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ACCELERATED ARTICLE

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Direct Monitoring of Heat-Stressed Biopolymers with Temperature-Controlled Electrospray Ionization Mass Spectrometry

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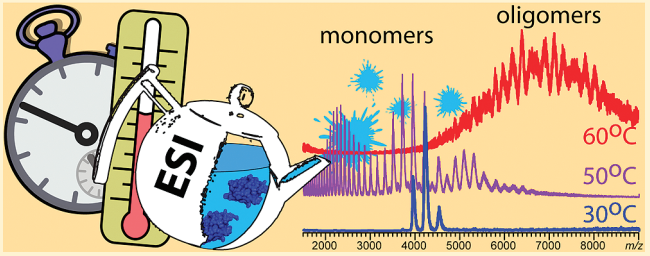
Supporting Information

ABSTRACT: The ability to monitor protein aggregation at the molecular level is critical for progress in many areas of life sciences ranging from understanding mechanisms of amyloidosis and etiology of conformational diseases to development of safe and efficient biopharmaceutical products. Despite the spectacular progress in understanding the mechanisms of protein aggregation in recent years, many aspects of the aggregating proteins behavior remain unclear because of the extreme difficulty in tracking evolution of these notoriously complex and heterogeneous systems. Here, we introduce a mass spectrometry-based methodology that allows the early stages of heat-induced aggregation to be studied by monitoring both conformational changes and formation of oligomers as a function of temperature. The new approach allows biopolymer behavior (both reversible and irreversible processes) to be monitored in a wide temperature range. Validation of the methodology is carried out by comparing temperature profiles of model proteins and nucleic acids deduced from mass spectrometry measurements and differential scanning calorimetry. Application of the methodology to study heat-induced aggregation of human glucocerebrosidase unequivocally links loss of conformational fidelity to formation of soluble oligomers, which serve as precursors to aggregation.



Protein aggregation is a phenomenon that is frequently encountered in many fields of biology and biophysics. The negative connotation that is commonly attached to this phenomenon is mostly due to the critical role it plays in a variety of neurodegenerative disorders,[1] but the occurrence of aggregation almost invariably leads to problems in many other areas as well. Despite the significant recent advances in deciphering the molecular mechanisms of biopolymer aggregation, numerous gaps in our understanding of this phenomenon still remain. One of the reasons for this is the extreme difficulty in tracking the aggregation processes using the existing experimental tools. Indeed, the populations of aggregating proteins are very complex, exhibiting a high degree of structural heterogeneity (both in terms of oligomerization state of individual species and conformation of their constituents). Furthermore, the highly transient nature of such systems makes it nearly impossible to track their behavior in real time using existing experimental techniques.

Electrospray ionization mass spectrometry (ESI-MS) has become an indispensable tool in the studies of protein higher order structure and dynamics because it allows macromolecular properties to be probed under a variety of conditions, including analysis of nonnative conformations of transient species, such as protein folding intermediates.[2] In recent years, ESI-MS has also been applied to study protein aggregation,[3,4] although such studies are typically limited to the analysis of various species that are populated at the end-point of the aggregation process. The ability of ESI-MS to provide information on biopolymer complexes in real time has been used in the past to monitor ordered dissociation and assembly of protein oligomers;[5−7] however, detailed characterization of the aggregation process remains out of reach of ESI-MS.

The transient nature of protein aggregation is its inherent feature that also makes it very difficult to provide accurate description of this process. Once the aggregation process is initiated, it proceeds very fast, making it extremely difficult to produce distinct snapshots. Even though ESI-MS provides an elegant way to obtain snapshots of various dynamic processes, a feature that has been used in the past to study phenomena ranging from protein interaction with their endogenous ligands to enzyme catalysis,[8] straightforward application of ESI-MS to online monitoring of aggregation processes is impractical due to unfavorable time scale. Therefore, it is not surprising that most studies of protein aggregation focus on the end points of this process, that is, early precursors to aggregation and the high molecular weight oligomers.

