A Q-ToF Mass Spectrometer Modified for Higher-Energy Dissociation Reduces Protein Assemblies to Peptide Fragments

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ABSTRACT

The gas-phase dissociation of protein assemblies is becoming crucial for the application of mass spectrometry to structural biology. However certain aspects of the dissociation mechanism remain elusive. Furthermore, many protein complexes resist dissociation at the energies accessible with current instrumentation. Here we report new insights into the collision-induced dissociation mechanism of protein assemblies. By holding activation energy constant and varying the charge state of the precursor ion, we show that the total charge of the precursor ion dramatically influences the internal energy required to dissociate monomers from the protein assembly. Furthermore, we have developed a modified Q-ToF instrument capable of accessing activation energies higher than previously possible. Under these conditions, protein assemblies eject subunits with excess internal energy that subsequently fragment into peptides. Together, these data indicate that the non-covalent dissociation is limited by the amount of charge available and not merely the activation energy, and project the exciting possibility of extracting sequence information directly from intact protein complexes in the gas phase

INTRODUCTION

Over the last decade mass spectrometry (MS) has become a *bona fide* approach to elucidating structural and dynamical aspects of macromolecular assemblies, primarily through preserving them intact in the gas phase.[1-7] Their subsequent collision-induced dissociation (CID) has become crucial for mining information regarding composition, stoichiometry, and oligomeric architecture.[8] To exploit fully such applications of CID a detailed understanding of the mechanism of dissociation is paramount, and has been an area of considerable research.

Although exceptions exist,[9, 10] the general mechanism that is emerging for CID of protein assemblies is one in which protein-protein complexes dissociate by successive removal of monomeric subunits from the intact complex.[11] Most data are consistent with a mechanism in which dissociation proceeds by charge-driven unfolding of a monomer which is eventually expelled after a sufficient number of inter- and intra-molecular non-covalent contacts have been compromised.[11-17] Nevertheless, many questions surrounding the mechanism of CID of protein complexes remain. For example, if protein complexes are activated using higher energies, will differences in mechanism be observed? What limits the number of subunits that can be removed sequentially from a protein complex? Here we have attempted to address these questions by performing CID of a protein complex at laboratory-frame energies (E_{lab}) considerably higher than those commonly accessible in Q-ToF type instrumentation.

In previous studies on the 396 kDa 24-mer of HSP16.5 from *Methanococcus jannaschii* we effected CID up to an E_{lab} of 9.4 keV,[11] where E_{lab} is the product of the charge state of the parent ion, and the accelerating voltage. We have examined the effect of increasing both variables by: i) modulating the charge-state distribution by manipulating the nanoelectrospray (nESI) parameters; and ii) customising a quadrupole time-of-flight (Q-ToF) mass spectrometer to allow higher accelerating voltages. We

demonstrate that, in a charge dependent manner, up to four monomeric subunits can be removed from the 24-mer. Moreover, by accessing an E_{lab} of ≈20 keV for this protein complex on our modified Q-ToF, we show that at this stage an additional dissociation mechanism becomes operative in which the monomeric subunits themselves fragment.

EXPERIMENTAL SECTION

Modifications made to the Q-ToF mass spectrometer

Several modifications were made to a Q-ToF Ultima (Waters, Milford MA, USA) in order to achieve higher E_{lab}s for protein complexes. The quadrupole was adapted to allow the selection and transmission of high m/z species,[18, 19] and, through fitting an additional power supply, the voltage available to the collision cell on the modified Q-ToF is twice that of the commercial instrument. Since ions now enter the collision cell at higher kinetic energies, we modified the inlet capillary that regulates gas flow into the collision cell to allow a higher ultimate pressure (100 μbar, collision gas line connected by PEEK tubing with 127 μm ID). The result is a Q-ToF instrument capable of acceleration voltages into the collision cell (termed 'collision energy') of 400 V. We are able to routinely observe ions up to collision energies of 350V (a 75% increase over the previous design). At voltages in excess of 350 V sensitivity is reduced, likely due to insufficient thermalization of the ions before the end of the collision cell.[2]

