Thermodynamics of Substrate Binding to the Chaperone SecB†

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ABSTRACT: The thermodynamics of binding of unfolded polypeptides to the chaperone SecB was investigated in vitro by isothermal titration calorimetry and fluorescence spectroscopy. The substrates were reduced and carboxamidomethylated forms of RNase A, BPTI, and α-lactalbumin. SecB binds both fully unfolded RNase A and BPTI as well as compact, partially folded disulfide intermediates of α-lactalbumin, which have 40−60% of native secondary structure. The heat capacity changes observed on binding the reduced and carboxamidomethylated forms of α-lactalbumin, BPTI, and RNase A were found to be −0.10, −0.29, and −0.41 kcal mol^{-1} K^{-1}, respectively, and suggest that between 7 and 29 residues are buried upon substrate binding to SecB. In all cases, binding occurs with a stoichiometry of one polypeptide chain per monomer of SecB. There is no evidence for two separate types of binding sites for positively charged and hydrophobic ligands. Spectroscopic and proteolysis protection studies of the binding of SecB to poly-L-Lys show that binding of highly positively charged peptide ligands to negatively charged SecB leads to charge neutralization and subsequent aggregation of SecB. The data are consistent with a model where SecB binds substrate molecules at an exposed hydrophobic cleft. SecB aggregation in the absence of substrate is prevented by electrostatic repulsion between negatively charged SecB tetramers.

Molecular chaperones have been shown to be involved in protein folding, assembly, and transport (*1*, *2*). The above events compete with nonproductive processes, which lead to protein aggregation. Chaperones bind the aggregation prone non-native state of a polypeptide funneling it to the functional native state. SecB is an *Escherichia coli* cytoplasmic, chaperone protein that is involved in protein translocation across the inner membrane. SecB binds to a subset of proteins destined for export and maintains them in a translocation competent state (*3*, *4*). The SecB-bound polypeptide is transferred first to the peripheral membrane protein SecA and subsequently to the integral membrane protein SecY (*5*−*7*). Little is known about the conformation of polypeptides when bound to SecB, but it has been proposed that these resemble the molten globule state (*8*). SecB is a homotetrameric protein having a molecular mass 68 kDa and is known to form stoichiometric complexes with precursor proteins (*9*). SecB has been shown to bind to a variety of proteins in the unfolded, molten globule and partially folded states but not to the native state. (*10*−*12*). It has previously been proposed that SecB has two types of binding sites for its preprotein ligands, one for positively charged ligands and another for hydrophobic ligands (*13*). Recent experiments on the interaction of SecB with SecA have suggested instead that a negatively charged region of SecB binds specifically to a positively charged region of SecA and is not involved in preprotein binding (*6*). Little is known regarding the secondary structure or peptide sequence motif recognized by SecB. SecB binding to its natural substrates takes place in competition with folding of substrate. Hence, it is difficult to obtain accurate thermodynamic data on binding to its natural substrates. Furthermore, the unfolded states of its natural ligands such as maltose-binding protein are highly aggregation prone (Ganesh, C., and Varadarajan, R., unpublished results). In the present work, we have, therefore, examined the thermodynamics of binding to a variety of model unfolded protein substrates. The binding constants of SecB for some unfolded polypeptides and model peptide ligands have been measured previously (*7*, *10*, *14*, *15*). In the current work, we have used completely unfolded CAM-RNase A,[1] CAM-BPTI, and partially folded disulfide intermediates of α-LA as model protein substrates to gain insights into the energetics of interaction between substrates and SecB. Isothermal titration calorimetry has been used to determine the enthalpy, entropy, and heat capacity of binding of SecB to these polypeptide ligands.

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[1] Abbreviations used: α-LA, alpha-lactalbumin; ANS, 8-anilino-1-naphthalene sulphonic acid; AR-RNase, acrylodan-labeled RNase A; AR-BPTI, acrylodan-labeled BPTI; AR-CAM-LA, 6th and 120th position in α-LA are labeled with acrylodan other cysteines are carboxamidomethylated; AR-CM-LA, 6th and 120th position in α-LA are labeled with acrylodan other cysteines are carboxymethylated; BPTI, bovine pancreatic trypsin inhibitor; BPN, denatured Subtilisin PJ9; CAM-RNase, carboxamidomethylated RNase A; CAM-BPTI, carboxamidomethylated BPTI; CAM-LA, carboxamidomethylated α-LA; 2S−S_{cam} LA, carboxamidomethylated α-LA with 2 disulphides intact; 3S−S_{cam}LA, carboxamidomethylated α-LA with 3 disulphides intact; CD, circular dichroism; DTT, dithiothreitol; ΔC_p, heat capacity change on binding; ITC, isothermal titration calorimetry; IPTG, 1-isopropyl 1-thio-β-D-galactopyranoside; MBP, maltose binding protein; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulphonyl fluoride; RNase A, ribonuclease A.

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EXPERIMENTAL SECTION

Materials. PMSF, iodoacetic acid, iodoacetamide, IPTG, Poly-L-Lys (56 kDa and 10 kDa) and DTT were from Sigma (Sigma Chemical Company, Missouri,U.S.A.). Fast flow Q-Sepharose, Sephacryl S-200, was from Pharmacia. Ultrapure GdnHcl and Ultrapure Tris were purchased from GIBCOBRL. All other chemicals were of analytical grade. CAM-LA, CM-LA, $3S-S_{cam}$, and $2S-S_{cam}$ were prepared as described (*16*−*18*). For $3S-S_{cam}$ and $2S-S_{cam}$ LA, reduction with DTT and subsequent titration with DTNB (*19*) confirmed the number of disulfides present. CAM-RNase A and CAM-BPTI were prepared as described (*7, 20*). Acrylodan-labeled BPTI and RNase A were prepared as described previously (*7*). For preparation of acrylodan-labeled CAM-LA and CM-LA, first $3S-S_{sh}$ was prepared (*16*−*18*) and subjected to acrylodan reaction. Further reduction and alkylation were carried out with either iodoacetamide or iodoacetate to obtain acrylodan-labeled CAM-LA or acrylodan-labeled CM-LA. Protein concentrations were calculated using an extinction coefficient at 280 nm of 27 200 M^{-1} cm^{-1} for CAM-LA and CM-LA (*16*−*18*). The extinction coefficients of $3S-S_{cam}$ LA and $2S-S_{cam}$ LA were taken to be 28 000 M^{-1} cm^{-1} and 27 500 M^{-1} cm^{-1}, respectively (*18*). The extinction coefficients for CAM-RNase A and for CAM-BPTI were taken to be 9300 and 9000 M^{-1} cm^{-1}, respectively (*7, 20*). The number of acrylodan molecules bound per protein molecule were estimated by taking the absorbance at 280 nm and at 360 nm where the extinction coefficient of acrylodan is 12 900 M^{-1} cm^{-1}. The net charges on the above modified protein substrates were estimated from protein sequence using the "Isoelectric" program of the Wisconsin package (*21*).

