

Unfolding Thermodynamics of the Tetrameric Chaperone, SecB[†]

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ABSTRACT: SecB is a cytosolic tetrameric chaperone in *Escherichia coli*, which maintains polypeptides, destined for export in a translocation competent state. The thermodynamics of unfolding of SecB was studied as a function of protein concentration, by using high sensitivity-differential scanning calorimetry and spectroscopic methods. The thermal unfolding of tetrameric SecB is reversible and can be well described as a two-state transition in which the folded tetramer is converted directly to unfolded monomers. Increasing the pH decreases the stability of the tetramer significantly, the T_m changing from 341.3 K at pH 6.5 to 332.6 K at pH 9.5. The value of ΔC_p obtained from measurements of ΔH_m as a function of T_m was 10.7 ± 0.7 kcal mol⁻¹ K⁻¹. The value of ΔC_p is among the highest measured for a multimeric protein. At 298 K, pH 7.4, the ΔG_u for the SecB tetramer is 27.9 ± 2 kcal mol⁻¹. Denaturant-mediated unfolding of SecB was found to be irreversible. The reactivity of the four solvent-exposed free thiols in tetrameric SecB is salt dependent. The kinetics of reactivity suggests that these four cysteines are in close proximity to each other and that these residues on each monomer are in chemically identical environments. The thermodynamic data suggest that SecB is a stable, well-folded, and tightly packed tetramer and that substrate binding occurs at a surface site rather than at an interior cavity.

The folding of newly synthesized proteins inside the cell depends on a set of conserved proteins known as molecular chaperones (1, 2). Molecular chaperones do not contain information specifying correct folding, rather they prevent nonproductive interactions between nonnative polypeptide chains and typically increase the yield but not the rate of folding reactions (2). In *Escherichia coli*, export of proteins from the cytoplasm into the periplasm and the outer membrane involves transport across the inner membrane. The export of a subset of periplasmic proteins is aided by the cytoplasmic chaperone SecB (3). Formation of stable folded structure inhibits translocation of proteins across the inner membrane (4, 5). SecB is a homotetrameric chaperone having a molecular mass of 68 kDa and is known to form stoichiometric complexes with precursor proteins (6). SecB is thought to bind to unfolded and partially folded polypeptides in the cytosol and mediate their entry into the export pathway (3). 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 (7–9).

Knowledge of the origins of the protein stability is essential to the understanding of structure and function. One of the methods to quantify stability of a protein is to populate the native and unfolded states by physical and chemical means and analyze the transitions as measured by calorimetric or spectroscopic methods (10). A large body of work on the

thermodynamics of small monomeric proteins has suggested that the hydrophobic effect and loss of conformational entropy are the major determinants of stability of the native state (11, 12). In contrast, there are fewer studies on multimeric proteins, and in the majority of the cases, they do not exhibit reversible denaturation. In addition to interactions within the polypeptide chain, oligomeric proteins are characterized by intersubunit interactions, which may contribute to the overall stability. In addition to providing the energetics of the unfolding process, equilibrium-unfolding studies can provide insights into the events that occur during the assembly of oligomers from individual chains. Thermal unfolding studies of multimeric proteins such as GroES, p53, and HIV protease have emphasized the role of quaternary association in the stabilization of the multimeric assembly (13–16).

In this work, we have examined the thermal and denaturant-mediated unfolding of SecB by means of DSC¹ and spectroscopic methods to gain insights into the thermodynamics and mechanism of disassembly of the tetrameric structure. We show that the thermal unfolding of the SecB tetramer is reversible and involves a single highly cooperative transition from the native tetrameric state to unfolded monomers, demonstrating that the unfolding of the protein is linked to subunit dissociation. We have also examined

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¹ Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; DTNB, 4-dithionitrobenzoic acid; DTP, 4-dithiopyridine; IPTG, 1-isopropyl 1-thio- β -D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride; TP, 4-thiopyridine; TNB, 4-thionitrobenzene; CGH10, citrate-glycine-HEPES buffer (10 mM each); ΔC_p , change in heat capacity; GdnHCl, guanidine hydrochloride; T_m , temperature at which fraction of unfolded protein is 0.5; ΔH_m , molar unfolding enthalpy at T_m ; ΔH_v , van't Hoff enthalpy; ΔG_u , Gibbs' free-energy change upon unfolding.

the reaction kinetics of the four solvent exposed free thiols in the SecB tetramer with 4-dithionitrobenzoic acid and 4-dithiopyridine. The kinetic data suggests that the cysteines are in close proximity and the correlation of this reactivity to the symmetry of the tetramer is discussed.

EXPERIMENTAL PROCEDURES

Materials. PMSF, iodoacetamide, IPTG, DTT, DTNB, and DTP were from Sigma (Sigma Chemical Company, Missouri). Fast flow Q-Sepharose and Sephacryl S-200 were from Pharmacia. Ultrapure grades of GdnHCl and Tris were purchased from GIBCOBRL. All other chemicals were of analytical grade.

Protein Purification. The SecB expression plasmid pJW25 in strain BL21 (DE3) was obtained from Prof. B. de Kruijff. SecB purification was done as reported previously (9). The purified protein was estimated to be 99% pure by SDS-PAGE as detected by silver staining (17) and analytical gel filtration HPLC. The extinction coefficient at 280 nm was taken to be $11\,900\text{ M}^{-1}\text{cm}^{-1}$ for monomeric SecB (18). All the SecB concentrations reported here are tetrameric unless otherwise stated.

