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Calorimetric analyses of the interaction between SecB and its ligands

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Abstract

SecB is a chaperone in *Escherichia coli* dedicated to export of proteins from the cytoplasm to the periplasm and outer membrane. It functions to bind and deliver precursors of exported proteins to the translocation apparatus before they fold into their native structures, thus maintaining them in a competent state for translocation across the membrane. The natural ligands of SecB are precursor proteins containing leader sequences. There are numerous reports in the literature indicating that SecB does not specifically recognize the leader peptides. However, two published investigations have concluded that the leader peptide is the recognition element (Watanabe M, Blobel G. 1989. *Cell 58*:685–705; Watanabe M, Blobel G. 1995. *Proc Natl Acad Sci USA 92*:10133–10136). In this work we use titration calorimetry to show that SecB binds two physiological ligands, which contain leader sequences, with no higher affinity than the same molecules lacking their leader sequences. Indeed, for one ligand the presence of the leader sequence reduces the affinity. Therefore, it can be concluded that the leader sequence provides no positive contribution to the binding energy.

Keywords: calorimetry; chaperones; 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). By binding to precursor proteins in a nonnative state, 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. Once the precursors have folded they cannot be exported. Thus, 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 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 results from the fact that precursors of exported proteins fold slowly, thus poising the kinetic partitioning toward binding of SecB and thereby export. To gain insight into the mechanism of binding we undertook studies using titration calorimetry.

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Results

Interaction of SecB with precursor and mature forms of ligands

The natural ligands for SecB are precursor proteins carrying amino terminal leader peptides that are proteolytically removed to generate the mature protein when the polypeptide is exported to the periplasmic space (Zwizinski & Wickner, 1980). There are numerous reports in the literature using several different approaches both in vivo and in vitro that conclude selective binding of precursors to SecB does not involve direct recognition of these leader sequences (Gannon et al., 1989; Lecker et al., 1989; Liu et al., 1989; Weiss & Bassford, 1990; De Cock et al., 1992). In addition, the region of contact between SecB, a tetramer of molecular weight 68,700 (Smith et al., 1996) and the mature form of each of three of its natural ligands as well as the precursor form of one ligand has been shown to be large, comprising minimally a length of 150 amino acid residues in the mature portion of the ligand (Topping & Randall, 1994; Khisty et al., 1995; Smith et al., 1997). Nevertheless, two studies carried out by Watanabe and Blobel (1989, 1995) conclude that SecB does specifically bind the leader peptide and has no direct interaction with the remainder of the polypeptide. To obtain further evidence that the binding of SecB to the mature region of the ligands is the biologically significant interaction we have used

titration calorimetry and have directly determined the affinity of SecB for the precursor form and for the mature form of two ligands.

In the presence of SecB, a nonnative ligand is in rapid equilibrium between the free and bound states and so partitions between binding and folding (Topping & Randall, 1997). This presents difficulties in performing titration calorimetry because only the unfolded form is active as a ligand. Thus, the polypeptides must be prevented from refolding during the titration, which takes several hours to complete. Fortunately we can modulate the rate of folding of two physiological ligands, maltose-binding protein and galactose-binding protein, so that the experiments are feasible. For the investigation of interaction with maltose-binding protein we have used the precursor and the mature forms of an altered species (MalE Y283D) that, as a consequence of substitution of an aspartate for tyrosine at position 283, folds very slowly (Liu et al., 1988). To study binding to galactose-binding protein we have taken advantage of the fact that this binding protein contains a Ca^{++} ion that stabilizes the native structure, and we have drastically reduced the rate of folding of the precursor and mature forms by including EGTA in the solutions.

Calorimetric titration of precursor maltose-binding protein with SecB was performed by successive injections of SecB into a solution of 3 μM precursor maltose-binding protein held at 7 °C in the reaction cell. Even though this altered species of maltose-binding protein folds more slowly than does the wild-type species (Liu et al., 1988), it was necessary to include guanidinium chloride in all solutions at 0.3 N to slow folding sufficiently to carry out the titration. Each injection of SecB resulted in an exothermic heat effect until the precursor maltose-binding protein was saturated, at which point the endothermic heat of dilution of SecB was observed (Fig. 1, upper). 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 is plotted in Figure 1, lower. The best fit of the data using a model of a single binding site gave a dissociation constant of 36 nM, a change of enthalpy (ΔH) of -31 kcal mol^{-1}, and a stoichiometry of 1:1, SecB tetramer: precursor MalE Y283D. The results from two titrations are given in Table 1.

