The observation of chaperone-ligand noncovalent complexes with electrospray ionization mass spectrometry

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(RECEIVED October 27, 1997; ACCEPTED January 13, 1998)

Abstract

Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) was applied for the study of noncovalent chaperone SecB-ligand complexes produced in solution and examined in the gas phase with the aid of electrospray ionization (ESI). Since chaperone proteins are believed to recognize and bind *only* with ligands with nonnative tertiary structure, this work required careful unfolding of the ligand and subsequent reaction with the intact chaperone (the noncovalent tetrameric protein, SecB). A high denaturant concentration was employed to produce nonnative structures of the OppA, and microdialysis of the resulting solutions containing the chaperone-ligand complexes was carried out to rapidly remove the denaturant prior to analysis. Multistage mass spectrometry was essential to the successful study of these complexes since the initial mass spectra indicated extensive adduction that precluded mass measurements, even after microdialysis. However, low energy collisional activation of the ions in the FTICR trap proved useful for adduct removal, and careful control of excitation level preserved the intact complexes of interest, revealing a 1:1 SecB:OppA stoichiometry. To our knowledge, these results present the first direct observation of chaperone-ligand noncovalent complexes and the highest molecular weight heterogeneous noncovalent complex observed to date by mass spectrometry. Furthermore, these results highlight the capabilities of FTICR for the study of such complex systems, and the development of a greater understanding of chaperone interactions in protein export.

Keywords: dissociation; Fourier transform ion cyclotron resonance; MS/MS; OppA; protein export; SecB; stoichiometry

Mass spectrometry has evolved to contribute to biological research on many frontiers (Ganem et al., 1991; Goodlett et al., 1993; Light-Wahl et al., 1993; Gale et al., 1994; Verentchikov et al., 1994; Little et al., 1995; Liu et al., 1995; Loo, 1995; Fitzgerald et al., 1996; Muddiman et al., 1996; Smith et al., 1996). The analysis of many types of biomolecules, including DNA, RNA, proteins, and their noncovalent complexes with mass spectrometry is rapidly becoming of great utility. However, protein biosynthesis, including all steps required to produce a fully folded and active protein, is not completely understood on a molecular level and many phases of the process are not yet clearly defined. While the translation of genetic code to the amino acid sequences that make

up specific peptides and proteins is probably the most known facet of protein biosynthesis and is widely used in recombinant methodologies, the phenomena that follow synthesis have proven to be topics not easily addressed by analytical techniques. Central questions of protein biosynthesis relate to the mechanisms of acquisition of the native structure of proteins and their proper localization. Both processes involve protein chaperones that specifically recognize polypeptides that have nonnative structure. During localization of proteins, the polypeptides often cross membranes. Studies with eukaryotic and prokaryotic systems have shown that secretion of protein via the endoplasmic reticulum, import of protein into the mitochondria, and export of protein into the periplasm of gram negative bacteria all involve transport of polypeptides through membranes before the protein folds into its native structure. Chaperones play a crucial role in these processes by interacting with the polypeptides during or shortly after synthesis, before they acquire their "native" structure, and facilitate transport. Our two research groups have worked closely together for the past few years to determine if mass spectrometry can provide useful information on this area of

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chaperone interaction. We have used SecB, a chaperone involved in protein export in *Escherichia coli*, as our model system. A prime directive for this work is the application of mass spectrometry to provide a greater understanding of both the role chaperone proteins play in the export of protein to the periplasm and their remarkable ability to recognize the nonnative structures of many different proteins with no consensus in primary sequence.

Previous work resulting from the collaboration between our groups has led to the determination of the oligomeric structure of SecB (Smith et al., 1996). This issue is key to the understanding of the unique capabilities of chaperones like SecB and had been difficult to definitively determine with other techniques. Crystallization of SecB has been problematic (Vrieling et al., 1995), and in addition, the complex may deviate from ideal spherical structure, complicating the interpretation of size-exclusion data.