The SecB expression plasmid pJW25 in strain BL21 (DE3) was obtained from Prof. B. de Kruijff. The cells were grown in LB at 30 °C and were induced at an A_{600} of 0.8−1. The cells were harvested by centrifugation at 4 °C at 4000 rpm. The cells were lysed after suspending them in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1 mM PMSF. The lysate was subjected to high-speed centrifugation at 45 000 rpm. The supernatant was applied to a Fast flow Q-Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.4. The column was washed with 3 column volumes each of 200 and 300 mM NaCl, 20 mM Tris-HCl, pH 7.4. The protein was eluted with 400 mM NaCl in 20 mM Tris-HCl, pH 7.4. The fractions containing SecB were pooled, concentrated and further purified on a Sephacryl S-200 gel filtration column equilibrated with 50 mM phosphate, pH 7.4, containing 0.1 mM PMSF. The fractions containing SecB were dialyzed against 20 mM Tris-HCl, pH 7.4, concentrated, and stored at −70 °C. The purified protein was estimated to be 99% pure by SDS-PAGE as detected by silver staining (*22*) and analytical gel filtration HPLC. Although SecB is a tetramer in solution, all SecB concentrations mentioned are monomer concentrations. The monomer extinction coefficient of SecB at 280 nm was taken to be 11 900 M^{-1} cm^{-1} (*23*).

Fluorescence and CD Measurements. The binding of various acrylodan-labeled derivatives of BPTI, RNase A, and α-LA to SecB was monitored by fluorescence spectroscopy. In a typical titration, 20−70 nM of the labeled substrate was taken in a cuvette and titrated against SecB by monitoring the increase in fluorescence emission at 480 nm (excitation wavelength used 391 nm). The emission slit-widths used for excitation and emission were 5 and 10 nm, respectively. The buffer used for fluorescence and CD was 100 mM potassium phosphate buffer, pH 7.4. The fluorescence and CD measurements were done using a Spex Flourolog-2 fluorimeter and a JASCO 720 spectropolarimeter, respectively. CD spectra were recorded using 1 mm path length rectangular quartz cuvettes at 25 °C at a scan speed of 10 nm min^{-1}. Data points were collected with a response time of 8 s and a bandwith of 2 nm. Each spectrum is an average of 4 scans. Buffer scans were recorded under the same conditions and subtracted from the protein spectra.

ITC Measurements. The titration calorimetry measurements were performed with a Microcal Omega Titration Calorimeter as described (*24*). All the substrates were dissolved in 10 mM HCl and then diluted into buffers, which had a final concentration of either 100 or 50 mM potassium phosphate, pH 7.4. SecB was dialyzed against double distilled water and then diluted into the buffer. All samples were centrifuged prior to titration to remove any visible precipitates. A typical titration consisted of injecting 15 μL aliquots of 500 μM SecB into 30−60 μM of derivatives of α-LA, CAM-RNase A, or CAM-BPTI in the appropriate buffer at pH 7.4. The time interval between consecutive injections was 2.5 min to ensure that the titration peak returns to the baseline prior to the next injection. Appropriate titrations were done at each temperature to determine the dilution heats of SecB into buffer that contained no substrates. The resulting, corrected data were evaluated to determine the binding stoichiometry, binding constant, and enthalpy using the ORIGIN software package for the OMEGA calorimeter, assuming single site binding. The reliability of determination of the binding constant depends on the dimensionless quantity C that is a product of the binding constant, K_b, and the total concentration of macromolecule in the cell $[M_t]$. The *C* values used here range between 1 and 20.

Proteolysis Experiments. Proteolysis experiments were performed in 10 mM HEPES, pH 7.6, on ice. To a 290 μL solution of 10 μM SecB, 10 μL of a 100 μg mL^{-1} from a stock solution of proteinase K was added in the presence and absence of the 5 μM poly-L-Lys (56 kDa or 8 kDa) and was incubated on ice. The reaction was stopped by adding 10 μL of 0.1 M PMSF after 20 min. The reaction mixture containing SecB and poly-L-Lys was centrifuged, and the pellet was suspended in 400 μL of loading buffer for SDS-PAGE analysis. To the 300 μL of supernatant 100 μL of loading buffer was added. The reaction mixture, which contained no poly-L-Lys, did not show any visible precipitate. To this mixture 100 μL of loading buffer was added. The samples were analyzed on 15% SDS-PAGE. Proteolysis experiments carried out on the complexes with BPTI have been done as previously described except that the samples were centrifuged prior to addition of loading buffer (*13*).