In initial instrument designs, we noticed breakdown in Ion Tunnel 1 when the collision voltage was maximized. This is because the absolute voltages on the source elements of the instrument are a combination of the set voltage and reference voltage on the collision cell. Therefore, in the current implementation, the collision cell is floated at a -150 V (in positive mode) offset with respect to the reference potential (ground) in order to limit breakdown in Ion Tunnel 1 (Figure 2). This was achieved by including a static offset to the reference voltages for the DC potentials of all of the electrodes in the instrument, including the resolving DC potentials applied to the quadrupole. The internal wiring was modified to remove the ground connection to the collision cell. An extra supply was needed to provide the floating voltage for the collision cell; this was achieved by reassigning one of the voltages used to supply a steering lens pair in the electrode set between the collision cell and the TOF analyzer. The remaining steering lens pair voltage was used to drive both lens electrodes. This action essentially

disabled the steering function of the lens; however, the steering function was found to be unnecessary to maintain instrument performance.

Protein preparation

HSP16.5 from *Methanococcus jannaschii* was a gift from Joseph Horwitz (Jules Stein Eye Institute, UCLA) and had been expressed in *E. coli* as described previously.[20] The protein was loaded onto a Superdex 200 10/300 GL gel filtration column (GE Healthcare) and eluted at 0.3 ml/min with 200 mM ammonium acetate at 6 °C. The resulting fractions corresponding to the 24-mer were combined to give a final protein concentration of 1.6 mg/ml.

Mass spectrometry conditions.

The MS spectra shown in Fig. 1 were acquired on a Q-ToF 2 (Waters, Milford MA, USA) which had been modified for the analysis of high-mass species.[19] Spectra were obtained by using a protocol described previously.[21] In MS mode, the following instrument parameters were used: capillary voltage 1.5 kV, cone gas 80 L/h, sample cone 170 V, extractor cone 20 V, accelerating voltage into the collision cell (termed 'collision energy' as per the manufacturer's terminology) 10 V, ion transfer stage pressure 6.5×10^{-3} mbar, ToF analyzer pressure 1.6×10^{-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 charge state of interest, and spectra were acquired over a range of accelerating voltages into the collision cell.

The spectra in figure 3 were obtained on the modified Q-ToF Ultima (see above). In MS mode, the following instrument parameters were used: capillary voltage 1.2 kV, cone gas 50 L/h, sample cone 300 V, RF lens 1 160 V, accelerating voltage into the collision cell ('collision energy') 5 V, inter-cone pressure 1.5 mbar, ToF analyzer pressure 1.3×10^{-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 charge

state of interest, and spectra were acquired over a range of accelerating voltages into the collision cell.

Data analysis

Data were processed using Masslynx software (Waters, Milford MA, USA) and exported into Excel and SigmaPlot (SPSS Software, Chicago IL, USA) for further analysis. The lower panel of figure 1 was generated by summing spectra obtained at all the different accelerating voltages, weighted to give the same E_{lab} irrespective of charge state. For example, to generate a fragmentation spectrum corresponding to an E_{lab} of 6.5 keV, data for 6 and 7keV can be combined and each weighted to 50%. To estimate the number of collisions, timescale of activation, and total accumulated internal energy an approach we described previously was used,[2] with HSP16.5 approximated by a sphere of diameter 120 Å.[22]

RESULTS AND DISCUSSION

A nESI MS spectrum of HSP16.5 is shown in figure 1A, and displays a charge state series centered on 8000 m/z with the most prevalent charge state being 50+, indicating that the 24-mer is transmitted intact through the mass spectrometer. The slightly higher charge states observed in this spectrum, than those reported previously,[11] were achieved by decreasing the distance between the nESI capillary and atmospheric-sampling orifice of the mass spectrometer (Fig. S1). Selection of the 50+ charge state and activation in an argon-filled collision cell gives rise to additional peaks observed at low m/z, corresponding to highly charged monomers (not shown), and in four distinct areas at high m/z corresponding to stripped oligomers of 23, 22, 21 and 20 subunits (Fig. 1B, upper). Therefore the 50+ charge state HSP16.5 24-mer can lose as many as four subunits. The removal of the fourth monomer is first observed at an E_{lab} of ≈8 keV (Fig. S2). At these conditions (50+, 8 keV) we estimate that the oligomer will undergo ≈23000 collisions over ≈370 μs in the collision cell, during which ≈7.9 keV of the kinetic energy will have been converted into internal modes.[2] However, when we had activated the 47+ charge state at an E_{lab} of 9.4 keV previously we only observed up to three successive monomer ejections from this complex.[11] Therefore our results here suggest, through comparison with those we published previously, that charge state appears to affect significantly the CID pathway.

