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Small heat shock protein activity is regulated by variable oligomeric substructure


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Abstract

The alpha-crystallins are members of the small heat shock protein (sHSP) family of molecular chaperones which have evolved to minimize intracellular protein aggregation, however they are also implicated in a number of protein deposition diseases. In this study we have employed novel mass spectrometry techniques to investigate the changes in quaternary structure associated with this switch from chaperone to adjuvant of aggregation. We have replicated the oligomeric rearrangements observed for in vivo disease-related modifications, without altering the protein sequence, by refolding the alpha-crystallins in vitro. This refolding results in a loss of dimeric substructure concomitant with an augmentation of substrate affinity. We show that packaging of sHSPs into dimeric units is used to control the level of chaperone function by regulating the exposure of hydrophobic surfaces. We propose that a bias toward monomeric substructure is responsible for the aberrant chaperone behaviour associated with the alpha-crystallins in protein deposition diseases.

Keywords

protein folding, mass spectrometry, small heat shock protein, chaperone

Disciplines

Analytical Chemistry | Biochemistry | Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences | Structural Biology

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SMALL HEAT SHOCK PROTEIN ACTIVITY IS REGULATED BY VARIABLE OLIGOMERIC SUBSTRUCTURE *

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The α -crystallins are members of the small heat shock protein (sHSP) family of molecular chaperones which have evolved to minimize intracellular protein aggregation, however they are also implicated in a number of protein deposition diseases. In this study we have employed novel mass spectrometry techniques to investigate the changes in quaternary structure associated with this switch from chaperone to adjuvant of aggregation. We have replicated the oligomeric rearrangements observed for post-translationally modified α -crystallins, without altering the protein sequence, by refolding the α -crystallins *in vitro*. This refolding results in a loss of dimeric substructure concomitant with an augmentation of substrate affinity. We show that packaging of sHSPs into dimeric units is used to control the level of chaperone function by regulating the exposure of hydrophobic surfaces. We propose that a bias toward monomeric substructure is responsible for the aberrant chaperone behaviour associated with the α -crystallins in protein deposition diseases.

The sHSPs α A- and α B-crystallin are most prevalent in the vertebrate eye lens, where they are found co-assembled and maintain lens transparency by preventing other proteins from forming light-scattering aggregates (1), but are also found in other tissues (2). It is clear that these proteins have the ability to sequester substrates that have become partially unfolded under conditions of stress, in order to preserve them in a state competent for refolding (3-6). However, molecular mechanisms of their function and regulation remain incompletely understood, particularly

as regards aberrant behavior in the transitions that occur to bring about disease states (7).

Structurally, the sHSPs are characterized by their low monomeric molecular mass, their assembly into oligomers, and the presence of a well conserved ' α -crystallin' domain (3-6). While crystal structures exist for some of the monodisperse members of the family (8), the extreme polydispersity of many of the mammalian sHSPs, including the α -crystallins, has hampered crystallographic analysis (4). Common to the sHSPs for which high resolution structures have been published, is the existence of dimers as 'building blocks' of the larger oligomer (3,6). We have reported that recombinant α A and α B form a range of oligomers with a notable preference for an even number of subunits (9,10). This suggests that dimeric substructure is a key characteristic of the sHSPs, and indeed dimers have been proposed as the active 'chaperoning unit' of the sHSPs (6). In our previous study on α B isolated from the lens under denaturing conditions we observed a difference in the proportion of oligomers containing an even or odd number of subunits (11). This observation, along with recent work showing structural changes in the α -crystallins upon renaturation from urea (12), suggests that *in vitro* refolding provides a means of modulating the quaternary structure of these proteins. Here, we exploit this phenomenon to examine the structural and functional properties of α -crystallins with identical sequence yet differing quaternary structure. Using novel mass spectrometry (MS) approaches we show that these proteins exist not only as different sized oligomers, but also that these oligomers have variable 'building blocks'. Moreover, we correlate the

loss of dimeric substructure with an augmentation in substrate binding, and suggest that it is these changes in local oligomeric organization which provide the molecular mechanism for sHSP regulation.

