Tandem Mass Spectrometry Reveals the Quaternary Organization of Macromolecular Assemblies

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Summary

The application of mass spectrometry (MS) to the study of progressively larger and more complex macromolecular assemblies is proving increasingly useful for structural biologists. The scope of this approach has recently been widened through the application of a tandem MS procedure. This two-step technique involves the selection of specific assemblies in the gas phase and inducing their dissociation through collisions with argon atoms. Here, we investigate the mechanism of this process and show that dissociation of subunits from a macromolecular assembly follows a sequential pathway, with the partitioning of charge between the dissociation products governed primarily by their relative surface areas. Using this basis of understanding, we highlight differences in the dissociation pathways of three related macromolecular assemblies and show how these are a direct consequence of changes in both local and global oligomeric organization.

Introduction

Various methods are used to achieve the principal goal of structural biology, namely determining the organization of biological macromolecular assemblies. Electrospray mass spectrometry (ES-MS) is rapidly becoming an attractive complementary methodology to traditional approaches such as X-ray crystallography, nuclear magnetic resonance, and electron microscopy [1, 2]. When used in isolation, ES-MS is able to determine the stoichiometry of macromolecular complexes, but when used in conjunction with other approaches, new insight into such complexes, and their subcomplexes, can often be obtained. For example, MS has provided key information regarding the overall stoichiometry of assemblies, enabling fitting of high-resolution structures to electron density maps [3, 4]. Furthermore, the applicability of MS to massive and highly complex molecular machines is highlighted by an MS study of intact ribosomes which defined the copy number of associated protein complexes [5]. Therefore, in the postgenomic era, MS is not only crucial in analyzing gene products [6], but it is also becoming increasingly valuable in studying the nature of their interactions.

Though spectra can be recorded for complexes in excess of 1 MDa, they are still, however, much smaller than the species in excess of 100 MDa which have been transmitted into the gas phase by ES [7]. A major reason why such large ions cannot be successfully measured by MS alone is due to the nature of the ES process whereby such ions carry a large and, moreover, variable amount of charge. Consequently, there is only very small separation between these adjacent charge states. (The predicted charge, where m is the assembly mass is given by the following relationship: z = 0.078 × m [8]. For example, a 100 MDa complex would therefore be expected to carry approximately 780 charges. This 780+ charge state hence would appear at 128,206 m/z, and the adjacent 781+ charge state at 128,042 m/z, a difference of 164 m/z. Such separation presents a significant challenge, as the spectra of intact noncovalent complexes is limited by the extent of desolvation that is possible without disruption of the complex [9].) This represents a major hurdle if the mass range of species amenable to study by MS is to continue to increase, particularly as issues of protein purity become more important as the stoichiometry of a macromolecular complex increases. (The probability of an oligomer of n subunits being composed entirely of pure monomer [Pm] is dependent on monomer purity [Pm] according to the following equation: Pm [\%] = [Pm (%)]^n/[100^n-1]. An oligomer composed of ten subunits, with a monomeric purity of 90% would result in 35% pure oligomer, whereas if the oligomer was composed of 10 subunits, the oligomeric purity would drop to 2.7 × 10^-3\%.) Further compounding these difficulties is the tendency of many biologically important assemblies to be naturally heterogeneous and polydisperse. To address this problem, we have recently developed a tandem MS (MS/MS) approach that employs the technique of collision-induced dissociation (CID) to dissociate specific macromolecular species, selected via their mass to charge (m/z) ratios [10]. We reasoned that an MS/MS approach would allow us the ability to select different regions of mass spectra obtained for heterogenous assemblies and consequently enable us to probe the stoichiometry of the interacting subunits. Moreover, dissociating the assembly would act to reduce the charge on the oligomeric species involved and thereby distribute the ion signal over a much wider m/z range than would be achieved by one-dimensional MS. This has provided a means of defining species that could not be adequately characterized by traditional structural approaches or by MS alone and has consequently opened a new avenue in the structural biology of macromolecular assemblies [11].

