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Thermal Dissociation of Multimeric Protein Complexes by Using Nanoelectrospray Mass Spectrometry

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The behavior of macromolecular systems at different temperatures is often crucial to their biological activity and function. While heat-induced changes of individual proteins are readily monitored by a number of spectroscopic methods, changes in noncovalent complexes of biomolecules are more challenging to interpret. Nanoelectrospray mass spectrometry is becoming increasingly powerful in the study of large noncovalent complexes, and here we describe the design, characterization, and application of a novel probe that allows the thermocontrol of the solution in the electrospray capillary. The transition temperature for the unfolding of the protein lysozyme is readily obtained and correlates closely with that measured by fluorescence spectroscopy, thereby demonstrating the validity of this approach. We apply this technique to the study of the 200-kDa complex of the small heat shock protein *Ta*HSP16.9, revealing both its dissociation into suboligomeric species and an increase in its size and polydispersity at elevated temperatures. In contrast, gas-phase activation of this complex is also carried out and yields a dissociation pathway fundamentally different from that observed for thermal activation in solution. As such, this probe allows the study of the reversible heat-induced changes of noncovalent complexes in a biologically relevant manner.

The gentle transfer of ions from the solution to the gas phase and their subsequent analysis, by using electrospray ionization (ESI) and mass spectrometry (MS),[1] has become invaluable in the study of biological macromolecules. While the primary applications are in the field of proteomics, this technique can be applied to the direct examination of the noncovalent complexes formed from these species and, consequently is also becoming increasingly important to structural biologists.[2] Conserving such noncovalent complexes intact in the gas phase has led to the direct mass measurement of species larger than 2 000 000 Da.[3] Moreover, this ability has allowed ESI-MS to be used in the investigation of the biochemical properties of such complexes and the manner in which they interact with each other as well as with ligands and cofactors.[4−9] Only a few studies however have addressed heat-induced changes to macromolecules and their noncovalent complexes so far, which in many cases have important biological significance.[5,10,11]

Maintaining intact noncovalent complexes of biological macromolecules during ESI-MS requires careful control of instrument conditions. Adjusting the pressures and acceleration voltages in the source region of the mass spectrometer (Figure 1A), and as a result the energy of collisions with the background gas, is crucial in desolvating the ions while still preserving noncovalent interactions.[12,13] Insufficient removal of solvent, and buffer ions, results in broadening of the charge states in the mass spectrum and consequently less accuracy in mass measurement. Conventional ESI probes often incorporate heating elements, as higher temperatures aid this desolvation, including some that directly heat the electrospray capillary and the solution within. This ability to control the temperature of the solution and analysis by using ESI has been exploited to study the thermal solution-phase unfolding behavior of a number of proteins on-line.[14−16] A miniaturized version of ESI, nanoESI, is often the ionization method of choice for the study of noncovalent complexes by MS.[2] This is due to the conditions of analysis possible, such as the use of aqueous

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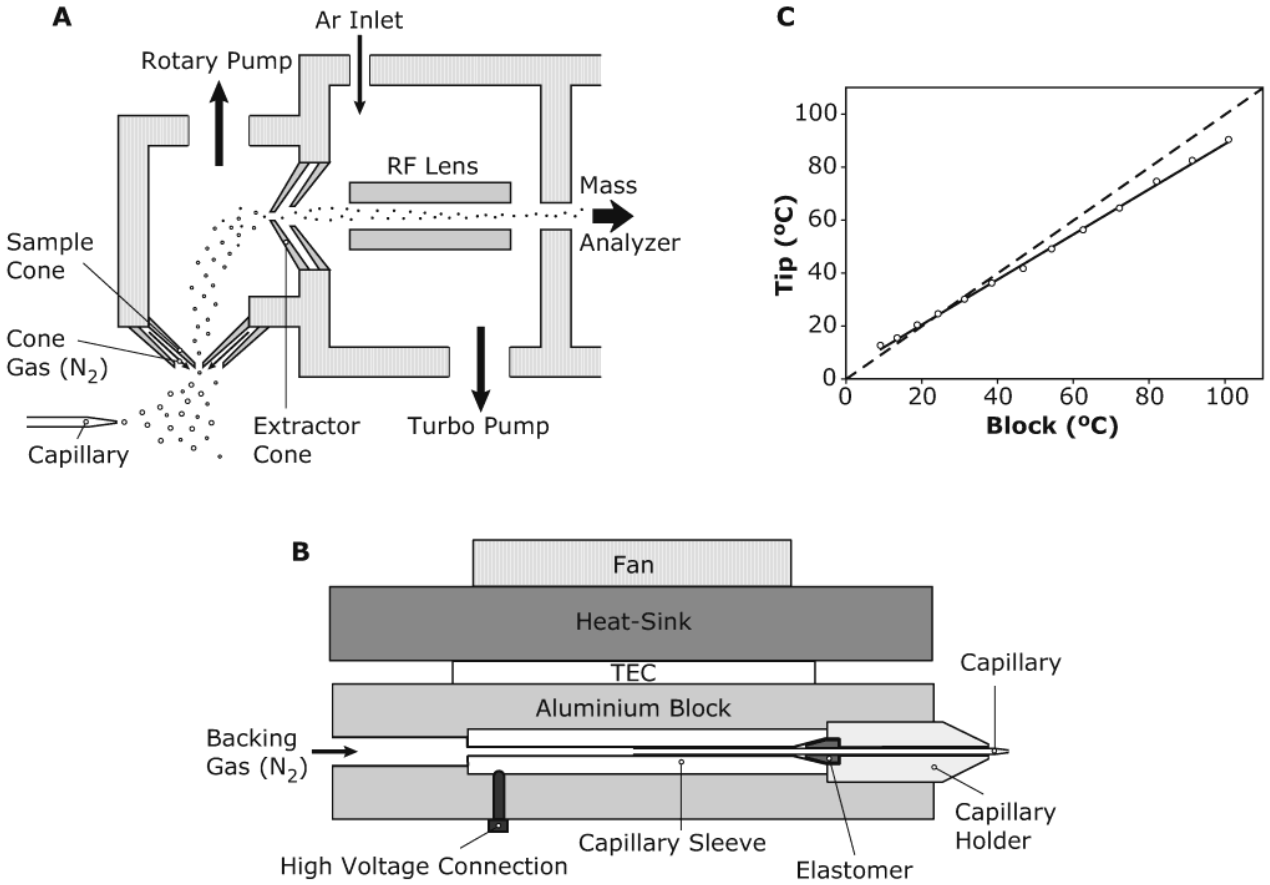