Another factor that greatly complicates the analysis of the aggregation process is the astonishing polymorphism exhibited by both its products and the intermediate states.[9] Earlier attempts to reduce the complexity of these ensembles by using a combination of separation techniques (e.g., size-exclusion chromatography, SEC) and ESI-MS were only partially successful, since the intermediate oligomeric species collected as SEC fractions are very dynamic and appear to continue to evolve after (and perhaps even during) the separation process.[4] As a result, a detailed mechanistic understanding of protein aggregation is still lacking, and the role of various nonnative protein states in this process remains a subject of ongoing debates.[10−15] In fact, molecular dynamics simulation in many cases

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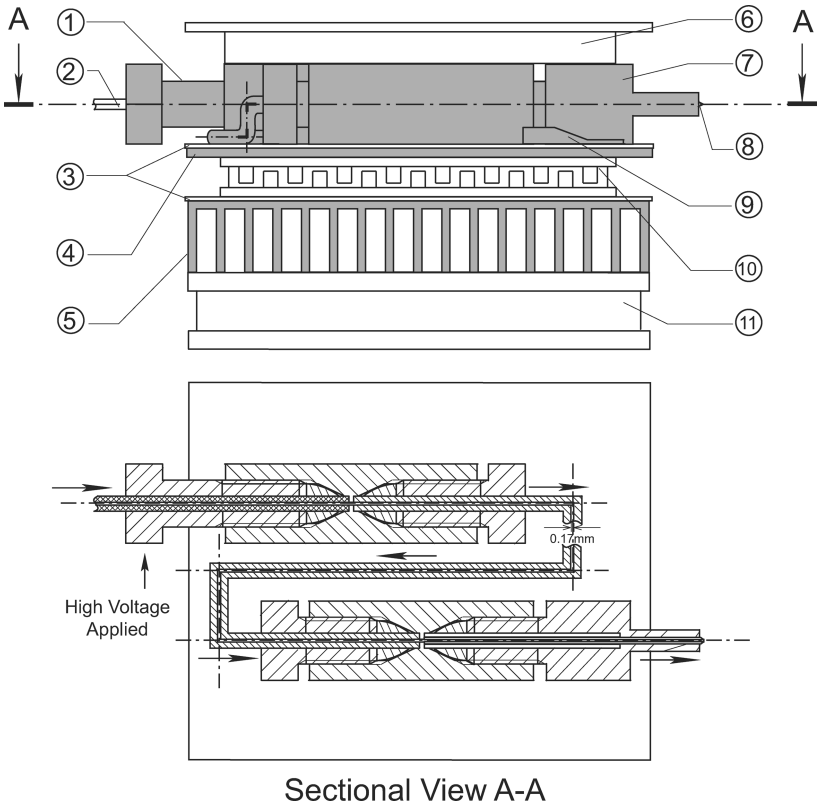

Figure 1. Schematic diagram of a heated ESI source: 1, electrospray head; 2, sample injection tubing; 3, thermal interface pad (thermal conductor, electrical insulator); 4, copper plate; 5, heat sink; 6, electrical insulation; 7, heat shield; 8, spray emitter; 9, control thermistor; 10, thermoelectric cooler; 11, fan.

is the only viable option as far as providing description of the aggregation process throughout the entire pathway.[16,17]

Protein aggregation can be modulated in vitro by a variety of factors, and protein solution temperature is one of the parameters that have a profound effect on this process. The effect of heat on protein behavior is widely exploited in biotechnology, for example, in accelerated stability studies, in which products are stressed by exposing them to high temperatures. However, dramatic changes in physical and chemical properties that frequently occur during stress-testing of proteins are difficult to interpret, which makes meaningful analysis extremely difficult. The capability of ESI-MS to explore both conformation and binding properties of biopolymers makes this technique an attractive tool for characterizing protein behavior under heat-stress conditions. Nonetheless, even though the idea to monitor protein behavior in solution as a function of temperature using ESI-MS was articulated nearly two decades ago,[18] very few examples of using ESI-MS to study protein behavior as a function of temperature have been reported so far.[19−22]

In this work, we approach the task of following the progress of aggregation by exploiting the correlation between the protein stability and temperature. As the temperature of the protein solution increases, loss of conformational integrity manifests itself in ESI-MS through a profound change in the ionic charge state distributions, while formation of oligomers can be easily detected by monitoring the masses of various ionic species. Although protein aggregation is almost always implicitly assumed to be an irreversible process, reversibility of some steps (particularly in the beginning) cannot be ruled out. In fact, the ability to shift equilibria during these early steps might become absolutely critical in designing efficient therapies for a variety of pathological conditions in which amyloidosis is implicated in disease etiology.[23−25] A new design of a temperature-controlled ESI source presented in this work allows both reversible and irreversible processes to be studied in heat-stressed biopolymers with high accuracy. The design features of the new source eliminate artifacts that interfere with the detection of temperature-induced changes of biopolymer properties. Validation of the new technique is carried out by performing parallel characterization of several model systems with differential scanning calorimetry (DSC) and temperature-controlled ESI-MS. The new source is also used to monitor heat-induced aggregation of a 63 kDa glycoprotein human glucocerebrosidase (GCase).