The separation via charge reduction that can be achieved through such an approach arises from the unique “asymmetric” nature of the dissociation of multimeric protein complexes. This phenomenon was recognized when an early study showed that activation of tetrameric proteins in the mass spectrometer results in the formation of complementary monomeric and trimeric...
products, with the monomers claiming a much larger proportion of the charge than might be expected [12]. Subsequent studies have shown that this asymmetric separation of mass and charge into highly charged monomers and lowly charged “stripped oligomers” occurs in the gas-phase dissociation of numerous protein complexes [9, 12–21]. The basic pathway of protein assembly dissociation in the mass spectrometer can therefore be described as follows:

\[ n^2 \rightarrow (n-1)^{q-x} + m^q, \]

where \( n \) is the number of subunits in the oligomer, \( q \) is the number of charges on the oligomer, and \( x \) is the average charge carried by a monomer \( m \). Moreover, it has been shown that this process can occur such that several monomers can be removed from an oligomer [11, 22–24], though the pathway of this multiple loss of subunits is not known. The result of these asymmetric dissociation events is that the resulting stripped oligomers are of much lower charge state than the parent oligomers. Consequently, the separation between adjacent charge states is vastly improved, and the possibility that individual species can be readily identified is greatly increased [11]. This approach has aided the characterization of species across many fields of structural biology, including ribosomal subcomplexes [5], membrane proteins [25], protein-degradation machinery [26], and molecular chaperones [27–29].

Though the use of this approach is already providing valuable insight into the organization of macromolecular assemblies and their components, the mechanism of dissociation of multimeric assemblies remains the matter of some debate, and consequently, the detailed application of this technique remains compromised. An early study on the gas-phase dissociation of small noncovalent protein complexes speculated that “dissociation of the [oligomer] may occur by a Coulombically driven process in which a monomer becomes ‘unraveled’ and ejected from the aggregate with a disproportionally large share of the charge” [12]. A large entropy gain during the dissociative transition state observed during the dissociation of a small pentameric protein complex [13], and investigations into the effect of flexibility within the components of the protein assembly [15, 16], provide evidence for this to be the case. It remains to be seen whether mechanistic details extrapolated from studies of smaller protein assemblies will translate to an understanding of the dissociation of larger systems. Therefore, it is of paramount importance for the continuing development of MS in the field of structural biology that the characteristics of the CID of a range of large macromolecular assemblies are examined, such that a proper basis for understanding can be achieved.

We report here detailed investigations into the dissociation of three proteins that form large noncovalent complexes: TaHSP16.9 from wheat, which is composed of 12 subunits [30]; MjHSP16.5 from Methanococcus jannaschii, which is composed of 24 subunits [31]; and bovine \( \beta \)-B-crystallin, which forms a polydisperse assembly centered around 28 subunits [11]. MS/MS experiments performed on these proteins result in their dissociation into monomers and various stripped oligomers. We show that this proceeds in a sequential manner with the charges redistributed in a Coulombically favorable fashion determined by their relative surface areas. This characteristic causes effective charge reduction and acts to increase peak separation by distributing the resulting spectrum over a broad \( m/z \) range. The power of this approach is exemplified in the MS/MS of \( \alpha \)-B-crystallin, where the stoichiometry of all the component oligomers can be determined, enabling a global description of the oligomeric organization despite an extreme level of heterogeneity. Furthermore, we show how, through a detailed examination of dissociation pathways, valuable insight into the local organization of macromolecular assemblies and their subunit interactions can be gained.

