SecB Binds Only to a Late Native-like Intermediate in the Folding Pathway of Barstar and Not to the Unfolded State†

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ABSTRACT: SecB is a cytosolic, tetrameric chaperone of *Escherichia coli* which maintains precursor proteins in a translocation competent state. We have investigated the effect of SecB on the refolding kinetics of the small protein barstar in 1 M guanidine hydrochloride at pH 7.0 and 25 °C using fluorescence spectroscopy. We show that SecB does not bind either the native or the unfolded states of barstar but binds to a late near-native intermediate along the folding pathway. For barstar, polypeptide collapse and formation of a hydrophobic surface are required for binding to SecB. SecB does not change the apparent rate constant of barstar refolding. The kinetic data for SecB binding to barstar are not consistent with simple kinetic partitioning models.

Molecular chaperones have been shown to be important for protein folding in the cell (*1*). Protein folding in vivo occurs at total protein concentrations much higher than those used in typical in vitro studies. The concentration of nascent polypeptide chains in the cytosol of the bacterial cell can reach 50 μM (*2*). While folding, proteins may form aggregation prone intermediates with exposed hydrophobic patches. A common property shared by chaperones is prevention of nonproductive intermolecular interactions between such intermediates (*3*). In *Escherichia coli*, export of proteins from the cytoplasm to the periplasm and the outer membrane involves transport across the inner membrane. Formation of stable folded structure inhibits translocation of proteins across the inner membrane (*4*, *5*).

The export of a subset of periplasmic proteins is aided by the cytoplasmic chaperone SecB. SecB is thought to bind to unfolded and partially folded polypeptides in the cytosol and mediate their entry into the export pathway (*6*). The interaction of SecB with one of its natural ligands, maltose binding protein (MBP[1]), has been studied extensively (*7*−*10*). While these studies have yielded considerable insight into the mechanism of SecB binding, little is known about the conformation of the chaperone-bound substrate. MBP is a large two-domain protein of 370 amino acids. It has several proline residues and displays complicated folding kinetics which are difficult to characterize in great detail. To obtain insight into the determinants of SecB binding, we have examined the binding of SecB to the small protein barstar.

Barstar is a small 89-amino acid protein which functions as an intracellular inhibitor of barnase in *Bacillus amyloliquefaciens.* The folding pathway of barstar has been extensively characterized using a variety of techniques (*11*−*13*). The folding, thermodynamics, and kinetics of several barstar mutants have also been studied (*14*−*16*), and crystal structures of both the free and bound protein are available (*17*, *18*). Of the two Pro residues in barstar, Pro48 occurs in the cis conformation in the folded state. Equilibrium unfolded barstar comprises 69% of the slow-folding (U_S) and 31% of the fast-folding (U_F) molecules in which the initial Pro48 conformations are trans and cis, respectively. The folding and unfolding pathways of barstar have been characterized as a function of denaturant concentration and temperature. At room temperature in 1 M GdnHCl, the major folding pathways of barstar are $U_F \rightarrow N$ (pathway 1) and $U_S \rightarrow I_{M1} \rightarrow I_{S1} \rightarrow I_N \rightarrow N$ (pathway 2), where I_{M1}, I_{S1}, and I_N are kinetic intermediates and N is the fully folded protein (*13*, *19*). In the first pathway, N is formed with a rate constant of 18 s^{-1} (half-life of 38 ms). In the second pathway, I_{S1} is formed fast and then converts to I_N with a

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[1] Abbreviations: MBP, maltose binding protein; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; CAM-V73A barstar, carboxamidomethylated mutant V73A of barstar; ANS, 8-anilino-1-naphthalenesulfonic acid; N, native barstar; N_S, amount of barstar formed during the slow phase of refolding; U_S, unfolded slow-folding species of barstar; U_F, unfolded fast-folding species of barstar; I_N, late intermediate on the slow-folding pathway of barstar; I_{M1}, collapsed unstructured barstar; I_{S1}, structured kinetic intermediate; preMBP, precursor MBP with a leader sequence; DTNB, dithionitrobenzoic acid; BPTI, bovine pancreatic trypsin inhibitor; proOmpA, precursor outer membrane protein of *E. coli*; $\Delta G°$, free energy of unfolding at zero denaturant concentration; C_m, denaturant concentration at which half the molecules are unfolded; PMSF, phenylmethanesulfonyl fluoride; IPTG, 1-isopropyl 1-thio-β-D-galactopyranoside.