ANS-binding and Scatter Experiments. ANS-binding experiments were done using a Spex Flourolog-2 fluorimeter. Excitation and emission slit-widths of 1.5 and 10 nm, respectively, were used. A solution of 0.6 μM SecB in 10 mM Hepes, pH 7.6, containing 10 μM of ANS was placed in a thermostated cuvette holder cooled using a water bath circulator. The temperature in the cuvette was maintained at 4 °C. Poly-L-Lys solution was prepared as described previously (*13*). Increasing concentrations of 56 kDa and 8

Table 1: Thermodynamic Parameters for Titrating Non-native Substrates in 100 mM Potassium Phosphate Buffer pH 7.4 with SecB[a]

substrate	T (°C)	n	$\Delta H°$ (kcal/mol)	K (M^{-1})	$\Delta G°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
CAM-BPTI	8.0	1.0	−6.0	2.6×10^5	−7.0	−1.0
	10.0	1.0	−5.8	3.0×10^5	−7.1	−1.3
	15.0	1.0	−7.4	3.3×10^5	−7.3	−0.1
	20.0	1.0	−8.9	3.2×10^5	−7.3	−1.5
	25.0	0.9	−10.8	3.4×10^5	−7.5	−3.3
CAM-RNase	10.0	1.0	−8.4	2.0×10^5	−6.9	−1.5
	15.0	1.0	−10.6	9.0×10^4	−6.5	−4.1
	20.0	1.1	−11.8	7.9×10^4	−6.6	−5.2
	25.0	1.0	−15.0	7.0×10^4	−6.5	−8.5
CAM-LA	10.0	1.0	−3.8	4.0×10^5	−7.3	3.4
	15.0	1.1	−4.4	2.6×10^5	−7.1	2.8
	20.0	1.0	−4.9	1.9×10^5	−7.0	2.2
	25.0	1.1	−5.4	1.1×10^5	−6.9	1.5
2S−S_{cam}LA	20.0	1.0	−5.4	9.05×10^4	−6.6	1.2
3S−S_{cam}LA	20.0	1.1	−6.8	1.07×10^5	−6.8	0.0

[a] Values of ΔC_p derived from the fits for eq 1 are −0.10, −0.29, and −0.41 kcal mol^{-1} K^{-1} for CAM-LA, CAM-BPTI, and CAM-RNase, respectively.

kDa poly-L-Lys were added and the fluorescence measured at 474 nm with excitation at 350 nm. Scattering experiments were done in a Jasco UV-spectrophotomer with a slit-width of 0.2 nm. The experiments were carried out as stated above except that 10 μM of ANS was not included. The increase in scatter of the solution was measured at 350 nm. Binding of the following positively charged substrates to SecB were also examined in a similar fashion: poly-L-Arg (15 kDa), melittin, polymixin B, Lys, Arg, spermidine, and cadaverine.

Refolding Kinetics of MBP at Room Temperature in the Presence of Poly-L-Lys and SecB. The refolding kinetic experiments were carried out using a Jasco FP 777 fluorimeter. The excitation and emission wavelengths used were 280 and 341 nm, respectively. The excitation and emission slit-widths were 1.5 and 10 nm, respectively. MBP was unfolded in 2 M Gdn-HCl, 10 mM HEPES, pH 7.4. To initiate folding, 100 μL of 1μM of unfolded MBP was diluted into 900 μL of 10 mM HEPES, pH 7.4, in the presence or absence of 0.4 μM SecB. Refolding experiments were also carried out in the presence of 0.4 μM poly-L-Lys (56 kDa) alone and with 0.4 μM SecB. For refolding experiments in the presence of 0.4 μM poly-L-Lys (56 kDa) and 0.4 μM SecB, the solution was centrifuged at 5000 rpm prior to addition of the unfolded MBP; 100 μL of 1μM of unfolded MBP was then diluted into 900 μL of the supernatant. The refolding of MBP was monitored by measuring the change in fluorescence intensity at 341 nm.

RESULTS AND DISCUSSION

Binding of SecB to CAM-RNase A and CAM-BPTI. CAM-RNase A and CAM-BPTI are completely unfolded polypeptides as monitored by far- and near-UV CD (*10, 20*). The binding of CAM-RNase and CAM-BPTI to SecB has been studied previously using fluorescence spectroscopy (*7, 10*). In the present work, we have investigated the interaction of SecB and the above model substrates using ITC. The binding reaction is an exothermic process and each monomer of SecB binds a single substrate molecule (Table 1 and Figure 1). The interaction with SecB a highly negatively charged protein (overall charge on protein −14.4 at pH 7.4) is

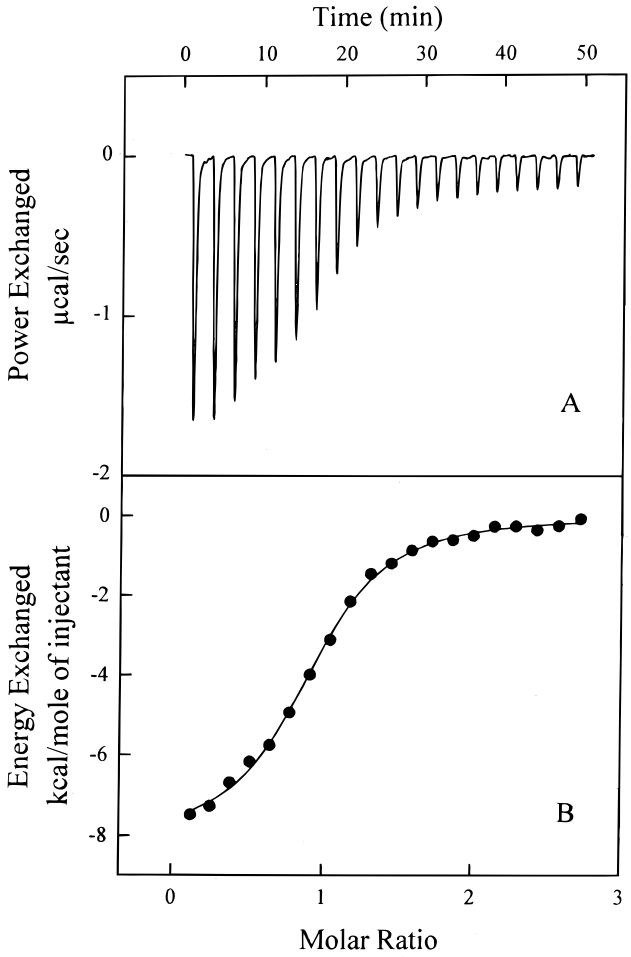

FIGURE 1: Exchange of heat on binding of CAM-BPTI to SecB. (A) A representative isothermal calorimetric titration of 508 μM SecB into 34 μM CAM-BPTI at 15 °C in 100 mM potassium phosphate buffer, pH 7.4. (B) The exothermic heats exchanged per mole of injectant as a function of the ratio of the SecB to CAM-BPTI. The data were fitted to a single site-binding model to obtain $\Delta H°$ and K of −7.46 kcal mol^{-1} and 3.33×10^5 M^{-1}, respectively.