Titration of the mature form of maltose-binding protein with SecB (Fig. 2) showed binding characterized by parameters very close to those observed for the interaction with the precursor form: K_d, 31 nM, ΔH, -30 kcal mol^{-1} and stoichiometry 1:1. The parameters obtained from three independent titrations are given in Table 1. Because the parameters for binding of SecB to the precursor and those for binding to the mature form, which lacks the leader peptide, are very similar it is certain that the determinants for binding do not reside solely in the leader sequence.

Typical binding isotherms for titration of precursor galactose-binding protein and of mature galactose-binding protein by SecB and the best fits of the integrated heats are shown in Figures 3 and 4. Comparison of the parameters from the best fits of four independent experiments for the precursor form and three for the mature form (Table 1) reveals a statistically significant difference in the binding constants. The mature galactose-binding protein has a 4-fold higher affinity for SecB than does the precursor form. This result again reinforces the conclusions that SecB does not selectively bind the leader and that the binding energy does not come exclusively from interaction with the leader. Indeed, we can conclude that the leader plays no significant part in the binding observed.

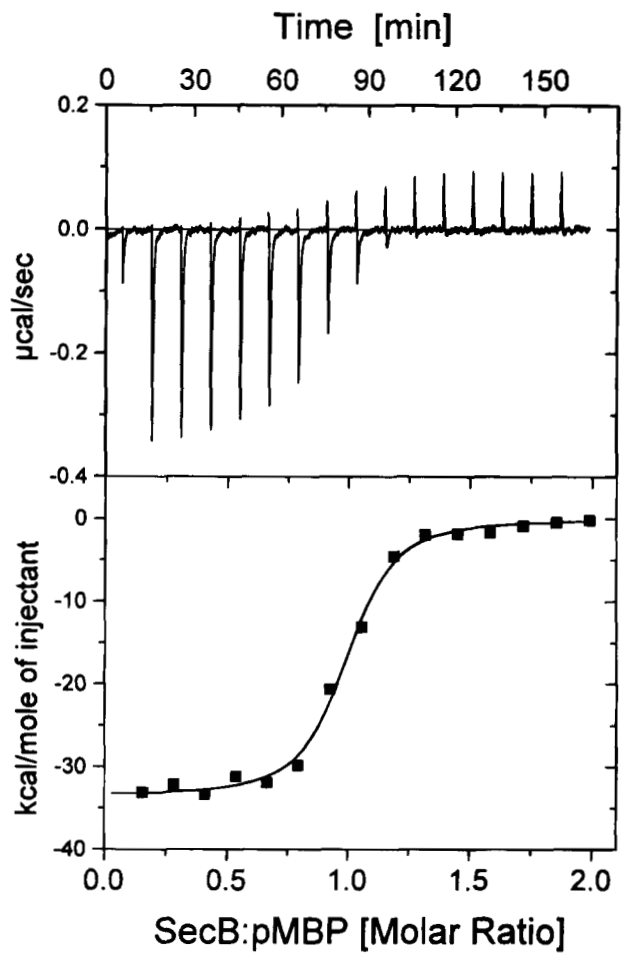

Fig. 1. Binding of precursor maltose-binding protein to SecB. Precursor maltose-binding protein (MalEY283D) was unfolded in 1.5 N GnHCl, 10 mM HEPES, 150 mM KAc, pH 7.6, and diluted to give final concentrations of 3.0 μM precursor MBP, 0.3 M GnHCl, 10 mM HEPES, 150 mM KAc, pH 7.6 immediately before loading into the cell (volume, 1.345 mL). SecB (held in the syringe at 63 μM tetramer) in the same buffer was added in a sequence of 15 injections, each of 8 μL, spaced at 10-min intervals. Determination of the heat of dilution of SecB was carried out identically except the protein was omitted from the reaction cell. The titration was carried out at 6.3 °C. Upper, raw data. Lower, integrated area of heat as a function of the mole ratio of the reactants. The points are the experimental data and the solid line is the calculated best-fit using a least-squares deconvolution algorithm. The binding parameters are summarized in Table 1.

