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The interaction between the chaperone SecB and its ligands: Evidence for multiple subsites for binding

LINDA L. RANDALL,[1] SIMON J.S. HARDY,[1,3] TRACI B. TOPPING,[1] VIRGINIA F. SMITH,[1,4] JAMES E. BRUCE,[2] AND RICHARD D. SMITH[2]

[1]Department of Biochemistry and Biophysics, Washington State University, P.O. Box 644660, Pullman, Washington 99164-4660
[2]Environmental and Molecular Sciences Laboratory, The Pacific Northwest National Laboratory, Richland, Washington 99352

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Abstract

The chaperone protein SecB is dedicated to the facilitation of export of proteins from the cytoplasm to the periplasm and outer membrane of *Escherichia coli*. It functions to bind and deliver precursors of exported proteins to the membrane-associated translocation apparatus before the precursors fold into their native stable structures. The binding to SecB is characterized by a high selectivity for ligands having nonnative structure but a low specificity for consensus in sequence among the ligands. A model previously presented (Randall LL, Hardy SJS, 1995, *Trends Biochem Sci 20*:65–69) to rationalize the ability of SecB to distinguish between the native and nonnative states of a polypeptide proposes that the SecB tetramer contains two types of subsites for ligand binding: one kind that would interact with extended flexible stretches of polypeptides and the other with hydrophobic regions. Here we have used titration calorimetry, analytical ultracentrifugation, and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry to obtain evidence that such distinguishable subsites exist.

Keywords: analytical ultracentrifugation; calorimetry; chaperones; Fourier transform ion cyclotron resonance mass spectrometry; SecB

SecB, a cytosolic tetrameric protein in *Escherichia coli*, is a chaperone that facilitates export of polypeptides to the periplasmic space and to the outer membrane (for review see Kumamoto, 1991; Collier, 1993; Hardy & Randall, 1993). SecB binds precursor proteins in the cytoplasm before they assume their native, stably folded structures and maintains them in a state that is compatible with transfer through the membrane. By binding proteins destined for export, SecB controls a kinetic partitioning between folding of the polypeptides to their native conformation in the cytoplasm, which is the wrong compartment, and export through the cytoplasmic membrane to their proper destination. Since folded precursors can neither bind SecB nor be exported, the proportion of the polypeptides that are properly localized is a function of the rate constant of folding relative to the rate constant of association with SecB (Randall & Hardy, 1995). The interaction of SecB with ligands is one of high affinity (Hardy & Randall, 1991) and is characterized by high rate constants for both association and dissociation (Fekkes et al., 1995; Randall & Hardy, 1995). Perhaps the most remarkable feature of this binding is that there is no apparent consensus in primary, secondary, or tertiary structure among the polypeptides that SecB has been shown to bind. Rather, the feature that distinguishes polypeptides as ligands for SecB is that they are in a nonnative conformation. A large part of the selectivity in vivo most likely results from the fact that precursors of exported proteins contain a leader sequence that slows the folding of the polypeptide thus poising the kinetic partitioning toward binding of SecB and thereby export. We have previously proposed a model based on studies with short peptide ligands to explain the ability of SecB to recognize a ligand as nonnative. This model depends on the existence of multiple subsites on the chaperone for interaction with different stretches of the polypeptide ligand. The overall high affinity for the ligand reflects the sum of the binding energies of interaction at the subsites. It was further proposed that there are two types of subsites on SecB, one that binds flexible stretches of the ligand and another that interacts with hydrophobic areas. Multiple occupancy of the sites for flexible stretches signals the chaperone that a nonnative ligand is bound causing a conformational change to expose the hydrophobic site and thereby provide additional sites of interaction. We made use of calorimetry, analytical ultracentrifugation, and mass spectrometry to obtain evidence for the existence of multiple subsites for binding.

Reprint requests to: Linda L. Randall, Department of Biochemistry and Biophysics, P.O. Box 644660, Washington State University, Pullman, Washington 99164-4660; e-mail: traci@membrane.chem.wsu.edu.

[3]On leave from the Department of Biology, University of York, York, United Kingdom.

[4]Present address: The Department of Chemistry, 152 Davey Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802-6300.