Table 2: Effect of Ionic Strength of Potassium Phosphate Buffer pH 7.4 and Salt Concentration on the Thermodynamic Parameters on Titration of Non-native Substrates with SecB at 20 °C

substrate	buffer mM (salt M)	N	$\Delta H°$ (kcal/mol)	K (M^{-1})	$\Delta G°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)
CAM-BPTI	100 (0.2)	1.0	−6.4	2.0×10^5	−7.1	0.7
CAM-RNase	100 (0.2)	1.0	−6.2	1.9×10^4	−5.7	−0.5
CAM-LA	100 (0.5)	1.0	−6.6	3.2×10^5	−7.4	0.8
CM-LA	100 (0.5)	1.0	−3.9	3.5×10^4	−6.1	2.1
CAM-BPTI	50 (0)	1.0	−8.0	1.0×10^6	−8.0	0.0
CAM-RNase	50 (0)	1.0	−15.9	7.9×10^4	−6.6	−9.4
CAM-LA	50 (0)	0.9	−3.2	8.7×10^4	−6.6	3.4

enthalpically driven, while the $T\Delta S$ component opposes binding. Since both CAM-BPTI (overall charge at pH 7.4 is +5.1) and CAM-RNase A (overall charge at pH 7.4 is +3.2) are both positively charged, there will be a favorable electrostatic driving force for complex formation. Consistent with this expectation, the inclusion of salt lowered the binding constant for both the substrates with the decrease being larger for CAM-RNase A (Tables 1 and 2). A significant amount of enthalpy−entropy compensation was also observed.

The temperature-dependence of the binding reaction was fitted to yield the heat capacity change on binding of SecB

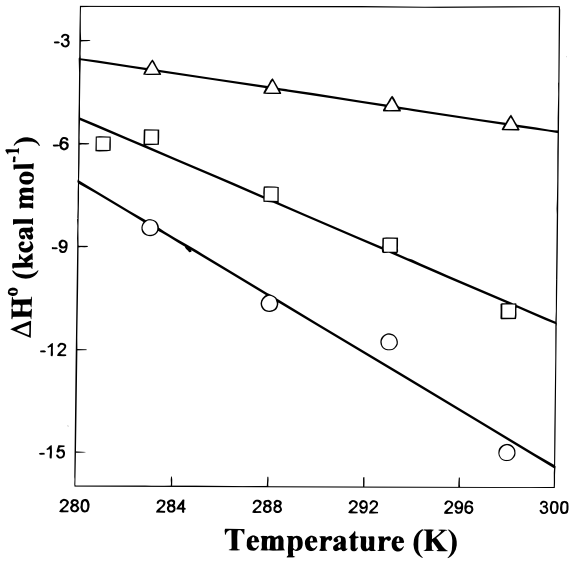

FIGURE 2: Temperature-dependence of the calorimetric enthalpy of the binding reaction (Table 1) between SecB and either CAM-RNase A (○), CAM-BPTI (□), or CAM-LA (△). The lines represent fits of the data to eq 1. Values of ΔC_p derived from the fits are −0.10, −0.29, and −0.41 kcal mol^{-1} K^{-1} for CAM-LA, CAM-BPTI, and CAM-RNase, respectively.

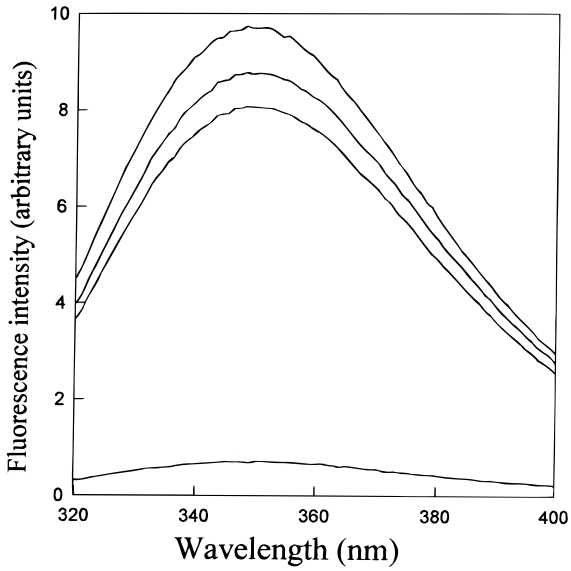

FIGURE 3: Binding of CAM-LA to SecB monitored by fluorescence spectroscopy at 25 °C, pH 7.4. From bottom to top, the spectra are as follows: 1 μM SecB, 8 μM CAM-LA, numerically added spectra of 1 μM SecB, and 8 μM CAM-LA, spectrum of a mixture containing 1 μM SecB and 8 μM CAM-LA.

to various substrates using the following equation:

$$\Delta H(T) = \Delta H\,(T) + \Delta C\mathrm{p}^{*}\,(T - T^{\circ}) \qquad (1)$$

The ΔC_p's of binding are −290 and −410 cal mol^{-1} K^{-1} for CAM-BPTI and CAM-RNase A, respectively (Figure 2).