Figure 1. (A) Schematic diagram of a Z-Spray source. Droplets emerge from the capillary and pass through sample and extractor cones toward the mass analyzer. Collisions with the background gas cause these droplets to shrink and lose solvent molecules, thereby desolvating the ions they contain. Increasing the voltages on the cones raises the energy of these collisions, thereby increasing the rate of desolvation and ultimately leading to gas-phase dissociation. (B) Schematic diagram of the custom-built nanoESI probe. The capillary fits closely into the holder and sleeve and is held in position by a section of elastomer. The temperature of the aluminum block is controlled by a thermoelectric cooler (TEC) coupled to a heat sink and fan. (C) Temperature in the center of the block versus that recorded at the tip of the capillary holder (solid black line). This demonstrates that when the TEC is used as a heater, there is a small temperature drop between the center of the block and the tip of the capillary holder. If there were no such heat loss, no difference would be observed (dashed line).

buffer solutions at neutral pH, and the reduced sample volumes required with nanoESI relative to conventional ESI. The decreased diameter of the capillary tip in nanoESI, and the resulting more facile generation of an electrospray and improved desolvation, however, mean that these probes do not require heating elements. Therefore, studying the effect of heating in solution phase on-line by nanoESI requires the design and construction of a novel probe.

In contrast to activation in solution, the collisions with the background gas in the source of the mass spectrometer that aid desolvation can be exploited to cause gas-phase collision-induced dissociation (CID). As the internal energy distribution of the ions in the source of the mass spectrometer is Boltzmann-like due to the high number of collisions that they experience, they can be assigned a characteristic temperature.[17] Adjusting the sample and extractor cone (Figure 1A) voltages affects the acceleration of the ions through the source and, consequently, the energy of the collisions with the background gas, thereby providing a way of controlling the internal energy of the sample ions. If sufficiently high voltages are used, the ions become essentially desolvated and additional gas-phase "thermal" dissociation occurs.[18] CID can also be conducted in a dedicated collision cell and is a more sophisticated experiment in that it allows the selection of a particular ion for dissociation.[19] A noncovalent complex of n components dissociated by using one of these CID techniques separates into one highly charged monomer and a lowly charged "stripped complex" of $n - 1$ components.[20−23] Such mass and charge asymmetry between the dissociation products is reminiscent of droplet fission during the electrospray process.[20] While these CID techniques are useful for determining the composition

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of noncovalent complexes,[2,6,18,19,23] the lack of solvation means that such gas-phase dissociation will not necessarily follow the same pathway as in solution, and it is these solution effects that are of primary biochemical interest.