Results

Evidence for multiple subsites for binding on SecB

In our working model of the mechanism of binding, the multiple subsites on a SecB tetramer are all occupied by long physiological polypeptide ligands such as the binding proteins. Thus, the stoichiometry of binding is one polypeptide to a tetramer. If we consider the binding of smaller ligands, too short to occupy simultaneously all of the subsites, then the stoichiometry is expected to be greater than one, as has been shown to be the case for natural and synthetic peptides of approximately 12 amino acids in length, for bovine pancreatic trypsin inhibitor, and for barnase (Randall, 1992; Fekkes et al., 1995; Stenberg & Fersht, 1997). If the postulated subsites on SecB had different binding parameters, that is, different affinities or perhaps the same affinity but with different contributions to the energy of binding coming from enthalpy, then titration calorimetry might be applied to the study of the interaction between SecB and ligands to provide support for the idea that different subsites exist. If all the subsites on SecB were occupied by one ligand, the titration would be symmetric regardless of which component were titrated into the other. However, if there were at least two types of subsites that had different heats of binding, and if we had a ligand that occupied each subsite separately, then the titration would show asymmetry. If SecB were in the calorimetry cell and the ligand were injected in increments, then the subsites with the highest affinity would be occupied in the early injections of ligand showing the heat change characteristic of that site (ΔH_1). The weaker sites would be titrated by later injections with a heat change related to that lower affinity site (ΔH_2). However, titration in the reverse direction would show a different pattern of heat effects. If the ligand were in the cell and SecB were added in increments, then in early injections the ligand would be present in great excess and thus both sites would be filled simultaneously. Consequently, the heat observed would be the sum of the heat from the two subsites ($\Delta H_1 + \Delta H_2$). At later injections SecB would be in excess and the ligand would be pulled from the weaker site to the site with higher affinity, thus the heat observed would be the difference of the heats ($\Delta H_1 - \Delta H_2$). Although one cannot predict the shape of the plots of the integrated heats, one can predict that the system would show asymmetry.

We have used calorimetry previously (Randall et al., 1998) to investigate the interaction of SecB with two of the physiological ligands which are long polypeptides. The titration calorimetry experiments using nonnative polypeptides as ligands must be carefully designed since only the unfolded form of a polypeptide is active as a ligand. Thus, polypeptides can be used as ligands only if folding can be prevented. In our previous work, we were able to analyze the interaction of SecB with the precursor and mature forms of two physiological ligands, maltose-binding protein and galactose-binding protein, by making use of a folding mutant of maltose-binding protein and taking advantage of the observation that galactose-binding protein, which contains a Ca^{++} ion in its folded structure, folds only slowly in the presence of EGTA. We showed in that study that the presence of the leader sequence in the precursor forms provides no positive contribution to the binding energy. For our study here we chose the mature form of galactose-binding protein as the long physiological ligand expected to fill all subsites at once. We also require a ligand that is smaller than the natural ligands and that would remain in a nonnative state. An ideal polypeptide to serve as the short ligand is the fragment of staphylococcal nuclease that has been described as a model for the denatured state of the nuclease by Alexandrescu et al. (1994). The 131-residue fragment, which was obtained by site-directed mutagenesis and which lacks residues 4–12 and 141–149 of the 149-residue protein, is referred to as Δ131Δ.

Analysis of the interaction of SecB with the long physiological ligand galactose-binding protein demonstrates that as predicted if the ligand were to occupy simultaneously all subsites, the titration is symmetric (Fig. 1). The binding parameters were essentially the same whether the galactose-binding protein was injected into SecB or the titration was carried out in the opposite direction (Table 1). Figure 2 demonstrates that indeed the titrations with the shorter ligand, Δ131Δ, showed asymmetry. However, we were not able to deconvolute the curves with the fitting functions available, probably because of the complexity of binding. Previous data indicate that there is more than one subsite of the type that binds extended stretches of polypeptide, and at least one of a different type that binds hydrophobic regions. Furthermore, binding at the latter appears to be highly cooperative; i.e., its affinity for ligand, indeed perhaps its very availability as a binding site, is dependent on occupation of the subsites that bind flexible regions (Randall, 1992). Thus, it is not surprising that we could not deconvolute the data to obtain believable values for the association constants, the heats of binding or the stoichiometries. Nonetheless, it is clear that there