Binding of SecB to Derivatives of α-LA. To examine the effect of protein conformation on binding to SecB, the binding of a number of partially folded and unfolded derivatives of α-LA were examined. The native state of α-LA is stabilized by four disulfide bonds and a single Ca^{2+} ion (*31*, *32*). At elevated temperatures, in the presence of intermediate concentrations of denaturants, or upon removal of the Ca^{2+} ion at acidic pH, α-LA adopts the molten globule conformation (*33*, *34*). This state is compact as judged by NMR and lacks tertiary interactions. The far-UV CD shows some of the secondary structure present in the native protein (*35*). The molten globule characteristics of various partially folded disulfide intermediates have been studied extensively (*17*, *18*, *36*). CAM-LA, 2S−S_{cam} LA, and 3S−S_{cam} LA have shown characteristics of molten globules under physiological conditions (*36*). 3S−S_{cam} LA is the most ordered of the three molten globules and CAM-LA is the least ordered. In contrast to the CAM derivatives, CM LA is largely unfolded and does not bind ANS. While there is an increase in the fluorescence of SecB on binding to CAM-LA (Figure 3), there is no change in the conformation of CAM-LA in complex with SecB as monitored by far-UV CD (data not shown). A single molecule of CAM-LA binds to a monomer of SecB. The ΔH° of binding is less exothermic than for CAM-BPTI or CAM-RNase, and the magnitude of ΔC_p is appreciably smaller for CAM-LA than for the latter two molecules. However, in contrast to the case for the above two proteins, the ΔS of binding is positive and hence the free energy of binding is similar in all three cases (Table 1). The differences in ΔH° and ΔS of binding for CAM-LA relative to those of CAM-RNase and CAM-BPTI may be interpreted as follows. Since CAM-LA is more compact and structured than either CAM-RNase or CAM-BPTI, there is a smaller loss of conformational entropy upon binding. The positive ΔS probably results from release of ordered solvent upon binding. In the case of CAM-RNase and CAM-BPTI, the larger loss of conformational entropy results in an overall negative ΔS of binding. However, the larger amount of surface area buried upon binding is reflected in more negative values of ΔH° and ΔC_p for CAM-RNase and CAM-BPTI relative to corresponding values for CAM-LA. The affinity of the various CAM-LA derivatives for SecB follow the order CAM-LA > 2S−S_{cam}LA > 3S−S_{cam}LA. Thus, progressive ordering of the CAM-LA molecule is accompanied by a decrease in binding affinity for SecB. However, there is no observable trend in the relative contributions of ΔH° and ΔS.

Electrostatic and Hydrophobic Contributions to SecB: Substrate Binding. It has previously been suggested (*13*) that SecB has specific binding sites for positively charged regions of the substrate. Hence, electrostatic interactions between SecB and substrates should be important for binding. It might, therefore, be expected that the affinity of SecB for positively substrates such as CAM-BPTI (charge +5.1) and CAM-RNase (charge +3.2) would be appreciably higher than for highly negatively charged substrates such as CAM-LA (charge −9.9). This is in contrast to the experimental observations, which show that SecB has a similar affinity for all three substrates. To further examine the importance of electrostatics in substrate recognition, titrations were carried out at different salt concentrations (Table 2). CM-LA does not bind SecB in 100 mM potassium phosphate buffer, pH 7.4, probably due to the eight extra negatively charged carboxylates that are present relative to CAM-LA. Binding is observed only in the presence of 0.5 M KCl, which probably screens the negative charges on both molecules (Table 2). Increasing the salt concentration results in a decrease in the binding constant of SecB for CAM-BPTI and CAM-RNase, but an increase in the binding constant for negatively charged CAM-LA. Decreasing the

Table 3: The Effect of Ionic Strength of Potassium Phosphate Buffer pH 7.4 and Salt Concentration on the Fluorescence-Derived Binding Constants of Unfolded Protein Substrates to SecB at 20 °C

substrate	buffer mM (salt M)	N	K (M^{-1})	K(ITC)/K (fluorescence)
AR-BPTI	100 (0.2)	1.0	1.5×10^7	0.013
AR-RNase	100 (0.2)	1.0	1.4×10^7	0.001
AR-CAM-LA	100 (0.5)	1.1	5.4×10^6	0.059
AR-CM-LA	100 (0.5)	1.2	1×10^6	0.035
AR-BPTI	50 (0)	1.0	6×10^7	0.016
AR-RNase	50 (0)	1.0	2.2×10^7	0.003
AR-CAM-LA	50 (0)	1.1	3.2×10^6	0.027

buffer concentration to 50 mM results in an increase in affinity for CAM-BPTI, no change for CAM-RNase, and a small decrease for CAM-LA. Taken together, the ionic strength dependence of the affinity suggests that though electrostatic interactions do play a role in substrate binding, these are not the major determinant of binding affinity. Whether the electrostatic interactions are a specific effect localized to particular regions of SecB and substrate or a nonspecific effect that is primarily determined by the overall charges of SecB and substrate cannot be determined from these experiments.

Discrepancies in the Fluorescence and Calorimetric-derived Binding Constants. Table 3 shows that the binding constants estimated from fluorescence are appreciably higher than those estimated from isothermal titration calorimetry (Table 2). The fluorescence measurements yielded binding constants of approximately 10^7-10^8 M^{-1}. These values are similar to that obtained previously (*7, 10*) and shows that there is nothing wrong with either the substrates or SecB used in the present studies. We have performed ITC experiments with the acrylodan-labeled BPTI and RNase A and have obtained identical thermodynamic data to those obtained by ITC with the CAM derivatives (data not shown). To confirm that the SecB substrates do not aggregate under the conditions of the experiment, the substrates were subjected to gel filtration using a Pharmacia Superose 6 column. The CAM-BPTI, CAM-RNase, and CAM-LA eluted at 17.3, 14.7, 15.2 mL, respectively. These elution volumes are considerably greater than the exclusion limit of the column (8 mL) and shows that the substrates are not aggregated. SecB elutes at 13 mL (concentration of SecB injected into column is 300 μM), showing that it is also not aggregated. The dilution heats of SecB into buffer measured during the titration calorimetry experiments (Figure 1) were also small, strongly suggesting that SecB is not aggregated under the conditions of the experiment. Similar discrepancies have been previously observed between thermodynamic-binding parameters measured by fluorescence and titration calorimetry in the RNase S fragment complementation system, in the interaction of human platelet profilin with actin, and binding of Hoechst33258 to duplex DNA (*25−28, 29*). A possible explanation for the discrepancy has been put forward by Haq et al. (*29*). The binding constant can only be determined accurately if the concentration of the protein in the titration cell is less than the dissociation constant of the interaction. However, in the present work, the titration calorimetry experiments were carried out at higher concentrations (μM) of non-native substrates than used in the fluorescence measurements (nM). It was not possible to carry out ITC experiments at lower substrate concentrations because of the low heats of binding. Since the substrate concentrations used for the present ITC experiments are higher than the dissociation constant, the binding constant values derived by ITC are probably inaccurate. Despite these problems, ITC can be used to accurately and directly measure the enthalpy of binding and the stoichiometry for the interaction, even in cases where the binding constants cannot be determined accurately (*29, 30*). In addition, the data in Table 3 show that although the absolute values of K determined by ITC and fluorescence differ, the trends in binding with salt concentration and chemical modification are very similar.