A number of biologically important complexes are known to undergo changes in structure and activity in response to thermal stimuli. Among these are the small heat-shock proteins (sHSPs), which have been shown to help protect the cell at elevated temperatures.[24−26] They are believed to accomplish their protective role by functioning as molecular chaperones, preventing detrimental protein associations, behavior that has been demonstrated in vitro.[26−28] Current models suggest that under non-heat-shock conditions the sHSPs are in a relatively inactive storage form, which typically is a multimer of 9−40 subunits, ranging in mass between 80 000 and 800 000 Da depending on the protein species, but under heat-shock conditions, they become activated and bind unfolded client proteins. Experiments conducted on sHSPs from yeast and *Synechocystis* suggest this activation involves the dissociation of the oligomer into suboligomeric forms.[26,28] A wheat sHSP, *Ta*HSP16.9, for which the crystal structure has been solved, consists of 12 identical subunits.[29] This protein has been shown to possess a very dynamic quaternary structure[8] and to undergo structural changes at heat-shock temperatures by using size exclusion chromatography (SEC)[29] and circular dichroism (CD).[30] The nature of these changes remains unclear, however, as SEC operation at elevated temperatures is problematic, and it is difficult to correlate CD measurements to quaternary structure.

In this study, we describe the design and construction of a nanoESI probe that allows efficient temperature regulation of the solution within the capillary and is compact and easy to operate. We characterize the feasibility of this device for the study of thermally induced macromolecular change by monitoring the unfolding behavior of hen egg white lysozyme and find that the results match well with other solution-phase measurements. By studying the behavior of the sHSP *Ta*HSP16.9 at a range of temperatures, we demonstrate that coupled to the precision of MS and the ability of nanoESI to preserve noncovalent interactions, this probe is able to provide new insight into reversible changes induced by the heating of large macromolecular assemblies.

EXPERIMENTAL SECTION

Design and Construction of the Thermocontrolled NanoESI Probe. Our fundamental design principle was to create a dedicated nanoESI thermocontrolled probe that would allow the regulation of the solution temperature in the capillary over a wide temperature range and, therefore, enable the study of heat-induced changes of macromolecules and their noncovalent complexes in solutions. A schematic of the probe is shown in Figure 1B. We designed the probe to fit onto the *xyz* stage of a Z-Spray source (Micromass, Manchester, U.K.) and to incorporate all the standard requirements including a backing-gas line, and a high-voltage power connection (Figure 1B). To adjust the temperature of the capillary we chose to use a thermoelectric cooler (TEC; Thermo Electric Devices, Moreton-in-Marsh, U.K.), which is capable of both heating and cooling by using the Peltier effect. Efficient and rapid heat transfer between the aluminum block and capillary is achieved by having direct contact between the two. The capillary holder and capillary sleeve were machined from stainless steel with a hole of 1.05-mm diameter in the center, just wide enough to accept a nanoESI capillary of 1-mm outer diameter. We incorporated a custom-built "K"-type thermocouple (Labfacility Ltd., Bognor Regis, U.K.) into the block to monitor the internal temperature. The high voltage required for electrospray is applied directly to the capillary sleeve, and the TEC and heat sink are electrically insulated from the block with nylon screws and thermally conductive, electrically nonconductive sheet (Warth International Ltd., East Grinstead, U.K.). As a safety precaution, a cover adapted from a standard nanoESI probe (Micromass) was fitted over the whole assembly.

Mass Spectrometry. All spectra were either aquired on a Q-ToF 2 modified for high-mass operation[19] or an LCT (both Micromass), under conditions optimized for the transmission of noncovalent complexes. NanoESI capillaries were prepared by using a micropipet puller (Flaming/Brown P-97, Sutter Instruments, Novato, CA) and borosilicate glass tubes of 1-mm outer and 0.5-mm inner diameter (Harvard Apparatus, Holliston, MA). These were then coated with a thin layer of gold by using an SEM sputter-coater (Polaron, Newhaven, U.K.) to make them electrically conductive. The pulled end of the capillary was clipped under a stereomicroscope, resulting in a tip inner diameter between 2 and 5 μm. A 1-μL aliquot of sample was injected into the capillary using GELoader tips (Eppendorf UK Ltd., Cambridge, U.K.). External calibration was achieved by using a 33 mg/mL aqueous solution of cesium iodide (Sigma). Data were aquired and processed with MassLynx software (Micromass), and all spectra are shown here without background subtraction and with minimal smoothing.

Analysis of Protein Unfolding. A 10 μM aqueous solution of hen egg white lysozyme (HEWL; L-6876, Sigma-Aldrich), adjusted to pH 2.0 with acetic acid (Sigma-Aldrich), was prepared. Aliquots of this solution were used in all the HEWL unfolding experiments.