To investigate the role of charge state on the dissociation process we performed the CID experiment on a range of charge states populated by HSP16.5. Compilations of CID spectra obtained for different charge states of the 24-mer, all at an average E_{lab} of 6 keV, are shown in the lower panel of figure 1B. By effectively decoupling the charge state from the E_{lab}, we can directly observe its influence on the dissociation profile. This also has the benefit of removing any differences due to the timescale of the experiment, i.e. 'kinetic window' effects. We find that a significant amount of 20-mers are only observed at 24-mer charge states ≥ 49+, meaning that, for a given E_{lab}, the extent of CID is determined

by the charge of the parent ion. This is likely due to lowly charged 24-mers carrying insufficient charge to unfold Coulombically a fourth subunit. In order to investigate this effect of charge state further we plotted the relative intensity of the 20-mers against the charge state of the precursor ion at different fixed E_{lab}s (Fig. 1C). The data clearly indicate that the higher charge states have a lower dissociation threshold in terms of E_{lab} when compared to lower charge states. Charge states lower than 49+ can, however, produce 20-mer at higher E_{lab}s. As such the extent of dissociation is governed not only by the E_{lab}, but also, as we have shown by parsing out its contribution to the E_{lab}, the charge state.

In order to access even higher E_{lab}s than described above we have modified a Q-ToF Ultima mass spectrometer (Waters, Milford, MA, USA) such that it is capable of acceleration voltages into the collision cell up to double those in the commercially available version, and can be operated at pressures up three times higher than normal (see Experimental Section). We thereby achieved accelerating voltages of up to 400 V without breakdown in the source region focussing elements (Fig. 2). CID spectra of HSP16.5 at accelerating voltages up to 200 V are identical to those in figure 1, with up to four monomer losses. As the voltage is increased beyond the previous 200 V maximum, over 25 new peaks are observed at low m/z (Fig. 3A). We identify these as singly and multiply charged *b* and *y* type fragment ions of HSP16.5. Cleavage products are observed up to 23 residues from the N-terminus, and 28 from the C-terminus, of the 147 residue monomer, providing an overall sequence coverage of ~35% (Fig. S3, Table S1). Plotting the relative intensity of intact monomers and their fragments as a function of E_{lab} shows that these peptides appear at ≈ 6 keV for the 56+ ion of the 24-mer (Fig. 3B). As only stripped oligomers composed of full-length monomers are observed, we conclude that these fragments arise from monomers ejected with excess kinetic energy undergoing subsequent dissociation of covalent bonds.

CONCLUSIONS

The results reported here provide new insights into the CID mechanism of macromolecular assemblies. By holding E_{lab} constant and varying the charge state of the precursor ion, we are able to show that the total charge of the precursor influences the internal energy required to dissociate monomers from the protein assembly. These data also indicate that the extent of non-covalent dissociation by CID is limited by the amount of charge available and not merely the activation energy. Increasing the charge state of the precursor ion presumably results in a 'Coulombically frustrated' ground state, thus lowering the activation energy barrier to subunit dissociation. Moreover, accessing even higher E_{lab}s eventually leads to fragmentation of monomeric subunits.

