed on a Q-ToF 2 instrument (Micromass UK Ltd) which had been modified for high mass operation (24). In MS mode, the following instrument parameters were used: capillary voltage 1.6 kV, cone gas 100 Lh⁻¹, sample cone 200 V, extractor cone 100 V, voltage applied to the collision cell 4 V, ion transfer stage pressure 9.0×10^{-3} mbar, quadrupole analyzer pressure 9.5×10^{-4} mbar, ToF analyzer pressure 1.7×10^{-6} mbar and 3.5×10^{-2} mbar of argon in the collision cell. For the tandem MS experiments, the quadrupole resolution was adjusted to encompass the entire peak of interest (30 m/z units), extractor cone voltage was reduced to 60 V and spectra were acquired over a range of voltages applied to the collision cell from 80 to 200 V. All spectra were calibrated externally using a solution of cesium iodide (100 mgml⁻¹), and processed with MassLynx software (Micromass UK Ltd). Spectra are shown here with minimal smoothing, and without background subtraction.

Simulated Spectra.

To demonstrate a theoretical deconvolution of the spectrum shown in figure 4A, individual charge-state envelopes for the different species involved were modeled

(Fig. 4*B*). The simulated spectra shown were constructed using SigmaPlot 2001 (SPSS Science). Three-parameter Lorentzian curves were used to model individual peaks, with a peak-width at half height of 12 m/z units, and the intensities of the peaks were based on the observed intensities of the different charge states in figure 4*A*.

Results and Discussion

α B-crystallin eluted as a single symmetrical peak of average molecular weight ~ 600 kDa when refolded from urea on a size exclusion column (Fig. 1A). The corresponding spectra recorded for the fractions indicated are shown in Fig. 1B. The spectrum of fraction 23 (top), gave rise to a signal in the range from 8000 to 15000 m/z. Subsequent fractions, analyzed under identical MS conditions, exhibited a trend towards lower m/z values, such that fraction 28 included well-defined peaks within the m/z range from 6000 to 12000. At the lower m/z values, oligomeric states of α B-crystallin species in the range of 10 to 18 subunits, i.e., 200 to 361 kDa, could be identified. The unresolved portion of the spectra, as evidenced by the elevated baseline above 8000 m/z, is due to the overlap of charge series arising from a range of increasingly large oligomers of α B-crystallin.

The individual fractions were pooled to give a representative sample of all oligomers in the sample. The mass spectrum of the combined fractions gave rise to signal in the range from 7000 to 15000 m/z and lacked the clearly defined peaks at lower m/z, observed for the individual later eluting fractions (Fig. 2A). In fact the smallest oligomer that could be assigned was a 16mer, suggesting this to be the smallest oligomer present in significant amounts. The amount of information available in this spectrum is limited, since the dominant peaks do not arise from multiple charging of a single species (as in a typical electrospray spectrum), but rather the overlap of several such series from different sized oligomers. This is demonstrated by the intense and narrow peak at 10040 m/z (indicated by an asterisk), which, since the molecular weight of a single subunit of α B-crystallin is 20080 Da, corresponds to oligomers carrying twice as many charges as subunits.

In an attempt to identify the underlying charge state series, MS spectra were acquired over the range of voltages applied to the collision cell from 80 to 200 V (Fig. 2B). In such an experiment, all ions formed during the electrospray process are indiscriminately subjected to collisions with argon atoms in the collision cell of the Q-ToF 2, resulting in the removal of excess solvent ions from the multimeric assemblies, thereby improving the quality of the spectrum. At a voltage applied to the collision cell of 100 V, there was an increase in desolvation and consequent improvement in definition of the peaks compared with the spectrum in Fig. 2A. Increasing the voltage applied to the collision cell to 120 V resulted in the appearance of monomers at around 1500 m/z, indicating the occurrence of a collision-induced dissociation (CID) process. It is established that noncovalent complexes subjected to CID result in the asymmetric dissociation of the oligomers into highly charged monomers and lowly charged 'stripped' oligomers (18, 26-29). As charge is conserved between dissociation products (27, 30), these stripped oligomers are observed at unusually high m/z ratios. In the spectrum obtained at 120 V stripped oligomers can be identified above 13000 m/z, including a relatively intense peak at 20080 m/z. The intensity of both the monomer and stripped oligomer signal increased upon raising the acceleration voltage in the collision cell, and at 200 V no peaks corresponding to the original intact oligomers were observed.