EXPERIMENTAL PROCEDURES

Expression and purification- The expression vector pET 21b(+) containing the genes for either bovine α A-crystallin or human α B-crystallin was introduced into the BL 21(DE3) strain of *E. coli* by standard methods. Protein expression and purification was performed using methods previously described (13).

ANS binding- α A and α B solutions of 13.5mg/ml were used. To effect unfolding of the protein, 12mg of urea was added to 20 μ l of each solution, giving a final volume of 30 μ l (6.67M urea), and incubated at room temperature for 30min. The proteins were refolded by dilution to 0.2mg/ml with 1320 μ l of PBS (100mM sodium chloride, 50mM phosphate, pH7.2). Matching controls were prepared by adding the urea after dilution. ANS (5 μ l of a 20mM stock in methanol) was added to each sample (resulting in a methanol concentration of 0.37% v/v in the samples) and the fluorescence emission was measured between 400 and 600nm at an excitation wavelength of 370nm using a Hitachi F4500 fluorimeter. The experiment was also performed, as above, using 200mM ammonium acetate instead of PBS as diluent.

Kynurenine binding- 60mg of urea was added to 100 μ l of 4mg/ml α B in PBS and left at room temperature for 30min. The protein was refolded by dilution to 1ml with PBS. Matching controls were prepared by adding the urea after dilution. Urea was removed from the diluted solutions by five consecutive concentration and dilution steps using Biomax concentrators (Millipore). 25 μ l of Kyn (5mg/ml in PBS) and 2 μ l of chloroform were added before being sealed and incubated at 37°C for 14 days. No significant proteolysis was observed to occur by MS analysis.

MS analysis- Nano electrospray MS of the Kyn-modified α B was performed using a Q-ToF Ultima (Waters). Samples were

desalted and denatured using C18 ZipTips (Millipore) prior to analysis.

Tandem MS experiments were performed on a modified Q-ToF 2 instrument as previously described (11). Prior to analysis ~2mg of α -crystallin (30mg/ml in PBS, with or without 6.67M urea) were loaded onto a Superdex 200 gel filtration column (GE Healthcare) and eluted at 0.3ml/min with 200mM ammonium acetate. Chromatography resulted in refolding and/or buffer exchange.

Chaperone assay- α Lac (0.5mg/ml final) was mixed with α B or α B_R (both 0.24mg/ml final) in the presence of 20mM DTT. The final sample volumes were 100 μ l, in 200mM ammonium acetate. The mixtures and a control of α Lac were transferred to identical cuvettes, placed in a heated (37°C) multi-cell block, and the apparent absorption due to light scattering at 360nm was monitored using a Cary 400 Scan spectrophotometer.

RESULTS

In vitro refolding enhances the substrate affinity of α B-crystallin via increased surface exposure

To structurally reorganize recombinant α B, we subjected the protein to *in vitro* refolding from a high concentration of urea. We have termed this protein α B_R. To investigate any surface changes in the oligomers associated with this refolding we employed two molecular probes: 1-anilino-8-naphthalene-sulfonic acid (ANS) which fluoresces in a hydrophobic environment; and the reactive small molecule, kynurenine (Kyn), which can covalently bind to exposed His residues in α B (14). Figure 1A shows the ANS fluorescence for equimolar concentrations of α B and α B_R in both PBS and ammonium acetate. In both cases the fluorescence intensity of the ANS probe was greater for the refolded protein relative to the original, indicating that a small but significant increase in exposed hydrophobic surface area had occurred. This is in agreement with a recent study examining the biophysical properties of α -crystallin (12). Figure 1B shows the relative binding stoichiometries of Kyn to the surface-exposed

His residues of αB , with αB_R demonstrating ~70% more total binding. Furthermore, while only doubly-modified chains were identified in the case of αB , a distinct proportion of the αB_R were triply-modified. These two probing techniques demonstrate that the refolded proteins are sufficiently different in structure such that they are more susceptible to both ANS binding and Kyn modification.