MATERIALS AND METHODS

The design of the temperature-controlled ESI source (Figure 1) incorporated a long metal heat shield to eliminate cooling of the protein solution during the sample introduction to the ESI interface. Utilization of a large-diameter capillary (20 μm) and the heat shield, which covers the entire ESI capillary, results in a dramatic decrease of heat exchange between the protein solution and the ambient prior to its introduction to the ESI interface. Enclosing of the entire ESI capillary inside a metallic heat shield provides thermal contact between the heating element and the capillary throughout its entire length, while the large diameter of the capillary reduces the heat loss and dissipation from its tip due to decreased surface-to-volume ratio. The heat shield also enhances heat propagation from the heating element through the bulk of the metal. Both phenomena greatly enhance the ability of the assembly to maintain uniform temperature throughout the entire length of the ESI capillary.

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The memory effect was eliminated by switching from a classic static nano-ESI setup to a continuous-flow scheme, in which a protein solution is pumped through the heated capillary to the ESI interface. An S-shaped metal capillary within the heated volume was used to increase the efficiency of the heating process. Temperature control in the ESI source was carried out using a thermoelectric cooler (TE Technology, Traverse City, MI), as described previously by Robinson et al.[20] The syringe supplying protein solution to the ESI source was not grounded to eliminate electrochemical changes of solution pH. The operator needs to wear insulating rubber gloves during all sample handling steps that involve touching the syringe or syringe pump to avoid the possibility of an electrical shock caused by the build-up of static electricity. The new source was retrofitted to a QStar-XL (ABI/Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer. All DSC measurements were performed with a VP-DSC (MicroCal LLC, Northampton, MA) microcalorimeter.

Cytochrome *c*, myoglobin, and lysozyme were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DNA single strands (ACAGGTAATCAGTATGACGAA and ATTCGTCATACTG-ATTACCTGT) were purchased from Eurofins MWG Operon (Huntsville, AL). Incongruent DNA strands were used to eliminate the possibility of signal interference in ESI-MS. GCase sample was provided by Shire HGT (Cambridge, MA). All other chemicals and reagents were of analytical grade or higher. All protein solutions were prepared in 20 mM ammonium acetate. The dsDNA sample was prepared by annealing the two complementary strands using a standard procedure; the DNA solution was prepared in 150 mM ammonium acetate.

RESULTS AND DISCUSSION

Optimization of the Temperature-Controlled ESI Source: Elimination of the Heat Loss, Redox Processes, and Memory Effect. The common shortcoming of existing methods to monitor biopolymer behavior directly in solution in a temperature-controlled fashion is the rapid cooling of the analyte solution during its transfer to the ESI interface region. Robinson et al.[20] and, later, Heck et al.[21] sought to minimize this effect by implementing a scheme in which small sample volumes were heated directly in the nano-ESI capillaries prior to the sample introduction to the ESI interface. This technique has been successful as a means to monitor irreversible processes in solution (e.g., dissociation and subunit exchange in large macromolecular assemblies). However, temperature-induced unfolding of monomeric proteins had never been demonstrated with this design, unless applied to systems that undergo thermal unfolding irreversibly (e.g., proteins with significant number of disulfide bonds under acidic conditions).

Our goal was to probe both reversible and irreversible changes in biopolymer higher order structure at elevated temperature. However, when the standard approach[20] was applied to study heat-induced conformational changes in proteins that unfold reversibly (e.g., myoglobin), no signs of thermal denaturation were observed in ESI-MS, even at very high temperature. Myoglobin is a small protein that undergoes thermal denaturation at 70.8 °C,[26] which results in both loss of native conformation and dissociation of the prosthetic heme group. The former process manifests itself in ESI-MS via dramatic changes in protein ion charge state distributions,[27] and the latter can also be easily detected because it results in a significant mass change. However, none of the expected changes were observed in ESI-MS even when the sample capillary was heated to 90 °C (see the Supporting Information for more detail). Among several possible reasons that make it difficult to observe reversible loss of higher order structure when using standard technique,[20] rapid cooling of the protein solution upon exiting the heated part of nano-ESI source seems most likely. Indeed, passage of the analyte solution through the part of the nanoemitter not covered by the heating element should result in a significant heat transfer to the ambient environment due to the very large surface-to-volume ratio of the emitter tip. As a result, reversibly unfolded proteins have enough time to refold prior to the moment when the protein solution is introduced to the ESI interface and the ionic signal is produced.