Here we present results that demonstrate the observation of noncovalent complexes between the *E. coli* chaperone protein, SecB (Watanabe & Blobel, 1989; Randall et al., 1990; Hardy & Randall, 1991; Randall, 1992; Vrieling et al., 1995; Smith et al., 1996; Randall et al., 1997) and a ligand, the periplasmic oligopeptide binding protein, OppA (Tame et al., 1994; Tame et al., 1995). To our knowledge, these results represent the first reported observation of an intact chaperone-ligand complex and the highest molecular weight heterogeneous complex (i.e., a complex of two different proteins) observed thus far by mass spectrometry. These results highlight the potential mass spectrometry has for providing information on biological systems that can be extremely difficult or intractable to study by other means. Finally, this work also demonstrates the utility of sample clean up techniques, both before electrospray ionization with microdialysis, and after accumulation in the mass spectrometer with in-cell clean up procedures.

Results and discussion

The *E. coli* chaperone protein SecB is itself a noncovalent tetramer composed of four identical subunits, each with a calculated average molecular weight of 17,147 kDa. It was previously observed that about half of the SecB monomers are covalently modified and exceed the predicted mass by 42 kDa (Smith et al., 1996). Using tryptic digestion and MALDI-TOF-MS, this modification was determined to be acetylation of the N-terminus (V.F. Smith and L.L. Randall, pers. comm.). Accurate molecular weight measurements of this tetramer have been reported with ESI-FTICR-MS with the utilization of low amplitude collisional activation to remove noncovalent adducts from the trapped ions (Pasa-Tolic et al., 1998). Figure 1A shows the ESI-FTICR mass spectra of SecB tetramer complexes without collisional activation to remove noncovalent adducts and resulted in a MW measurement of $68,757 \pm 50$ Da (95% confidence limits, $n = 3$). Resolution sufficient for detection of tetramer species having varying numbers of acetylated monomers was not achieved due to the effective degradation of resolution resulting from the high levels of adduction. Therefore, the average MW (taken as the centroid of the broad peaks) is likely to reflect an average of the various levels of acetylated monomer incorporation and should be higher than predictions based on the nonacetylated monomer MW (68,588 kDa). Selected-ion accumulation (SIA) (Bruce et al., 1995) and application of the in trap cleanup method for the 16+ charge state of the tetramer induced significant adduct removal and resulted in higher resolution spectra revealing tetramers with various numbers of acetylated monomers in addition to the unmodified tetramer (i.e., with no acetylated

Fig. 1. ESI-FTICR mass spectra of SecB (44 micromolar in 10 mM NH₄OAc). **A:** Spectrum showing the dominant tetramer ions. **B:** MS/MS spectrum of the 16+ tetramer, illustrating asymmetric dissociation of tetramers to yield trimer and monomer ions with nearly equal charge states. Also observed in the CAD of the tetramers are the covalent modifications of SecB monomers (42 Da) reported previously, indicating that the modification does not prevent tetramer formation. Q = SecB tetramer charge state; M = SecB monomer charge state; T = SecB trimer charge state.

monomers) and a MW of 68,620 kDa (Pasa-Tolic et al., 1998). The MS/MS spectrum in Figure 1B illustrates the SIA and dissociation of the 16+ tetramer to give predominantly the 8+ monomer and 8+ trimer ions, similar to the asymmetric dissociation previously observed with streptavidin tetramers (Schwartz et al., 1995). While 1 Da mass resolution was not obtained on the complex itself, collisional activation of the complex did produce high resolution spectra for the monomeric species. The average molecular weight from these measurements is $17,146 \pm 1$ and $17,187 \pm 1$ Da (95% confidence limits $n = 3$) for the unmodified and acetylated monomer, respectively. These results indicate that the calibration is less effective in the higher m/z region where the tetramer is observed (an issue that arises due to the difficulty in obtaining suitable reference compounds for this region), and possibly that additional noncovalent adduction presently serves to decrease mass accuracy for measurements of these larger complexes.

Under the conditions employed to obtain the spectra shown in Figure 1, the predominant species observed in the primary spectra was the intact SecB tetramer. Experiments performed at similar concentrations but at lower pH, showed predominantly the monomer species. However, experiments with 10 mM NH₄OAc and higher SecB concentrations (400 micromolar), revealed dimeric and trimeric complexes of SecB tetramers with a combined molecular weight of $206,310 \pm 50$ Da (95% confidence limits, $n = 5$) for the trimer of tetramers, as indicated in Figure 2. This is significantly higher than the predicted MW of such a complex (206,016 kDa for a complex with 50% inclusion of modified monomers), and likely indicates substantial adduction persists in this case. These complexes should not be considered physiologically relevant because they were observed only at concentrations well above the estimated intracellular concentration of SecB (1–3 micromolar). However, these results do illustrate the capability of ESI-FTICR-MS for the analysis of high molecular weight noncovalent complexes. This represents an important attribute for pro-