The higher affinity of SecB for the mature form of galactose-binding protein compared to the precursor form could also be demonstrated using gel filtration chromatography, which resolves complexes of SecB and its ligands from the free forms. When solutions containing equimolar quantities of precursor and mature galactose-binding protein in 1 N GuHCl were diluted into a solution that contained a limiting quantity of SecB, the ratio of the ligands recovered in complex with SecB showed an enrichment for the mature form (Fig. 5). When the precursor was in a twofold molar excess over the mature, the representation of each species in the complex was approximately equal. These results are consistent with the data obtained using titration calorimetry indicating that the affinity of SecB for the mature form of galactose-binding protein is higher than that for the precursor form.

Table 1. *Parameters for interaction of SecB and ligands*

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	7.7	27 ± 7	1.2	−10.3	−25[c] ± 0.5	−14.7
precursor MBP	6.3	36 ± 7	1.0	−10.1	−31 ± 0.5	−20.9
SecB into	6.5	30 ± 8	1.0	−10.2	−33 ± 0.6	−22.8
mature MBP	7.5	31 ± 5	1.0	−10.2	−30 ± 0.3	−19.8
	8.0	38 ± 8	0.9	−10.1	−33 ± 0.5	−22.9
SecB into	7.2	17 ± 5	0.9	−10.6	−35 ± 0.7	−24.4
precursor GBP	7.2	35 ± 7	1.0	−10.2	−36 ± 0.5	−25.8
	7.1	45 ± 12	1.0	−10.0	−36 ± 0.6	−26.0
	7.0	56 ± 13	1.0	−9.8	−37 ± 0.6	−27.2
SecB into	6.8	5.0 ± 1	1.1	−11.3	−29 ± 0.2	−17.7
mature GBP	9.4	10 ± 4	1.0	−10.9	−30 ± 0.6	−19.1
	10	13 ± 3	1.4	−10.7	−31[c] ± 0.5	−20.3

[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]The ΔH given was calculated assuming $n = 1.0$.