Titrations with ANS and SDS-PAGE Analysis. The present calorimetric and fluorescence titrations show no evidence for more than one kind of binding site. Earlier studies (*13*) have asserted that SecB has separate and specific binding sites for positively charged ligands and hydrophobic ligands. Both of these subsites were thought to be involved in specific recognition of substrate by SecB. Positively charged ligands were shown to bind SecB with an affinity in the micromolar range (*37*). Binding of such ligands to SecB was shown to increase the fluorescence of the hydrophobic dye ANS (*13*). To characterize the binding thermodynamics of positively charged ligands with SecB, we attempted to carry out calorimetric titrations using either 8 kDa poly-L-Lys or 56 kDa poly-L-Lys. However, in both cases, titration with SecB resulted in precipitation of the SecB-poly-L-Lys complex. A fluorescence titration of SecB was also carried out in the presence of ANS under conditions identical to that described earlier (*13*). There is an increase in ANS fluorescence as reported earlier (*13*). However, there was also a concomitant increase in scatter measured at 350 nm due to precipitation of the complex of SecB and pol-L-Lys (Figure 4). The previous studies also showed that poly-L-Lys binding caused SecB to become resistant to proteolysis by proteinase K and were interpreted in terms of a ligand induced change in SecB conformation. The protease protection experiments were repeated in the present work. After protease digestion, samples were centrifuged prior to electrophoresis. This additional step removes aggregated species of SecB from solution. When the mixture of SecB, proteinase K, and poly-L-Lys was centrifuged, all of the SecB was in the pellet. In contrast, when the mixture of SecB, proteinase K, and CAM BPTI was centrifuged, all the SecB was retained in the supernatant (Figure 5). This clearly shows that the apparent protection in the presence of poly-L-Lys is due to aggregation of SecB upon poly-L-Lys binding and not because of a conformational change. The aggregated species probably have exposed hydrophobic surface, which leads to ANS binding. In contrast to the situation with pol-L-Lys, the protease protection observed in the presence of CAM-BPTI is not due to aggregation.

We have also studied the interaction of CAM-BPTI with SecB as monitored by ANS fluorescence and light scattering. As shown in Figure 6A and B, there is a negligible increase in ANS fluorescence or scatter at 360 nm on binding of CAM-BPTI to SecB. However, on addition of poly-L-Lys (56 kDa) to the SecB−CAM-BPTI complex, there is a large enhancement in ANS fluorescence and concomitant increase in scatter of the solution. This indicates that the increase in ANS fluorescence is due to aggregate formation. SDS-PAGE of the sample before and after centrifugation confirmed that

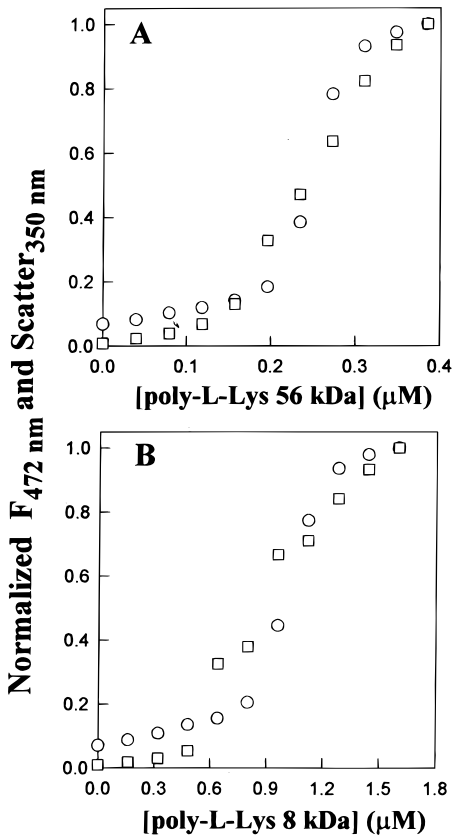

FIGURE 4: Spectroscopic characterization of poly-L-Lys binding to SecB at pH 7.6, 4 °C. (A) Increasing concentrations of 56 kDa poly-L-Lys were added to a 0.6 μM solution of SecB in 10 mM Hepes, pH 7.6 containing either zero (□) or 15 μM (○) ANS. Normalized values of either scattering intensity at 350 nm (□) or ANS fluorescence at 472 nm (○) are plotted as a function of total poly-L-Lys concentration. (B) Experiments identical to those in (A) but using 8 kDa poly-L-Lys.