To assess the effect of this structural reorganization on sHSP function, αB and αB_R were assayed for their ability to prevent precipitation of the aggregation-prone substrate, reduced apo- α -lactalbumin (αLac) (Fig. 1C). In the absence of chaperone, a rapid increase in apparent absorbance due to light scattering was observed, indicative of the aggregation of αLac . When αB was added the onset of αLac aggregation was considerably delayed, demonstrating the protective ability of this protein. αB_R was found to further reduce the level of aggregation, suggesting αB_R had a higher substrate affinity than αB . Therefore, taking our chaperone and surface probing experiments together, it appears that *in vitro* refolding results in an increase in substrate affinity or capacity via the exposure of new surfaces.

Tandem MS characterizes the polydisperse sHSP assemblies

Over the last decade MS analysis of intact protein assemblies has become a valuable addition to the structural biologists' tool kit, allowing the determination of both structural and dynamical parameters (15). To examine the quaternary arrangement of the α -crystallins we used a tandem MS (MS/MS) approach we previously developed whereby the range of oligomers which comprise the polydisperse ensemble may be identified (11). Figure 2A shows MS/MS spectra of αB_R resulting from the selection and activation of ions in the peak at 10080 m/z (Fig. S1). This peak corresponds to all oligomers, each carrying twice as many charges as subunits. At an accelerating voltage of 110V some undissociated ions remain, but two additional areas of signal are also observed, in the ranges

of 12000-17000m/z and 17000-28000m/z (lower panel). At 170V the same groups of peaks are observed, however those at higher m/z dominate the spectrum (upper panel). These two ranges correspond to singly and doubly stripped oligomers, that is, parent oligomers having lost one or two monomers respectively. Monitoring the relative abundance of ions as a function of the accelerating voltage reveals that this dissociation is a sequential process and allows us to determine the voltages at which the different dissociation steps occur (Fig. 2B).

Figure 2C shows an expansion of the area of the spectrum where doubly stripped oligomers carrying two charges less than subunits are found, i.e. $[(\alpha B_R)_{(n-2)}]^{[n-4]+}$ where n is the number of subunits in the original, parent oligomers. Doubly stripped species of 23 to 32 subunits, which correspond to original oligomers with 25 to 34 subunits, are clearly observed. In this way not only can the different oligomeric species which comprise the heterogenous assembly be identified, despite differing by less than 5% in mass, but also, from their intensities, their relative abundances can be quantified.

In vitro refolding of αA and αB results in the loss of dimeric substructure

Figure 3A shows the oligomeric distribution of αA and αB prior to and after *in vitro* refolding. These histograms were obtained from MS/MS experiments as described above. Intensities for peaks corresponding to the charge states of the different doubly stripped oligomers were quantified, and from these, the relative abundances of the original oligomers were obtained. αA and αB exhibit a distribution of oligomeric stoichiometries centered on a 26mer and a 28mer respectively. Moreover, both proteins display a marked preference towards assembling into oligomers with an even number of subunits. αA_R and αB_R are centered on a 24mer and 28mer respectively and are therefore comparable with their wild type equivalents in number of subunits. Notably, however, these proteins have lost their preference for forming even-numbered species. Thus the dimeric

substructure of the wild-type proteins, evidenced by an excess of oligomers with an even number of subunits, is lost upon refolding.

Distinct substructures within protein types are found by gas-phase dissociation

To further investigate the difference between the quaternary organization of these proteins, we employed a recently developed method of probing substructure by MS (16). This approach involves a detailed examination of the dissociation pathway of protein assemblies during MS/MS. Specifically we have examined the ease of the dissociation step in which an additional monomer is removed from the singly stripped oligomers, the rationale being that different local quaternary arrangements might effect differences in dissociation behavior.

The percentage of species which are doubly stripped (relative to singly stripped), for the four different proteins at a range of accelerating voltages, representing the ease of gas-phase removal of the second subunit is shown in figure 3B. Additionally, they have been separated according to whether they contain an even or odd number of subunits. In all cases the percentage of doubly stripped oligomers follows a sigmoidal profile. From the first derivative we obtain the turning points shown in Table S1. For αA_R and αB_R we do not observe a significant difference between the dissociation pathway for odd- and even-numbered oligomers, suggesting there is no difference in substructure between them. This agrees with the evidence from the histograms which suggests that their sub-oligomeric organization is exclusively monomeric (Fig.3A). In the case of αA and αB , however, there is a significant difference between the odd and even oligomers, namely the transition from singly to doubly stripped oligomers occurs at higher initial kinetic energies in the case of the even oligomers. These results suggest that there is a fundamental difference in the substructure of even and odd oligomers. Furthermore, the turning points of the curves are similar for the odd oligomers of both αB and αB_R , and for αA and αA_R , whereas they