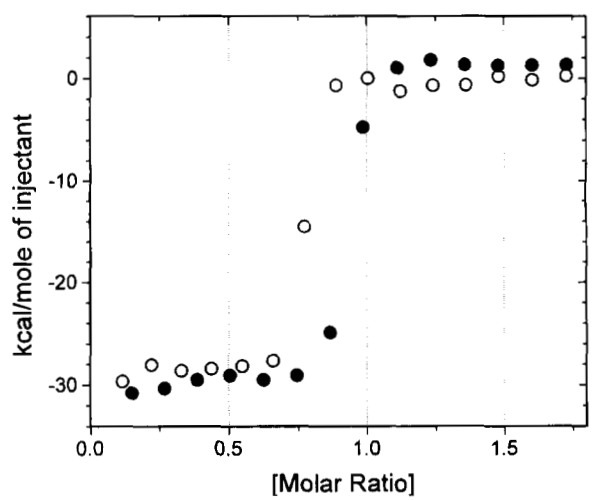

Fig. 1. Comparison of titration of SecB by galactose-binding protein with titration of galactose-binding protein by SecB. The titration of mature galactose-binding protein by SecB (●) was carried out as follows: Galactose-binding protein was unfolded in 1.0 N GnHCl, 10 mM Hepes, 150 mM KAc, pH 7.6 and diluted to give final concentrations of 6.2 μM GBP, 0.2 N GnHCl, 10 mM Hepes, 150 mM KAc, 0.4 mM EGTA, pH 7.6 immediately before loading into the cell. SecB tetramer (held in the syringe at 140 μM) in the same buffer was added in a sequence of 14 injections, each of 7 μL spaced at 10 min intervals. The titration was carried out at 7.2 °C. [These data were published previously (Randall et al., 1998) and are included here for direct comparison.] The titration of SecB by galactose-binding protein (○) was as follows: SecB at 11.6 μM was held in the cell in 0.2 N GnHCl, 10 mM Hepes, 150 mM KAc, 0.4 mM EGTA, pH 7.6. Galactose-binding protein was unfolded in 1.0 N GnHCl, 10 mM Hepes, 150 mM KAc, pH 7.6 and diluted to give final concentrations of 111 μM galactose-binding protein, 0.2 N GnHCl, 10 mM Hepes, 150 mM KAc, 0.4 mM EGTA, pH 7.6 immediately before loading into the syringe, which was held at 4 °C. The galactose-binding protein was added in a sequence of 15 injections, each of 18 μL, spaced at 11 min intervals. The titration was carried out at 8.6 °C.

Table 1. *Parameters for interaction of SecB and galactose-binding protein*

Direction of titration	T (°C)	K_d (nM)	n	ΔG[a] (kcal mol^{-1})	ΔH (kcal mol^{-1})	$T\Delta S$[b] (kcal mol^{-1})
SecB into	6.8	5.0 ± 1	1.1	−11.3	−29 ± 0.2	−17.7
mature GBP[c]	9.4	10 ± 4	1.0	−10.9	−30 ± 0.6	−19.1
	10	13 ± 3	1.4	−10.7	−31 ± 0.5[d]	−20.3
Mature GBP	8.6	5.5 ± 1	0.9	−11.2	−23 ± 0.2	−11.8
into SecB	11	16 ± 6	0.8	−10.6	−33 ± 0.5	−22.4

[a]Calculation of ΔG was from $\Delta G = -RT \ln K_a$ and $K_a = 1/K_d$.
[b]$T\Delta S$ was calculated from $\Delta G = \Delta H - T\Delta S$.
[c]These data were previously published in Randall et al. (1998) and are presented here to facilitate comparison.
[d]The ΔH given was calculated assuming $n = 1.0$.

are subsites on SecB that bind ligands with different affinities. To determine the stoichiometry of binding of the fragment of staphylococcal nuclease to SecB, we turned to analytical ultracentrifugation and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry.

Analytical ultracentrifugation

Boundary sedimentation velocity experiments were carried out on samples containing SecB alone or SecB mixed with Δ131Δ in the molar ratios of Δ131Δ:SecB tetramer of 1:1, 2:1, 3:1, and 4:1. The centrifuge cell was filled with the sample solution at uniform concentration and centrifugal force applied so that the proteins would sediment with a velocity proportional to their sedimentation coefficients (s values). The boundary of proteins, which moves away from the meniscus, was observed during centrifugation using a scanning absorption optical system. A boundary analysis method, developed by van Holde and Weischet (1978), was used to remove the effect of diffusional spreading on the sedimenting boundary and allow analysis of a sample containing more than one sedimenting component. The basis of this analysis is the fact that whereas sedimentation is proportional to the time of centrifugation, diffusion is proportional to the square root of the time. In the analysis each boundary is divided into 20 horizontal segments and an apparent sedimentation coefficient s^* is calculated for each boundary division. Extrapolation to infinite time in the plots of s^* for each boundary division as a function of the inverse square root of time eliminates the effects of diffusion. When the population sediments as one species, as when SecB was analyzed alone (Fig. 3), the extrapolated values for s^* converge at the y-axis. A plot of these extrapolated s^* values vs. fraction of the boundary gives a vertical s distribution at 4.4 S (Fig. 4, ■). When Δ131Δ was added at a molar equivalence to the SecB tetramer, one component was observed with a sedimentation coefficient (4.9 S) significantly higher than that of SecB alone (Fig. 4, ▲). Further increases in sedimentation coefficient occurred when Δ131Δ was in excess of SecB at 2:1 (5.1 S, Fig. 4, ♦) and 3:1 (5.4 S, Fig. 4, ●) molar ratios indicating the formation of progressively larger complexes. However, addition of Δ131Δ at 4:1 (Fig. 4, ▼) molar ratio did not cause the complex to sediment with an s value significantly higher than