Among the several small proteins tested, only acidified lysozyme showed clear signs of unfolding, as suggested by the appearance of high charge-density protein ions in mass spectra acquired at elevated temperature (see the Supporting Information for more detail). We note, however, that the extent of multiple charging exhibited by lysozyme ions under these conditions is similar to that observed in protein molecules affected by disulfide scrambling (a process that is known to be greatly catalyzed at high temperature[28]), which locks the protein irreversibly in an unfolded state. Therefore, the anomalous behavior of lysozyme highlights another problem with using conventional static nanospray in temperature-controlled ESI-MS measurements—namely, the memory effect. Indeed, placing the protein solution in a small, heated capillary for the entire duration of the experiment may cause uncontrolled protein degradation. This may obviously interfere with the measurement process and is likely to obfuscate evaluation of protein conformation and its other characteristics as a function of temperature, rather than heat exposure.

We approached the goal of building a temperature-controlled ESI source for studies of both reversible and irreversible processes in solution by modifying Robinson's original design,[20] with the aim of suppressing the heat loss and eliminating the memory effect. Rapid cooling of the protein solution in the ESI emitter was eliminated by using a metal heat shield covering the entire ESI capillary (Figure 1) and large-diameter (20 μm) capillaries as ESI emitters. This resulted in a dramatic decrease in heat exchange between the protein solution and the ambient prior to its introduction to the ESI interface due to a lower surface-to-volume ratio of the tip of the emitter. Enclosing the entire ESI capillary inside a metallic heat shield provides thermal contact between the heating element and the capillary throughout its entire length, and the increased capillary diameter reduces the heat loss and dissipation from its tip due to increased surface-to-volume ratio. This greatly enhances the ability of the ESI source to maintain uniform temperature throughout the entire length of the capillary. The memory effect was eliminated by switching from the "static" nano-ESI setup to a continuous-flow scheme in which a protein solution is pumped through the heated capillary to the ESI interface. These new design features resulted in dramatic improvement of our ability to observe heat-induced denaturation in small proteins (e.g., see the results of myoglobin unfolding presented in the Supporting Information).

Another problem that was encountered during the design of the temperature-controlled ESI source was the need to eliminate electrochemical effects leading over time to lowering the solution pH and, as a consequence, protein unfolding. Grounding the syringe supplying protein solution to the ESI source (a standard procedure recommended by the instrument manufacturer to avoid build-up of static electricity) resulted in apparent acidification of the solution, followed by protein unfolding even at room temperature, a phenomenon that was previously observed and reported by Van Berkel[29] and Konermann.[30] To eliminate the solution pH changes leading to false-positive signals of protein

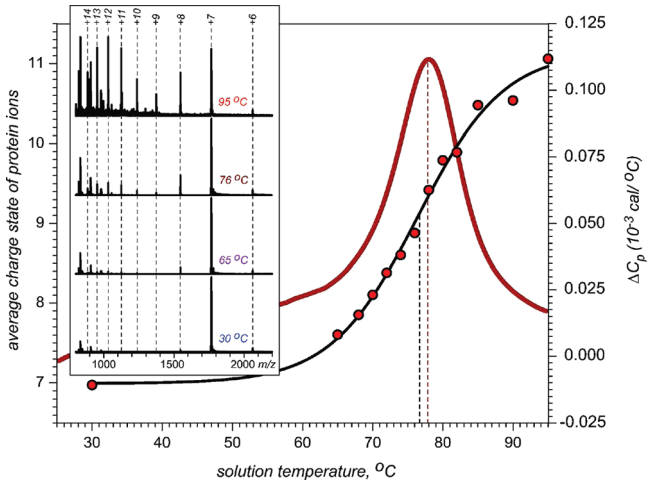

Figure 2. Thermal unfolding of cytochrome c (1 μM in 20 mM ammonium acetate, pH 4.7) monitored with temperature-controlled ESI-MS (red dots/black curve) and DSC (brown curve). The two dashed lines show midpoints of transitions determined by the two techniques. ESI mass spectra of cytochrome c at several different temperatures are shown in the inset.