are significantly different in the case of the even pairs (Table S1). This suggests that the substructure of the different oligomers of αB_R , as well as αB with an odd numbers of subunits is the same, but that a different substructure is present for αB with an even number of subunits. The same properties were observed for αA_R and αA . Combining this with the evidence in figure 3A, that dimeric substructure is lost upon *in vitro* refolding, we suggest that wild-type αA and αB exist as a combination of forms, the extremes of which are oligomers with monomeric substructure, and even oligomers with dimeric substructure.

DISCUSSION

Previously we have shown that the substructure of αA and αB can be altered by post-translational modification. Specifically, phosphorylation of αB (9,17) and truncation of αA (10) caused a decrease and increase in the amount of dimeric substructure respectively. Here we have extended this work to show that the native proteins themselves exist as an apparent equilibrium between different substructural states, namely with dimeric or monomeric building blocks (Fig.4A). As such, these proteins are heterogeneous in terms of both local and global quaternary organization.

By causing shifts in this equilibrium without sequence modification, we have observed that disruption of the dimeric sHSP substructure to form oligomers composed of monomeric building blocks results in an augmentation of chaperone activity. Moreover the chaperone function of these two states is different: the monomeric substructural state has greater exposed hydrophobic surface area, and is consequently more active in protecting against protein precipitation. Consequently, it is not the changes in primary structure caused by post-translational modification, but rather the alterations in substructure that they engender, which determine chaperone activity.

A recent model of sHSP chaperone function suggests that sHSP oligomers exist in two forms with differential activity; a low affinity

state and a high affinity state (3,18). We propose here that in the case of the α -crystallins these two forms correspond to oligomers with dimeric and monomeric substructure respectively (Fig. 4A). We suggest that it is the ratio of these two forms which determines the overall efficacy of the chaperone ensemble. Stressors, or irreversible protein modification, affect this ratio by causing sufficient destabilization such that dimeric substructure is perturbed. This leads to an increase in substrate affinity by exposing the former interfacial regions to unfolded substrate proteins. This ability to package subunits into dimers for subsequent activation represents an elegant mechanism of regulating the chaperone activity of these sHSPs.

There is a growing body of literature in which α B has been reported to be involved in protein deposition diseases (7), potentially due to co-precipitation of the chaperone with substrate proteins *in vivo* (19). An explanation for this which arises from the work presented here is that mutation, post-translational modification, or some other type of alteration may disrupt the dimeric substructure of α B such that a critical amount of binding surfaces is

exceeded. Indeed, we have shown previously that phosphorylation of α B can result in a loss of dimeric preference and consequent uncontrolled co-aggregation behaviour (9). Similarly, an inheritable R120G mutation in α B has been shown to result in protein deposition disorders including cataract and cardiomyopathy (20,21). *In vitro* measurements of R120G versus wild-type α B indicated that a dramatic increase in substrate affinity was responsible for R120G forming insoluble co-aggregates with the client proteins (19).

We hypothesize that this chaperone hyperactivity associated with some mutations and post-translational modifications is the result of a shift in the ratio of dimeric to monomeric substructure of α B. If we consider the level of α B function to be determined by position on a continuum of this ratio, then aberrant chaperone behaviour occurs when the ideal substructural balance is exceeded (Fig. 4B). Thus the chaperone function of sHSPs might be viewed as an exquisite balancing act which is, in the case of some protein misfolding disorders, tipped such that the chaperone itself contributes to the disease.