Spectroscopic Measurements. Thermal unfolding of SecB monitored by optical probes was carried out using a JASCO J-500 spectropolarimeter, JASCO UV/VIS 7850 spectrophotometer, and in a JASCO FP-777 spectrofluorimeter. The changes in secondary structure were followed by wavelength scans from 200 to 250 nm at various concentrations of protein and were performed in a 5 mm rectangular quartz cuvette at $1\text{ }^{\circ}\text{C min}^{-1}$. Changes in tertiary structure were monitored by scanning from 300 to 250 nm in a 1 cm path length quartz cuvette at $1\text{ }^{\circ}\text{C min}^{-1}$. Changes in absorbance upon thermal unfolding were monitored using a 1 cm path length cuvette placed in a thermo-coupled device with a stirring assembly for heating the sample at $1\text{ }^{\circ}\text{C min}^{-1}$. Unfolding transitions obtained by spectroscopic methods were analyzed as described (14) to obtain F_u . Reversibility of SecB unfolding as monitored by fluorescence emission was carried out using $1\text{ }\mu\text{M}$ of native and refolded SecB. Denaturant-mediated unfolding was carried out using urea and guanidine hydrochloride in CGH10 buffer, pH 7.4. SecB ($1\text{ }\mu\text{M}$) was incubated in CGH10 overnight in varying concentrations of denaturants and the equilibrium unfolding monitored by measuring the change in fluorescence intensity at 353 nm with excitation used at 280 nm.

Differential Scanning Calorimetry. All calorimetric experiments were carried out in a Microcal MC-2 ultrasensitive calorimeter. DSC measurements were carried out as a function of scan rate, protein concentration, and pH. The data were analyzed using Origin software supplied by the DSC manufacturer. The raw data of the calorimetric scans were baseline corrected and concentration normalized. Progress baseline connection of the pre- and posttransition baselines was generated for estimation of the heat capacity change. Analysis of the DSC scans was done using the single two-state transition with subunit dissociation model, as described in the manual supplied by the manufacturer. All protein solutions were dialyzed extensively against large volumes of desired buffer and degassed prior to use. The sample pH was checked before and after the scan. The buffer used was CGH10 for the entire pH range 6.5–9.5.

DTNB and DTP Assays. DTNB assays were carried out in a JASCO spectrophotometer. For monitoring the unfolding kinetics of SecB, $100\text{ }\mu\text{L}$ solution of $6.6\text{ }\mu\text{M}$ solution of SecB was added to a $500\text{ }\mu\text{L}$ solution of $200\text{ }\mu\text{M}$ DTNB in denaturant contained in CGH10, pH 7.4. The reactivity of Cys residues to DTNB was monitored by measuring the increase in absorbance at 412 nm. The molar extinction coefficient of TNB released in the reaction was taken to be $13\,600\text{ M}^{-1}\text{cm}^{-1}$ (19). For equilibrium experiments, $700\text{ }\mu\text{L}$ of a $1.1\text{ }\mu\text{M}$ solution of SecB was incubated overnight either in GdnHCl or urea contained in CGH10, pH 7.4. The number of free thiols labeled were measured 4 h after adding $20\text{ }\mu\text{L}$ of a 5 mM solution of DTNB to SecB. For salt dependence of the reactivity of the solvent-exposed Cys, the concentration of KCl in CGH10, pH 7.4, was varied from 50 to 900 mM. A total of $20\text{ }\mu\text{L}$ of a 5 mM solution of DTNB in CGH10, pH 7.4, was added, and the increase in absorbance at 412 nm was monitored as a function of time. DTP assays were carried out in an identical manner to that stated above except that $0.7\text{ }\mu\text{M}$ of SecB was used and the release of the thio-pyridine moiety was followed at 324 nm. The molar extinction coefficient of the released TP moiety was taken to be $19\,600\text{ M}^{-1}\text{cm}^{-1}$ (19).

Gel Filtration Experiments. Gel filtration experiments were carried out in a Superose 6 column (Pharmacia) with a flow rate of 0.5 mL min^{-1} . The buffer used was CGH10 with 200 mM NaCl. The column was equilibrated with the denaturant prepared in CGH10 and 200 mM NaCl. The refractive index of the eluate solution was measured to check the equilibration of the column with the denaturant (20). A total of $100\text{ }\mu\text{g}$ of protein in $20\text{ }\mu\text{L}$ of denaturant in CGH10 pH 7.4 incubated overnight was injected, and the elution of the protein was monitored by absorbance at 280 nm.

RESULTS AND DISCUSSION

Thermodynamic Parameters Characterizing SecB Stability. We have characterized the stability of SecB as function of protein concentration and pH by high-sensitivity DSC, fluorescence, and absorption spectroscopy. The transitions were reversible under all conditions studied, as demonstrated by repeated scans of the same sample. Figure 1 shows the buffer-corrected partial molar excess heat capacity data for $86.4\text{ }\mu\text{M}$ of SecB in CGH10, pH 7.4, scanned at $60\text{ }^{\circ}\text{C h}^{-1}$. The dotted line indicates the calculated baseline excess heat capacity of the system. Examination of the areas under the scan and rescan reveals a high reversibility of 83%. The effect of scan rate was studied by carrying out DSC scans at $20\text{ }^{\circ}\text{C h}^{-1}$. The data indicate that both T_m and ΔH_m are independent of scan rate. For all DSC scans and optical measurements, the transition temperature is defined as the temperature at which $F_u = 0.5$. Figure 2 shows a set of DSC scans obtained in the protein concentration range $16.9\text{--}86.4\text{ }\mu\text{M}$ in CGH10, pH 7.4. The thermodynamic parameters characterizing unfolding, at different concentrations of SecB, are summarized in Table 1. It is clear from Figure 3 that the transition temperature increases with protein concentration. At the highest concentration tested of $86.4\text{ }\mu\text{M}$, the transition occurs at 344.1 K with an excess heat capacity change of $12.9\text{ kcal mol}^{-1}\text{ K}^{-1}$. Each of the DSC scans was analyzed as described above. The value of n (number of subunits) was systematically changed so as to get the value of n that best fits the experimental data. A value of $n = 4$ best described