A series of mass spectra at different temperatures (over the range of 10−90 °C) were acquired on the Q-ToF 2 using otherwise identical experimental conditions: capillary voltage 1.5 kV; sample cone 200 V; extractor cone 50 V; collision energy 7 V; ion energy 1.5 V; 100 L/h cone gas (N_2); hexapole ion guide stage pressure 6.0×10^{-3} mbar; quadrupole analyzer pressure 1.4×10^{-5} mbar; and time-of-flight (TOF) analyzer pressure 5.0×10^{-7} mbar. The solution was introduced into the back of the capillary, so that once inserted into the probe, the solution was approximately in the center of the aluminum block. There it was allowed to equilibrate for 1 min. The probe was moved into sampling position, the high capillary voltage applied, and a solvent flow initiated by a backing pressure of 0.7 bar. The spectra shown in Figure 2A were obtained by combining the first 2 min of scans once the signal had stabilized. It was found that the temperature of the probe drifted

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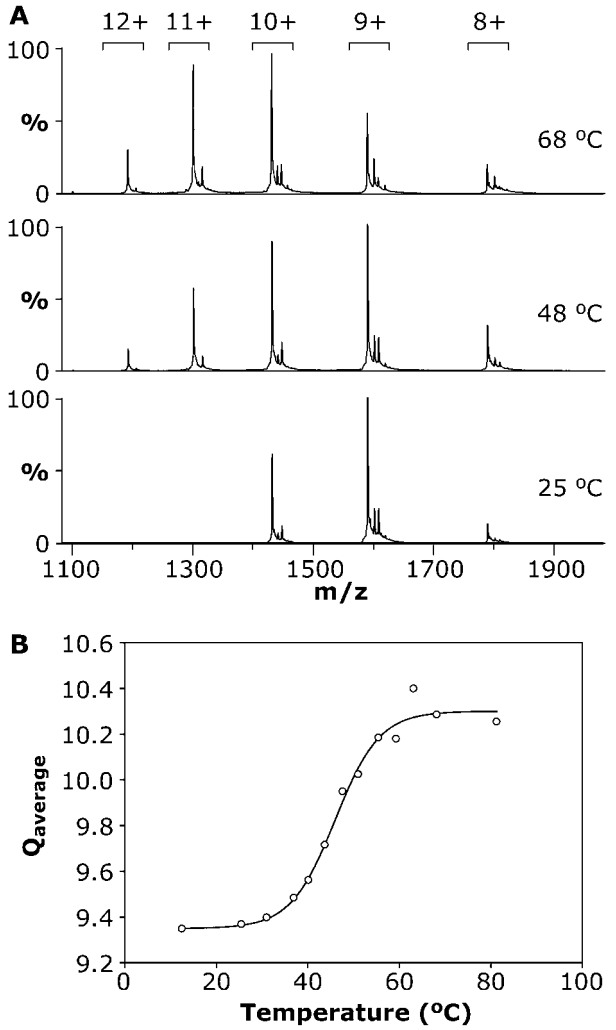

Figure 2. (A) Spectra of hen egg white lysozyme at room temperature (lower panel), 48 °C (middle panel), and 68 °C (upper panel). A shift to higher charge states with increasing temperatures reveals unfolding of the protein structure. (B) Average charge state ($Q_{average}$) versus the temperature measured by the embedded thermocouple. A clear unfolding transition is observed and a melting point of 43.0 ± 0.6 °C determined from the first derivative (corrected according to Figure 1C).

very slightly during electrospray (a maximum of ±0.7 °C), so the temperatures quoted are averages of the temperatures recorded as the acquisition started and once the acquisition had finished. To quantify the shift to higher charge states, we computed the average charge state ($Q_{average}$) by using eq 1, where N is the

$$Q_{\text{average}} = \sum_{i}^{N} Q_i W_i / \sum_{i}^{N} W_i \qquad (1)$$

number of observed charges states in the spectrum, Q_i is the net charge of the ith charge state, and W_i is the signal intensity (peak height) of the ith charge state.

Two separate acquisitions were obtained at each temperature, the $Q_{average}$ was calculated for each, and the mean of the two was used in Figure 2B.

Thermocontrolled fluorescence measurements were carried out on a fluorometer (LS 50, Perkin-Elmer, Beaconsfield, U.K.) coupled to a heated circulator bath (C35, Haake, Karlsruhe, Germany). A cuvette of 10-mm path length was used, and excitation and emission slit widths were kept at 5 and 3.5 mm, respectively. One milliliter of the HEWL solution was excited at 280 nm, resulting in an emission spectrum featuring a broad band centered around 340 nm. HEWL unfolding was monitored by recording the emission intensity at 318 nm, as the temperature was ramped from 20 to 90 °C over a 3-h period. Fluorescence data were aquired with FL WinLab software (Perkin-Elmer) and processed with SigmaPlot (SPSS UK Ltd., Woking, U.K.).

MS Analysis of a Multimeric Protein Complex. The *Ta*HSP16.9 sample was a gift from Elizabeth Vierling and coworkers (University of Arizona, Tucson, AZ) and had been expressed in *Escherichia coli* and purified as described previously.[29] The sequence is given by GenBank accession number S21600, with one sequence change T7S and the absence of the N-terminal Met. The protein was provided at a concentration of 5 μg/μL, stored at −20 °C, and thawed just before use. The sample was buffer exchanged twice into 200 mM ammonium acetate (pH 7). This was achieved by using microchromatography columns (Bio-Spin, Biorad, Hemel Hempstead, U.K.), which had been preequilibrated with the desired buffer.