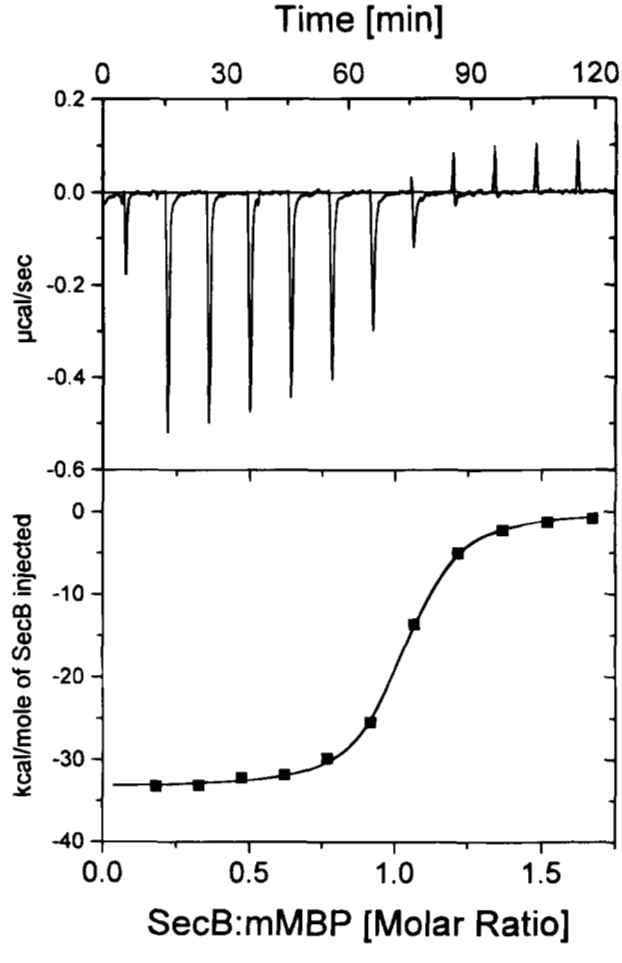

Fig. 2. Binding of mature maltose-binding protein to SecB. Titration of the mature form of maltose-binding protein (MalEY283D) by SecB was carried out as described in the legend to Figure 1 for titration of the precursor except: the concentrations were 4.5 μM maltose-binding protein in the cell and 110 μM SecB tetramer in the syringe, there were 15 injections and the temperature was 7.5 °C.

Discussion

SecB is a chaperone dedicated to export. Thus, the idea that SecB recognizes its physiological ligands via the amino-terminal leader sequence, which all of them possess, is an attractive one. Although two reports support this notion (Watanabe & Blobel, 1989, 1995), there are many studies that directly (Randall et al., 1990; Hardy & Randall, 1991; De Cock et al., 1992; Van Raalte et al., 1996) and indirectly (Bankaitis & Bassford, 1984; Collier et al., 1988; Gannon et al., 1989; Collier et al., 1990) indicate that SecB binds to internal sections of nonnative precursors rather than to the leaders. Here we reinforce the conclusion that the leader sequence does not contribute significantly to the binding of SecB by measuring directly the dissociation constants of SecB for two different ligands, each one with and without a leader sequence. The data, obtained by titration calorimetry, show clearly that for maltose-binding protein the presence of a leader makes no appreciable difference to the affinity for SecB. For galactose-binding protein the presence of the leader decreases the affinity by a factor of 4. This result is supported by competition experiments showing that SecB, when in limiting supply, binds mature GBP in preference to precursor GBP. Although this difference in affinity is not likely to have physiological significance in terms of binding to SecB, it is of interest to note that the difference in free energy of stability (ΔG) of the complexes between SecB and precursor GBP and SecB and mature GBP results from a more unfavorable entropy term in the case of the complex with the precursor (Table 1). A possible explanation is that the hydrophobic residues of the leader are partially buried in the nonnative precursor, which is free in solution, and they become more exposed to the solvent upon formation of a complex with SecB. The mature species lacks the hydrophobic leader sequence and, thus, there might be less of a difference in entropy between the free and complexed forms. The binding frame for SecB has been shown to be contained within the mature region of three polypeptide ligands and, thus, it is reasonable to suppose that in the complex the leader sequence would be exposed to solvent. Further support for this notion is that the leader sequence must be accessible for binding by SecA, the next component along the export pathway.