**Results and Discussion**

**A Common Dissociation Pathway for Macromolecular Assemblies**

Mass spectra of the small heat shock proteins (sHSPs) TaHSP16.9 and MjHSP16.5, introduced by using a miniaturized version of ES known as nanoES [32], are shown in Figures 1A and 1B. In each case, a single species is observed, corresponding to masses of 200,790 Da and 395,107 Da, respectively. From comparison with the
masses calculated from the polypeptide sequences, we can conclude that TaHSP16.9 and MjHSP16.5 form monodisperse 12-mers and 24-mers, respectively, and that these native stoichiometries [30, 31] can be preserved in the gas phase of the mass spectrometer without dissociation.

When the accelerating voltages and vacuum are raised in the source region of the mass spectrometer, thereby increasing the ions' acceleration between collisions with surrounding gas molecules, a spectrum of MjHSP16.5 shows peaks corresponding to monomers at low m/z and stripped oligomers (23-mers and 22-mers) at high m/z (Figure 1C). Similarly, activation for TaHSP16.9 results in the formation of monomers and 11-mers [33]. This technique is known as "in-source" CID. Alternatively, ion activation can also be achieved downstream in the spectrometer by accelerating the ions into a gas-filled collision cell. A spectrum obtained when MjHSP16.5 is dissociated in this way is shown in Figure 1C. Again monomers, 23-mers, and 22-mers are observed. This demonstrates that the products, and hence likely the mechanism, of dissociation of these large protein complexes are essentially the same despite the activation occurring at different stages of the mass spectrometer. As such, the mechanistic conclusions we draw below apply to dissociation performed in both the source region or collision cell.

Though the two proteins have different overall architecture (TaHSP16.9 is disc-shaped [30], MjHSP16.5 is spherical [31]), they are both composed of dimeric "building blocks" [34]. Heating TaHSP16.9 in solution leads to the observation of these dimers [33], and subunit exchange experiments monitored by mass spectrometry indicate that the reaction proceeds via loss of dimers from the oligomeric complex [35]. The dissociation experiments presented here, showing the loss of monomers, are in direct contrast to this (Figure 1), demonstrating that while dissociation may exhibit the same characteristics, irrespective of where activation occurs in the instrument, it follows a fundamentally different pathway in the gas phase to that which takes place in solution. One of the main advantages of this dissociation is, however, that the masses of the monomers, oligomers, and stripped oligomers are all measured within the same experiment. Therefore, it is possible to determine the overall stoichiometry, even in cases where the sequence of the component monomeric subunit is unknown.

Sequential Removal of Subunits Allows Characterization of Oligomeric Species

To investigate the mechanism of the dissociation process, we performed MS/MS experiments wherein a particular charge state of a species is selected and dissociated. The dissociation pathway of the TaHSP16.9 12-mer is shown in Figure 2. At the lowest collision energies, 12-mers remain intact. Increasing the accelerating voltage results in the formation of monomer and stripped complexes comprising 11 subunits. At the highest collision energies 10-mers are also observed (Figure 2A). These 10-mers are only formed once the accelerating voltage reaches 120 V. At this point, 12-mers are no longer observed, and the relative abundance of 11-mers starts to decay, having reached a maximum (Figure 2B). Therefore, the 10-mers must be formed from the 11-mers, which have, in turn, been formed from the 12-mers. This indicates that this process is occurring in a sequential manner (Figure 2C). Similarly, the loss of multiple subunits is also observed for the dissociation of the MjHSP16.5 24-mers (Figure 3). The onset of dissociation into monomers and 23-mers occurs at approximately 70 V, and, at slightly higher collision energies, 22-mers are formed. At the highest collision energies, 21-mers are also observed, showing that in this
case as many as three subunits can be removed from the parent oligomer (Figure 3A). The results presented here clearly show that multiple loss of subunits during CID is a sequential process rather than a concerted one.