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rate constant of 18 s^{-1} (*13*). Hence, after about 150 ms, 31% of the molecules are in the native state and the remaining 69% are in state I_N. The I_N state converts slowly to N with a single apparent rate constant of 0.009 s^{-1} (half-life of 77 s). I_{M1} is a burst-phase intermediate which has been shown to be a compact globule lacking any secondary and tertiary structure; I_{S1} is an early intermediate with some tertiary structure because its fluorescence properties are different from those of U_S and I_{M1}, and I_N is a native-like intermediate capable, like N, of inhibiting barnase (*11*). All the above kinetic intermediates bind the hydrophobic dye ANS.

In this work, we have studied the blockage of barstar refolding by SecB in the presence of 1 M GdnHCl at pH 7 and 25 °C. We show that SecB does not bind to the unfolded state of barstar but binds to a late intermediate in barstar folding. The binding kinetics are not consistent with simple kinetic partitioning models and suggest that thermodynamic rather than kinetic factors are important in the binding of SecB to barstar.

EXPERIMENTAL PROCEDURES

Materials. PMSF, iodoacetamide, IPTG, and DTT were from Sigma (St. Louis, MO). 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.

The SecB expression plasmid pJW25 in strain BL21(DE3) was obtained from B. de Kruijff. The cells were grown in LB containing ampicillin at 100 μg/mL 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 and lysed after they were suspended in 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 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 20 mM Tris-HCl (pH 7.4) containing 200 and 300 mM NaCl, respectively. 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 (*20*) 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} (*21*). The procedure for purification of barstar has been previously described (*12*). The molar extinction coefficient of wild type barstar and the V73A mutant of barstar were taken to be 23 000 M^{-1} cm^{-1} (*12*).

Buffers. All kinetic experiments were carried out in 50 mM sodium phosphate, 250 μM EDTA, and 1 mM DTT containing 1 M GdnHCl at pH 7 and 25 °C. All solutions were passed through a 0.45 μm filter, and the pH was checked before they were used. The concentrations of GdnHCl stock solutions were determined by refractive index measurements (*22*) on an Abbe refractometer.

Preparation of Carboxamidomethylated V73A Barstar (CAM-V73A). A 200 μM solution of the barstar mutant, V73A, was unfolded in 6 M GdnHCl (pH 7.5) in 100 mM potassium phosphate buffer containing 1 mM EDTA and 400 μM DTT for 1 h. To this solution was added iodoacetamide to a final concentration of 2 mM, and the mixture was incubated in the dark for 30 min. The reaction mixture was then rapidly desalted on a PD-10 column equilibrated in 50 mM potassium phosphate (pH 7). The derivative did not show any free thiol groups as estimated by titration with DTNB (*23*).

Equilibrium Experiments. The equilibrium unfolding of CAM-V73A barstar as a function of GdnHCl concentration was monitored by fluorescence on a SPEX Fluorolog-2 fluorimeter using 287 nm as the excitation wavelength with a bandwidth of 1.5 nm and an emission wavelength of 320 nm having a bandwidth of 5 nm. The measured fluorescence intensities were analyzed in terms of a two-state transition to obtain the fraction of unfolded protein and free energy of unfolding as a function of denaturant as described previously (*24*).