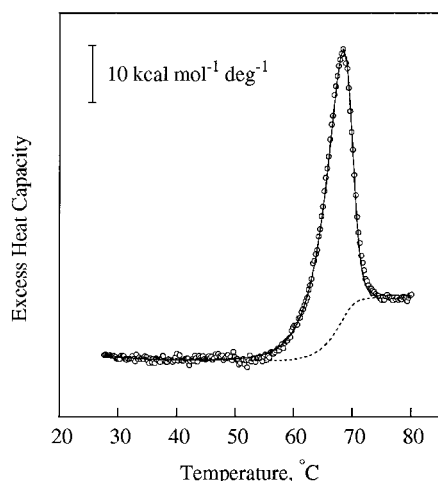


FIGURE 1: Representative DSC scan of 86.4 μM of SecB at pH 7.4 at a scan rate of 60 deg h^{-1} . The data points are shown as open circles (\circ). The two-state tetramer self-dissociating model fit to the data is shown as a thin line. The calculated baseline is shown as a dotted line.

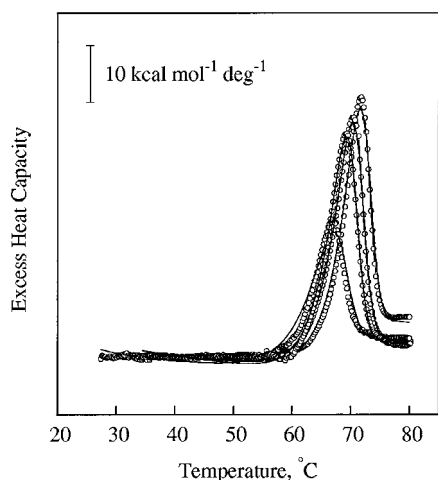


FIGURE 2: Excess heat capacity of SecB at different protein concentrations. SecB concentrations used from left to right are 16.9, 25.6, 53.2, and 86.4 μM . The data points are shown as open circles (\circ). The continuous lines represent theoretical curves generated with two-state unfolding coupled to subunit dissociation. The thermodynamic parameters corresponding to the fits are shown in Table 1.

Table 1: Thermodynamic Parameters for the Unfolding of the SecB Tetramer as a Function of Protein Concentration at pH 7.4

[SecB] (μM)	T_m (K)	ΔH_m^a (kcal mol $^{-1}$)	ΔC_p^a (kcal mol $^{-1}$ K $^{-1}$)
8.3	339.5	199	10.9 (± 0.4)
16.9	339.8	208	9.4 (± 0.4)
25.6	341.6	237	9.8 (± 0.2)
53.2	342.8	283	11.7 (± 0.3)
86.4	344.1	292	12.9 (± 0.3)

^a The average uncertainties in the measurements of the ΔH_m and ΔC_p are 1.1 and 3%, respectively.

the data. This is in accordance with the tetrameric status of the SecB oligomer (21). There is cooperative unfolding of the tetramer to unfolded monomers without any detectable intermediates. Thermal transition curves at protein concentrations lower than 8.3 μM were examined by CD and absorbance experiments, as the calorimetric response does not allow accurate measurements at such low concentrations. The change in ellipticity at 222 nm and increase in absor-

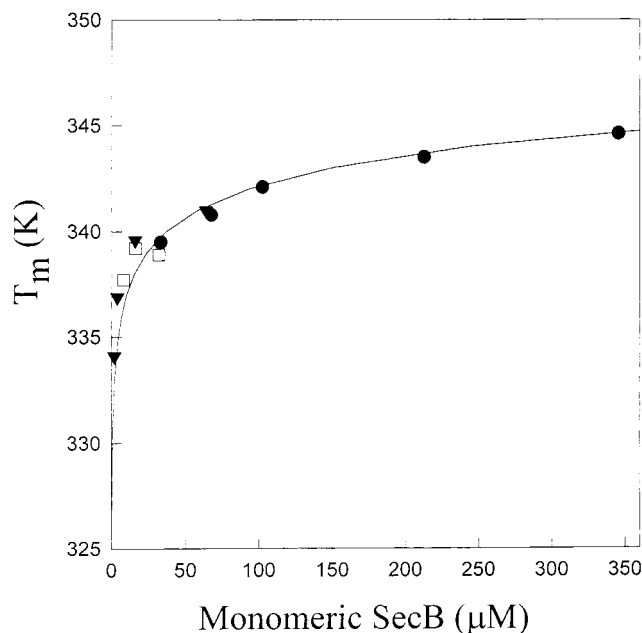


FIGURE 3: Unfolding transition temperature (T_m) of SecB as a function of tetramer concentration. The solid circles (\bullet) are from DSC-determined data, solid triangles (\blacktriangle) are from CD experiments and the open squares (\square) are from absorbance experiments. For all the data, the T_m is defined as the temperature at which $F_u = 0.5$. The solid line is the predicted dependency according to the two-state tetramer self-dissociating model.