The spectra of the stripped oligomers show that despite the fact that very narrow m/z ranges were selected from the mass spectra (typically isolation windows 30 m/z wide), the resulting MS/MS spectra are distributed over much wider m/z ranges (12,000 and 18,000 m/z, respectively) (Figures 2A and 3A). The sequential loss of highly charged subunits results in the charge reduction of the oligomeric components, and subsequently, the separation between adjacent peaks is greatly increased. Whereas adjacent peaks corresponding to the intact 24-mer of MjHSP16.5 are separated by ~190 m/z (Figure 1C), the removal of successive monomers results in separations of ~360 m/z, ~790 m/z, and ~1650 m/z (for 23-mers, 22-mers, and 21-mers, respectively). The potential of this characteristic is exemplified by performing dissociation on another sHSP, αB-crystallin. Unlike its monodisperse plant and bacterial counterparts described above, this protein forms a polydisperse assembly centered around 28 subunits [11]. An MS spectrum of this protein features overlapping charge state series from 6,000 to 14,000 m/z corresponding to all the different contributing components of this polydisperse assembly (Figure 4A). We performed MS/MS on the peak corresponding to an overlap of all the oligomers, each carrying twice as many charges as subunits (10,040 m/z) [11]. As the accelerating voltage is increased, up to three monomers can be removed from the different oligomers (Figure 4B). The peaks corresponding to the charge states of the variously stripped oligomers originating from a parent 28-mer56+ were monitored and show a sequential reaction pathway, similar to that of MjHSP16.5 (Figure 4C). The result of the charge reduction effected by this consecutive loss of subunits is that whereas peaks could not be sufficiently separated by MS alone (Figure 4A), after stripping of highly charged subunits, individual assemblies can be resolved (Figure 4B, inset), and consequently their relative populations quantified [11].

Such charge reduction is increasingly important as progressively larger and more complex species are studied by means of MS. As well as distributing the signal over a wider m/z range, the peak width also decreases upon dissociation, most likely as a result of removal of adducted buffer ions and water molecules [9]. This further improves the likelihood of resolving individual species. Moreover, the demonstration that the pathway of the loss of multiple subunits is sequential, paves the way for detailed use of this approach to probe the arrangement of components within macromolecular assemblies [28, 36].

### Structural Information from Charge Partitioning during Dissociation

In the first dissociation steps of the two monodisperse assemblies (TaHSP16.9 and MjHSP16.5), 46% and 29% of the charge is apportioned to the dissociated monomers, respectively. This is a surprisingly large proportion of the charge since the individual subunits comprise only 8.3% and 4.2% of the total mass respectively (Table 1). In other words, during dissociation, there is a large asymmetry in the charge partitioning over the CID products, relative to their mass. However, as the charges on gas-phase proteins are more likely to be found on surface of the ion, rather than buried within the molecule, analyzing the dissociation patterns with respect to surface area rather than mass (or volume) is perhaps more appropriate [37, 38]. Therefore, we estimated the surface areas of both the stripped oligomers...
and the monomers that result from this dissociation process. These estimated surface areas are for monomers in an unfolded state, as previous studies have shown this to be their likely conformation during CID [13, 15, 16] (Table 1).

These results show that the surface areas (\(\sim 40\%\) and \(\sim 30\%\)) of the two unfolded monomers from TaHSP16.9 and MjHSP16.5 are closely similar to values for charge partitioning to the monomer detailed above (46\% and 29\%). Moreover, the results of all subsequent calculations show that the partitioning of charge between monomers and stripped oligomers closely follows the ratios of their surface areas. In other words, the surface area charge density is constant over the two product ions. This means that while dissociation is asymmetric with respect to the mass of the ions, it is symmetric with respect to the surface area of the ions. Furthermore, because of the unfolding of the ejected monomer, the total surface area of the products is projected to be larger than that of the parent oligomer. Consequently, the surface area charge density decreases with removal of monomers such that the whole process is Coulombically favorable. Therefore, we suggest that the main driving force behind the dissociation process for large