Fig. 2. ESI-FTICR mass spectrum of SecB (400 micromolar in 10 mM NH_4OAc) illustrating the higher MW complexes (likely due to aggregation in this case) that can be studied with ESI-FTICR-MS. Higher order complexes like the dimer (D) and trimer (TT) of tetramers (combined MW = ~206 kDa) were observed at relatively high mass-to-charge ratio as indicated.

spective MS-based chaperone studies since, complexes targeted for future study include the ligand-SecB-SecA ternary complexes which are the next species of interest along the protein export pathway, having a molecular weight of ~273 kDa plus the ligand mass. For example, a complex containing the ligand periplasmic oligopeptide binding protein OppA would have a MW of ~330 kDa.

The oligopeptide-binding protein (OppA) is a periplasmic substrate-binding protein that binds peptides of 2 to 5 amino acids in length and delivers them to a peptide transport system in the cytoplasmic membrane. As such, OppA is an example of a protein synthesized within the cell and exported to the periplasm space for function. The chaperone SecB promotes the efficient export of certain proteins, including OppA, by maintaining them in an unfolded state and delivering them to the translocase, a complex comprising the peripheral membrane protein SecA, mentioned above, and several integral membrane proteins (den Blaauwen & Driessen, 1996). Figure 3 illustrates ESI-FTICR-MS spectra of OppA obtained in 100 mM acetic acid and in a 10 mM NH_4OAc buffer (pH = 6.7). Relatively high charge states are typically observed with low pH solutions and this observation is likely due to the greater charging allowed by the less compact structure (Light-Wahl et al., 1994), as well as exposure of charge sites from interior sections of the molecule. The spectra obtained under acidic conditions lead to a molecular weight measurement of $58,809.3 \pm 0.6$ Da (95% confidence limits, $n = 10$ most abundant charge states) in excellent agreement with the predicted MW of 58,808 kDa.

Previous studies of SecB in complex with maltose-binding protein (Diamond et al., 1995; Diamond & Randall, 1997), galactose-binding protein (Khisty et al., 1995; Topping & Randall, 1997), and oligopeptide-binding protein (Smith et al., 1997), have shown that SecB interacts with these proteins only in their denatured state. Thus, the ligand oligopeptide-binding protein was denatured with guanidium chloride, as described in the experimental section. Figure 4 shows spectra illustrating the results obtained following the unfolding, mixing, dialysis, and analysis protocol outlined below. Figure 4A shows the high mass-to-charge region in which SecB-OppA complexes were observed with higher trapping voltages. As discussed above, the high levels of adduction present even after

Fig. 3. ESI-FTICR spectrum of the protein OppA. **A:** Illustrates the higher charge states observed in 100 mM acetic acid solution that lead to substantial protein unfolding. **B:** Illustrates a spectrum resulting from SIA of a single charge state and demonstrates the higher resolution achieved by limiting the trapped ion population to a smaller number of different species. **C:** Shows the lower charge states observed at higher pH (10 mM NH_4OAc solutions, pH 6.7).

dialysis can preclude mass measurements, and even observation of complex. However, the application of in-cell clean up with low amplitude swept excitation serves to remove much of this adduction, resulting in clearly resolved SecB-OppA complexes (Fig. 4B). Mass measurements for the complexes indicate an average molecular weight of $127,468 \pm 91$ Da (95% confidence limits, $n = 4$) in good agreement with the theoretical average complex MW of 127,394 kDa. These measurements provide the first MS measurements for chaperone-ligand complexes and indicate a 1:1 stoichiometry for these complexes. Further improvements in our measurement capabilities are likely to result from even more extensive adduct removal and implementation of proper calibrants in the higher m/z region.