In addition to these mechanistic insights, our results project exciting new possibilities for the CID of protein complexes. Chief among these is the tantalizing prospect of identifying and characterising the protein chains which comprise an unknown macromolecular assembly solely by MS. Our data shows that within one experiment it is possible to characterize three strata of protein organization, namely the oligomeric, monomeric, and peptide levels. This amounts to an extension of the 'top-down proteomics' approach,[23-25] and is particularly significant given that the current bottleneck in determining the architecture of protein complexes involves simultaneously identifying protein subunits within the context of the intact assembly.[26] As such, this approach provides the possibility of determining the organization and identity of an unknown protein complex using a single MS experiment. Several challenges remain however, including efficient dissociation of all subunits within the assembly by either higher-energy CID or alternative gas-phase dissociation techniques.[12, 27-31] Developments in instrumentation, together with refinements to database search algorithms for the analysis of 'top-down' sequencing data,,[32] suggest that approaches such as the one described here will become an integral part of future structural genomics initiatives.

ACKNOWLEDGMENT

We thank Joseph Horwitz (UCLA) for the HSP16.5. JLPB is a Royal Society University Research Fellow; BTR is a Waters Research Fellow; FS is funded by the Biotechnology and Biological Sciences Research Council and Engineering and Physical Sciences Reseach Coucil; and CVR is a Royal Society professor.

SUPPORTING INFORMATION

The effect of needle positioning, the dissociation profile of HSP16.5, details of the protein-backbone fragmentation.

FIGURE CAPTIONS

Figure 1: Oligomers of HSP16.5 formed during CID. **A** nESI mass spectrum of HSP16.5 shows a major series of peaks around 8000 m/z corresponding to a complex composed of 24 copies of the full length protein. The low intensity intermediate peaks arise due to 24-mers which contain one copy of a low abundance monomer (<0.5%) which had been truncated at residue 27. **B** Upper panel: tandem MS of HSP16.5 of the 50+ charge-state (red) at a range of E_{lab}s. Oligomers are observed having lost one (orange), two (green), three (blue) and four (magenta) subunits, in an energy-dependent sequential manner. Lower panel: analogous experiments on different charge states, but compiled at the same average E_{lab} of 6 keV. The charge state distribution of the different components shifts as the accelerating voltage is varied, but as each species is both enhanced and depleted by the sequential dissociation process ascertaining precise details as to the partitioning of charge between monomers and stripped oligomers is not possible. **C** Graph of relative abundance of the HSP16.5 20-mers versus the charge state of the precursor ion at different E_{lab}s. This shows that the extent of dissociation is governed by not only the E_{lab}, but also the charge state of the precursor.

Figure 2: A schematic diagram of the modified Q-ToF Ultima instrument (upper) and associated potential energy surface (lower). The black trace indicates the energy surface of the unmodified instrument, capable of accelerating voltages into the collision cell up to 200V. Through the addition of an extra power supply and floating the collision cell, the modified instrument (red) is capable of accelerating voltages up to 400V without breakdown in ion tunnel 1.

Figure 3: A Tandem MS data acquired for the 56+ ion of HSP16.5 using the modified Q-ToF Ultima at

E_{lab}s of 15.7, 11.2 at 6.7 keV (upper, middle and lower panels, respectively). Intact monomer (red), and multiply (green) or singly (blue) charged peptide fragment ions are observed. Selected fragment ions are labeled. **B** A graph showing the disappearance and appearance of the 24mer, monomer, and peptides as a function E_{lab}. This reveals how fragmentation of the peptide backbone occurs at E_{labs} significantly higher than those required to dissociate monomers from the oligomers.