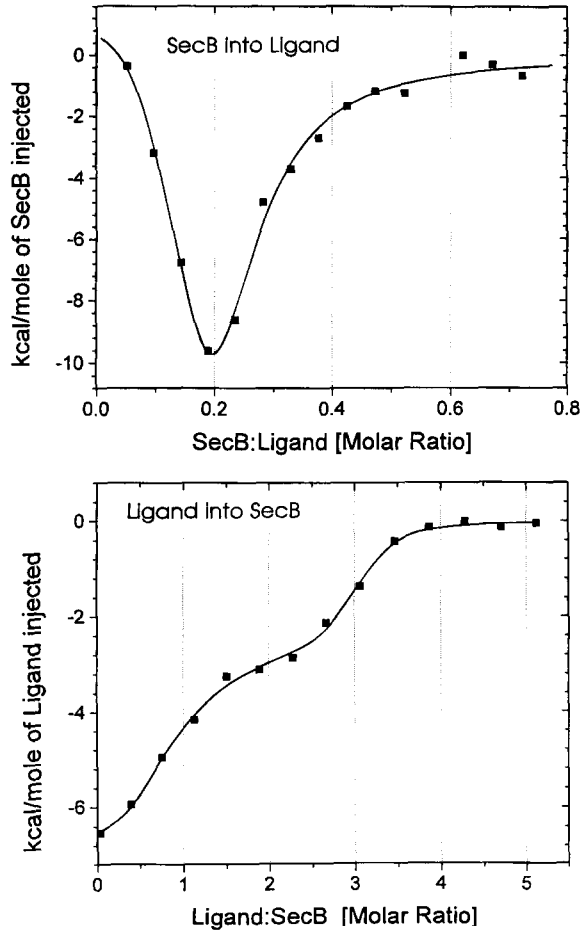

Fig. 2. Comparison of titration of SecB by Δ131Δ with titration of Δ131Δ by SecB. The titrations of SecB into Δ131Δ were carried out in 10 mM Hepes, 150 mM KAc, pH 7.6 with Δ131Δ held in the cell at 52 μM. SecB tetramer, in the syringe at 0.25 mM, was added in a sequence of 14 injections, each of 12 μL, spaced at 8 min intervals. The reverse titration was carried out in the same buffer with SecB tetramer in the cell at 60 μM. Staphylococcal nuclease Δ131Δ, in the syringe at 0.36 mM, was added in a sequence of 14 injections of 20 μL each spaced at 8 min intervals. Both titrations were carried out at 9.4 °C.

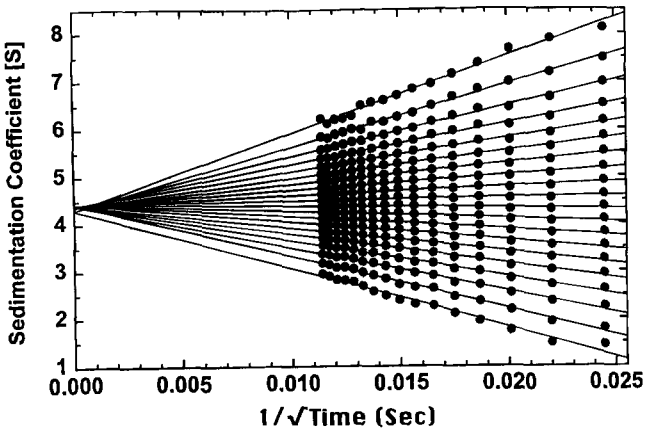

Fig. 3. Analysis of sedimentation of pure SecB. The 7 μM SecB in 10 mM Hepes pH 7.6, 150 mM KAc was loaded into one of two chambers in an analytical ultracentrifuge cell with reference buffer in the other chamber and centrifuged at 42,000 rpm at 15 °C. Continuous scans of the cell were taken every 7 min with a radial step size of 0.001 cm. Fifteen successive scans, beginning after the sedimenting boundary had fully cleared the meniscus were subjected to analysis by the method of van Holde and Weischet (1978), with the middle 90% of each boundary being divided into 20 sections, and with a smoothing factor of 5. Each point is the apparent sedimentation coefficient of each fraction of the boundary from a particular scan. Each vertical array of 20 points represents the 20 fractions of one boundary. Each line is the best fit through the points for a particular boundary fraction.

that seen at 3:1. This suggests that SecB is saturated when it binds three molecules of the nuclease. If a sample is heterogeneous as would be expected to be the case in the mixture of Δ131Δ and SecB at 4:1, the extrapolated s^* values of the boundary fractions should yield multiple y-intercepts. However, analysis of the mixture at 4:1 showed a vertical distribution plot (Fig. 4, ▼). It is likely that the free Δ131Δ was not detected since it would account for only 8% of the absorbance at 280 nM. At a ratio of 5:1, 50% to 60% of the sedimentary boundary had an s value of 1.5 S, characteristic of free Δ131Δ; however, only 40% of the expected absorbance was observed in the cell indicating that most of the SecB in complex had precipitated and therefore the remaining absorbance was enriched in free Δ131Δ (data not shown).

