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Electrospray mass spectrometric investigation of the chaperone SecB

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Abstract

Electrospray ionization mass spectrometry was used to investigate the structure of the *Escherichia coli* chaperone protein SecB. It was determined that the N-terminal methionine of SecB has been removed and that more than half of all SecB monomers are additionally modified, most likely by acetylation of the N-terminus or a lysine. The use of gentle mass spectrometer interface conditions showed that the predominant, oligomeric form of SecB is a tetramer that is stable over a range of solution pH conditions and mass spectrometer interface heating (i.e., inlet capillary temperatures). At very high pH, SecB dimers are observed. SecB contains a region that is hypersensitive to cleavage by proteinase K and is thought to be involved in conformational changes that are crucial to the function of SecB. We identified the primary site of cleavage to be between Leu 141 and Gln 142. Fourteen amino acids are removed, but the truncated form remains a tetramer with stability similar to that of the intact form.

Keywords: chaperone; electrospray mass spectrometry; noncovalent interactions; SecB

Molecular chaperones are found throughout nature participating in a wide range of processes, including folding, oligomerization, and subcellular localization of polypeptides (Randall & Hardy, 1995). All molecular chaperones selectively bind nonnative polypeptides with no affinity for proteins that have acquired their native state. The mechanism allowing chaperones to recognize a polypeptide as a ligand by virtue of the fact it is nonnative is of great interest. Although detailed structural information would be enormously valuable in understanding how a chaperone works, the structures of only a few chaperones have been determined at high resolution: GroEL (Braig et al., 1994) and Pap D (Holmgren & Braenden, 1989) by X-ray crystallography, and domains of Hsc 70 (Morshauser et al., 1995) and DnaJ (Szyperski et al., 1994) by NMR.

We report here the first use of electrospray ionization mass spectrometry (ESI-MS) to investigate the quaternary structure of SecB, a chaperone protein of *Escherichia coli*. SecB is the only chaperone among the several identified in *E. coli* that is dedicated to the facilitation of protein export across the cytoplasmic membrane (Randall & Hardy, 1995). Besides binding to and promoting the export of a subset of precursor proteins in vivo, SecB has also been shown to bind a number of proteins and peptides that possess nonnative structure even though they are not natural ligands found in *E. coli*. (Hardy & Randall, 1991). Efforts to crystallize SecB in several laboratories have not produced diffraction-quality crystals (Vrielink et al., 1995; Dodson, Guy G., pers. comm.), thus we have no information related to the 3D structure. SecB has been shown by CD to contain a high percentage of β structure, as well as regions with no defined secondary structure (Breukink et al., 1992; Fasman et al., 1995). The initial evidence that SecB is oligomeric came from analysis by size-exclusion chromatography and electrophoresis of the native protein through polyacrylamide gels. However, a range of molecular weights have been reported: 64 kDa (Watanabe & Blobel, 1989), 79 kDa (Weiss et al., 1988), 90 kDa (Kumamoto et al., 1989), and 115 kDa (Weiss et al., 1988), and thus the oligomeric state has not been determined definitively. The range of values obtained would be explained if SecB deviated from the ideal spherical structure because the techniques used base the determination of molecular weight on the separation of molecules according to their radii of gyration. Here we use ESI-MS to determine the mass of the monomeric and oligomeric species of SecB. (The ESI-MS methodology is discussed in more detail in the Results and the Discussion.) SecB is shown to be a tetramer that is stable over a range of electrospray interface conditions (i.e., inlet capillary heating temperatures) and solution pHs. A

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modified form of SecB that has the carboxyl-terminal 14 amino acids removed by treatment with proteinase K was also a tetramer of stability comparable to that of intact SecB.