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FIGURE 1

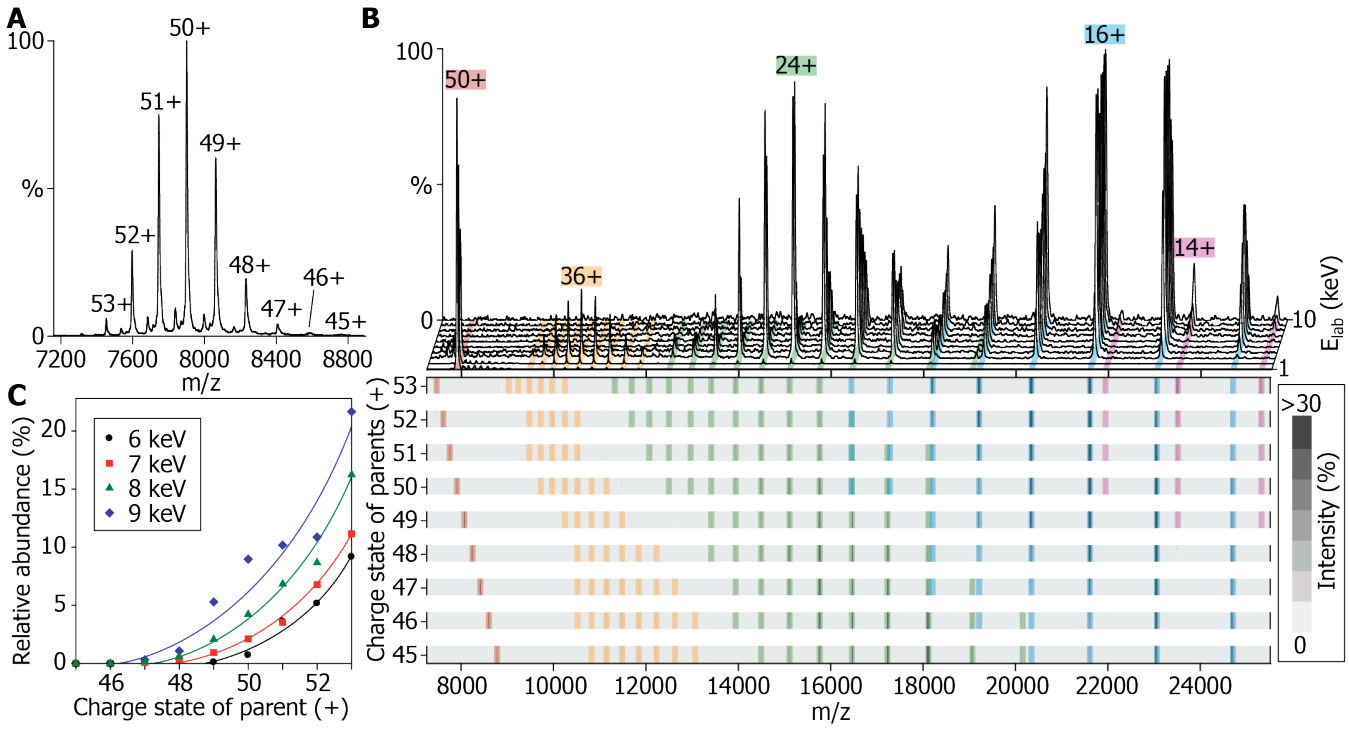
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FIGURE 2