A series of spectra at different temperatures were acquired on the Q-TOF 2 using otherwise identical experimental conditions (Figures 3 and 4 and the upper panel of Figure 5): cone 200 V; extractor 50 V; capillary 1.7 kV; collision energy 4 V; ion energy 1.5 V; cone gas 100 L/h; backing gas pressure 0.4 bar; sample equilibration time of 1 min; hexapole ion guide stage pressure 7.0×10^{-3} mbar; quadrupole analyzer pressure 2.5×10^{-5} mbar; and TOF analyzer pressure 5.3×10^{-7} mbar.

The spectrum in the lower panel of Figure 5, showing in-source CID of the sHSP, was acquired on the LCT under the following conditions: cone 200 V, extractor 0 V, capillary 1.6 kV, ion energy 36 V, cone gas 150 L/h; backing gas pressure 0.4 bar; and pressures of 8.3 and 1.0×10^{-6} mbar in ion source and TOF analyzer stages, respectively.

RESULTS AND DISCUSSION

Performance of the Thermocontrolled NanoESI Probe. By varying the potential difference across the TEC, the temperature of the block, as measured by the embedded thermocouple, was successfully controlled between 8 and 100 °C. As the solution is equilibrated in the center of the block, this corresponds to the temperature range accessible using this probe. Measuring the temperature of the solution inside the capillary directly was not possible, due to the small inner diameter. Figure 1C shows the difference between the temperature measured at the end of the capillary holder (in the absence of a capillary) and that measured by the embedded thermocouple. In an ideal design, there would be no difference in these temperatures, but due to the exposure of the capillary holder to air at ambient temperature there is a discrepancy, though always of less than 10%. This graph, therefore, also enables the characterization of the temperature drop, which can then be taken into account in a quantitative manner. These results demonstrate a successful validation of the probe as a tool to control the temperature of the analyte solution.

MS Analysis of Protein Unfolding. To assess the validity of this technique in the study of thermally induced macromolecular change, we examined the unfolding of a protein by both fluores-