Results

SecB has not proven amenable to study by high-resolution techniques such as X-ray crystallography and multidimensional NMR; however, recent advances have made mass spectrometry an attractive alternative for providing structural information. ESI-MS has emerged as one of the most effective and sensitive methods for the analysis of large biopolymers due to the production of multiply charged ions from solution (Fenn et al., 1989; Smith et al., 1990a). This multiple-charging phenomenon allows the observation of ions at relatively low mass-to-charge ratios ($m/z < 2{,}500$), such that mass spectrometers with limited m/z range can be utilized for the study of high molecular weight biomolecules. ESI-MS can also be used to detect noncovalent complexes if solution conditions and instrumental parameters are appropriately adjusted. For large multimeric proteins, the observation of the native oligomeric form requires a compromise between providing adequate heating/activation for ion desolvation and maintaining gentle enough interface conditions to avoid disruption of the complex (Light-Wahl et al., 1994). Electrospray ionization of multimeric proteins from aqueous solutions buffered near physiological pH generally results in less gas-phase charging observed for the protein, with ions at higher m/z compared to the use of more conventional acidic solutions. This is ascribed to Coulombic constraints to charging when the protein is maintained in its native or more compact state (Light-Wahl et al., 1994). Therefore, in contrast to ESI-MS analyses of proteins under denaturing conditions, which generally allow observation of large polypeptides under m/z 2,000, analyses of these intact oligomeric proteins typically necessitate the use of a mass spectrometer capable of extended m/z range (>3,000) detection.

There are several important variables in the electrospray atmosphere–vacuum interface region that can be adjusted to either preserve noncovalent complexes or, alternatively, to promote their dissociation by thermal or collisional activation (Loo et al., 1988; Smith et al., 1990b; Rockwood et al., 1991; Smith & Light-Wahl, 1993). The inlet capillary heating temperature, although not directly reflecting either solution temperature or the degree of internal excitation of the polypeptide during the millisecond timescale relevant to the ESI process, provides a relative measure of heating and the stability of the noncovalent association. In general, milder interface conditions than typically employed for ESI-MS (i.e., lower capillary heating temperatures and capillary-skimmer voltage offsets, ΔCS) are necessary in order to observe noncovalent associations.

Determination of the mass of the SecB monomer

SecB is a soluble protein with a deduced primary sequence of 155 amino acids (Kumamoto & Nault, 1989) and a calculated M_r of 17,278. Edman sequencing, however, suggested that the N-terminal methionine is removed posttranslationally (L.L. Randall & V.F. Smith, unpubl. results; Kumamoto et al., 1989); the predicted M_r for SecB modified in this way is 17,147. The protein contains four cysteine residues and determination of the content of sulfhydryl groups using Ellman's reagent indicates that there are no disulfide bonds (data not shown). Analysis of SecB by ESI-MS on the triple quadrupole instrument in the negative ion mode reveals two species by deconvolution of the monomeric charge states observed between 500 and 3,000 m/z (Fig. 1A,B). One had a mass of 17,146 Da, and is likely to represent the polypeptide from which the N-terminal Met has been removed. A larger species of 17,189 Da had slightly greater abundance. *N*-acetylation of either the amino terminus or the ϵ-NH_2 of one of the three lysines in SecB is the most likely source of the additional 42 Da. The low sequencing yield obtained from Edman degradation is consistent with blockage of the amino terminus by an acetyl group in a subpopulation of SecB.

Determination of the oligomeric state of intact SecB

The oligomeric state of SecB was investigated with the extended m/z range single quadrupole mass spectrometer. In the studies, capillary heating was varied between 20 W and 35 W and the ΔCS was held constant at 80 V. In a 10 mM NH_4OAc, pH 6.7, solution at 20 W, ions consistent with 16^- to 14^- charge states (Q^{16-} to Q^{14-}) were observed as shown in Figure 2A. Considering the low resolution of the instrument and the likely presence of residual adduction/solvation, the experimentally determined