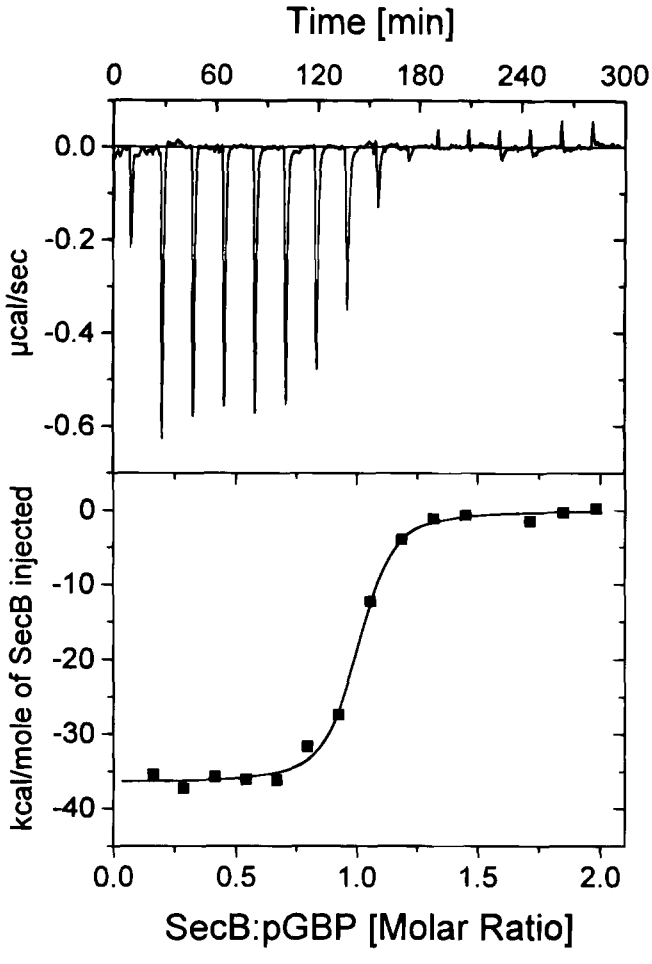

Fig. 3. Binding of precursor galactose-binding protein. Precursor galactose-binding protein was unfolded in 1.0 N GnHCl, 10 mM HEPES, 150 mM KAc, pH 7.6, and diluted to give final concentrations of 4.8 μM precursor GBP, 0.1 N GnHCl, 10 mM HEPES, 150 mM KAc, 0.4 mM EGTA, pH 7.6, immediately before loading into the cell. SecB tetramer (held in the syringe at 140 μM) in the same buffer was added in a sequence of 15 injections, each of 7 μL, spaced at 10-min intervals. The titration was carried out at 7.2 °C.

The values of approximately 30 nM obtained for the dissociation constants of the complex of SecB with precursor galactose-binding protein and with precursor maltose-binding protein are reasonable considering that the concentration of SecB in vivo is approximately micromolar. If we assume that only 10% of cellular SecB is uncomplexed at any given time, the concentration of free SecB would be sufficiently above the K_d so that almost all newly synthesized precursor would be bound. Furthermore, the binding constants for the interaction of these precursors with SecB are of approximately the same magnitude as that determined for the binding to SecA of the precursor form of OmpA in complex with SecB (Hartl et al., 1990).

In summary, studies of the formation of complexes between SecB and two physiological ligands indicate that the leader sequences supply neither specificity nor energy of binding to these interactions.

Materials and methods

Protein purification

The proteins were purified using the published procedures as follows: SecB (Randall et al., 1990); the mature form of the altered species of maltose-binding protein, MalE Y283D (the product of the allele *malEY283D* (Chun et al., 1993)); precursor and mature forms of galactose-binding protein (Khisty et al., 1995; Topping & Randall, 1997). The precursor form of MalE Y283D was purified from a *secA*^{ts} Δ*malE312* strain of *E. coli* (Chun & Randall, 1994) harboring plasmid pTT7 that contains the *malEY283D* gene under control of the tac promoter. Cells were grown at 30 °C in Luria broth containing 50 μg/mL ampicillin to a density of 5×10^8 cells/mL and then diluted threefold with medium held at 42° containing 0.5 mM IPTG. Growth was continued for 2 h at 42° to express the defect in SecA and cause accumulation of precursor MalE Y283D. The cells were harvested, washed once with 10 mM TrisCl pH 7.6, 0.3 M NaCl, and the cell pellet frozen at −70 °C.