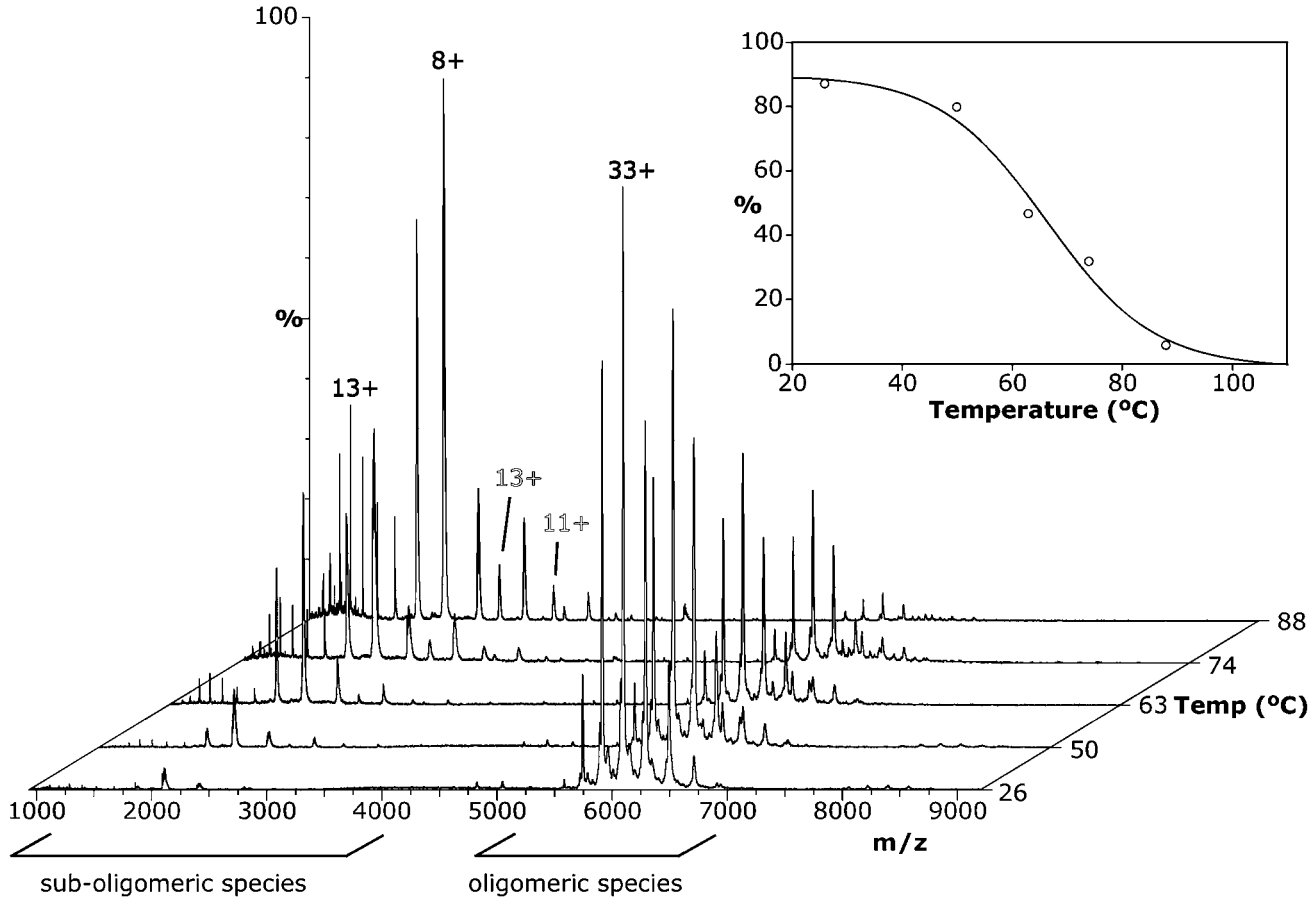

Figure 3. Dissociation of a multimeric protein complex by using the thermocontrolled nanoESI probe. Spectra of *Ta*HSP16.9 at different temperatures show the dissociation on heating of the dodecamer (~6000 *m*/*z*, selected charge state labeled in solid type) into suboligomeric species (at low *m*/*z*). Selected charge states for monomers (solid type) and dimers (outlined type) are labeled. Inset: Percentage of dodecamer versus temperature. The signal intensity corresponding to dodecamer charge states is expressed as a percentage of total signal intensity. A transition is observed, with a melting point of 60 ± 2 °C determined from the first derivative (corrected according to Figure 1C).

cence spectroscopy, a well-characterized technique in such studies, and nanoESI-MS. HEWL is a protein of mass 14 305 Da that has been extensively studied by using ESI-MS.[31,32] Thermal unfolding of HEWL is reversible, and it generally refolds on a millisecond time scale,[31] making it a suitable test system for characterization of the probe.

The intrinsic fluorescence often displayed by proteins frequently allows the observation of conformational changes by monitoring the variation in tryptophan emission spectra. HEWL contains six tryptophan residues, and its conformational properties have been comprehensively studied by using fluorescence spectroscopy.[32,33] Intrinsic tryptophan fluorescence of HEWL has been shown to undergo a red shift with increasing temperature,[32] consistent with a certain degree of unfolding of the HEWL structure. The unfolding reaction was monitored by recording the emission intensity at 318 nm, as the temperature was ramped from

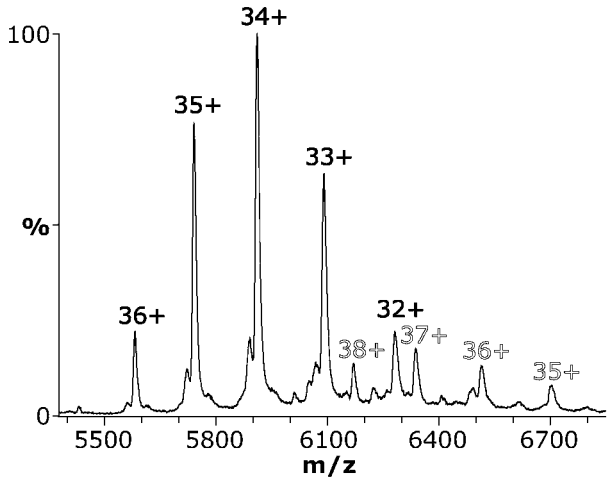

Figure 4. Expansion of the high-*m*/*z* region of the spectrum of *Ta*HSP16.9 recorded at a solution temperature of 74 °C. On increasing the temperature, an increase in polydispersity and oligomer size is observed. Tetradecamers (outlined type) as well as dodecamers (solid type) are clearly observed at higher temperatures, whereas the protein is exclusively dodecameric at room temperature.