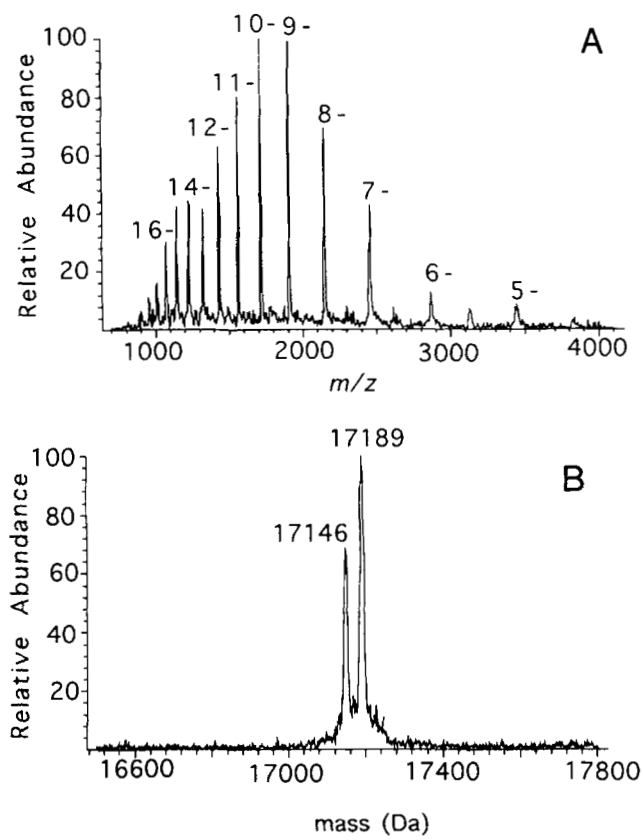

Fig. 1. Mass of the SecB monomer. Negative ion ESI mass spectrum of SecB (60 μM monomer) in 10 mM ammonium acetate, pH 6.7. Inlet capillary temperature was set to 180 °C and electrospray potential was −2.6 kV. Shown are **(A)** mass spectrum from 500 to 4,000 m/z and **(B)** deconvolution of charge states in mass spectrum **(A)** from 500 to 3,000 m/z.

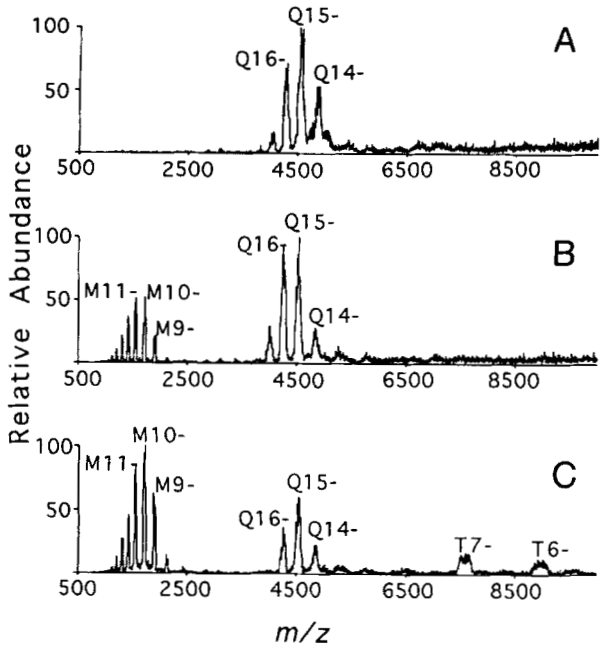

Fig. 2. Oligomeric state of intact SecB. Comparison of negative ion ESI mass spectra for SecB in 10 mM ammonium acetate, pH 6.7 at (**A**) 20 W, (**B**) 27 W, and (**C**) 35 W resistive heating applied to the inlet capillary. The labeling scheme for all spectra is as follows: D, dimer; M, monomer; T, trimer; Q, tetramer. The superscript denotes the charge state of the ion.

mass of 68,610 ± 70 Da correlates well with the predicted mass of 68,588 Da for the nonacetylated tetramer and 68,672 Da for a 1:1 ratio of the acetylated and nonacetylated forms. When the capillary heating was increased to 27 W (Fig. 2B), a small population of monomeric species became apparent at lower *m/z*. Ions corresponding to the monomeric (M^{13-} to M^{8-}) charge states were observed between *m/z* 1,300 and 2,200, and were more prominent at higher capillary temperatures (35 W); appearance of the monomeric charge states is attributed to thermally induced dissociation of the tetramer into its individual subunits, as shown in Figure 2C. The trimer species (T^{7-} and T^{6-}) observed in Figure 2C result from gas-phase dissociation of the tetramer and do not reflect solution-state association. Similar behavior has been observed in the ESI-MS analysis of other tetrameric proteins, such as avidin and hemoglobin, when interface conditions were made harsher (Light-Wahl et al., 1994).

pH dependence of stability of the SecB tetramer

A series of experiments was performed using the extended *m/z* range quadrupole mass spectrometer to investigate the pH dependence of the stability of the SecB tetramer. A comparison of the mass spectra obtained at four different pHs under identical interface conditions (27 W) is provided in Figure 3A, B, C, and D. At pH 5.5 and pH 6.7, the Q^{16-} to Q^{14-} charge states of the tetramer were the predominant species. At pH 9.0, Q^{13-} and Q^{12-} became more intense, but the distribution of species clearly still favored the tetrameric state. At pH 11.0, however, there was a significant contribution from monomeric (M^{12-} to M^{7-}) and dimeric (D^{11-} to D^{9-}) species. At a higher capillary temperature (35 W) (Fig. 4A,B,C,D), there was also a small contribution from the monomeric species at pH 5.5 and 6.7 and no significant contribution from dimeric species. At pH 11.0, the monomeric charge states predominated, but there was a significant dimer contribution. Efforts to examine the complex at pH 5.0 were unsuccessful because this sample produced an unstable electrospray, possibly due to reduced solubility of SecB near its isoelectric point, determined to be 3.95–4.1 (Weiss et al., 1988).