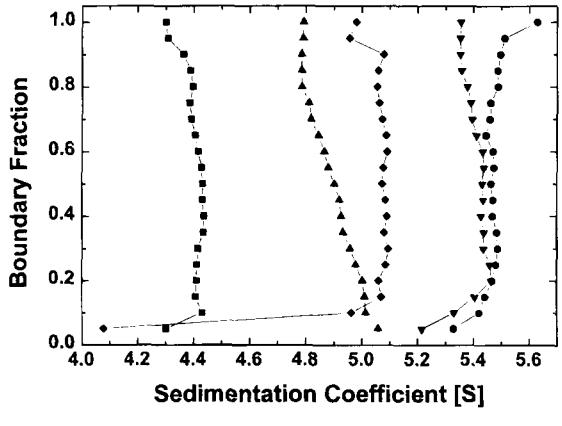

Fig. 4. Distribution of sedimentation coefficients of SecB complexes with Δ131Δ. Δ131Δ and SecB in 10 mM Hepes pH 7.6, 150 mM KAc were mixed at molar ratios of 0:1 (■); 1:1 (▲); 2:1 (♦); 3:1 (●); 4:1 (▼), and the mixtures were subjected to centrifugation and analysis as shown in Figure 3. The intercept of each line of the van Holde–Weischet analysis with the y axis is plotted against the boundary fraction to which the line pertained. Except for the sample containing SecB alone where the concentration was 7 μM, the concentration of SecB was 5 μM. The deviation to low values in the lowest boundary fraction for the molar ratio of 2:1 (♦) does not represent free material sedimenting slowly, but rather is the result of noise in data near the baseline.

Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry

When analyzed under conditions designed to preserve noncovalent interactions, the mass spectra of the SecB tetramer consist primarily of low charge state ions of the tetrameric species, usually between 13+ and 17+. This low charging is commonly observed with noncovalent complexes and is normally attributed to the compact nature of the complex. A typical mass spectrum (Fig. 5) shows SecB tetramers with charge states ranging from 13+ to 16+. Deconvolution gives rise to a molecular mass of 68,750 ± 20 Da (95% confidence limits, n = 4). In separate studies of SecB and of complexes between SecB and a polypeptide ligand, the oligopeptide-binding protein (Bruce et al., 1998; Pasa-Tolic et al., 1998), it was shown that gentle ion activation in the ion cyclotron resonance cell resulted in improved mass resolution and excellent agreement with predicted values; thus, it is likely that the mass reported here is higher than the predicted mass of 68,585 Da because of adduction of small species, presumably from the buffer solution.

Mass spectrometry of Δ131Δ carried out under conditions similar to those used for SecB tetramers produced spectra showing both higher charge states and a wider charge distribution than those that were typically observed for SecB tetramers (Fig. 6A). It has previously been suggested in line with broad experience (Light-Wahl et al., 1994) that such broad distributions are indicative of extended or flexible structures. Deconvolution of the mass spectrum to zero charge reveals the isotopic distribution of molecular mass of Δ131Δ (Fig. 6B). Comparison of the predicted isotopic envelope of Δ131Δ based on the amino acid sequence (Fig. 6B, dashed line) with the data (Fig. 6B, solid line) shows excellent agreement between the most abundant isotopic species (14,873.78 Da) measured and that predicted (14,873.91 Da).

Mass spectra for solutions of SecB mixed with Δ131Δ at ratios of Δ131Δ:SecB tetramer of 1:1, 2:1, and 3:1 show SecB tetramer ions in complex with one or two molecules of Δ131Δ, as well as free SecB tetramer (Fig. 7; Table 2). In some experiments, free Δ131Δ ions were also observed in the lower m/z range, indicating that additional Δ131Δ ions were available for binding. However, we did not observe any peaks in the mass spectra arising from complexes containing more than two ions of Δ131Δ for one SecB tetramer.

Discussion

Our working model, based on studies of the binding of peptides to SecB (Randall, 1992), explains the ability of SecB to bind nonnative polypeptides with high specificity and high affinity in terms of interaction of the individual ligand at a number of different subsites on SecB. These subsites are of at least two kinds: the subsites of one type bind short, flexible sequences; the other kind of subsite, which only becomes exposed after all or some of the first kind are filled, binds hydrophobic regions of the ligand. Such