The charge state distribution (CSD) of the monomers was found to shift towards higher m/z when the voltage applied to the collision cell was increased (Fig. 2C). The average charge state of the monomer, calculated as described previously (18), was found to be

18+ at 120 V, 16+ at 160 V, and 15+ at 200 V. These values were consistent with the successive loss of monomers from the oligomers, resulting in the observed stripped oligomers[§]. These observations can be summarized by the following equations:

$$\begin{aligned}n^q &\rightarrow [n-1]^{q-x} + m^x \\n^q &\rightarrow [n-2]^{q-y} + 2m^y \\n^q &\rightarrow [n-3]^{q-z} + 3m^z\end{aligned}$$

where n is the number of subunits in the oligomer, q is the number of charges on the oligomer, x , y and z are the average charge carried by a monomer m at a given voltage applied to the collision cell. This observation of three distinct product ion series formed consecutively upon increasing the voltage applied to the collision cell, shows that CID of α B-crystallin results in the dissociation of an oligomer of n components into monomers and stripped oligomers of $n-1$, $n-2$ and $n-3$ subunits.

Although the definition of the peaks arising from the stripped oligomers in Fig. 2B was greater than for the original oligomer series, the spectra remain extremely complex and cannot be interpreted unambiguously. In order to obtain information about the relative populations of the various oligomers in the sample, a dissociation experiment of the polydisperse assembly was carried out using MS/MS. This experiment involved isolation of the peak at 10040 m/z (encompassing all species with two charges per subunit), and subsequent CID. A series of spectra were acquired over a range of voltages applied to the collision cell from 80 V to 200 V, the combined spectrum of which is shown in Fig. 3. The precursor ion, isolated for collisional activation, is visible at 10040 m/z . Between 12500 and 16500 m/z , ions arising from the removal of a single monomer from each oligomer ($n-1$) are present. Another series of ions, attributed to the doubly-stripped oligomers ($n-2$), between 17500 and 26000 m/z , dominates the high m/z region of the spectrum, while further minor species ($n-3$) can be seen around 30000 m/z . A schematic illustrating the observed multiple dissociation of monomers is presented in Fig. 3.

The doubly stripped oligomer series (Fig. 3) exhibited the highest definition of peaks, least charge state series overlap, and thereby presented no ambiguity in interpretation. Thus this series was used to determine the distribution of oligomeric states in the α B-crystallin assembly. Fig. 4A shows an expansion of the MS/MS spectrum obtained at a voltage applied to the collision cell of 130 V. At this voltage the amount of doubly-stripped oligomers was at its greatest, with the amounts of singly-stripped and triply-stripped oligomers approximately equal. Though the spectrum remains complex, charge states corresponding to different oligomeric α B-crystallin species can readily be identified. Mass measurement allowed the unambiguous assignment of the different doubly-stripped oligomers.

The dominant peak observed at 20080 m/z corresponds to doubly-stripped oligomers carrying one charge per subunit, i.e., an oligomer consisting of $n-2$ subunits carries $n-2$ charges. The group of peaks situated either side of the central overlapping peak are not discreet CSDs, but rather the result of contributions from the range of different sized

doubly-stripped oligomers. To illustrate how this arises, a deconvolution of the experimental data in Fig. 4A was performed by simulating the individual CSDs for the eight major, doubly-stripped oligomers (Fig. 4B). These simulations show how the dominant peak at 20080 m/z in Fig. 4A arises from the overlap of peaks at this m/z from all the different doubly-stripped oligomers. Either side of this m/z value, the charge states do not overlap, hence groups of peaks are observed. These simulated spectra indicate the peak series that were used to calculate the corresponding oligomer masses, and demonstrate how contributions from individual components combine to give the spectrum observed for a polydisperse assembly. The ability to resolve peaks arising from the doubly-stripped oligomers allowed us to quantify their relative populations. By calculating the intensities of each peak series in the region of the spectrum shown (Fig. 4A), we observed a symmetrical distribution of doubly-stripped oligomers differing in size by one subunit, from 22 to 31 subunits, with a modal value of 26 subunits (Fig 5). This corresponds to a distribution of oligomers in the original α B-crystallin sample of 24 to 33 subunits with a modal value of 28 subunits.