Determination of the mass of proteinase K-cleaved SecB monomer

It was previously observed that in a solution of low ionic strength, SecB could be quantitatively cleaved by low levels of proteinase K to produce a fragment that had an apparent molecular weight of about 12 kDa when analyzed by SDS-PAGE (Randall, 1992). At high ionic strength or in the presence of a peptide ligand, however, SecB was insensitive to digestion by proteinase K, presumably due to a conformational change that rendered the cleavage site inaccessible. Because the conformational change is probably related to the mechanism of ligand binding, the location of the hypersensitive site is of interest. Therefore, ESI-MS analysis of the proteolyzed monomer was performed in order to identify the cleavage site(s) on SecB.

ESI mass spectra of proteinase K-treated SecB were obtained using the triple quadrupole instrument. Deconvolution of the mass spectrum revealed a series of paired species, the larger and smaller ion of each pair differing by an average of ~42 Da (Fig. 5), similar to the pattern observed for intact monomeric

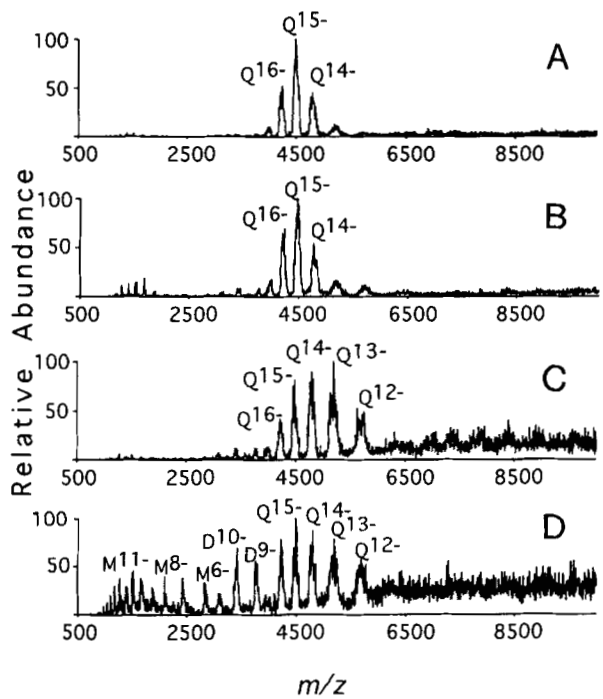

Fig. 3. Dependence on pH of the stability of the tetramer. Comparison of negative ion ESI mass spectra for SecB (60 μM monomer) in 10 mM ammonium acetate at: (**A**) pH 5.5, (**B**) pH 6.7, (**C**) pH 9.0, and (**D**) pH 11.0. Resistive inlet capillary heating was 27 W.

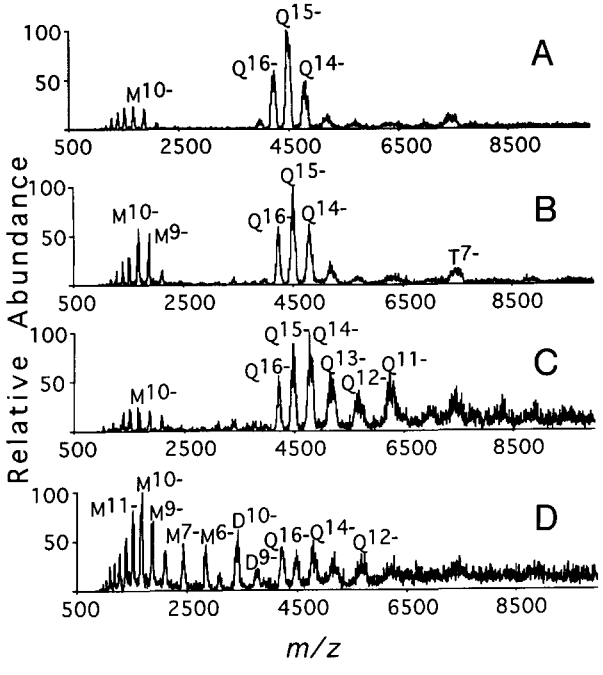

Fig. 4. Thermal dissociation of the tetramer at various pHs. Comparison of negative ion ESI mass spectra for SecB (60 μM monomer) in 10 mM ammonium acetate at: (**A**) pH 5.5, (**B**) pH 6.7, (**C**) pH 9.0, and (**D**) pH 11.0. Resistive inlet capillary heating was 35 W.