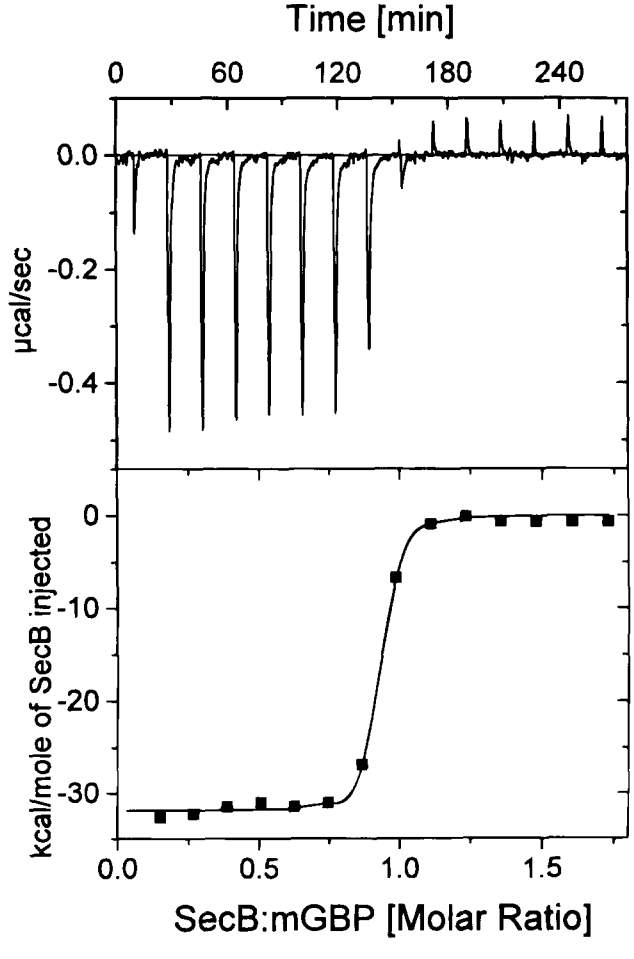

Fig. 4. Binding of mature galactose-binding protein to SecB. Titration of the mature form of galactose-binding protein by SecB was carried out as described in the legend to Figure 3 for titration of the precursor except that the cell contained 6.2 μM mature GBP and there were 14 injections. The titration was carried out at 6.8 °C.

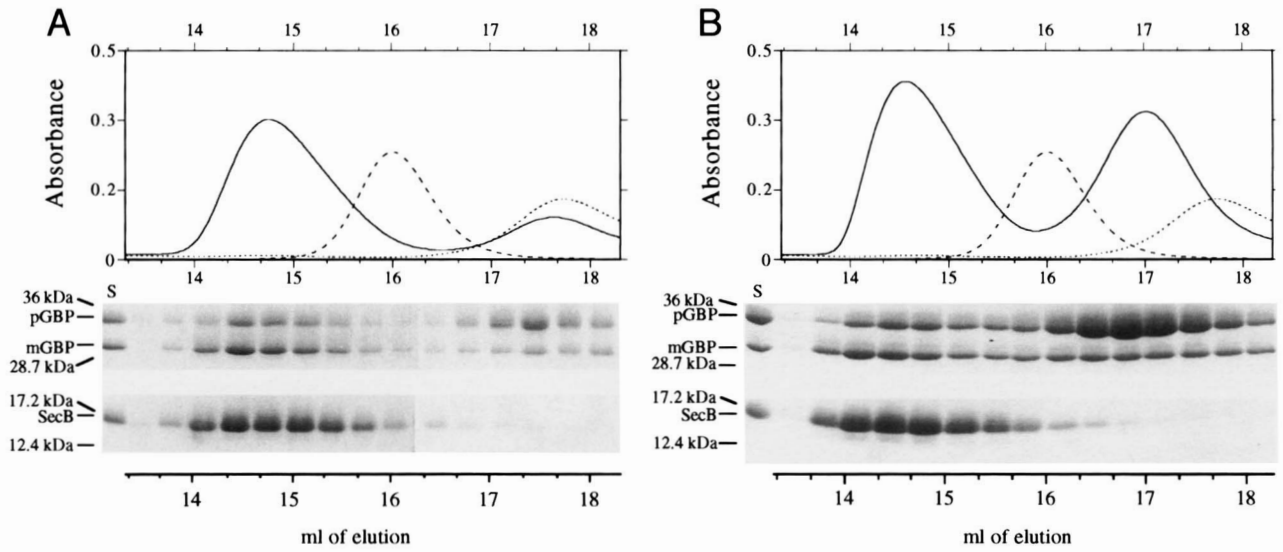

Fig. 5. Complexes between SecB and galactose-binding protein. **A:** Complex at a molar ratio of 3 μM precursor GBP: 3 μM mature GBP: 3 μM SecB. **B:** Complex at a molar ratio of 6 μM precursor GBP: 3 μM mature GBP: 3 μM SecB. Upper panels: absorbance profiles of complexes of precursor GBP, mature GBP, and SecB resolved by size exclusion chromatography. HPLC of protein mixtures were carried out as described in Materials and methods. In each case, the solid line represents the mixture of SecB and the unfolded GBP species, the dashed line represents SecB only and the dotted line represents a mixture of the unfolded GBP species. Lower panels: SDS-polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.33-mL fractions, starting after elution of 13 mL. Lanes S contain 2% of the quantity of sample applied to the column. The positions and masses (in kDa) of molecular weight markers, glyceraldehyde 3-phosphate dehydrogenase 36, carbonic anhydrase 28.7, myoglobin 17.2, cytochrome *c* 12.4, and the positions of SecB, mature GBP (mGBP), and precursor GBP (pGBP) are indicated.