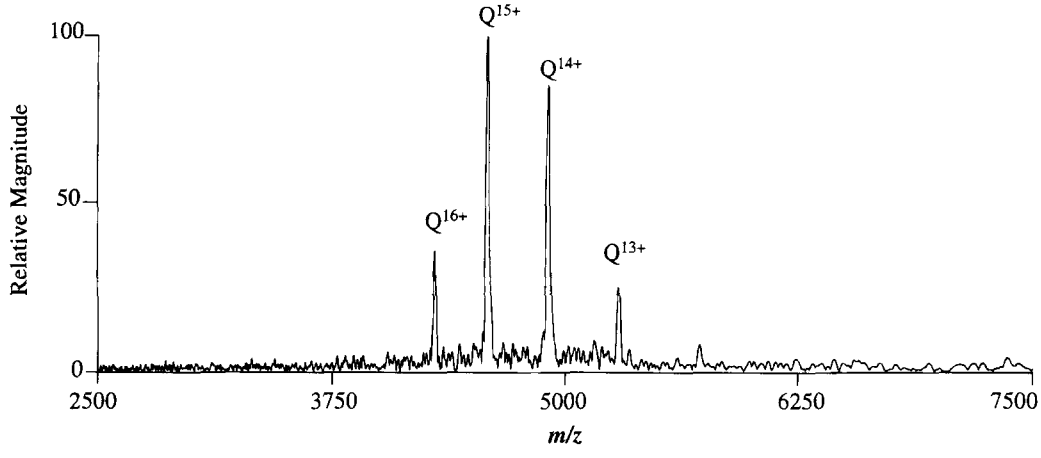

Fig. 5. Mass spectrum of SecB tetramer. This typical spectrum was obtained using a sample containing SecB tetramer at 150 μM in a 10 mM NH_4OAc solution. Q denotes a tetramer species.

a binding device is exquisitely designed for selectively binding nonnative slowly folding proteins. Not only would SecB specifically select nonnative proteins based on flexibility of structure and accessibility of hydrophobic regions, but the hydrophobic surface on SecB would be exposed only when binding had commenced, thereby avoiding aggregation of SecB that is not in a complex. This model predicted that the binding site for SecB on a polypeptide ligand would be large because the ligand must bind to multiple subsites on SecB. That was shown to be the case when the binding frames of three different ligands, maltose-binding protein (Topping & Randall, 1994), galactose-binding protein (Khisty et al., 1995), and oligopeptide-binding protein (Smith et al., 1997) were defined: all three encompass a sequence of at least 150 residues. In this study another aspect of the model is shown to be correct,

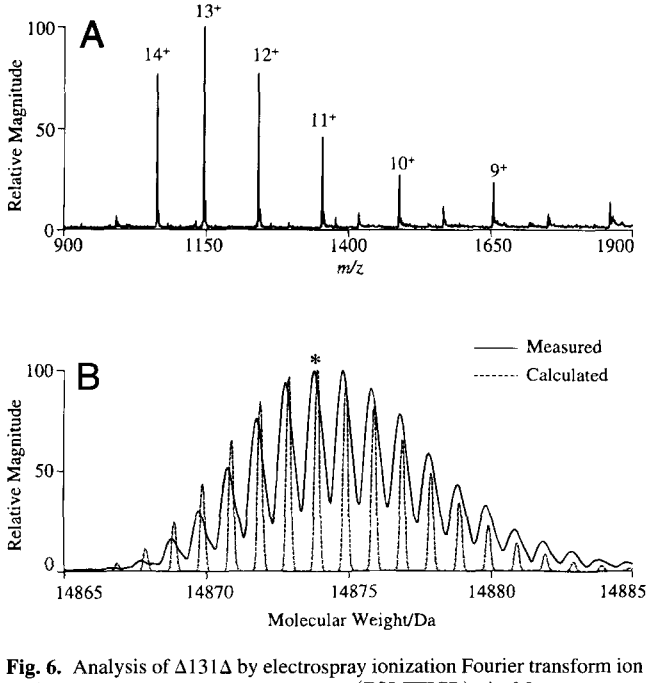

Fig. 6. Analysis of Δ131Δ by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR). **A:** Mass spectrum of Δ131Δ. The spectrum was acquired with a protein concentration of 65 μM in 10 mM NH_4OAc solution. **B:** Deconvolution of the mass spectrum shown in **A**. The observed data are represented by the solid line and the broken line represents the predicted isotopic distribution of Δ131Δ based on the amino acid sequence. The most abundant isotopic species is denoted *.