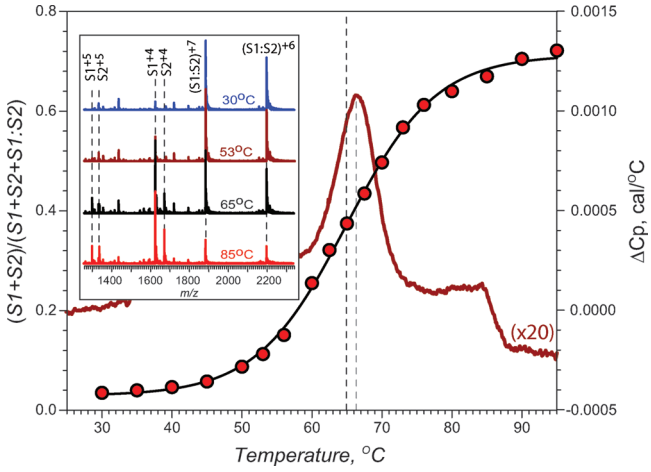

Figure 3. Thermal dissociation of dsDNA composed of complementary strands S1 and S2 (10 μM in 150 mM ammonium acetate, pH 6.8) monitored with temperature-controlled ESI-MS (red dots/black curve) and DSC (brown curve). The two dashed lines show midpoints of transitions determined by the two techniques. ESI mass spectra of dsDNA at several different temperatures are shown in the inset.

unfolding, the syringe was kept ungrounded during all measurements. This resulted in complete elimination of the solution acidification (as suggested by the measurement of the pH of the sprayed solution) and any false-positive signals of protein unfolding (see the Supporting Information for more detail).

Precision of Detecting Reversible Changes in Biopolymer Higher-Order Structure: Small Protein Unfolding. Accuracy of the new source vis-à-vis detection of reversible conformational changes was evaluated by comparing the melting temperatures obtained using temperature-controlled ESI-MS and DSC. As was already mentioned in the preceding section, monitoring protein ion charge state distributions provides an easy and reliable way to monitor the integrity of protein conformation, in which the appearance of high-charge-density ions in the mass spectra signals a large-scale conformational transition.[31] These transitions also result in significant changes in the heat capacity, enabling their detection by DSC.[32]

Cytochrome *c* is a small protein, with a covalently attached heme group, that is known to undergo reversible thermal denaturation.[33] A DSC profile of cytochrome *c* reveals a single transition at 78 ± 1 °C (Figure 2). Monitoring charge state distribution of cytochrome *c* ions in ESI-MS using the same solution parameters as in DSC experiments suggests that the protein remains folded until the solution temperature is raised above 65 °C, at which time the emergence of the high-charge-density protein ions provides a clear indication that a fraction of the protein molecules begin to populate non-native (less compact) states (inset in Figure 2). Plotting the average charge of cytochrome *c* ions as a function of temperature in the 65−95 °C range results in a sigmoidal curve with a transition midpoint at 76 ± 2 °C. This value is within the experimental error of the melting point generated by DSC.

Precision of Detecting Reversible Changes in Biopolymer Higher-Order Structure: Dissociation of DNA Duplex. Monitoring ionic mass in ESI-MS provides a very effective way to monitor the integrity of biopolymer assemblies.[34] An example of a reversible heat-induced dissociation of a biopolymer assembly is presented in Figure 3, where melting of a DNA duplex (made by annealing two complementary strands) is monitored over a 25−95 °C temperature range. Evolution of the fraction of singlet vs total (singlet and duplex) DNA signal is used to calculate the transition midpoint as 64.9 ± 2 °C. DSC analysis of this DNA duplex carried out under the same conditions as ESI-MS measurements (solvent composition and oligonucleotide concentration) yields a melting temperature of 66.4 ± 1 °C (Figure 3), which is within the experimental error of the value produced by ESI-MS.