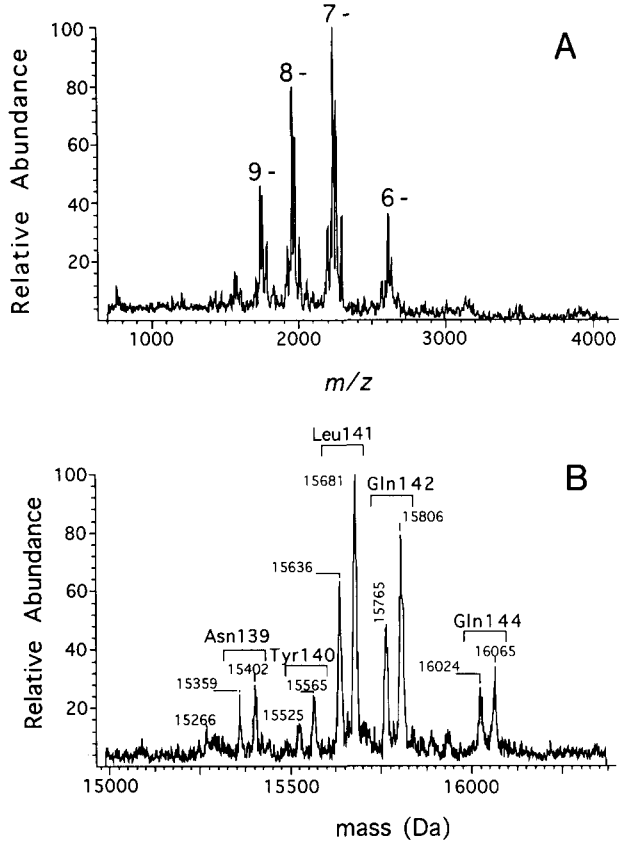

Fig. 5. Mass of the proteinase K-cleaved monomer. Negative ion ESI mass spectrum of proteinase K-treated SecB (60 μM monomer) in 10 mM ammonium acetate, pH 6.7, at 180 °C capillary temperature and −2.6 kV electrospray potential. Shown are (**A**) mass spectrum from 800 to 4,000 *m/z* and (**B**) a deconvolution of the mass spectrum from 800 to 4,000 *m/z*. Each pair of ions represents an unmodified and a putatively *N*-acetylated cleavage product. The C-terminal residue of each pair is indicated above the peaks.

SecB. Because it was shown previously that the proteinase K-cleaved form of SecB had the same N-terminal sequence as the intact form (Randall, 1992), it was possible to deduce the cleavage site(s) by inspection of the primary sequence of SecB. The species with mass of 15,636 Da is most likely the product of cleavage after Leu 141 (predicted M_r = 15,638). (In numbering the amino acids, we have taken the N-terminal Met as residual.) The ion with mass of 15,681 Da would be the putatively *N*-acetylated form of that same cleavage product (predicted M_r = 15,681). Similarly, the next most abundant pair at 15,765 and 15,806 Da probably represent the unmodified and *N*-acetylated products of cleavage after Gln 142, corresponding closely to the predicted masses of 15,766 and 15,808 Da, respectively. Less preferred cleavage sites at Asn 139 (peaks at 15,359 and 15,402 Da), Tyr 140 (15,525 and 15,565 Da), and Gln 144 (16,024 and 16,065 Da) were also identified. No significant cleavage after Gln 143 was observed.

Size-exclusion chromatography of SecB

As discussed in the Introduction, techniques that base the determination of molecular weight on the radius of an equivalent hydrodynamic sphere give an overestimate of the size of SecB. Figure 6 demonstrates that SecB elutes from a size-exclusion column with an apparent molecular weight greater than 100,000, significantly greater than the expected 68,000. The proteinase K-cleaved form elutes as an oligomer with an apparent molecular weight of 82,000. The mass removed from the tetramer is predicted to be only 6,040 Da, thus it seems that removal of the 14 amino acyl residues from each monomer changed the hydrodynamic radius significantly. Because size-exclusion chromatography cannot be used to reliably determine the oligomeric state, we analyzed the proteinase-K treated SecB on the extended *m/z* range single quadrupole mass spectrometer to demonstrate that it remained tetrameric.