REFERENCES

1. Brady, J. P., Garland, D., Douglas-Tabor, Y., Robison, W. G., Jr., Groome, A., and Wawrousek, E. F. (1997) *Proc. Natl. Acad. Sci. USA* **94**(3), 884-889
2. Kappe, G., Franck, E., Verschuure, P., Boelens, W. C., Leunissen, J. A., and de Jong, W. W. (2003) *Cell Stress Chap.* **8**(1), 53-61
3. Haslbeck, M., Franzmann, T., Weinfurtner, D., and Buchner, J. (2005) *Nat. Struct. Mol. Biol.* **12**(10), 842-846
4. Horwitz, J. (2003) *Exp. Eye Res* **76**(2), 145-153
5. Narberhaus, F. (2002) *Microbiol. Mol. Biol. Rev.* **66**(1), 64-93; table of contents
6. van Montfort, R., Slingsby, C., and Vierling, E. (2002) *Adv. Protein Chem.* **59**, 105-156
7. Sun, Y., and MacRae, T. H. (2005) *Febs J* **272**(11), 2613-2627
8. Haslbeck, M., Franzmann, T., Weinfurtner, D., and Buchner, J. (2005) *Nat. Struct. Mol. Biol.* **12**(10), 842-846
9. Aquilina, J. A., Benesch, J. L. P., Ding, L. L., Yaron, O., Horwitz, J., and Robinson, C. V. (2004) *J. Biol. Chem.* **279**(27), 28675-28680
10. Aquilina, J. A., Benesch, J. L. P., Ding, L. L., Yaron, O., Horwitz, J., and Robinson, C. V. (2005) *J. Biol. Chem.* **280**(15), 14485-14491
11. Aquilina, J. A., Benesch, J. L. P., Bateman, O. A., Slingsby, C., and Robinson, C. V. (2003) *Proc. Natl. Acad. Sci. USA* **100**(19), 10611-10616
12. Saha, S., and Das, K. P. (2007) *Protein J.* **26**(5), 315-326
13. Horwitz, J., Huang, Q. L., Ding, L., and Bova, M. P. (1998) *Methods Enzymol.* **290**, 365-383

14. Aquilina, J. A., and Truscott, R. J. (2001) *Biochem. Biophys. Res. Commun.* **285**(5), 1107-1113
15. Robinson, C. V., Sali, A., and Baumeister, W. (2007) *Nature* **450**(7172), 973-982
16. Benesch, J. L.P., Aquilina, J. A., Ruotolo, B. T., Sobott, F., and Robinson, C. V. (2006) *Chem. Biol.* **13**(6), 597-605
17. Ecroyd, H., Meehan, S., Horwitz, J., Aquilina, J. A., Benesch, J. L., Robinson, C. V., Macphee, C. E., and Carver, J. A. (2007) *Biochem. J* **401**(1), 129-141
18. Claxton, D. P., Zou, P., and McHaourab, H. S. (2008) *J. Mol. Biol.* **375**(4), 1026-1039
19. Bova, M. P., Yaron, O., Huang, Q., Ding, L., Haley, D. A., Stewart, P. L., and Horwitz, J. (1999) *Proc. Natl. Acad. Sci. USA* **96**(11), 6137-6142
20. Vicart, P., Caron, A., Guicheney, P., Li, Z., Prevost, M. C., Faure, A., Chateau, D., Chapon, F., Tome, F., Dupret, J. M., Paulin, D., and Fardeau, M. (1998) *Nat. Genet.* **20**(1), 92-95
21. Rajasekaran, N. S., Connell, P., Christians, E. S., Yan, L. J., Taylor, R. P., Orosz, A., Zhang, X. Q., Stevenson, T. J., Peshock, R. M., Leopold, J. A., Barry, W. H., Loscalzo, J., Odelberg, S. J., and Benjamin, I. J. (2007) *Cell* **130**(3), 427-439

FOOTNOTES

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FIGURE LEGENDS

Fig. 1: Binding of ANS (a) to α B and α B_R in both PBS (dark grey) and ammonium acetate (light grey) reveals that the refolded protein has more exposed hydrophobic surfaces. The fluorescence observed was 343 and 394 fluorescence units in PBS, and 1152 and 1310 fluorescence units in ammonium acetate, for α B and α B_R respectively. Similarly, an increase in the amount of Kyn binding (b) was observed for the refolded protein. Assaying the chaperone activity of α B towards α Lac (c) reveals that both proteins reduce the rate of aggregation relative to the control (circles). α B_R (triangles) reduces aggregation more efficiently than α B (squares), however, reflecting increased substrate affinity or capacity.