The frozen pellet was thawed and suspended at a density of 3×10^{10} cells/mL in 20 mM TrisCl pH 7.6, 2 mM EDTA, and 100 μg/mL lysozyme. The cell suspension was sonicated to disrupt the cells and break the DNA and then centrifuged for 20 min at 27,000 $\times$ *g*. The pellet, which contained membrane and precursor MalE Y283D in inclusion bodies, was suspended at an equivalent cell density of 3×10^{10} cells/mL in 20 mM TrisCl pH 7.6, 3% (w/v) Triton X-100 and 5 mM EDTA to solubilize the membrane proteins. The inclusion bodies, collected by centrifugation for 20 min at 27,000 $\times$ *g*, were solubilized in a minimal volume (approximately 6×10^{10} cell equivalents/mL) of 6 M urea, 25 mM bis-Tris pH 6.7, and subjected to chromatofocusing using a Pharmacia MonoP HR5/20 column. The gradient was formed with Polybuffer 74 diluted 1/10 in 6 M urea and adjusted to pH 5.3 with HCl. Multiple runs were necessary to avoid exceeding the capacity of the column. Fractions containing the precursor MalE Y283D as detected by SDS gel electrophoresis were pooled and dialyzed against 1.5 M GuHCl, 10 mM HEPES-KOH pH 7.0. Concentration was achieved by multiple rounds of reducing the volume by a factor of 4 in a SpeedVac (Savant) and dialyzing against 1.5 M GuHCl, 10 mM HEPES-KOH pH 7.0. The final dialysis was against 1.5 M GuHCl, 10 mM HEPES-KOH pH 7, 150 mM potassium acetate and the concentrated, denatured protein was stored at -70 °C in this buffer. The maximum achievable concentration for precursor MalE Y283D in this buffer was 5.9 mg/mL.

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 precursor MalE Y283D and denatured mature MalE Y283D, 63,440 M^{-1} cm^{-1}; denatured mature galactose-binding protein and denatured precursor galactose-binding protein, 37,410 M^{-1} cm^{-1}.

Titration calorimetry

All calorimetric titrations were carried out using the OMEGA titration calorimeter from MicroCal, Inc. (Northampton, MA) and the Origin software supplied with the instrument. The system has been described in detail in 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. The details of the titrations are given in the legends to the figures.

Size-exclusion chromatography

Mature and precursor galactose-binding protein were each unfolded by incubation in 1 M GnHCl, 10 mM HEPES, 1 mM EGTA, 300 mM KAc, pH 7.0 for 1 h at room temperature. Mature and precursor galactose-binding protein were mixed in ratios as given for each experiment and then added to SecB held in a buffered solution on ice such that the final concentrations were 10 mM HEPES, 0.16 M GnHCl, 1 mM EGTA, 300 mM KAc, pH 7.0. Immediately following mixing, a portion of the solution (200 μL) was analyzed by size-exclusion high-performance chromatography using a TSK3000.SW column equilibrated in 10 mM HEPES, 1 mM EGTA, 300 mM KAc, pH 7.0, at a flow rate of 1.0 mL/min and absorbance at 280 nm was monitored. Chromatography was carried out at 4 °C. Pure maltose-binding protein (1 μg) was added to each fraction as carrier and protein was precipitated by the addition of 5% trichloroacetic acid. Precipitates were collected by centrifugation, washed with acetone, suspended in sample buffer, and analyzed by electrophoresis on 14% SDS-polyacrylamide gels as described with the exception that the sodium phosphate in the

stacking gel was 27.5 mM (Randall & Hardy, 1977). The gels were stained with Coomassie brilliant blue.

Acknowledgments

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