**Table 1. Partitioning of Charge, Mass, and Surface Area upon Dissociation of TaHSP16.9 and MjHSP16.5**

<table>
<thead>
<tr>
<th></th>
<th>TaHSP16.9</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12-mer</td>
<td>11-mer</td>
<td>Monomer</td>
<td>10-mer</td>
<td>Monomer</td>
</tr>
<tr>
<td>Charge ((z))a</td>
<td>32.0</td>
<td>17.4</td>
<td>14.6</td>
<td>10.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Charge (%)b</td>
<td>54.4</td>
<td>45.6</td>
<td>59.8</td>
<td>40.2</td>
<td></td>
</tr>
<tr>
<td>Mass (Da)c</td>
<td>200948</td>
<td>184267</td>
<td>16726</td>
<td>167397</td>
<td>16726</td>
</tr>
<tr>
<td>Mass (%)b</td>
<td>91.7</td>
<td>8.3</td>
<td>90.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Surface area ((\text{Å}^2))d</td>
<td>30591</td>
<td>29223</td>
<td>16,708–23,094</td>
<td>27855</td>
<td>16,708–23,094</td>
</tr>
<tr>
<td>Surface area (%)b</td>
<td>55.9–63.6</td>
<td>36.4–44.1</td>
<td>54.7–62.5</td>
<td>37.5–45.3</td>
<td></td>
</tr>
</tbody>
</table>

|                       | MjHSP16.5 |          |           |          |          |
|                       | 24-mer    | 23-mer    | Monomer   | 22-mer    | Monomer  |
| Charge (\(z\))a       | 47.0      | 33.4      | 13.6      | 22.0      | 11.4     |
| Charge (%)b           | 71.0      | 29.0      | 65.9      | 34.1      |           |
| Mass (Da)c            | 395107    | 378852    | 16452     | 362241    | 16452     |
| Mass (%)b             | 95.9      | 4.2       | 95.6      | 4.3       |           |
| Surface area (\(\text{Å}^2\))d | 45239    | 43966     | 16316–21360 | 42682     | 16316–21360 |
| Surface area (%)b     | 67.3–72.9 | 27.1–32.7 | 66.7–72.3 | 27.7–33.3 | 66.0–71.7 |

**Footnotes:**

a The charge was determined from the average charge states of the stripped oligomers in Figures 2A and 3A averaged over all charge states. Monomer charge states were determined by subtracting the stripped oligomer charge state from its parent oligomer’s charge state.

b Percentage charge, mass, and surface area refers to the partitioning of each of these attributes that occurred during the dissociation event (e.g., 12-mer → 11-mer + monomer, or 11-mer → 10-mer + monomer).

c The masses quoted are those determined from the spectra in Figures 2A and 3A.

d The upper and lower bounds for the surface area of the unfolded monomers are the largest and smallest estimated by the different approaches detailed in the Experimental Procedures.
noncovalent protein-protein complexes is the enthalpic stabilization resulting from a minimization of Coulombic repulsion.

This detailed knowledge of the mechanism at work in the CID of large protein complex ions can allow for extrapolations of ion surface area from charge partitioning ratios. As the surface area charge density is constant over the products, we can, by measuring the partitioning of charge and estimating the surface area of an unfolded monomer, calculate the surface area of the stripped oligomers with the following relationship:

\[
\frac{Z_{\text{SO}}}{SA_{\text{SO}}} = \frac{Z_{\text{M}}}{SA_{\text{M}}}, \text{ and therefore} \\nonumber
\frac{SA_{\text{SO}}}{SA_{\text{M}}} = \frac{Z_{\text{M}} Z_{\text{SO}}}{Z_{\text{M}}}.
\]

where \(Z\) is charge, \(SA\) is surface area, and the subscripts \(SO\) and \(M\) refer to stripped oligomers and monomers, respectively. This value could then be applied to estimate the surface area of the parent oligomer. This approach, and others based on surface areas [38, 39], may well prove useful in the future in distinguishing between different possibilities when the structure of a macromolecule is unknown.