Table 2: Thermodynamic Parameters for the Unfolding of the SecB Tetramer as a Function of pH

pH	T_m (K)	ΔH_m^a (kcal mol $^{-1}$)	ΔC_p^a (kcal mol $^{-1}$ K $^{-1}$)
9.50	327.1	100	8.5 (± 0.6)
9.00	330.8	124	7.2 (± 0.8)
8.75	332.9	141	10.7 (± 0.7)
8.50	333.6	157	9.1 (± 0.9)
8.38	334.5	177	5.8 (± 0.4)
8.25	335.1	180	7.2 (± 0.5)
8.00	338.6	198	8.7 (± 0.3)
7.80	339.4	210	14.4 (± 0.4)
7.40	340.9	238	13.7 (± 0.2)
7.00	341.0	245	11.3 (± 0.3)
6.50	341.3	256	12.7 (± 0.2)

^a The average uncertainties in the measurements of ΔH_m and ΔC_p are 1.6 and 5.5%, respectively.

bance at 253 nm upon SecB unfolding were used to monitor unfolding of SecB. The unfolding monitored by change in absorbance and in secondary CD at the same concentration of protein is essentially overlapping, showing that the unfolding is truly a two-state process (data not shown). Another indicator of two-state unfolding is the fact that all the DSC transitions fit well to a single transition involving dissociation of the tetramer to unfolded monomers. We have measured the ΔH_m as a function of T_m by carrying out DSC scans at different pHs. The DSC scans were carried out as a function of pH in the pH range 6.5–9.5 and the results are summarized in Table 2. Figure 4 shows the baseline-subtracted fits at two extremes of pH 6.75 and 9. Below pH 8.75, all DSC scans could be well fit to subunit dissociation coupled to unfolding where $n = 4$. Above pH 8.75, the DSC scans were found to fit both $n = 2$ and $n = 4$ equally well (Figure 4). This is in accordance with a previous observation that dimers are populated at alkaline pH (21). In all the cases, the data are well fit to a two-state model. The free energy

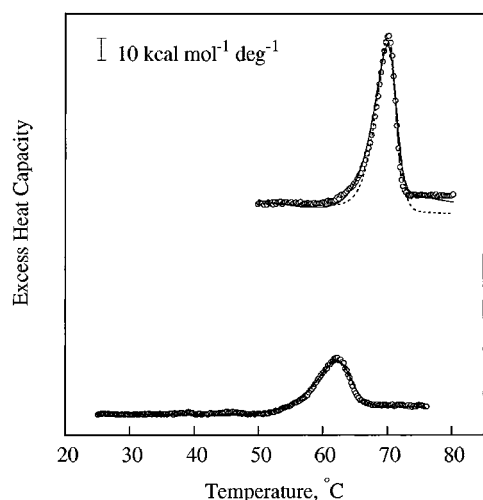


FIGURE 4: DSC scans of 25 μM SecB at pH 6.5 (bottom) and 9 (top). The data points are shown as open circles (O). The two-state self-dissociating model fit to the data is shown as a thin solid line for $n = 4$ and dotted line for $n = 2$.

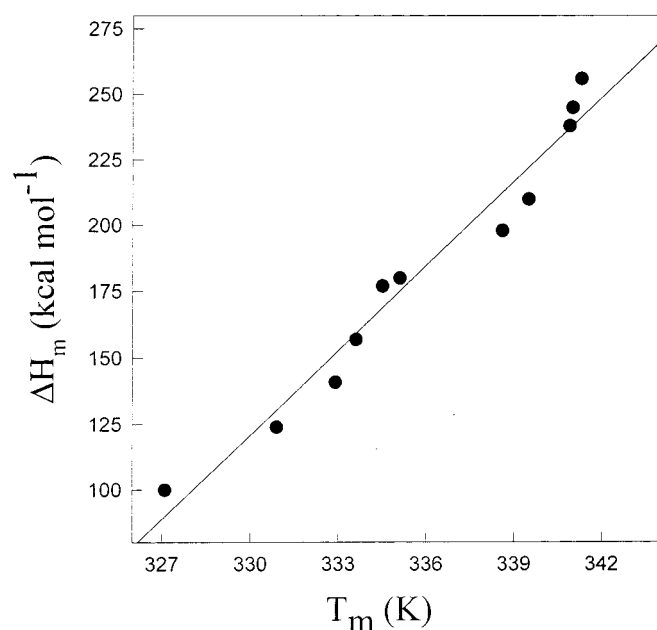


FIGURE 5: ΔC_p estimation from the linear dependence of ΔH_m on T_m . The ΔH_m and T_m values from Table 2 are fitted to a straight line. The slope yields a ΔC_p value of $10.7 \pm 0.7 \text{ kcal mol}^{-1} \text{ K}^{-1}$.

of unfolding of a protein can be obtained as a function of temperature from a single DSC experiment. However, it is difficult to get an accurate estimate of the ΔC_p from a single scan (22). We have estimated the ΔC_p value from a plot of ΔH_m as a function of T_m (Figure 5). The slope ($d\Delta H_m/dT_m$) provides an estimate of ΔC_p of $10.7 \pm 0.7 \text{ kcal mol}^{-1} \text{ K}^{-1}$. The data fit well to a straight line (correlation coefficient 0.96). This indicates that ΔC_p is independent of temperature and pH within the range of this study.