Rapid Kinetic Experiments. Rapid mixing fluorescence experiments were carried out on a Biologic SFM-3 stopped-flow machine. The excitation wavelength was 287 nm with a bandwidth of 20 nm. Emission intensity was monitored at 320 nm using a band-pass filter with a bandwidth of 10 nm. Two detection channels were used to monitor folding kinetics in two different time domains for the same mixing event. Barstar was unfolded in 6 M GdnHCl for at least 2 h prior to measurements. Refolding of barstar in the absence of SecB was initiated by 6-fold dilution of 90 μM unfolded barstar in 6 M GdnHCl into 50 mM potassium phosphate buffer (pH 7). The final concentration of GdnHCl was 1 M. Refolding of barstar in the presence of SecB was carried out as described above except that the barstar solution in 6 M GdnHCl was diluted into a 40 μM SecB solution in 50 mM potassium phosphate buffer (pH 7). The fluorescence contribution of SecB was determined by 6-fold dilution of 6 M GdnHCl into a 40 μM SecB solution in 50 mM potassium phosphate buffer (pH 7). This fluorescence was then appropriately subtracted from the refolding traces.

Manual Mixing Experiments. The manual mixing experiments were carried out on a Spex Fluorolog-2 fluorimeter. Folding was initiated by diluting a 3 μM barstar solution unfolded in 6 M GdnHCl to a final concentration of 0.5 μM in native buffer containing SecB. The final GdnHCl concentration was 1 M. The SecB concentration was varied between 2 and 30 μM. No inner-filter effect was seen under the conditions used for the manual mixing experiments as the path length of the cuvette was 2 mm. The excitation wavelength was 287 nm with a bandwidth of 1.5 nm, and the emission wavelength was 320 nm with a bandwidth of 5 nm. The fluorescence contribution of SecB was subtracted from the refolding traces prior to analysis.

Delayed Mixing Experiments. Barstar refolding was initiated by making a 6-fold dilution of 20 μL of a 25 μM solution of unfolded barstar in 6 M GdnHCl into 100 μL of native buffer in a siliconized microfuge tube. After variable times of incubation, the solution was transferred to a fluorescence cuvette containing 880 μL of a 20 μM SecB solution in 1 M GdnHCl containing buffer, and the fluorescence intensity at 320 nm was monitored as described above.

The final concentrations of barstar and SecB were 0.5 and 17.6 μM, respectively. The SecB contribution to the observed fluorescence was determined by transferring 120 μL of 1 M GdnHCl into 880 μL of 20 μM SecB in buffer containing 1 M GdnHCl. This fluorescence contribution was subtracted from all folding traces in the presence of SecB.

Fitting of Kinetic Data. In the stopped-flow experiments, the refolding kinetics of barstar both in the presence and in the absence of SecB fit well to the sum of two exponentials:

$$F(t) = F(\infty) - F_1 \exp(-\lambda_1 t) - F_2 \exp(-\lambda_2 t) \quad (1)$$

where λ_1 and λ_2 are the apparent rate constants of the slow and fast phases and F_1 and F_2 are their amplitudes, respectively. In the manual mixing experiments, the observed slow phase of refolding of barstar was fit to a single exponential to obtain the rate constant λ_1.

Kinetic Partitioning Models for the Interaction between Barstar and SecB. The intermediates bound to SecB are in fast equilibrium with free SecB. This is similar to the model proposed earlier for binding of apo-α-lactalbumin to GroEL (*25*). The kinetic scheme is given by Scheme 1

$$\mathrm{I} + \mathrm{S} \leftrightarrow \mathrm{IS}$$

$$\mathrm{I} \rightarrow \mathrm{N}$$

Here S (free SecB) binds to I with an equilibrium constant K, and free I refolds to N with a rate constant k_f. If P_T is the total concentration of barstar, it can be shown that:

$$\frac{d[N]}{dt} = k_f[I] = \frac{k_f([P_T] - [N])}{1 + K[S]} \quad (2)$$

Scheme 2

$$\mathrm{I} + \mathrm{S} \rightarrow \mathrm{IS}$$

$$\mathrm{I} \rightarrow \mathrm{N}$$

I binds irreversibly to S with a rate constant k_S, and I folds to N with a rate constant k_f.