A previous investigation into the effect of the conformational flexibility on charge partitioning during dissociation led to the qualitative correlation of ease of protein unfolding with the amount of charge transferred to the monomers [15]. Our proposal that the relative surface areas of products are determinants of charge partitioning is consistent with this finding as the surface area of a protein is proportional to its degree of unfolding. It is therefore feasible to envisage that charge partitioning data, particularly coupled with an examination of the energetics of activation during CID, may lead to a quantitative assessment of protein folding parameters and the strength of interactions between subunits.

Interactions between Subunits Revealed from Details of the Dissociation Pathway

It is interesting to note that loss of a second subunit from stripped \(Mj\)HSP16.5 oligomers happens at collision energies only slightly higher than those required to detach the first (the difference in voltage between when the maximum amount of 23-mer and 22-mer are observed is only 20 V). This suggests therefore that loss of the second subunit is a relatively easier dissociation process than loss of the first (Figure 3B). Since it is established that unfolding of the monomer is the crucial step in the dissociation process [13, 15], we can conclude that in the case of \(Mj\)HSP16.5, unfolding of the second monomer is a facile process compared to unfolding of the first. This observation possibly arises from the dimeric substructure revealed by the crystal structure [31]; once the first monomer of a dimer is removed, the second has fewer interactions to break in order to unfold and detach from the oligomer.

By contrast, the closely related \(\alpha\beta\)B-crystallin, if isolated under chemically denaturing conditions, does not contain dimeric substructure; rather, monomers appear to be the basic unit of organization [11]. For this protein, however, the voltage difference between the maxima of the relative abundances of singly and doubly stripped oligomer is 40 V, compared to only 20 V for \(Mj\)HSP16.5. This difference is more marked if one takes into account the charge state of the parent ions, thereby comparing the initial kinetic energy of the ions as they enter the collision cell (Figure 5). From differences in initial kinetic energy of the two species, it can be clearly seen that a much smaller increase is required to dissociate the second monomer from the \(Mj\)HSP16.5 oligomer than from \(\alpha\beta\)-crystallin. We propose that this is a consequence of the dimeric substructure of \(Mj\)HSP16.5 and the absence of similar interactions in isolated \(\alpha\beta\)-crystallin. This example highlights how tandem MS can be used to elucidate structural information not only in terms of the overall stoichiometry but also on a local level by probing interactions between neighboring subunits.

Significance

ES-MS is fast becoming an accepted methodology for establishing the stoichiometry of macromolecular assemblies and is widely applied in conjunction with conventional high-resolution structural biology approaches [1]. The capabilities of MS have recently been augmented by the application of tandem MS, which acts to remove highly charged monomers from macromolecular assemblies. This not only provides further validation of the stoichiometry of the assembly but, by virtue of charge reduction, also allows characterization of assemblies that cannot be resolved by one-dimensional MS [11]. Here, we have investigated the mechanism of this gas-phase dissociation and established that loss of multiple subunits is a sequential process. We have also ascertained a correlation between the relative surface areas of the products and division of the charge during dissociation. This provides a basis for interpreting tandem MS data and enables elucidation of the global oligomeric organization as well as unambiguous determination of stoichiometry. Furthermore, by careful monitoring of the sequential loss of monomers during the dissociation processes, we have also shown that we can probe the underlying interactions between neighboring subunits allowing us to deduce local structural
organization. These experimental observations from the dissociation products and the details of the dissociation pathway, can lead to an array of structural information on both global and local oligomeric levels (Figure 6). This study has increased our understanding of the dissociation process as applied to large macromolecular assemblies and highlights the exciting possibility of obtaining important structural information, both locally and globally, for simple homogenous assemblies and complex heterogenous ones. Coupled with the suitability of ES-MS for the study of transient interactions of these species [28, 35, 40], this further highlights the ever-increasing role of MS-based technologies in the analysis of macromolecular complexes.