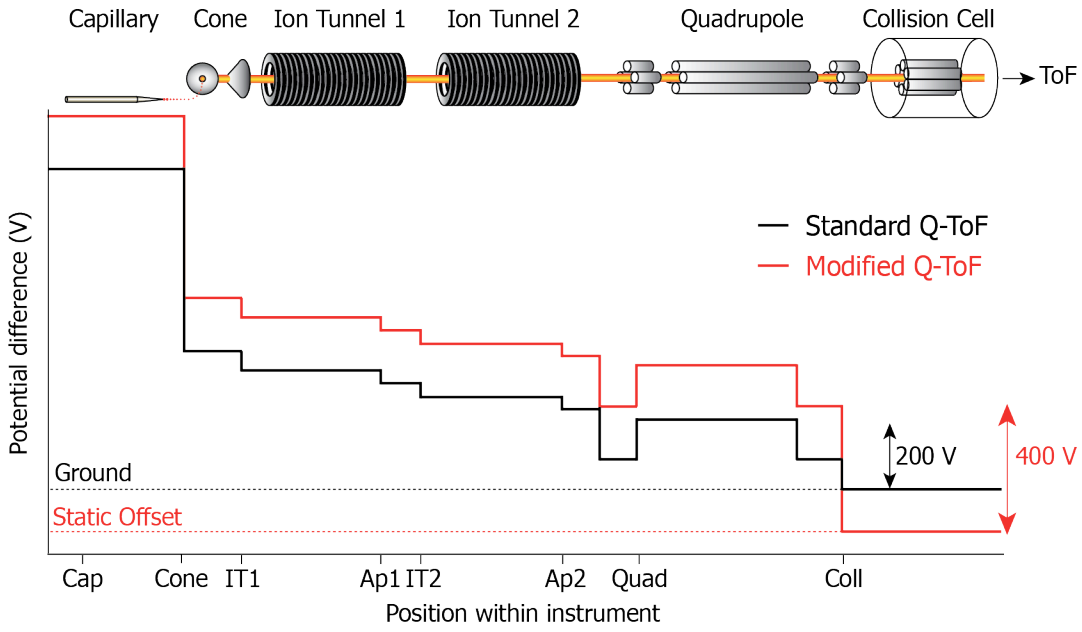
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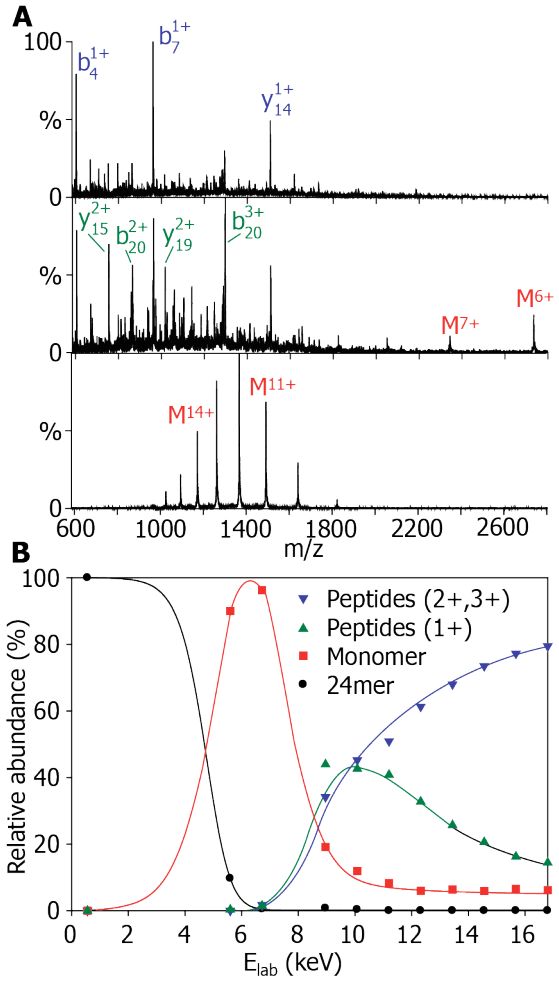
FIGURE 3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M^{6+}
M^{7+}
M^{11+}
M^{14+}
600
1000
1400
1800
2200
2600
m/z
B
Relative abundance (%)
Peptides (2+,3+)
Peptides (1+)
Monomer
24mer
E_{lab} (keV)

SUPPORTING INFORMATION

A Q-ToF Mass Spectrometer Modified for Higher-Energy Dissociation Reduces Protein Assemblies to Peptide Fragments

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This supporting information contains a description of the effect of needle positioning on charge state (page S2), the dissociation profile of the 50+ charge state (page S4), sequence coverage details (page S5), and supporting references (page S8)