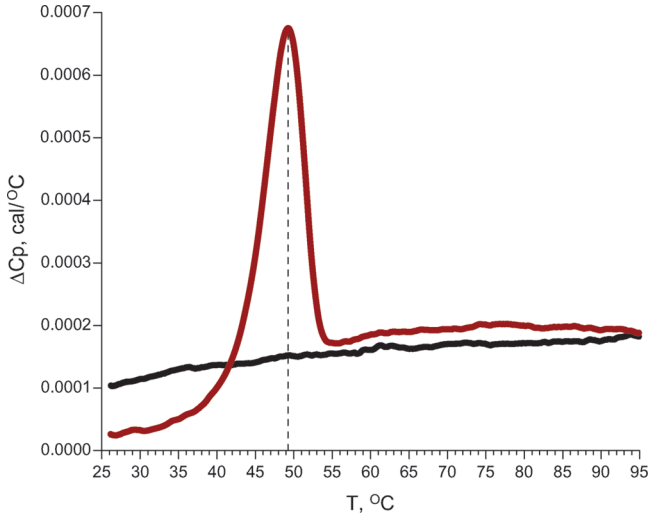

Figure 4. DSC profile of GCase (1 μM in 20 mM ammonium acetate, pH 4.7) showing a transition at 49 °C (brown trace). The black trace represents a rerun of the DSC experiment for the GCase sample that already went through a single cycle of DSC measurements.

Heat-Induced Protein Aggregation: Probing Convoluted Irreversible Processes with Temperature-Controlled ESI-MS. Following the validation of the new temperature-controlled ESI source with parallel DSC measurements, the new technique was applied to probe the behavior of larger protein systems prone to aggregation. Figure 4 shows a DSC profile of human glucocebrosidase (GCase), a 63 kDa glycoprotein, which became a standard treatment of the Gaucher's disease.[35] As is the case with all biopharmaceutical products, certain environmental factors may