Experimental Procedures

Proteins
TahSP16.9 from wheat and MJSP16.5 from Methanococcus jannasch were expressed in E. coli and purified as described previously [30, 41]. $\beta$-Crystallin was isolated and purified from bovine lenses as described previously [11]. The same buffer exchange procedure was employed for the three proteins: they were loaded onto a Superdex 200HR10/30 gel filtration column (Amersham Pharmacia) and eluted at 0.3 ml min$^{-1}$ with 200 mM ammonium acetate at 4°C. The resulting fractions corresponding to the protein oligomers were combined to give final protein concentrations of 0.7 mg ml$^{-1}$ ($\beta$-crystallin), 1.6 mg ml$^{-1}$ (TahSP16.9), and 1.2 mg ml$^{-1}$ (MjHSP16.5). These samples were analyzed directly by mass spectrometry.

Nanoelectrospray Mass Spectrometry
Experiments were conducted with a Q-ToF 2 mass spectrometer (Waters/Micromass UK, Ltd.), which has been modified for high-mass operation [42]. Typically, 2 $\mu$l of solution were electrospayed from gold-coated glass capillaries prepared in house. All spectra were calibrated externally with a solution of cesium iodide (100 mg ml$^{-1}$) and processed with MassLynx software (Waters/Micromass UK, Ltd.). Spectra are shown here with minimal smoothing and without background subtraction.

In MS mode, the following instrument parameters were used for the analysis of TahSP16.9/MJSP16.5/$\beta$-crystallin: capillary voltage 1.65/1.65/1.6 kV, cone gas 100 Lh$^{-1}$, sample cone 200 V, extractor cone 10/10/100 V, accelerating voltage into the collision cell 4 V, ion transfer stage pressure 3.9/6.6/9.0 $\times$ 10$^{-3}$ mbar, quadrupole analyzer pressure 9.0/9.1/9.5 $\times$ 10$^{-4}$ mbar, ToF analyzer pressure 1.7 $\times$ 10$^{-5}$ mbar, and 3.5 $\times$ 10$^{-2}$ mbar of argon in the collision cell. For the tandem MS experiments, the quadrupole resolution was adjusted to encompass the entire charge state of interest, and spectra were acquired over a range of accelerating voltages into the collision cell. In-source dissociation of MJSP16.5 was accomplished by increasing the extractor cone voltage to 100 V and reducing the pressure in the ion transfer stage pressure to 1.8 $\times$ 10$^{-3}$ mbar.

Surface Area Estimations
Estimating the surface area of the unfolded monomers released during CID was performed by three different methods. (1) The surface area of the folded monomers was determined by using SwissPDB DeepView software. An estimate of the surface area for an unfolded monomer was then determined by using a relationship between the sequence length and change in surface area upon unfolding [43]. (2) An online utility (http://roselab.jhu.edu/utils/unfolded.html), which estimates lower and upper bounds for the surface area in the unfolded state, based both on an analysis of known structures and hard sphere Monte Carlo simulations [44, 45], was used. (3) Surface areas were also estimated by simple shape approximations: a sphere to model a globular protein and a cylinder to model a completely extended polypeptide backbone. The predicted radius of the sphere was calculated from a relationship with the sequence length and radius of gyration of unfolded proteins [46]. The length of the cylinder was determined from the sequence length multiplied by the backbone length of an amino acid residue (3.57 Å, from the average distance between adjacent $\alpha$-carbons in the crystal structure). Its radius was estimated at 6.4 Å from the average dimensions of the amino acids (weighted according to sequence).

The surface area for the TahSP16.9 12-mer was estimated by approximating its structure to a cylinder of radius 47.5 Å and height 55 Å [30], while the structure of MJSP16.5 was approximated as a sphere of radius 60 Å [31]. The surface areas of the stripped oligomers were estimated through scaling down the volumes of these shapes. These estimated surface areas match well within the limits imposed by calculations from charge-based estimations [38, 39].
The surface areas determined by these methods agree within approximately 15% of their average to each other and, therefore, provide estimations adequately precise for the purpose of these studies.

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