By its very nature, that is, isolation of a narrow m/z window, an MS/MS experiment does not take into account all of the information in the MS spectrum and hence the sample. Consequently, this population distribution does not fully reflect the range of oligomers in α B-crystallin, as is evidenced by the fact that the 16mer observed in the MS spectrum of the pooled fractions (Fig.2A) was not observed in the MS/MS spectrum. Similarly, information from the high m/z region of the spectrum may have been excluded by this experiment. To investigate this, the MS (rather than MS/MS) spectrum acquired at a voltage applied to the collision cell of 200 V (Fig. 2B), was examined more closely. This spectrum (Fig. 6) is complicated by the fact that the groups of peaks corresponding to those observed in Fig. 4A contain a broader distribution of oligomers, thus they overlap, leading to ambiguity in interpretation of the entire spectrum. However, peaks additional to those in the MS/MS spectrum, which do not overlap, are observed close to the central peak at 20080 m/z. Mass measurement using these peaks revealed that α B-crystallin was composed of minor oligomers ranging up to 40 subunits ($n-2 = 38$) in size. Thus, when the low and high m/z regions are taken into account, the results from this study show that α B-crystallin exists as a continuum of species from 10 to 40 subunits, centered around the most highly populated assembly containing 28 subunits.

Conclusions

The use of collision-induced dissociation in a mass spectrometer to remove up to three monomers from each α B-crystallin assembly has enabled us to define the precise oligomeric nature of this polydisperse protein, for which previously, only a range of molecular masses have been reported. The advantage of using mass spectrometry compared with other molecular weight determination techniques is that we are able to measure the relative populations of individual oligomeric assemblies. Moreover, we are able to define species at the extremes of the distribution, revealing oligomers as small as 10mers, and as extensive as 40mers. This approach has the potential to determine the number of α B-crystallin subunits involved in chaperone action, and yield insights into the mechanism of substrate interaction.

sHSPs from plants, yeast and bacteria have been shown to consist of dimeric building blocks, which have been proposed as the active unit engaged in chaperone activity, and to contribute to their monodispersity (9, 31). By contrast, the formation by α B-crystallin of oligomers containing odd and even numbers of subunits strongly implies that the monomer is the basic building block of this assembly. This intrinsic polydispersity and difference in substructure may reflect broader substrate specificity for α B-crystallin compared with the more specialized non-mammalian sHSPs. Furthermore, monomers, or variable clusters thereof, may constitute the active units *in vivo* and contribute to an enhanced chaperone efficacy.

These results highlight the exciting possibility of defining subunit composition for other heterogeneous complexes for which to date only a range of molecular weights have been reported. Moreover, together with the ability to monitor reactions in real-time (17), this technique will contribute to the understanding of the dynamics of such species, and to a clearer view of their mechanism of action.

Footnotes

[§]An α B-crystallin oligomer of ~600 kDa (the mean molecular weight as estimated by SEC in Fig. 1A), composed of 30 subunits would be in a 60+ charge state at 10040 m/z. Due to the conservation of charge between dissociation products, loss of an 18+ monomer from a $[30\text{mer}]^{60+}$ would result in the stripped oligomer $[29\text{mer}]^{42+}$, which would appear at 13865 m/z. This lies within the m/z range observed for the stripped oligomers in the spectrum obtained at 120 V. At higher voltage applied to the collision cell however, the dominant stripped oligomers appear at much higher m/z values, which can only be accounted for by the loss of more than one monomer from the original oligomers. At 160 V, the loss of two monomers carrying an average of 16+ each from a $[30\text{mer}]^{60+}$ would result in the doubly stripped oligomer $[28\text{mer}]^{28+}$ at 20080 m/z. Similarly the peaks above 27000 m/z can be accounted for by the loss of three monomers, resulting in the formation of triply stripped oligomers.