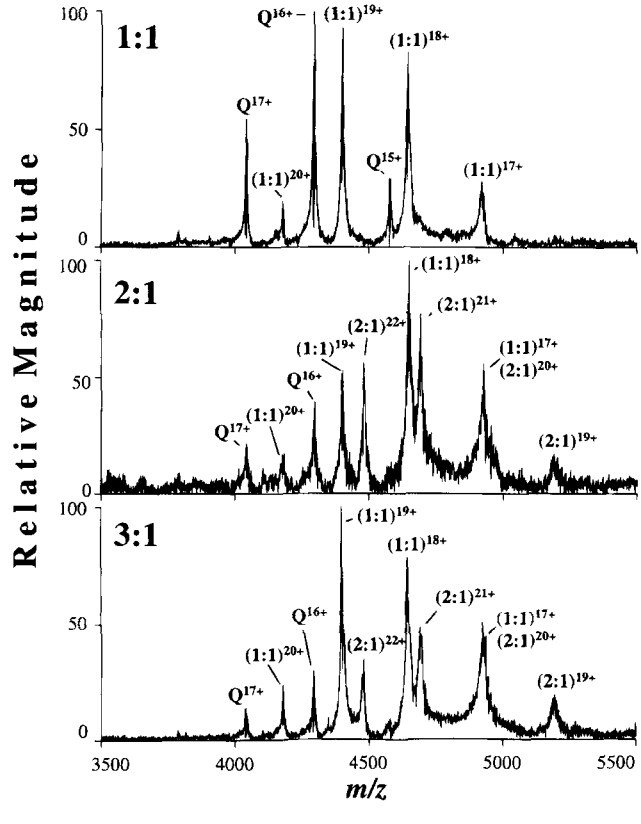

Fig. 7. ESI-FTICR mass spectra of mixtures of Δ131Δ and SecB. The solutions analyzed contained Δ131Δ and SecB at the molar ratios indicated in each panel.

Table 2. *Mass of noncovalent complexes between SecB and Δ131Δ*

	Calculated mass[a]	Observed mass[b]
SecB tetramer (Q)	68,585	68,582 ± 10
SecB:Δ131Δ (1:1)	83,460	83,464 ± 20
SecB:2Δ131Δ (1:2)	98,339	98,436 ± 240

[a]The masses were calculated using the mass of the SecB monomer species that has the N-terminal methionine removed but no N-terminal acetylation. Smith et al. (1996) showed that the purified SecB has all been post-translationally processed to remove the methionine and approximately 50% of the population was acetylated.

[b]Confidence limits of 95% are given for the measured masses. The resolution achieved was sufficient to distinguish SecB tetramers that included acetylated monomers from those that contained only unmodified monomers for free SecB and for SecB in 1:1 complexes, but not for the SecB in 1:2 complexes. For the 1:2 complexes, the centroid of the large distribution was taken as the average value and, therefore, the measured masses of these complexes are expected to be higher than the predicted values that are based on nonacetylated monomer masses. The larger 95% confidence limits observed for these complexes can be ascribed to the fact that fewer peaks and weaker signals were observed, as well as to the uncertainty in peak definition due to the inability to define the monomer distribution. Overall, the assignments made to the peaks observed in these experiments fall well within the observed 95% confidence limits.

namely that there are different kinds of subsites on SecB for binding polypeptide ligands. To demonstrate this we used a short polypeptide ligand, Δ131Δ, that allowed us to fill the subsites separately and assessed the interaction by titration calorimetry. The difference in the shape of the curves of integrated enthalpy vs. molar ratio obtained when the titrations were carried out in opposite directions (SecB added to Δ131Δ and Δ131Δ added to SecB) indicates that there are different kinds of binding sites on SecB. We are unable to assign reliable values to the binding parameters as a consequence of the complexity of the binding but this does not diminish the significance of the qualitative conclusion.

Previous studies of the interaction of peptides with SecB indicated that there are four equivalent binding sites for flexible peptides. The longest of the ligands tested was bovine pancreatic trypsin inhibitor at 58 residues. The protein Δ131Δ is 131 amino acyl residues long, close to the size determined for the binding frame on physiological ligands. Unlike the short peptide ligands, Δ131Δ should be long enough to span more than one of the subsites and perhaps interact with part of the hydrophobic patch if it were available. Thus, it was of interest to measure the stoichiometry of interaction between Δ131Δ and SecB. Analysis of sedimentation velocity in the analytical ultracentrifuge of mixtures of SecB and Δ131Δ showed that the rate of sedimentation increased with increasing ratios of Δ131Δ to SecB up to 3:1 indicating that SecB can bind at least three molecules of the fragment of staphylococcal nuclease under these conditions. The stoichiometry obtained from the mass spectrometric analyses was two molecules of Δ131Δ bound to a tetramer of SecB. Since the mass spectrometry is carried out in vacuum, it seems likely that the binding of the third molecule that was observed by centrifugation involves hydrophobic interactions, which would not be stable in the absence of H_2O. Our earlier mass spectrometric analysis of SecB indicated that at high pH the SecB tetramer dissociated into dimers (Smith et al., 1996). If SecB were a dimer of dimers, we might account for the binding of three molecules of Δ131Δ as follows: one ligand molecule might bind to each dimer with each of the ligand molecules occupying two subsites for flexible chains as identified by the short peptide ligands, and the third molecule of Δ131Δ might be associated with the hydrophobic site. The best fit of the calorimetric data of the titration of SecB into Δ131Δ was 3 mol of Δ131Δ bound, two at one type of site and the third at a site with different enthalpy, and is consistent with this interpretation. We were unable to use titration calorimetry to examine the interaction of SecB with the short peptide ligands originally used to define the flexible binding sites because saturation of these sites causes precipitation of the complex, which although problematical is consistent with our model that a hydrophobic patch is exposed.

In summary, the binding energy that stabilizes a complex between SecB and long nonnative polypeptide ligands is derived from interaction among multiple sites along the polypeptide ligand and subsites on the SecB tetramer that are of at least two sorts as indicated by differences in the enthalpy of binding of short polypeptides that occupy the subsites separately.