MODULATING THE CHARGE OF PROTEIN COMPLEX IONS BY ALTERING THE ELECTROSPRAY EMITTER POSITION

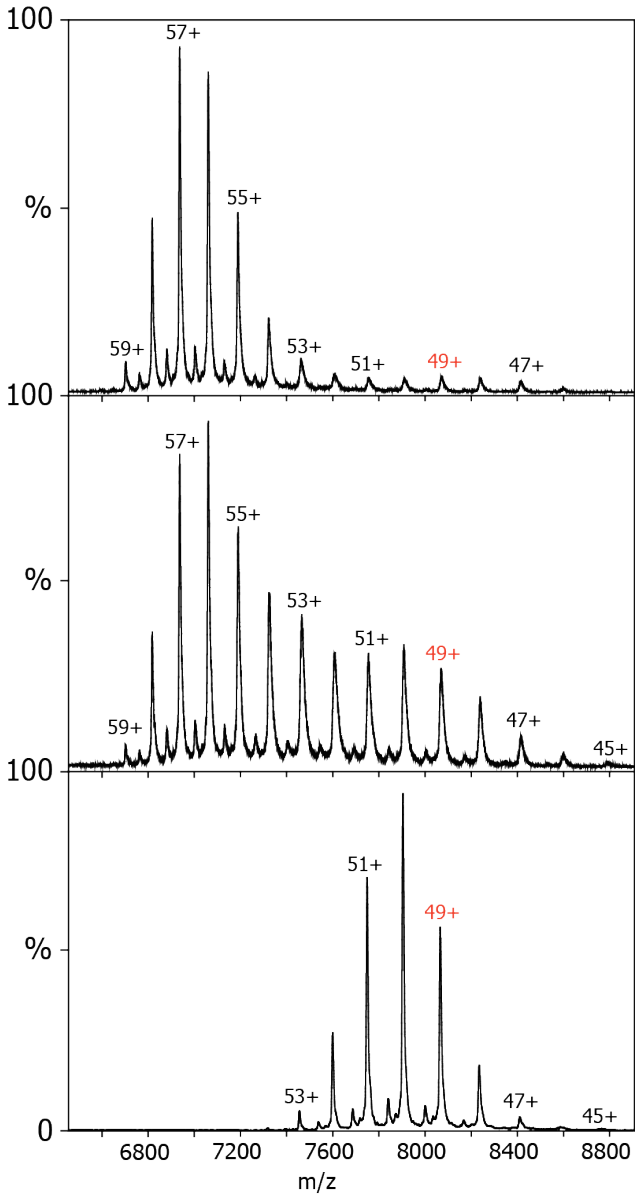

Figure S1. Mass spectra acquired of HSP16.5 using different distances between the electrospray emitter and sampling orifice for the instrument (decreasing distance from lower to upper panel). The 49+ charge state (red) is the modal charge state of the complex as per the charge-residue model.[1]

The charge state distributions of monomeric proteins in electrospray ionization have been an active area of research for many years.[2] Much of the evidence collected to date suggests that width and average value of the charge state distribution for monomeric proteins can be used as an indicator of the folded state of the protein, with higher average charge states and wider distributions being indicative of unfolded states in solution.[2]

There has been less research concerning the relationship between charge state distributions of intact protein complexes and assembly structure.[3] Early electrospray mass spectra of large protein and polymer systems were used as evidence of the charge-residue model of electrospray ionization,[1] and experimental data suggest that the charging of protein complexes is related to their available surface area.[4] Also, the influence of small amounts of solution-phase additives on the charge state distribution of protein complexes has been reported.[5, 6]

In our experiments, we altered the relative position of the electrospray emitter to the sampling orifice of the mass spectrometer (Fig S1). Typical distances where low charge states are observed are 1-3 cm from the sampling orifice (Fig S1, bottom) and all experiments were conducted using a 90 º angle between the emitter and the sampling orifice. The highest charge states were observed when the emitter was placed just a few millimeters from the sampling cone (Fig S1, top).

The origin of the change in charge state distribution observed in figure S1 is still under investigation in our laboratory, but is likely due to a reduced number of collisions and larger ultimate droplet sizes when the needle is positioned close to the sampling orifice.[7]

DISSOCIATION OF UP TO FOUR MONOMERS FROM HSP16.5

The loss of several monomeric units upon collisional activation has been reported for a number of protein complexes.[8] Isolation of the 50+ charge state of the HSP16.5 24-mer, and activation leads to the generation of the 23, 22, 21, and 20-mer (Fig. 1) resulting from the loss of up to four monomers. Figure S2 shows the appearance and disappearance as a function of E_{lab}, suggesting the sequential nature of the loss of multiple subunits.