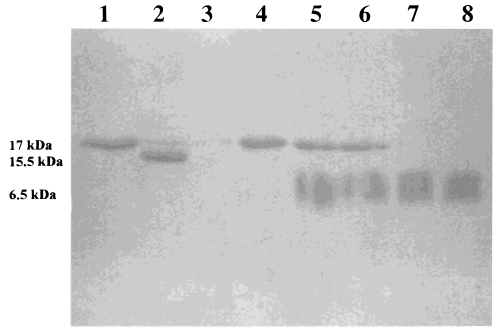

FIGURE 5: Resistance of SecB to proteolysis in the presence of poly-L-Lys or CAM-BPTI. Digestion conditions are indicated in Experimental Section. (1) SecB alone, (2) Sec B + proteinase K, (3) [SecB + proteinase K + poly-L-Lys (56 kDa)] supernatant, (4) [SecB + proteinase K + poly-L-Lys (56 kDa)] pellet, (5) SecB + CAM-BPTI, (6) [SecB + proteinase K + CAM-BPTI] supernatant, (7) CAM-BPTI, (8) CAM-BPTI + proteinase K.

(as in Figure 5) all of the BPTI and SecB formed an insoluble aggregate in the presence of poly-L-Lys. The salt dependence of the poly-L-Lys induced aggregation of SecB was also examined. As shown in Figure 7, both increase in salt concentration and increase in SecB concentration promote aggregation. Other salts such as calcium chloride, magnesium sulfate, sodium chloride, and lithium chloride also promote the aggregation poly-L-Lys dependent SecB aggregation (data not shown). The observation that aggregation can occur at

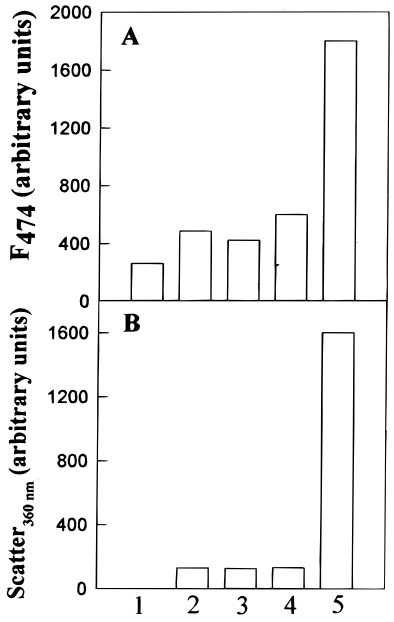

FIGURE 6: Characterization of ligand binding to SecB by ANS fluorescence and light scattering. (A) ANS fluorescence emission intensity at 474 nm, excitation at 350 nm: (1) 10 μM ANS alone, (2) 5 μM SecB + 10 μM ANS, (3) 5 μM BPTI + 10 μM ANS, (4) complex of 5 μM SecB and 5 μM BPTI +10 μM ANS, and (5) complex of 5 μM SecB and 5 μM BPTI with 0.25 μM poly-L-Lys 56 kDa +10 μM ANS. (B) Light scattering was measured at 360 nm of samples 2−5. 10 μM ANS was not included in these measurements.

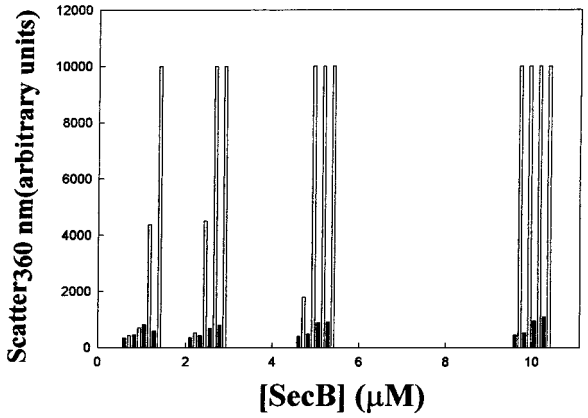

FIGURE 7: Salt and SecB concentration dependence of poly-L-Lys induced aggregation of SecB. At SecB concentrations of 1, 2.5, 5, and 10 μM, the concentration of salt was varied from 0 to 300 mM KCl and the intensity of scattered light at 320 nm was measured in the presence (empty bars) and absence (filled bars) of 0.25 μM poly-L-Lys (56 kDa). At each concentration of SecB, the vertical bars from left to right represent 0 mM KCl, 0 mM KCl, 20 mM KCl, 20 mM KCl, 50 mM KCl, 50 mM KCl, 300 mM KCl, and 300 mM KCl.

low salt concentrations if the SecB concentration is sufficiently high suggests that aggregation is not due to a salt-induced conformational change in SecB. We also examined the effects of other polycations on the aggregation of 2.5 μM SecB. Both poly-L-Arg and the positively charged peptide melittin, at concentrations of 2.5 μM lead to aggregation of SecB. In contrast, polyamines such as spermidine and cadaverine as well as the amino acids Arg and Lys did not lead to aggregation even at concentrations as high as 1 mM. Thus, the poly-L-Lys induced aggregation of SecB appears to require the presence of an amide bond

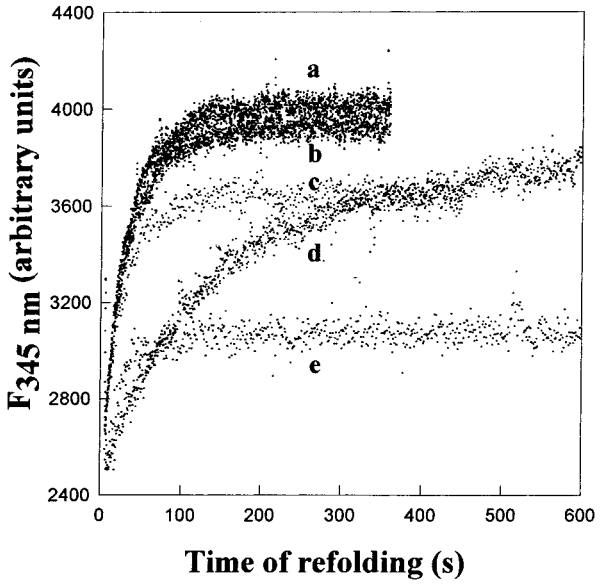

FIGURE 8: Effect of SecB and poly-L-Lys on the refolding kinetics of MBP in 0.2 M Gdn HCl, pH 7.4. (a) 0.1 μM of MBP. (b) 0.1 μM of MBP refolding in the presence of 0.4 μM μM poly-L-Lys (56 kDa). (c) A solution of 0.4 μM of SecB and 0.4 μM of poly-L-Lys (56 kDa) was subjected to centrifugation at 5000 rpm. Unfolded MBP was diluted into the supernatant to a final concentration of 0.1 μM. (d) 0.1 μM of MBP in the presence of 0.4 μM of SecB. (e) 0.1 μM of MBP refolded in 0.2 M Gdn-HCl in 10 mM HEPES, pH 7.4, containing 0.4 μM of SecB and 0.4 μM of poly-L-Lys (56 kDa). Conditions were as in (c), except that the centrifugation was omitted.