Charge stripping and/or backbone cleavage of the monomers may occur in the CID process, however for the purposes of this study these phenomena have not affected the use of the monomer CSDs to interpret the dissociation products.

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Figure Legends

Figure 1: Survey of the changes in mass spectra of consecutive fractions of α B-crystallin separated by size exclusion chromatography. (A) Elution profile of bovine α B-crystallin refolded on column from 6 M urea into 200 mM ammonium acetate. The fractions indicated were collected and kept on ice prior to MS analysis. (B) Electrospray mass spectra of fractions 23 (upper) through 28 (lower) from (A). A shift in the distribution of ions on the m/z scale, from high to low values, was observed across the elution profile indicating a significant decrease in the oligomeric size of α B-crystallin between fractions at the onset and tail of the peak.

Figure 2: (A) Electrospray mass spectrum of the combined fractions of bovine α B-crystallin from size exclusion chromatography. Signal is observed between 7000 and 15000 m/z , with the most intense peaks at around 10000 m/z . These peaks are not due to a charge state series of a single oligomer, but rather the overlap of several such series from different sized oligomers. The spectrum was acquired at a voltage applied to the collision cell of 4V. (B) Mass spectra obtained for a range of collision energies demonstrate that α B-crystallin oligomers undergo multiple loss of monomers; from one at a voltage applied to the collision cell of 120 V up to three at 200 V. (C) Expansion of the monomer region at voltages applied to the collision cell of 120, 160 and 200 V. The average charge state of the dissociated monomers was observed to decrease, from 18+ at 120 V to 16+ at 160 V and 15+ at 200V.

Figure 3: Tandem MS of the overlapping components in the isolated peak (indicated by an asterisk) at 10040 m/z (Fig. 2A) resulted in dissociation of the oligomers into highly charged monomers at low m/z , and various stripped oligomers at high m/z values. Spectrum showing a summation of the acquisitions for a series of voltages applied to the collision cell of 80 to 200 V. Overlaid is a schematic showing a possible pathway for the multiple loss of monomers from a representative oligomer, and the resultant mass and charge asymmetry during the gas phase collisionally-induced dissociation. The schematic shows a sequential pathway, however we cannot rule out the simultaneous dissociation of two and three monomers from a single oligomer.

Figure 4: (A) Expansion of the region of the spectrum corresponding to oligomers with $n-2$ subunits, obtained by tandem MS analysis at a voltage applied to the collision cell of 130V. Peaks corresponding to the charge states of doubly-stripped oligomers with different numbers of subunits ($n-2$) are observed. The overlapping peak at 20080 m/z results from oligomers carrying the same number of charges as subunits. The groups of peaks at lower m/z than this central peak result from oligomer populations carrying more than one charge per subunit ($n-1$, n , $n+1$), whereas those at higher m/z arise from oligomers carrying less than one charge per subunit ($n-3$, $n-4$, $n-5$). The upper spectrum (grey) represents the sum of the simulated spectra presented in Fig. 4B. (B) A theoretical deconvolution of the spectrum demonstrates how several different sized stripped oligomers combine to give the observed spectrum (A). Spectra for different sized doubly-stripped oligomers ($23 \leq [n-2] \leq 30$) were simulated from the theoretical m/z

values for each oligomer ion series and the observed intensities for the different charge states in (A).

Figure 5: Histogram showing the relative populations of the different doubly-stripped oligomers ($n-2$) formed by α B-crystallin, calculated from intensities of the peaks in the tandem MS spectrum. The population of oligomers in the original sample (n) was calculated from the doubly-stripped ($n-2$) data.

Figure 6: Expansion of the spectrum containing the $n-2$ oligomer series acquired at a voltage applied to the collision cell of 200 V (Fig. 2B), without selection of a precursor ion window. In this experiment the entire population of ions present in the sample was subjected to high energy gas phase collisions, giving rise to stripped oligomers for the full range of constituent assemblies. Non-overlapping peaks proximal to the peak at 20080 m/z provided additional information regarding the largest oligomers present in this sample.