Model for Two-State Unfolding of SecB. A two-state concentration-dependent self-dissociating model for thermal unfolding shown below was used to analyze the data as described (23, 14). In the case of folded SecB (S_4), unfolding to four unfolded monomers (U) with no intermediate states the transition is described by



$$K = [U]^4/[S_4] \quad (2)$$

$$\Delta G_u^\circ(T) = -RT \ln K \quad (3)$$

$$\Delta G_u^\circ(T) = \Delta H^\circ(T_o) - T\Delta S(T_o) + \Delta C_p(T - T_o - T \ln(T/T_o)) \quad (4)$$

where K is defined as the equilibrium constant for the tetrameric state converting directly to the (monomeric) unfolded state, without folded monomeric intermediate, and T_o is some reference temperature. The total protein concentration (P_T) in monomer units can be expressed as

$$P_T = [U] + [S_4]4 \quad (5)$$

The fractional populations in the native (F_N) and unfolded (F_U) states are given by

$$F_N = 4[S_4]/P_T \quad (6)$$

Using eqs 3–6 we obtain,

$$F_U = [U]/P_T \quad (7)$$

$$F_U^4 + KF_U/4P_T^4 - K/4P_T^4 = 0 \quad (8)$$

Such a model would predict a dependence of T_m (the temperature at which F_U is 0.5) on the monomeric SecB concentration. Figure 3 shows the T_m as a function of monomeric SecB concentration. The solid line in Figure 3, which predicts the dependence of the T_m as a function of SecB concentration, was generated in the following way. At each temperature T in the range 250–400 K, values of ΔG_u° and K are calculated from eqs 4 and 3, respectively, using the value of ΔC_p of $10.7 \text{ kcal mol}^{-1}$, $\Delta H^\circ(T_o)$ and $\Delta S(T_o)$ determined from calorimetric data using 341 K as reference temperature. The value of K is substituted into eq 8 with $F_U = 0.5$ to give a corresponding value for P_T . As seen from Figure 3 the experimental data fits well to T_m s predicted from the self-dissociation coupled with unfolding model. These studies show that SecB appears to unfold as a single cooperative unit and that unfolding of the monomers is coupled to subunit dissociation. Such reversible two-state unfolding thermal unfolding behavior has been shown in only one other multimeric chaperone system: co-chaperonin GroES. GroES is a heptamer of 10 kDa subunits and the thermal unfolding of GroES has been shown to involve a highly cooperative reversible transition between folded heptamers and unfolded monomers (15). The thermodynamics of unfolding studies of chaperone GroES has provided insights into the stabilization and assembly of these large multisubunit structures. Analysis of the high-resolution structure of GroES indicated that the isolated folded monomers buried a significantly smaller fraction of their total surface than monomeric proteins of similar molecular mass. Thus the stabilization of the oligomeric structure was derived from intersubunit interactions rather than intramolecular interactions (15). In the cases of dimeric proteins, Arc repressor and core histone H2A–H2B, intersubunit interactions have been shown to stabilize the dimeric structure (13, 24).

Table 3: Thermodynamic Parameters for Some Oligomeric Proteins that Undergo Unfolding Coupled with Subunit Dissociation

protein	N_{res}^a	oligomeric state	ΔC_p [kcal (mol K) ⁻¹]	ΔC_p [cal (mol K residues) ⁻¹]	ΔG_u° (298 K) (kcal mol ⁻¹)	ΔG_u° (298 K) [cal (mol residues) ⁻¹]	ref
BZIP GCN4	112	dimer	0.7	6.5	13.5	120.8	31
ROP	126	dimer	2.5	19.5	17.1	135.7	29
Arc repressor	106	dimer	1.6	15.1	11	103.8	13
adenylate kinase	582	trimer	2.9	4.9	30	51.5	27
HIV protease	198	dimer	3.2	16.2	14.2	71.7	16
H2A-H2B	255	dimer	2.8	11	8.4	32.9	24
p53	252	tetramer	1.7	6.7	30.3	120.2	14
Mellitin	104	tetramer	1.2	11.5	NR ^b	NR	23
archaeal histones	138	dimer	2	14.4	14	101.4	32
GroES	679	heptamer	7	10.3	63	92.8	15
SecB	620	tetramer	10.6	17	27.9	45.2	

^a N_{res} is the total number of residues in the oligomer. ^b NR, not reported. All thermodynamic parameters are expressed per mole oligomer.

Comparison of ΔC_p of SecB with Other Multimeric Proteins. Thermal unfolding studies have been extensively carried out on small monomeric proteins because their unfolding transitions are highly reversible. However, there are fewer studies on the thermal unfolding of multimeric proteins because of their tendency to undergo irreversible denaturation. SecB is a large tetrameric protein, which displays two-state reversible, thermal folding. It is therefore of interest to see how the thermodynamic parameters governing SecB unfolding compare with other large monomeric and multimeric proteins. ΔC_p is the thermodynamic quantity closely related to the hydrophobic driving force and the burial of nonpolar surface area upon folding. Using the data on unfolding thermodynamics of small monomeric proteins, attempts have been made to correlate the total accessible surface area (ΔA_{SA}) or the nonpolar surface ($\Delta A_{\text{SA}_{\text{np}}}$) buried upon unfolding and ΔC_p (25). The extent of polar and nonpolar surfaces that are buried from the solvent is a major determinant of the stability of the native state. In the case of the p53 tetramer, the tetramerization domain buries a larger fraction of nonpolar surface than most globular proteins (14). Similar observations have been made in case of the assembly of heptameric GroES and coiled coil GCN4 (15, 26). According to Myers et al. (25) for monomeric proteins, ΔA_{SA} is linearly dependent on the number of residues (N_{res}) in the protein and ΔC_p is linearly dependent on the ΔA_{SA} as follows:

$$\Delta A_{\text{SA}} = -907 + 93N_{\text{res}} \quad (9)$$

$$\Delta C_p = -119 + 0.2\Delta A_{\text{SA}} \quad (10)$$

Hence,

$$\Delta C_p = -300.4 + 18.6N_{\text{res}} \text{ cal mol}^{-1} \text{ K}^{-1} \quad (11)$$