Fig. 2: MS/MS of α B_R (a) at accelerating voltages of 110V (lower panel) and 170V (upper panel). Dissociation of monomers from the parent oligomers results in singly and doubly stripped oligomers, with more of the latter at the higher voltage. (b) Plotting the relative abundance of the species as a function of acceleration voltage demonstrates the sequential nature of the dissociation. (c) An expansion of the area boxed in (a) shows the very clear separation of peaks achieved. Doubly stripped species of 23 to 32 subunits are clearly resolved.

Fig. 3: Histograms derived from MS/MS data (a) show the relative abundances of the oligomeric species which comprise α A and α B. The lower histograms correspond to the proteins before *in vitro* refolding, the upper ones after. In each case the tendency to favor even-numbered oligomers that was evident for the native proteins (lower) was abolished (upper) by the process of *in vitro* refolding. The percentage of doubly stripped oligomers (relative to singly stripped) as a function of initial kinetic energies (b) for α A and α B before an after *in vitro* refolding. Oligomers containing an even number of subunits are represented by black circles and a solid line, whereas those with odd numbers are represented by white circles and a dashed line. The dissociation profiles show that oligomers of the α -crystallins with an even number of subunits show differential dissociation behavior to odd-numbered oligomers, and that this difference is lost upon refolding.

Fig. 4: We propose that the α -crystallins can exist as oligomers with dimeric or monomeric substructure, and combinations thereof (a). The transition from a relatively inactive, low-affinity state, to an active high-affinity state, is dictated by the ratio of the types of substructure in the assembly. Dissociation of dimers to monomers leads to the exposure of surfaces in the former contact regions thereby increasing substrate affinity. We propose (b) that at an ideal ratio of substructures the net protection conferred by the proteins is maximized. The observation that the α -crystallins are present in a number of age-related protein deposition diseases indicates, however, that they can also be hyper-activated, giving rise to co-precipitation phenomena *in vivo*. We suggest that sequence changes such as post-translational modifications or mutations disrupt their dimeric substructure sufficiently such that a critical level of substrate affinity is exceeded. At this point the proteins lose the ability to limit the number of substrates they bind, leading to aberrant chaperone activity and co-precipitation with client proteins.