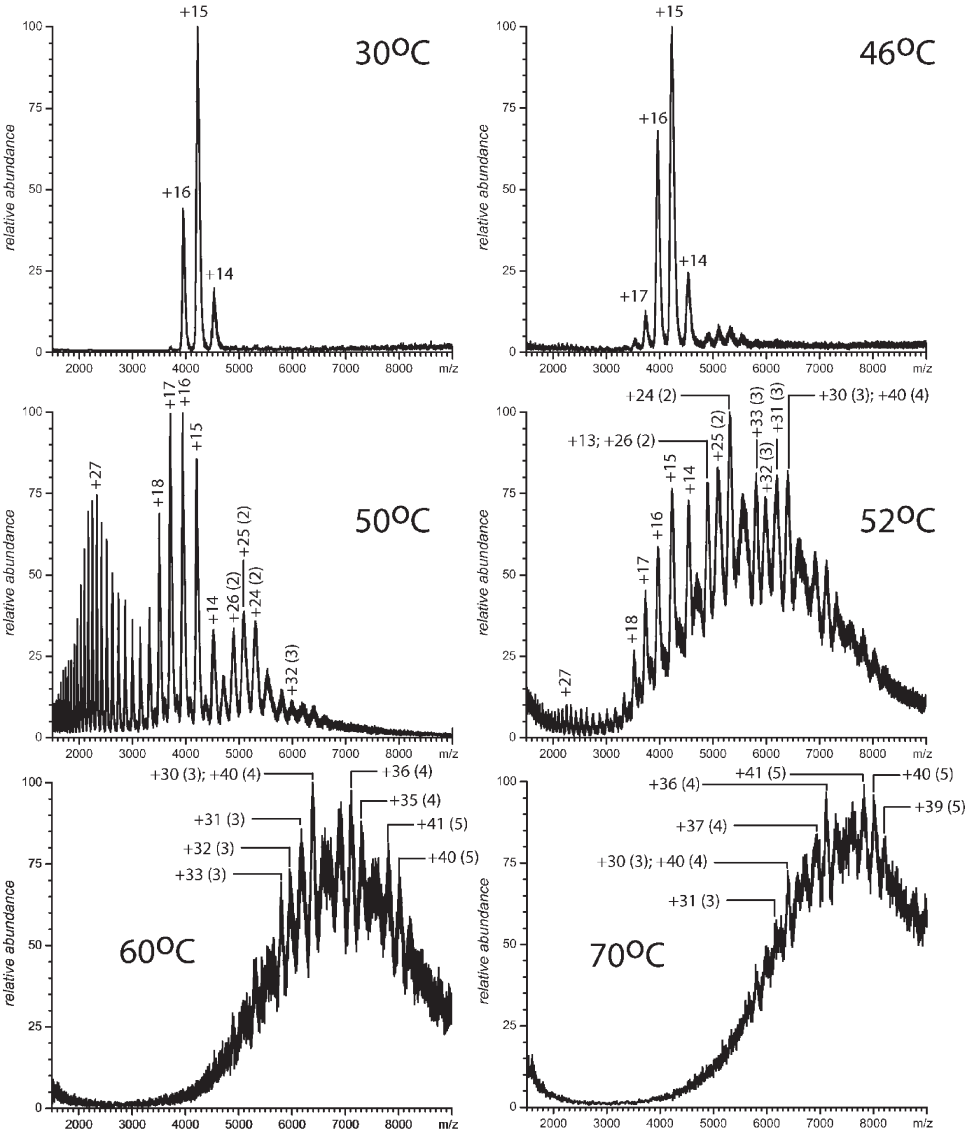

Figure 5. ESI mass spectra of GCase (1 μM in 20 mM ammonium acetate, pH 4.7) recorded at various solution temperatures. The numbers without parentheses indicate charge states of the GCase monomers; the numbers in parentheses indicate the size of the protein associations.

trigger GCase aggregation, a process that is of primary concern when stability, efficacy, and safety of protein-based therapies are considered.[36] Heat stress is frequently used to test protein instability; however, classical biophysical techniques that are commonly employed to detect the onset of GCase loss of stability in response to elevated temperature (such as fluorescence spectroscopy[37] or DSC[38]) do not provide a detailed description of this process at a molecular level. For example, the DSC profile of GCase (Figure 4) clearly shows a well-defined transition at 49 °C, in agreement with previously published work.[38] The asymmetric shape of the peak suggests that the transition is irreversible, a notion that is confirmed by the essentially flat appearance of the "second pass" DSC profile. However, very little can be said about the nature of the underlying processes based on the DSC data alone.

On the other hand, examination of the behavior of heat-stressed GCase with temperature-controlled ESI-MS provides very rich information content. Indeed, it not only allows the unfolding events to be detected but also yields detailed information regarding specific molecular events that accompany such transitions (Figure 5). Although changes in the extent of multiple charging of protein ions are relatively minor in the 25−46 °C range, further increase of the protein solution temperature clearly leads to a large-scale loss of structure, as evidenced by the dramatic changes in the protein ion charge state distribution (i.e., the appearance of the high-charge-density protein ion peaks centered around charge state +27). This loss of structure at the level of GCase monomers coincides with the onset of GCase oligomerization. Although the extent of oligomerization observed at 50 °C is relatively modest, further temperature increase results in progressive growth of their sizes and abundance. Thus, temperature-controlled ESI-MS provides a unique ability to monitor the emergence and evolution of soluble protein oligomers, which are likely to play important roles in aggregation.

The protein solution flow rate was maintained at 1.0 μL/min for all measurements presented in Figure 5, which corresponds to 2.0 min of heat exposure. The duration of the heat stress can be easily changed simply by varying the flow rate, and Figure 6 shows the behavior of heat-stressed GCase at 55 °C as a function of the stress duration. One can clearly see that the unfolded monomers (represented in ESI-MS