The empirical eq 11 given by Myers et al. (25) is based on a correlation analysis of the data from 45 monomeric proteins. It would, thus, be of interest to examine if oligomeric proteins behave in a similar fashion. In a recent study, Backmann et al. (27) reported an analysis of the calculated versus experimentally observed ΔC_p values. They concluded that ΔC_p values of oligomeric proteins reported in the literature tend to be low values compared with the values calculated by eq 11. Karantz et al. (28) have suggested that incomplete unfolding is responsible for the relatively low heat capacity change. High values of ΔC_p were found for only a few oligomeric proteins (13, 29, 30). In the

case of the SecB tetramer for which the N_{res} is 620, we obtain a ΔC_p value of 11.2 kcal mol⁻¹ K⁻¹ from eq 11. This is close to the experimentally determined value of 10.7 ± 0.7 kcal mol⁻¹ K⁻¹. This suggests that the unfolded state of SecB is probably not compact and is devoid of any structure contributing to the heat capacity change. Table 3 summarizes the thermodynamic data for a few oligomeric proteins that undergo unfolding coupled with subunit dissociation. As seen from Table 3, the ΔC_p per moles of residue for SecB is 17.2 cal deg⁻¹ (mol residue)⁻¹. This is higher than for typical small monomeric proteins and also the listed multimeric proteins. This suggests that a large protein such as the SecB tetramer, buries a larger fraction of its total ASA than smaller proteins. It has been suggested previously that SecB is a malleable macromolecule, which may be converted to a rigid state upon interaction with target substrates (33). The present data suggests instead that SecB has a well packed tetrameric structure, even in the absence of a bound substrate.

Oligomeric Status of SecB in the Cell. The concentration of SecB in vivo has been estimated to be 4 μM (34, 35). Gel filtration experiments even at the lowest possible concentrations (nanomolar) were unable to detect the presence of any intermediate dimeric species. Hence, it was concluded that the equilibrium constant for the conversion to dimeric species is lower than nanomolar (35). The stability curve, which represents the variation with temperature of the change in the standard Gibbs energy of unfolding, is shown in Figure 6. The ΔG_u° (298) per SecB tetramer at 298 K as estimated from the stability curve is found to be 27.9 ± 2 kcal mol⁻¹. Thus, at 298 K, the equilibrium constant between dissociated, unfolded monomers and folded tetramers is 10⁻²⁰ M³. The present data suggests that, under physiological conditions, the functional form of SecB is stable as a tetramer within the cell. The temperature of maximal stability of SecB is 314 K. This is higher than for smaller, monomeric globular protein predictions, consistent with a recent prediction of the size dependence of this parameter (36). All the thermodynamic data suggest that the SecB tetramer is a tightly packed, well-folded and stable protein. Hence, substrate binding is likely to occur on the surface rather than in an interior cavity as in the case of the GroEL/GroES system.

Denaturant-Mediated Unfolding and Reactivity of Cys Residues in SecB. Equilibrium unfolding of chaperone SecB was carried out using urea and GdnHCl in CGH10, pH 7.4. Intrinsic protein fluorescence methods and reactivity of the cysteine residues to the thiol specific reagent DTNB was

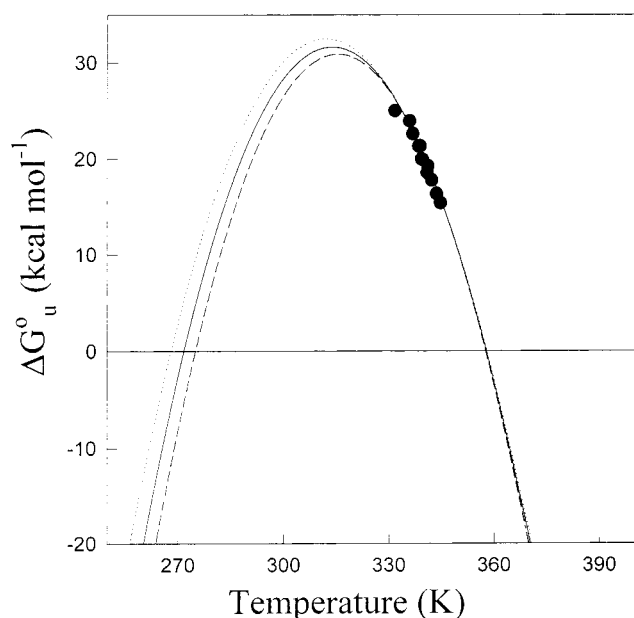


FIGURE 6: ΔG_u° for unfolding of SecB as a function of temperature at pH 7.4. The stability curve (solid line) was calculated from eq 4 using the following parameters: $\Delta H^\circ(T_0) = 323.6 \text{ kcal mol}^{-1}$, $\Delta S(T_0) = 0.890 \text{ kcal mol}^{-1} \text{ K}^{-1}$, $T_0 = 341 \text{ K}$, and $\Delta C_p = 10.7 \text{ kcal mol}^{-1} \text{ K}^{-1}$. The two dotted lines represent the effect on the stability curve due to the error in the measurement of ΔC_p of $\pm 0.7 \text{ kcal mol}^{-1} \text{ K}^{-1}$. The filled circles (●) represent values of ΔG_u° for unfolding using eqs 8 and 3 obtained using experimentally observed T_m s at various P_T s.