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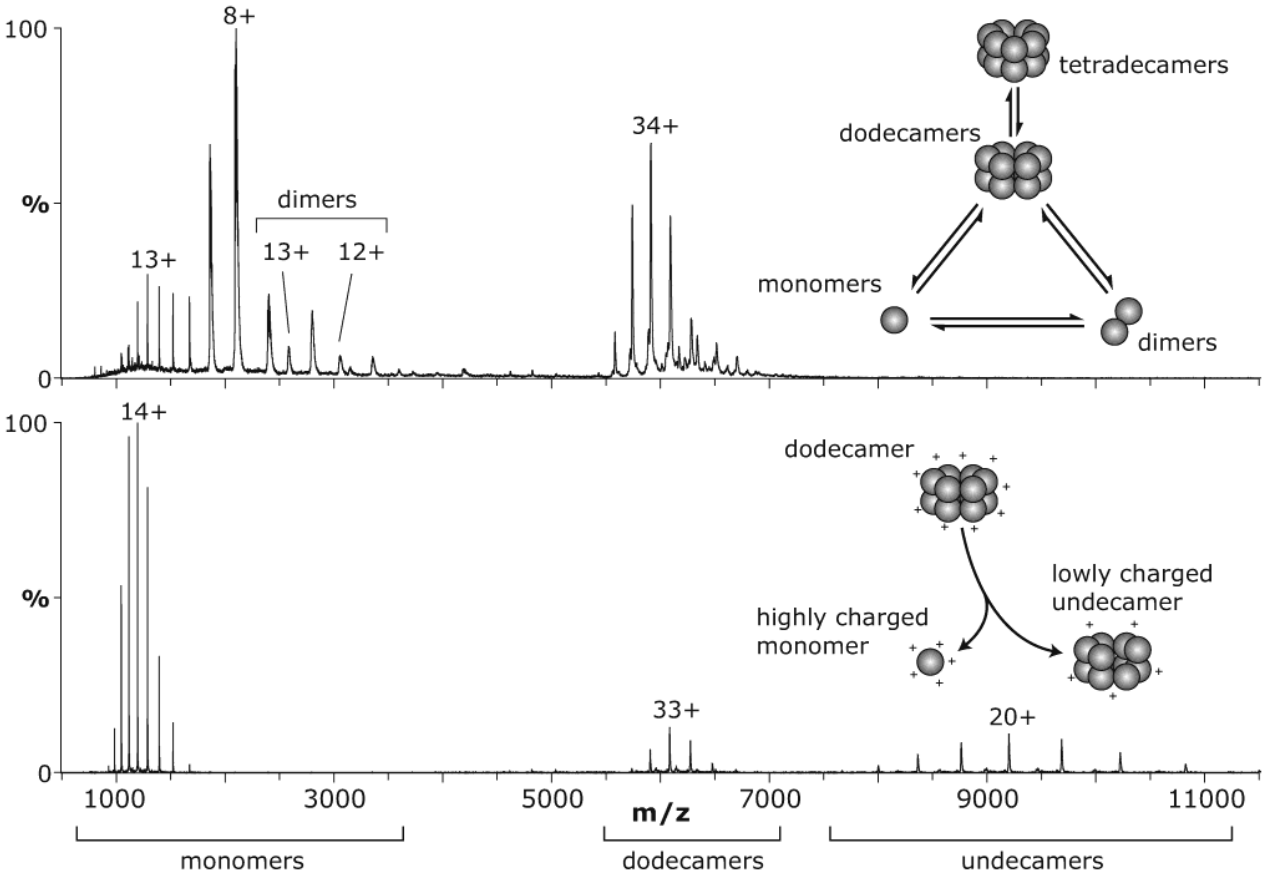

Figure 5. Solution-phase versus gas-phase activation of *Ta*HSP16.9. Heating in the solution phase (upper panel, spectrum at 74 °C) results in dissociation of the dodecamers into monomers and dimers and also the formation of tetradecamers. This allows the delineation of solution-phase equilibria (inset, upper panel) which shift away from the dodecamers as the temperature is increased. Activation in the gas phase by CID (lower panel, spectrum at 24 °C) results in the dodecamers dissociating into highly charged monomers and lowly charged undecamers.

20 to 90 °C. The transition temperature (T_m) for the unfolding of HEWL under these conditions was found to be 43.8 ± 0.3 °C (data not shown).

To monitor the unfolding by using nanoESI-MS, spectra were acquired at 13 different temperatures (Figure 2). The spectra show a shift to higher charge states as the temperature is increased. This observation is consistent with an unfolding transition, as from the same solution conditions, an unfolded protein has more accessible sites available for protonation than a native one and, consequently, occupies a higher charge-state distribution.[34] To quantify this effect, we computed the average charge state ($Q_{average}$) (see Experimental Section), which has previously been used as an indicator of conformation in the interpretation of protein mass spectra.[35] A plot of $Q_{average}$ versus temperature is shown in Figure 2B and, from its first derivative, gives a T_m of 43.0 ± 0.6 °C (corrected according to Figure 1C). This compares well with the result obtained from fluorescence spectroscopy, demonstrating that this technique is valid for the study of thermal effects on macromolecules and can consequently provide a powerful alternative to spectroscopical methods. Morevover, this good correlation between the results obtained by using these two different techniques shows that solution-phase properties, such as in this case the T_m, can be conserved during the electrospray process and be monitored by using MS.