Determination of oligomeric state of proteinase K-treated SecB

The spectra of the cleaved SecB are similar in appearance to those of the intact SecB except that the peaks are broader, reflecting the heterogeneity of the monomers. Spectra showing the ions corresponding to the tetrameric 15^- to 13^- charge states (Q^{15-} to Q^{13-}) for a sample in 10 mM NH_4OAc, pH 6.7, at 20 W are displayed in Figure 7A. The mass of 62,780 ± 85 Da determined experimentally from the three charge states correlates well with the predicted mass of 62,724 Da for the *N*-acetylated form (based on cleavage after Leu 141). Increasing the capillary heating caused the tetramer to dissociate into monomers. Ions corresponding to the monomeric charge states M^{13-} to M^{7-} were observed as shown in Figure 7B and C. The dissociation of the proteinase K-cleaved tetramer showed an interface capillary temperature dependence similar to that of the intact SecB

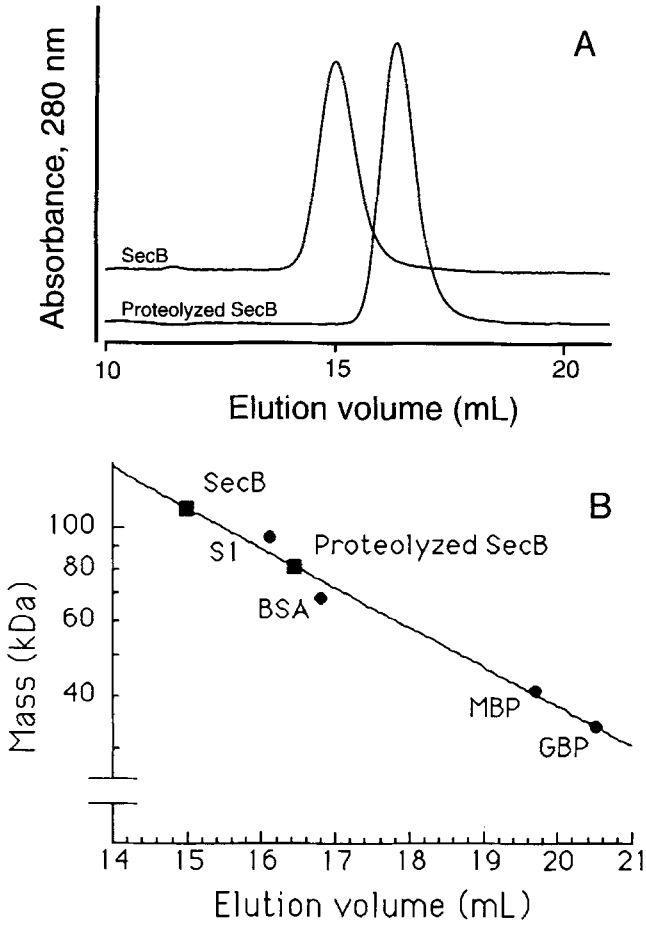

Fig. 6. Size-exclusion chromatography of SecB. Intact and proteinase K-cleaved SecB (20 μg each) were subjected to HPLC on a TSK3000SW (Toso Haas) size-exclusion column (**A**). A mass standard curve (**B**) was generated for this column by plotting the log of the mass against elution volume for the following proteins (indicated by filled circles): myosin subfragment 1 (S1), 95 kDa; bovine serum albumin (BSA), 68 kDa; maltose-binding protein (MBP), 41 kDa; and galactose-binding protein (GBP), 34 kDa. The masses of the two species of SecB (filled squares) can be estimated from the standard curve using the elution volumes obtained in A. All chromatography was carried out at 10 °C in 20 mM HEPES, 150 mM potassium acetate, pH 7.0, at a flowrate of 1 mL/min.