used to monitor the denaturant mediated unfolding of SecB. Figure 7A shows the unfolding of SecB as monitored by the change in fluorescence at 358 nm. As evident from the figure, there is an increase in fluorescence on unfolding of SecB. Folded SecB exhibits an emission maximum at 305 nm while unfolded SecB has an emission maximum at 355 nm when excited at 280 nm (data not shown). However, in both urea and GdnHCl-mediated unfolding, the transition was found to be irreversible as monitored by fluorescence methods. The formation of soluble aggregates could lead to irreversible unfolding of SecB. We tested this hypothesis by carrying out gel filtration experiments as a function of denaturant concentration. In the absence of any denaturants, tetrameric SecB elutes at 13.7 mL in a Superose 6 column. Bruce et al. (21) have detected dimers of SecB at alkaline pH. We have also been able to fit DSC scans to $n = 2$ for experiments in the alkaline range. At concentrations lower than 0.75 M of GdnHCl SecB elutes also at 13.7 mL. Muren et al. (35) have reported mutations in SecB which were able to populate dimers of SecB and result in loss of SecB function. We have not been able to detect dimers of SecB at low concentration of denaturants by gel filtration experiments (data not shown). At concentrations higher than 1.2 M GdnHCl, SecB aggregates and elutes at 8 mL, which is within the void volume of the column (data not shown). Another method used to monitor the denaturant-mediated unfolding reaction was the reactivity of cysteine residues to thiol specific agents. We have used DTNB and DTP as thiol solvent accessibility probes in our experiments. The reactivity of cysteine has been used previously to monitor the kinetics of protein unfolding. Also, equilibrium unfolding and refolding of proteins has been studied by monitoring the reactivity of cysteine residues (37–39). DTNB reacts with solvent

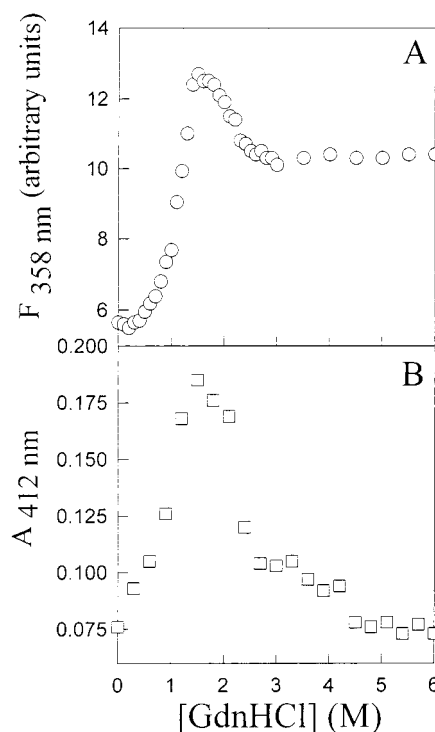


FIGURE 7: (A) GdnHCl denaturation curve of $1.1 \mu\text{M}$ SecB at 25°C and pH 7.4. GdnHCl-induced denaturation was followed by monitoring the fluorescence emission at 358 nm on excitation at 280 nm. (B) DTNB reaction of the thiols of $1.1 \mu\text{M}$ SecB incubated overnight in GdnHCl at 25°C and pH 7.4. The reaction with DTNB was carried out for 4 h and then the absorbance at 412 nm was recorded. An absorbance of 0.06 at 412 nm corresponds to 4 thiols labeled per tetramer of SecB under these conditions.

accessible free cysteine in proteins by a thiol-disulfide exchange reaction. The increase in absorbance at 412 nm due to the TNB ion released can be used to estimate the number of thiols labeled in the protein (19). After overnight incubation at a given denaturant concentration, samples of SecB were assayed for thiol reactivity by incubating the protein with DTNB for 4 h. As shown in Figure 7B the number of Cys labeled increases with increase in denaturant concentration up to 1.5 M GdnHCl and 2 M Urea. However beyond 1.5 M GdnHCl and 2 M urea, there is a decrease in the number of Cys getting labeled, probably because of the formation of aggregates. Cys in different environments should react with different rates and hence would exhibit multiexponential kinetics of reactivity to DTNB (40). SecB has four Cys per monomer. The reactivity of the four Cys residues per monomer was assayed by reaction with DTNB as a function of denaturant and salt concentration. The kinetics of unfolding as monitored by reactivity of the thiols to DTNB was carried out. Below 1 M of GdnHCl, 1 thiol per monomer reacts with DTNB (data not shown). As shown in Figure 8, upon an increase in the GdnHCl concentration beyond 1 M, all 16 thiols in SecB tetramer are labeled. The labeling occurs with slow multiexponential kinetics showing that the Cys are still in different environments and the protein is unfolding while labeling is occurring. At high denaturant concentration, the reaction becomes single exponential, showing that the environment of all the Cys residues is the same, i.e., that complete unfolding has occurred before labeling. The reactivity of the four Cys, which are solvent exposed, was found to be dependent on salt concentration.