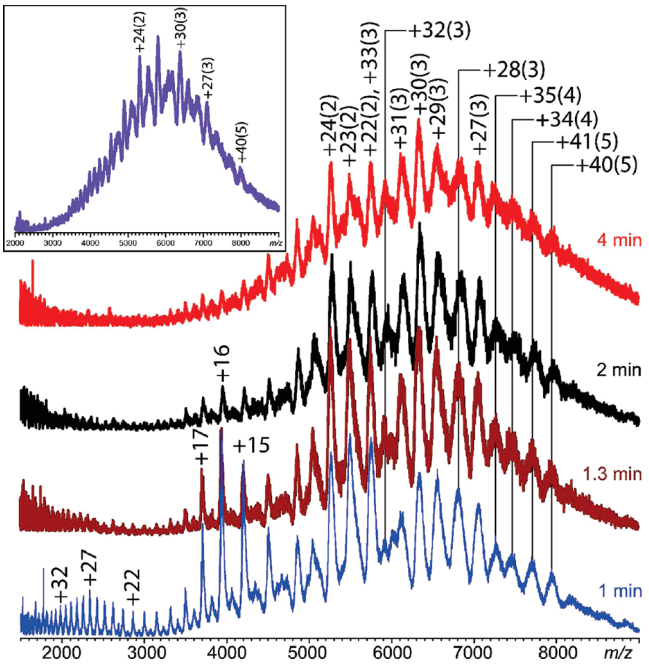

Figure 6. Kinetics of heat-induced aggregation of GCase at 55 °C (1 μM in 20 mM ammonium acetate, pH 4.7): heat expose time is varied by changing the protein solution flow rate in the ESI source. The inset shows an ESI mass spectrum of GCase incubated at 55 °C for 1 h, followed by cooling the solution to room temperature (the mass spectrum was acquired at room temperature).

by charge states higher than +18) are present in solution only following relatively brief heat exposure, but prolonged heating results in nearly complete elimination of such species and an increase in the abundance of ionic species representing GCase oligomers. A reference mass spectrum for the end-point of heat stress at 55 °C was obtained by incubating GCase at this temperature for 1 h, followed by cooling the protein solution to room temperature and acquisition of ESI-MS data at room temperature. The resulting reference spectrum (inset in Figure 6) is very close to the mass spectrum of GCase exposed to 55 °C for 4 min (e.g., complete absence of the protein monomer peaks and intensity distribution of oligomers peaks).

Analysis of the behavior of GCase as a function of both solution temperature and exposure time to heat clearly suggests that protein unfolding is the initial step leading to large-scale oligomerization of the heat-stressed GCase. The transient nature of this process is emphasized by the fact that the unfolded GCase monomers (represented in ESI-MS by high charge density protein ions at m/z < 3000) are observed only in the narrow temperature range around 50 °C (Figure 5). Appearance of unfolded GCase monomers coincides with the onset of protein oligomerization, and a further temperature increase results in a further shift of quasi-equilibrium toward larger oligomeric species. No unfolded monomers are seen in ESI-MS at (and above) 55 °C under the quasi-equilibrium conditions, suggesting that they become converted to the oligomeric species on a faster time scale. Indeed, decreasing the heat exposure time at this higher temperature (Figure 6) leads to the reappearance of high-charge density monomer ions in ESI-MS, suggesting that unfolded GCase monomers are transiently populated, even above the transition temperature. Taken together, these observations provide unequivocal evidence that unfolded GCase monomers are obligatory intermediates en route to aggregation.

The study of GCase behavior in response to a heat stress presented in this work demonstrates that the temperature-controlled ESI-MS measurements allow the initial stages of protein aggregation to be characterized in great detail. Indeed, the ability to make a distinction between various biopolymers species (based on the differences in their masses) and their conformers (based on the differences in their charge state distributions) allows the temperature-controlled ESI-MS measurements to be carried out in complex systems with very high degree of specificity. This unique feature of the new experimental technique (coupled with the ESI-MS ability to obtain mass information on very large biopolymers and their assemblies) makes it very appealing to the biotech and biopharmaceutical sectors, where the need to engineer/formulate stable biopolymer-based products (e.g., protein or nucleic acid drugs) places a premium on the ability to characterize their behavior as a function of temperature with a high degree of precision and accuracy. Likewise, the ability to observe and interpret changes in aggregation pathways caused by small molecules and therapeutic proteins will be invaluable for the ongoing efforts to design effective medicines targeting amyloidosis-related disorders.

CONCLUSIONS

Understanding the molecular mechanisms of protein aggregation critically depends on availability of reliable and robust methods to monitor these processes. Heat stress remains one of the most popular approaches to evaluate the stability of aggregation-prone proteins in the biopharmaceutical industry, but the experimental techniques that are currently used in such studies do not provide detailed information on protein aggregation at the molecular level. The ESI-MS-based methodology presented in this work allows early stages of heat-induced protein aggregation to be studied by monitoring both conformational changes and formation of oligomers as a function of temperature. Validation of the new methodology with model proteins and nucleic acids confirms its ability to obtain accurate information on reversible, solution-phase processes. Furthermore, the new technique allows both reversible and irreversible processes involving biopolymers to be studied in a time-dependent fashion, in which unfolding and formation of oligomers are correlated with both temperature and duration of heat exposure. These capabilities of the new methodology are highlighted by using it to study heat-induced aggregation of a biopharmaceutical product, human glucocerebrosidase.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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