FIGURE 1

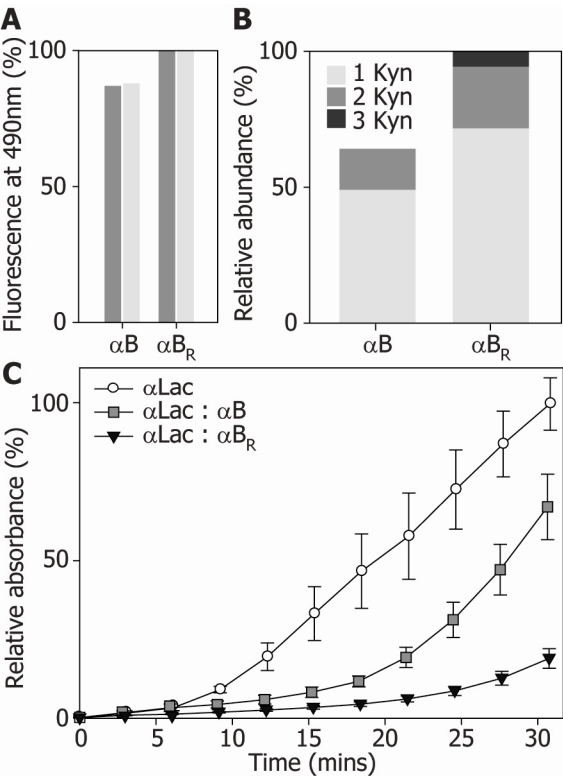


FIGURE 2

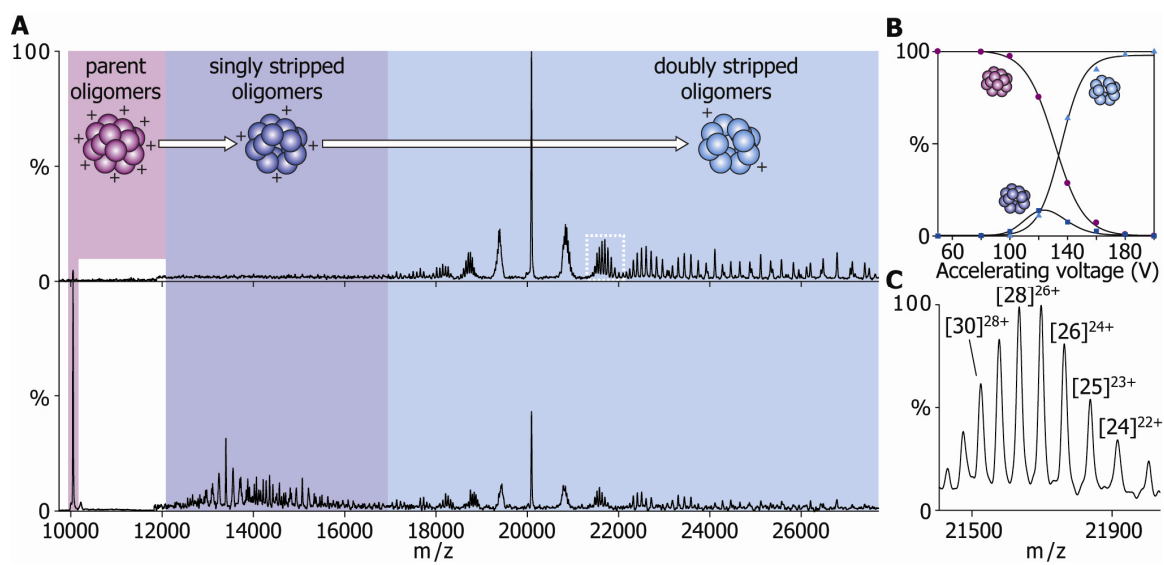


FIGURE 3

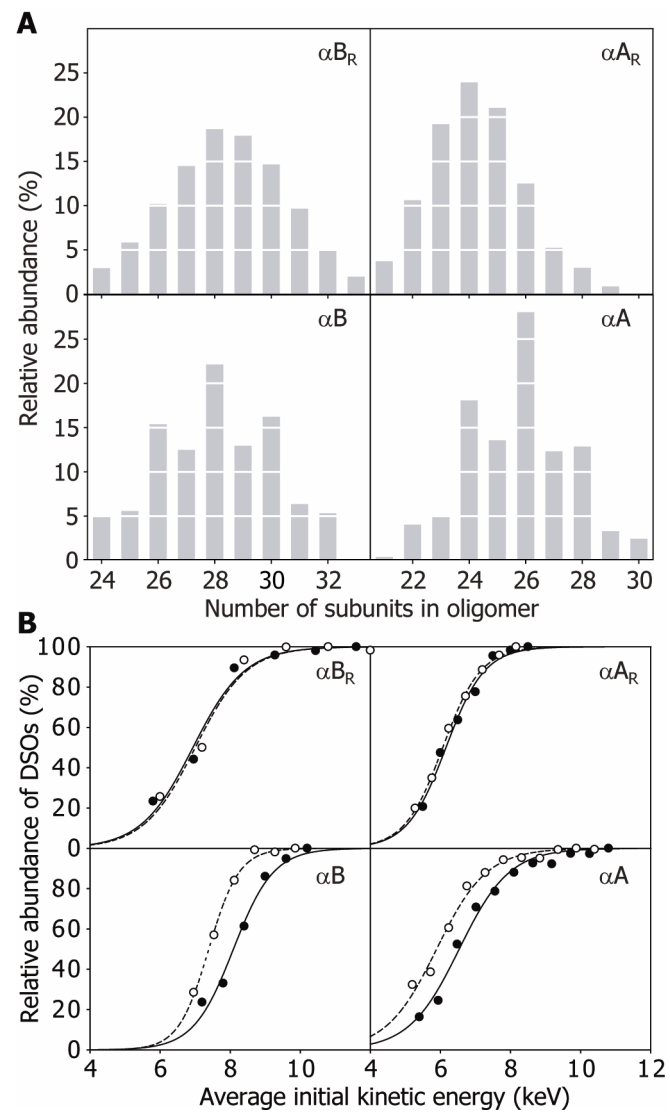


FIGURE 4