Materials and methods

Protein purification

The proteins were purified using published procedures as follows: SecB (Randall et al., 1990), precursor and mature forms of galactose-binding protein (Topping & Randall, 1997; Khisty et al., 1995). Staphylococcal nuclease Δ131Δ was purified from strain AR120 containing a plasmid with the λP_L promoter and the coding sequence for Δ131Δ (gift from D. Shortle; see Shortle & Meeker, 1989). Cells were grown overnight at 40° in Luria broth and the protein purified, lyophilized, and stored as described (Alexandrescu et al., 1994).

The concentrations of the purified proteins were determined spectrophotometrically at 280 nM using coefficient of extinctions as follows: SecB tetramer, 47,600 M^{-1} cm^{-1}; denatured, mature galactose-binding protein and denatured, precursor galactose-binding protein, 37,410 M^{-1} cm^{-1}; and staphylococcal nuclease Δ131Δ, 15,800 M^{-1} cm^{-1}.

Titration calorimetry

All calorimetric titrations were carried out using the OMEGA titration calorimeter from MicroCal, Inc. (Northampton, Massachusetts) and the Origin software supplied with the instrument. The system has been described in detail (Wiseman et al., 1989). The instrument was modified by MicroCal, Inc. so that we can maintain the contents of an injection syringe at constant temperature by circulating chilled water through a jacket around the syringe housing.

Calorimetric titration of one protein with another was performed by successive injections of one into a solution of the other held in the reaction cell. Each injection resulted in an exothermic heat effect until the protein held in the cell was saturated, at which point the endothermic heat of dilution of the injected protein species was observed. The reaction heat, obtained by integration of the deflection from baseline for each injection and corrected by subtraction of the integrated heat of dilution, was normalized to the moles of injectant and plotted. The details of the titrations are given in the legends to the figures.

Analytical ultracentrifugation

Sedimentation coefficients of SecB and complexes between SecB and Δ131Δ were obtained using sedimentation velocity ultracen-

trifugation. Mixtures containing various stoichiometric ratios of SecB and Δ131Δ in 10 mM hepes, 150 mM KAc pH 7.6 were made on ice. SecB concentrations were either 5 or 7 μM and Δ131Δ concentrations varied from 5 to 20 μM. The SecB was the last addition to each mixture. At a ratio of Δ131Δ:SecB of 4:1 addition of SecB caused a precipitate to form. These mixtures were incubated at room temperature until the precipitate appeared to have dissolved. This typically took several hours. Each mixture was loaded into a separate cell of the XL-A Ultracentrifuge (Beckman Instruments, Palo Alto, California) and subjected to centrifugation at 42,000 rpm for up to 5 h at 15 °C. Radial scans to measure the absorbance profile of the column of liquid in each of the three cells in a rotor were taken at regular intervals and the sedimentation coefficient of each sedimenting boundary was measured by the method of van Holde and Weischet (1978) using the XLA Data Analysis Program Version 2.93 from Borries Demeler (University of Texas Health Science Center, San Antonio, Texas). The values used for the density and viscosity of the buffer relative to water were 1.0082 and 1.036. A minimum of 15 radial scans of each mixture was subjected to this analysis.

Mass spectrometry conditions

All experiments were performed at Pacific Northwest National Laboratories using an electrospray ionization source coupled to a 7 T Fourier-transform-ion cyclotron resonance mass spectrometer described by Winger et al. (1993). The techniques used to allow the observation of noncovalent complexes have been described before (Bruce et al., 1995; Pasa-Tolic et al., 1998; Bruce et al., 1998) and are only briefly summarized here. The electrospray ionization source was optimized for low analyte solution flow rates of 0.3 μL min^{-1}. The mass spectrometer interface consists of a heated capillary for ion desolvation. Ions are guided from the electrospray capillary at atmospheric pressure to the ion cyclotron resonance cell by two rf-only quadrupoles. Ions were selectively accumulated and irradiated in the ion cyclotron resonance cell, whose pressure was adjusted with N_2 gas. All spectra were acquired in the positive ion mode.

To remove nonvolatile salts, which are incompatible with the electrospray process, the protein mixture was exchanged into either 10 or 50 mM ammonium acetate as indicated in the legends to the figures using a microdialysis apparatus described elsewhere (Liu et al., 1996). The flow of the exchange buffer was 2 mL min^{-1} using gravity feed; the sample was pumped through the hollow fiber at 5 μL min^{-1} using a syringe pump.

All manipulations of mass spectrometry data, including Fourier transformation, apodization, zerofilling, and deconvolution or mass transformation, were carried out using our own software called ICR-2LS (Anderson & Bruce, 1998). Molecular weight and isotopic distribution calculations based on the amino acid sequence of SecB or Δ131Δ were performed with Mercury (Rockwood & Van Orden, 1996) now a module within ICR-2LS.

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