A distinct advantage of the utilization of cyclotron excitation for adduct removal is the m/z selectivity and careful control of excitation levels attainable with such an approach, allowing the dissociation of the interfering adducts while preserving both the SecB tetramer and the SecB-OppA complex, as shown in Figure 4. Figure 5A shows a spectrum obtained by selectively accumulating a narrow region within the broad distribution of ions, in this case using lower trapping potentials and SIA from 3,000–6,000. A 1:1

Fig. 4. ESI-FTICR spectra of SecB-OppA complexes. **A:** Shows a typical spectrum obtained with this system. In the absence of adduct removal, mass measurements and observation of complexes were not possible. **B:** Illustrates the effects observed by activating the trapped ions with low amplitude cyclotron excitation. In this case, ions were excited with a low amplitude (28 Vpp) swept frequency covering the m/z range from 3,000 to 10,000 at 1 Hz/ μ s. 1:1 Complexes were observed for this system as indicated in this spectrum.

mixture of SecB tetramer and OppA (10 micromolar each) was prepared using the protocol outlined below. Single frequency excitation corresponding to irradiation of ions centered around m/z 4,645 was used for this accumulation to produce the spectrum

Fig. 5. **A:** Spectrum showing SIA of a lower m/z region obtained with lower trapping potentials and BBQE from m/z 3,000–6,000. Solution condition employed for these data included a 1:1 ratio of SecB tetramer to OppA (10 micromolar each). **B:** A narrow region of the broad distribution was selected for collisional activation (i.e., MS/MS) employing single frequency quadrupolar excitation (corresponding to m/z 4,645). **C:** More extensive excitation resulted in dissociation of the adducts and chaperone-ligand complex, as well as partial dissociation of SecB tetramer ions as indicated by the observation of SecB monomer species. A = OppA charge state; Q = SecB tetramer charge state; M = SecB monomer charge state; T = SecB trimer charge state.

shown in Figure 5B. Much greater S/N was obtained with these conditions by filling the trapped ion cell to capacity with species only covering an m/z range of ~ 50 Da. Cyclotron excitation at this frequency resulted in noncovalent adduct removal, as well as dissociation of SecB-OppA complexes, as shown in Figure 5C. Partial dissociation of SecB tetramer species was also observed, as indicated by appearance of SecB monomer and trimer charge states. Importantly, the precise control of dissociation afforded with the application of low amplitude cyclotron excitation combined with the multi-stage mass measurement capabilities of FTICR allows the application of mass spectrometry to complex systems such as the present chaperone-ligand complexes that were otherwise, intractable.

Conclusion

The present results demonstrate the unique capabilities afforded by FTICR-MS in conjunction with ESI for the study of biological systems involving large, fragile noncovalent complexes. The approach demonstrated here illustrates that these complexes can be studied with mass spectrometry by employing measures for sample clean-up, both microdialysis before ESI and injection into the mass spectrometer and in-cell activation for further adduct removal. The precise control provided by the excitation amplitude promotes nearly any chosen level of dissociation ranging from adduct removal, to disruption of the chaperone-ligand complex to dissociation of SecB tetramers. These capabilities have proven invaluable in the study of fragile complexes from complex solution conditions, and are expected to be of greater utility for the study of larger systems.

Anticipated future directions include the utilization of these capabilities in conjunction with time-resolved studies (Konermann et al., 1997) to follow protein folding and unfolding. Specifically, we will address the ability of SecB to bind to ligands that are in various stages of refolding following dilution from denaturant. As the time allowed for refolding increases, the ligand will acquire more structure and eventually be unable to bind to SecB. At that point, it will be no longer possible to observe the complex in the mass spectra. By conducting H/D experiments in parallel to the complex observation studies, formation of structural elements in the refolding intermediates can possibly be identified. Combining the results of these experiments should allow us to correlate the acquisition of structure by a ligand with the loss of its ability to bind SecB, shedding light on the mechanism of chaperone-ligand recognition.

Materials and methods

Protein preparation

SecB was purified as reported previously (Randall et al., 1990). The ligand protein, OppA, was provided by Jeremy Tame (University of York) and has been purified according to published methods (Tame et al., 1994). Initial concentrations of both SecB and OppA were determined by measurement of absorbance at 280 nm using extinction coefficients of 47,600 M⁻¹ cm⁻¹ and 122,300 M⁻¹ cm⁻¹, respectively. Concentrations are given as tetrameric SecB and monomeric OppA. Ultrapure guanidinium chloride (GuHCl) from ICN Biomedical was used to denature OppA; all other reagents were standard reagent quality.