and is not completely nonspecific. However, since binding of poly-L-Lys, poly-L-Arg, and of other highly positively charged peptides such as melittin leads to aggregation of SecB, it is unlikely that this reaction is of physiological significance and earlier conclusions drawn from the results of apparent binding of poly-L-Lys (*13, 37*) are unlikely to be correct.

The data suggest that SecB contains a relatively exposed hydrophobic binding site. Aggregation of SecB molecules through this hydrophobic patch normally does not occur because of electrostatic repulsion between negatively charged SecB molecules. However, because of its high negative charge, SecB also has an affinity for highly positively charged peptides. Residues 20−24 and 74−80 of SecB are thought to be involved in binding to the highly positively charged C-terminal region of SecA (*38*). It is possible that these residues may also be involved in binding other positively charged ligands such as poly-L-Lys. The present work shows that charge neutralization of SecB by positively charged peptide ligands leads to aggregation of SecB whether or not authentic substrates are present.

Effect of SecB and Poly-L-Lys on the Refolding of MBP. MBP is one of the physiological ligands for SecB. At room temperature in vitro, SecB slows down the folding of MBP, but does not block folding of the protein. However, in the presence of poly-L-Lys, SecB has previously been shown to block folding of MBP at room temperature. It has been asserted that poly-L-Lys binding causes a conformational change in SecB that exposes a hydrophobic patch, allowing it to bind refolding MBP (*37*). Figure 8 shows the effects of SecB and poly-L-Lys on the refolding kinetics of MBP. As shown in the previous section, poly-L-Lys binding leads to aggregation of SecB. When the solution of SecB and poly-L-Lys is centrifuged prior to addition of MBP there is very little blockage of MBP refolding. However, if the solution is not centrifuged, then an appreciable blockage of MBP refolding occurs as was observed previously. Hence, the SecB−poly-L-Lys aggregates appear to block MBP refolding. This probably occurs because the aggregates contain exposed hydrophobic surfaces competent to bind partially folded MBP. However, it is unlikely that these results have any bearing on the mechanism of action of intact, tetrameric SecB. To confirm that the apparent blockage of MBP refolding was due to the presence of SecB aggregates, SDS-PAGE of the mixture was carried out before and after centrifugation of the sample as in Figure 5. In the presence (but not in the absence) of poly-L-Lys, all of the MBP and SecB were found to be in the form of insoluble aggregates.

Comparison With Binding Data For Other Chaperones. The chaperone system for which the most detailed structural and thermodynamic data exist is the chaperone GroEL. The chaperone GroEL has been shown to bind the compact flexible disulfide folding intermediates of α-LA, which has exposed hydrophobic surfaces (*36*). The interactions of GroEL with CAM-LA and denatured pepsin have been investigated recently by ITC (*39*). The interaction of CAM-LA with GroEL was shown to be driven by the hydrophobic effect reflected from the highly negative ΔC_p of −1 kcal mol^{-1} K^{-1}. A similar study using denatured BPN as substrate (*40*) also showed the hydrophobic effect to be the major driving force for interaction of substrate with GroEL. However, the interaction with denatured pepsin exhibited a very small negative heat capacity change of − 0.047 kcal mol^{-1} K^{-1}. This showed that the hydrophobic effect might not always be the dominant driving force for binding to GroEL (*39*). In the case of SecB, the values of ΔC_p range from −0.1 to −0.4 kcal mol^{-1} K^{-1} and the binding of both positively and negatively charged unfolded protein substrates is ionic strength dependent. For the same substrate (CAM-LA), the value of ΔC_p is much smaller in absolute magnitude for binding to SecB than to GroEL (−0.1 vs 1 kcal mol^{-1} K^{-1}). This suggests that surface area buried upon binding is much smaller in the case of SecB than in GroEL.

Model For Substrate Recognition By SecB. A large body of work (*41*) suggests that proteins that bind a variety of unfolded and partially folded peptide and protein substrates typically bind to an extended conformation of the substrate. These systems include MHC molecules, SH2 and SH3 domains, and the periplasmic oligopeptide binding protein (*42*−*48*). Crystallographic studies on chaperone peptide complexes have so far shown the only mode of peptide binding to be in an extended state. The chaperones PapD and DnaK bind peptides in an extended state mediated by hydrogen bonds involving the backbone amides of the substrate (*49, 50, 51*). Isothermal titration calorimetry studies of protein−peptide complexation (*46, 52, 53*) show that binding is typically enthalpically driven. Polar interactions involving main chain atoms of the substrate often contribute significantly to the binding energetics. The present calorimetric data indicate a relatively low ΔC_p of binding. If it is assumed that the substrate binds in an extended conformation (*4*), then using the relationships of Myers et al. (*54*) this value of ΔC_p corresponds to burial of approximately 7, 20, and 29 residues of CAM-LA, CAM-BPTI, and CAM-RNase per

SecB monomer, respectively. These estimates are somewhat smaller than the binding frame estimates of 150 residues per SecB tetramer from earlier proteolysis protection experiments (*55*, *56*). The present data suggest that only a small fraction of the total accessible area of the substrate is buried upon binding to SecB. The data are consistent with a model in which SecB binds substrate molecules at a relatively exposed hydrophobic cleft rather than sequestered in a central cavity as in the case of GroEL. SecB aggregation in the absence of substrate is prevented normally by electrostatic repulsion between negatively charged SecB tetramers.

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