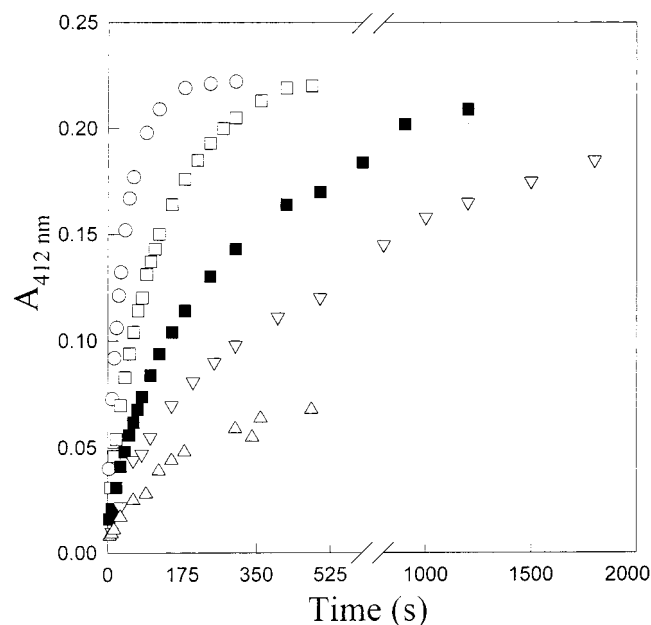


FIGURE 8: Unfolding kinetics as a function of GdnHCl as monitored by the reactivity of exposed thiols. Kinetic traces of unfolding of SecB as monitored by increase in absorbance at 412 nm at 1 M (Δ), 2 M (∇), 3 M (\blacksquare), 4 M (\square), and 5.2 M (\circ) GdnHCl concentrations contained in CGH10 pH 7.4. A 100 μ L solution of 6.6 μ M SecB was added to a 500 μ L solution of 200 μ M DTNB contained in appropriate concentration of GdnHCl in CGH10, pH 7.4, such that the final denaturant concentration would be as stated above. An absorbance of 0.06 at 412 nm corresponds to four thiols labeled per tetramer of SecB under these conditions.

As shown in Figure 9A, in low ionic strength buffer CGH10, pH 7.4, the four Cys react slowly with multiexponential kinetics. With increase in the KCl concentration the reaction can be well fit to a single exponential function. Thus, at low ionic strength the environment surrounding the four Cys is not apparently identical. However, in the presence of 100 mM KCl in CGH10, pH 7.4, the reaction fits well to a single exponential function, indicating the environment surrounding the Cys has become identical. Such salt-dependent reactivity can be explained as follows: DTNB is a negatively charged molecule. If one assumes that the four Cys are in close proximity, the reaction of the first Cys would be fast and the reaction of the subsequent Cys would be slower due to the presence of the negatively charged carboxylate of TNB on the reacted Cys (40). As the negative charge is screened due to the presence of salt, the reaction becomes single exponential. Hence, the apparent rate constant increases from 0.0013 (100 mM KCl) to 0.0042 (800 mM KCl). As shown in Figure 9B, the reactivity of the uncharged molecule, DTP, is independent of salt concentration and fits well to a single exponential (apparent rate constant is 0.013). This suggests that the environments surrounding the solvent-exposed Cys in the SecB tetramer are very similar or identical. Also, the reactivity of the both thiol-reactive probes indicates that the cysteines are partly accessible to solvent. This observation coupled with observations that thermal unfolding is two state and that SecB contains four identical binding sites for small unfolded substrates (34) suggests that, at neutral pH, SecB is a symmetrical tetramer. This differs from results from mutational studies (35), which suggested that SecB is a dimer of dimers. In the present experiments, we do not observe dimeric intermediates in SecB unfolding. It is only at alkaline

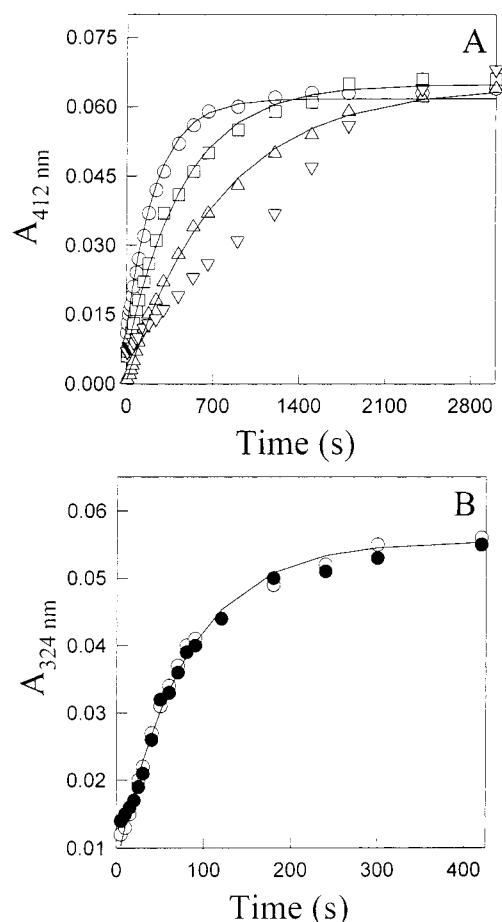


FIGURE 9: (A) Salt dependence of the reactivity of the solvent exposed Cys to DTNB. The reaction kinetics of DTNB to the exposed Cys in native SecB (1.1 μ M) was monitored at 412 nm as a function of KCl in CGH10, pH 7.4. (\circ) 600 mM KCl, (\square) 200 mM KCl, (Δ) 100 mM KCl, and (∇) 0 mM KCl. An absorbance of 0.06 at 412 nm corresponds to four thiols labeled per tetramer of SecB under conditions stated. (B) Reaction kinetics of the solvent exposed Cys in SecB (0.7 μ M) to DTP was carried out in 0 mM KCl (\bullet) and 600 mM KCl (\circ) contained in CGH10, pH 7.4, in an identical manner as stated in panel A. However, the reaction kinetics was monitored at 324 nm. An absorbance of 0.055 at 324 nm corresponds to 4 thiols labeled per tetramer of SecB under conditions stated. The solid lines in both panels A and B represent single-exponential fits to individual traces.

pH that the denaturation data fit equally well to both tetramer and dimer dissociation models. The thiol reactivity data are qualitative in nature, and hence it is still possible that SecB may be a dimer of dimers, and rather than a symmetrical tetramer, a definitive answer must await structure determination of SecB by crystallography or NMR.

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