Chaperone-ligand complex formation

A subtle, yet challenging aspect of the application of mass spectrometry to chaperone protein studies is related to the nonnative states of the ligand that must be surveyed. SecB readily binds to many proteins and peptides in nonnative states, but has no affinity for completely folded proteins. Thus, a complication of the study of chaperone-ligand binding is related to the production of nonnative ligand states while preserving the tetrameric complex of SecB and, furthermore, employing conditions conducive to observation of the complex with ESI-MS. One option that can be employed involves the use of permanently denatured ligands that are unable to assume tightly folded states and, thus, bind to SecB with high affinity under a variety of solution conditions. This is an option currently under exploration, but eliminates the possibility of examining dynamic effects on chaperone affinity observed as a function of the degree of ligand folding. The approach that we have employed for the present work is to unfold the ligand protein with the use of the denaturant GuHCl (3 M), rapidly dilute the denatured protein away from the denaturant in the presence of SecB to allow interaction, and then dialyze the solution to remove the high salt concentration immediately prior to mass spectral analysis. In addition, to slow the refolding of the OppA and prolong the existence of the complex, the rapid microdialysis step (Liu et al., 1996) of SecB-OppA complexes was carried out at 0°C. The interaction of SecB with its ligands is characterized by a rapid equilibrium between the bound and free states. Because the ligand is continually sampling the free state, eventually the unfolded ligand partitions completely to a folded form that can no longer interact with SecB.

The ligand OppA was denatured by diluting a stock solution of the protein into 20 μ L in which the final concentrations were 0.14 mM OppA, 2 mM DTT, and 3 M GuHCl. This solution was then allowed to stand for 2 h at room temperature. Reaction mixtures were then prepared using the denatured OppA solution and stock solutions of SecB (145 micromolar) and DTT (10 mM), such that the final concentrations of SecB and OppA were 10 and 18 micromolar, respectively. The final concentration of GuHCl was 0.2 M. Immediately after mixing, samples were dialyzed against a solution of 10 mM NH_4OAc and 10 mM DTT at 0°C, as mentioned above, and then analyzed immediately by ESI-FTICR-MS. With the microdialysis setup employed (Liu et al., 1996) dialysis of reaction mixtures was carried out in 2–4 min.

Mass spectrometry

All experiments were performed with a 7 T Fourier transform ion cyclotron resonance mass spectrometer. Since this mass spectrometer and many of the techniques that have been applied to allow the observation of noncovalent complexes have been described in detail elsewhere (Winger et al., 1993; Bruce et al., 1995; Pasa-Tolic et al., 1998), only a brief description is given here. Ions were introduced to the vacuum system of the mass spectrometer with an electrospray ionization (Yamashita et al., 1984; Aleksandrov et al., 1985; Fenn et al., 1989) (ESI) source developed at PNNL. The ESI spray emitter tip was 100 micron i.d. polyimide coated fused silica capillary that had been stripped of the coating and pulled to a smaller i.d. (approximately 5–10 microns as described by Gale & Smith, 1993 and Emmett & Caprioli, 1994). Solution flow rates were 300 nL/min and were controlled with a Harvard Apparatus syringe pump. Ions are guided by quadrupole ion optics from the

atmospheric pressure electrospray ionization source through five stages of pumping and injected into the high magnetic field with the aid of three quadrupole ion guides. A piezoelectric pulsed valve was used to introduce N_2 for accumulated trapping of ions injected into the ion trap and for ion activation, where applied.

A common constraint upon the application of mass spectrometry to biological problems involves the solution conditions necessary and the resulting high levels of adduction that can preclude mass measurements for higher molecular weight species. Methods for adduct removal based on infrared laser irradiation have recently been reported (Little et al., 1994). Also described elsewhere, in-cell heating (Pasa-Tolic et al., 1998) of trapped ions, in the form of low amplitude single frequency, or swept frequency dipolar excitation has been employed for adduct removal resulting in improved mass measurements of large noncovalent complexes. The present work demonstrates that these adduct removal techniques significantly improved, and in some cases, enabled mass measurements of noncovalent complexes.

Acknowledgments

The authors wish to acknowledge helpful discussions with Dr. Ljiljana Pasa-Tolic. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the United States Department of Energy through contract No. DE-AC06-76RLO 1830. This work was supported in part by Public Health Service Grant GM29798 to L.L.R. from the National Institute for General Medical Sciences. Research by V.F.S. was supported by Biotechnology Training Grant T32 GM08336 from the National Institute of General Medical Sciences.

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