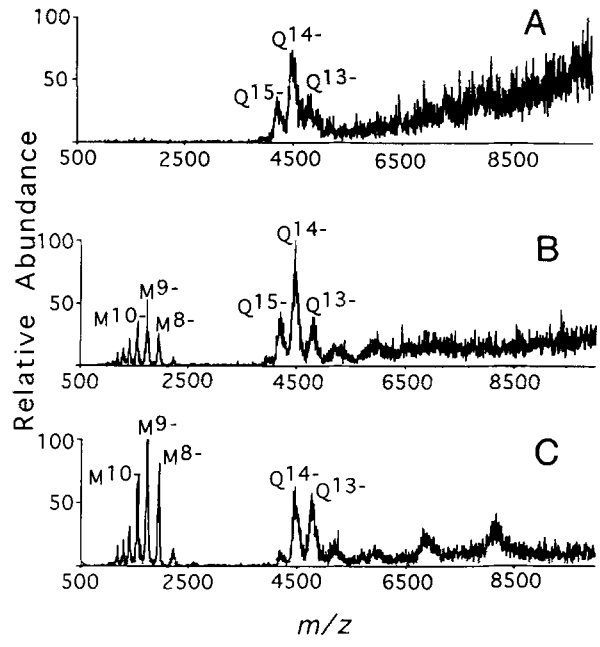

Fig. 7. Thermal dissociation of the proteinase K-treated SecB tetramer. Comparison of negative ion ESI mass spectra for proteinase K-treated SecB (60 μM monomer) in 10 mM ammonium acetate, pH 6.7, at: (**A**) 20-W, (**B**) 27-W, and (**C**) 35-W resistive heating applied to the capillary.

tetramers. Comparison of spectra obtained under identical experimental conditions reveals that the relative abundances of the monomeric charge states, which reflect dissociation of the tetramer, are approximately equal for the proteinase K-cleaved (Fig. 7A,B,C) and intact (Fig. 2A,B,C) forms of SecB.

Discussion

When using ESI-MS to study noncovalent associations, appropriate control experiments are necessary to establish that the species detected in the gas phase correspond to the species that exist in solution (Light-Wahl et al., 1994). It is especially important to show that ions indicative of a specific complex of defined stoichiometry are more abundant than ions due to dissociated subunits or possibly arising from random aggregation (Smith & Light-Wahl, 1993; Light-Wahl et al., 1994). Further support for the specificity of the associations is provided if the noncovalent complexes are disrupted into individual subunits using experimental conditions, either in the solution or at the electrospray interface, that are unfavorable for preserving the complex (Loo et al., 1988). We have satisfied both of these conditions for the SecB tetramer. The SecB tetramer, detected as a series of ions with m/z values corresponding to predicted tetrameric charge states, is the predominant species over a range of interface capillary heating temperatures and in solutions of different pH. Further, there is no evidence of random aggregation (i.e., trimers, pentamers, hexamers, etc.) occurring in solution or arising from the electrospray process. Disruption of the tetramer by high capillary temperature (or at high pH) results in the production of ions observed with m/z values corresponding to the monomeric or dimeric charge states, providing further evidence for the identity of the higher m/z ions and the noncovalent nature of their association.

The tetramer can be dissociated directly into monomers by heating in the mass spectrometer interface with no evidence of intermediate species, behavior similar to dissociation observed for other tetrameric proteins studied by ESI-MS (Light-Wahl et al., 1994). Dissociation into dimeric intermediate species followed by dissociation to monomers would not be observed if the dissociation is the result of thermal denaturation of the polypeptide structure, because such denaturation would probably occur without first destroying specific interface contacts. However, a dimeric species is populated if disruption is effected through change in pH. The tetramer is stable in the pH range 5.5–9.0, but, at pH 11.0, the dimeric ion species is observed. The dramatic change in distribution of ions at pH 11.0 suggests that lysine residues (intrinsic $pK_a \cong 10$) are involved in interactions at an interface between dimers and contribute to stability of the tetrameric form. Because there are only three lysine residues in

SecB, site-directed mutagenesis can be used to determine which of the residues are involved.

The proteinase K-cleaved forms of SecB were determined to be a heterogeneous mixture with the predominant form arising from cleavage at Leu 141. Cleavage at this site is consistent with the known specificity of proteinase K (i.e., a preference for cleaving after aliphatic and aromatic residues [Kraus et al., 1976]). The observation of the species 42 Da larger than the predicted mass indicates that the modification, presumably acetylation, does not occur between residues 142 and 155, in accord with the idea that it is the N-terminus or a lysine that is modified because the three lysines (residues 19, 34, and 41) are present in the proteinase K-cleaved form. The truncated form remains tetrameric, with stability similar to that of the intact form, indicating that the C-terminal 14 residues are not crucial to the sites of interaction between monomers.