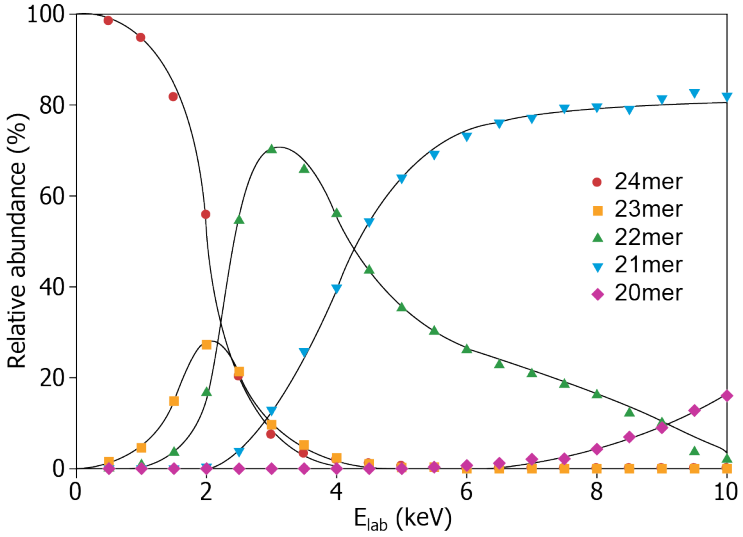

Figure S2. Appearance and disappearance of the different oligomeric species of HSP16.5 observed during CID of the 50+ charge state (see Fig. 1). Up to four subunits are released

ID	Charge State(s)	Molecular Mass (Da)
b_3	1	493.85
b_4	1	604.47
b_7	1,2	962.40
b_{11}	1	1437.05
b_{14}	1,2	1871.89
b_{16}	2	2126.30
b_{19}	2	2490.24
b_{20}	2	2590.95
b_{23}	2,3	2919.10
y_6	1	670.36
y_7	1	798.00
y_{10}	1	1084.19
y_{12}	1	1283.60
y_{14}	1,2	1508.00
y_{15}	1,2	1620.71
y_{16}	1	1733.44
y_{17}	1	1832.05
y_{18}	1	1918.91
y_{19}	1,2	2031.56
y_{21}	1	2187.23
y_{22}	1	2300.80
y_{23}	1	2429.29
y_{24}	1	2576.14
y_{25} - CO	1	2830.84
y_{28}	1	2933.00

Using higher acceleration voltages we were able to observe dissociation of covalent bonds within the HSP16.5 monomer, most likely following ejection from the intact complex. Similar observations have been made recently, primarily using surface-induced dissociation (SID) to activate protein complexes.[9] Peptides were identified using freely available web-based tools (http://prospector.ucsf.edu/) (Table S1).

Table S1. The most 25 most intense peptide identified from 'top-down' fragmentation of HSP16.5.

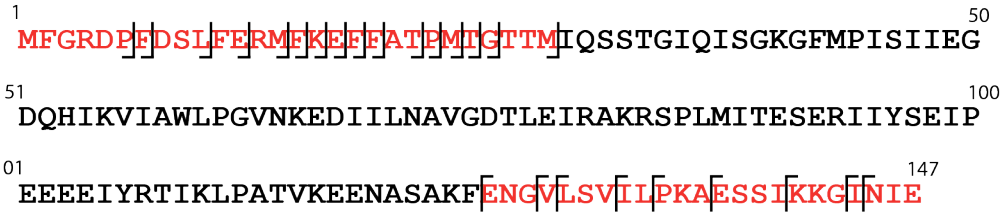

Figure S3. Sequence coverage observed for the 'top-down' fragmentation of HSP16.5 monomers. Peptides containing the residues labelled in red were observed in our experiments, and the black lines demonstrate the cleavages.

The sequence coverage reported (34.7 %, Figure S3) is derived entirely from this assignment, and includes data only from *b* type and *y* type ions observed. Those identified correspond to the most intense

b and *y* type ions (S/N>3) and some intense ion signals were not included as they did not correspond to *b* or *y* type ions predicted from the HSP16.5 sequence. Therefore, the sequence coverage presented should be viewed as a conservative estimate of the sequence information present in the data collected. Poor sequence coverage for core residues is a common observation made for top-down sequencing of proteins,[10, 11] and is consistent with the pattern of coverage that we observe for HSP16.5 monomers.

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