MS Analysis of a Multimeric Protein Complex. Spectra of *Ta*HSP16.9 at a range of temperatures are shown in Figure 3. At 26 °C, the dominant species is a dodecamer (12 subunits) of 200 790 Da, in agreement with the crystal structure[29] and previous nanoESI measurements.[8] Increasing the temperature, however, results in dissociation of the dodecamer into smaller species. These species are predominantly monomers, though there are also significant amounts of dimers. The most intense peaks assigned to the monomers are centered about the 8+ charge state, but at the highest temperatures, a second, more highly charged, monomer distribution centered on the 13+ state appears, suggesting an additional, less structured conformation. An increase in temperature also results in the observation of larger oligomers at higher m/z than the dodecamers (Figure 4). These larger species are almost exclusively tetradecamers (14 subunits). By plotting the decrease in signal intensity of dodecamers as a function of total signal intensity, we can obtain a transition curve for the dissociation, giving a corrected T_m of 60 ± 2 °C (Figure 3 inset).

As an alternative, we also investigated the gas-phase activation of *Ta*HSP16.9, by using in-source CID (see introduction) (Figure

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5). Such activation of the *Ta*HSP16.9 dodecamer results in the appearance of highly charged monomers and lowly charged undecamers (11 subunits) (Figure 5, lower panel). This is a dramatic example of the uneven separation of both mass and charge observed during CID. By contrast, there are no undecamers observed in solution-phase dissociation and no dimers or tetradecamers in the gas-phase technique. While monomers are observed in both spectra, they are very different in appearance. Those formed during gas-phase dissociation are highly charged, with the most intense peak corresponding to the 14+ charge state, whereas the principal monomeric charge states observed during solution-phase thermal dissociation are centered on the 8+ charge state. The second monomer distribution that appears during dissociation in solution at the highest temperatures is also highly charged, with the most intense peak belonging to the 13+ state. There are two possible explanations for this additional distribution. Either it arises due to a different conformation of the protein in solution or is the result of gas-phase dissociation. The absence of undecamers, which would be observed during the latter dissociation pathway, suggests that this higher charge-state distribution corresponds to partially unfolded *Ta*HSP16.9 monomers in solution. Overall, comparing the spectra obtained by using the solution- and gas-phase thermal activation techniques shows that they give very different results.

The solution-phase thermal activation suggests there to be an equilibrium in solution between *Ta*HSP16.9 dodecamers and monomers and dimers. Increasing the temperature causes this equilibrium to shift toward dissociation as the temperature is increased. Subunit exchange experiments showed dimers to be the predominant units of exchange but also demonstrated that these dimeric interactions must at some stage be disrupted.[8] The thermal studies presented here suggest that, when free in solution, monomers, rather than dimers, are the predominant suboligomeric form of *Ta*HSP16.9. The formation of tetradecamers upon raising the temperature is intriguing. The increase in oligomerization could be a result of the heated electrospray process wherein the *Ta*HSP16.9 monomers and dodecamers cluster together nonspecifically.[36,37] If this were the case, however, one would expect to see predominantly the formation of tridecamers rather than tetradecamers. This increase in oligomerization could be a result of self-chaperoning activity, whereby intact dodecamers bind unfolded monomers to prevent their aggregation. However, as unfolded monomers remain in solution at elevated temperatures (Figure 5, upper panel), an alternative and more likely explanation is that at higher temperatures some of the *Ta*HSP16.9 dodecamers incorporate additional subunits to increase their oligomeric size and polydispersity. This could potentially cause an increase in substrate specificity and improved chaperone efficiency.

These observations of *Ta*HSP16.9 over a range of temperatures allow the delineation of the equilibria for the protein complex in solution (Figure 5, top panel inset). At room temperature, the protein forms a specific dodecamer, but as the temperature is raised, the equilibria shift away from this oligomeric form. The first distinct change observed at elevated temperatures is dissociation into dimers and monomers. Such dissociation of sHSPs at heat-shock temperatures has been reported previously[26,28] and is thought to have important functional implications.[24,25] The second change that occurs is an increase in size and polydispersity of the protein at higher temperatures. The related α-crystallins have been shown to increase in size as the temperatures is increased,[38,39] but such a distinct shift away from monodispersity has not been observed previously and may have important consequences for the chaperone action of this protein.

CONCLUSIONS

In this report, we have detailed the construction and characterization of a nanoESI probe that can be used to control the temperature of the sample solution. By monitoring the unfolding of a protein we have shown this probe gives results that agree well with fluorescence spectroscopy, and by examining the behavior of an sHSP complex at a range of temperatures, we have demonstrated its applicability to the study of multimeric protein complexes. Moreover, this probe has given insight into the behavior of a protein complex that has remained elusive by using other techniques. The results detailed here extend the applications of nanoESI in the study of biochemical properties of noncovalent complexes to include temperature-dependent structural changes in solution.

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