The discrepancy between the apparent molecular weight of the SecB oligomer (>100,000) as determined by size exclusion chromatography and the true mass of the tetramer (68,610) as determined by mass spectrometry indicates that SecB does not have a compact globular structure. Removal of the C-terminal 14 amino acyl residues causes SecB to elute at a volume closer to that expected; however, the change from elution with an apparent molecular weight greater than 100,000 to elution with an apparent molecular weight of 82,000 is greater than can be accounted for by the loss of 6,040 Da in mass. These results suggest that SecB has an elongated, nonspherical structure. However, it is possible that SecB is spherical, but loosely packed relative to most proteins. This would agree with the proposal that SecB is malleable and interacts with its ligands, which are loosely folded non-native polypeptides, in a manner resembling folding interactions (Randall & Hardy, 1995). If this were the case, one might expect SecB to have an expanded volume relative to the volume of tightly folded proteins of the same mass.

Materials and methods

The purification of SecB has been described previously (Topping & Randall, 1994). Protein concentration was 1 mg mL^{-1} (15 μM for the tetramer) in 10 mM ammonium acetate buffer. The pH of the ammonium acetate buffer solution was adjusted as necessary with either ammonium hydroxide or acetic acid. The proteinase K-cleaved form of SecB was prepared by incubating 1 mg mL^{-1} SecB in 10 mM NH_4OAc, pH 6.7, with 2.8 μg mL^{-1} proteinase K for 20 min at 4 °C. The reaction was stopped with 0.1 mM (final concentration) PMSF. Proteinase K and PMSF were purchased from Sigma Chemical Company.

The high m/z spectra of the SecB oligomer were collected in the negative ion mode using a low frequency extended mass range single quadrupole mass spectrometer with an approximate m/z limit of 45,000 (Winger et al., 1993). Spectra were collected in the negative ion mode because SecB exhibited more charging compared to positive ion mode (data not shown). The greater charging in the negative ion mode is probably due to the large number of acidic residues on SecB. The instrument characteristics include low-resolution (~100) and discrimination against low m/z ions (<1,000). The ESI interface consisted of a resistively heated stainless steel capillary (1.59-mm o.d., 0.5-mm i.d., 20-cm long) similar to the design of Chowdhury et al. (1990), as modified by Rockwood et al. (1991). Important interface parameters are the capillary heater power (reported in watts), which was varied as indicated, and the voltage offset between the capillary and skimmer lens (ΔCS), which was held constant at 80 V. A ΔCS of 80 V has proven to be optimal for preserving noncovalent complexes using this instrument. The electrospray source implemented in these studies used a coaxial sheath flow of SF_6 and allowed low sample flow rates (0.3 μL min^{-1}) (Gale & Smith, 1993). The ESI source voltage was −2.6 kV. Capillary heater powers of 20, 23, 27, and 35 W correspond to approximately 200, 225, 255, and 305 °C, respectively, as measured by a thermocouple on the outside of the capillary. (The actual heating of the electrosprayed solutions is brief due to the short residence time in the capillary.) A heated countercurrent flow of N_2 gas at atmospheric pressure was also employed to assist electrospray desolvation. A Teknivent Vector One data system was used for data acquisition. Spectra presented are the average of four 2-min scans acquired over a 500–10,000 m/z range in 5 m/z steps. The 12^+ to 1^+ charge states of horse heart cytochrome c in 10 mM ammonium acetate, pH 6.7, were used as m/z standards for instrument calibration. The charge states of experimental samples were assigned using the method described by Smith et al. (1990b).

A commercial Finnigan MAT (San Jose, California) TSQ 7000 triple-quadrupole mass spectrometer was used to collect negative-ion ESI-MS data for the SecB monomer to verify sample purity and determine molecular weight. The source utilized on the single-quadrupole instrument was also used with this instrument. Experimental conditions used were a sample flow rate of 0.3 μL min^{-1}, electrospray potential of −2.6 kV, coaxial sheath gas of SF_6, capillary temperature of 180 °C, and spectrum acquisition over an 800–4,000 m/z range with 5 s/scan for 5 min. The TSQ7000 was operated at unit resolution and the mass range was calibrated with apomyoglobin in a 50:50 methanol:water/1% acetic acid solution.

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