$$\frac{d[N]}{dt} = k_f[I] \quad (3)$$

$$\frac{d[I]}{dt} = -k_f[I] - k_S[I][S] = -(k_f + k_S[S])[I] \quad (4)$$

If S is present in large excess, its concentration does not change with time. Hence,

$$I = I_o e^{-(k_f + k_S[S])t} \quad (5)$$

$$\frac{d[N]}{dt} = k_f[I_o] e^{-(k_f + k_S[S])t} \quad (6)$$

Integrating the above equation gives

$$[N] = \frac{k_f[I_o]}{k_f + k_S[S]}[1 - e^{-(k_f + k_S[S])t}] \quad (7)$$

Thus, the apparent rate constant of formation of N will increase with increasing [S], and the final amount of N will decrease with increasing [S].

Determination of N_S in Manual Mixing Experiments. Let N_S and N_{S0} be the final concentrations of N formed during the slow phase of barstar refolding in the presence and absence of SecB, respectively. N_{S0} is equal to 70% of the total barstar concentration, N_T, since 70% of the molecules fold during the slow phase. In the presence of SecB, some of the barstar binds to SecB and is prevented from folding to N. Since the fluorescence of barstar bound to SecB is the same as that of barstar in the I_N state (see below), N_S can be determined as follows. If ΔF_{S0} and ΔF_S are the fluorescence amplitude changes occurring in the slow phase in the absence and presence of SecB, respectively, then

$$\Delta F_S/\Delta F_{S0} = N_S/N_{S0} = N_S/0.7N_T \quad (8)$$

If ΔF_{T0} and ΔF_{TS} are the total fluorescence amplitude changes (sum of the changes in fast and slow phases) occurring in the absence and presence of SecB, respectively, then $\Delta F_{S0} = 0.3\Delta F_{T0}$ and $\Delta F_S = \Delta F_{TS} - 0.7\Delta F_{T0}$ because the fluorescence amplitude change occurring in the fast phase ($0.7\Delta F_{T0}$) is unaffected by SecB. Substituting these values in eq 8, one obtains

$$N_S = 0.7N_T(\Delta F_{TS} - 0.7\Delta F_{T0})/0.3\Delta F_{T0} \quad (9)$$

RESULTS

We have characterized the binding of SecB to barstar by carrying out refolding studies of barstar in 1 M GdnHCl at pH 7.0 both in the presence and in the absence of SecB. SecB is stable under these conditions for up to 3 h. Under these conditions, the fluorescence as well as secondary and tertiary CD spectra of SecB are identical to corresponding spectra in native buffer at pH 7 without denaturant. Furthermore, we have shown that SecB is able to block the refolding of one of its natural substrates, MBP, under these conditions (data not shown). These data demonstrate that SecB is folded and active at this denaturant concentration. The barstar intermediate I_N is maximally populated in 1 M GdnHCl. At lower denaturant concentrations, the folding of barstar is more complicated as alternate folding pathways become available. At higher denaturant concentrations, I_N is destabilized and barstar folds primarily through the pathway $U_S \leftrightarrow U_F \rightarrow N$ (*13*). We have monitored the binding of barstar to SecB by Trp fluorescence. Trp fluorescence provides a convenient method for monitoring the binding reaction even in the presence of excess SecB. Barstar contains three Trp residues, and SecB contains a single, exposed Trp residue (*26*). The fluorescence of folded barstar is approximately 15 times that of an equal number of moles of SecB monomers at 320 nm.

Interaction of SecB with the Denatured State of Barstar. The mutant V73A of barstar is destabilized with respect to the wild type protein and has a C_m of 1.5 M GdnHCl (J. Srinivasan and J. B. Udgaonkar, unpublished results). Wild type barstar has a C_m of 1.9 M GdnHCl. To further destabilize the mutant, chemical modification of the two Cys residues with iodoacetamide was carried out. The resulting carboxamidomethylated derivative of this mutant barstar has a C_m of 0.89 M GdnHCl. In 1 M GdnHCl, more than 90% of the molecules of CAM-V73A barstar are unfolded (Figure 1) as monitored by Trp fluorescence (wavelength of maximal emission is 355 nm.). The numerically added spectra of

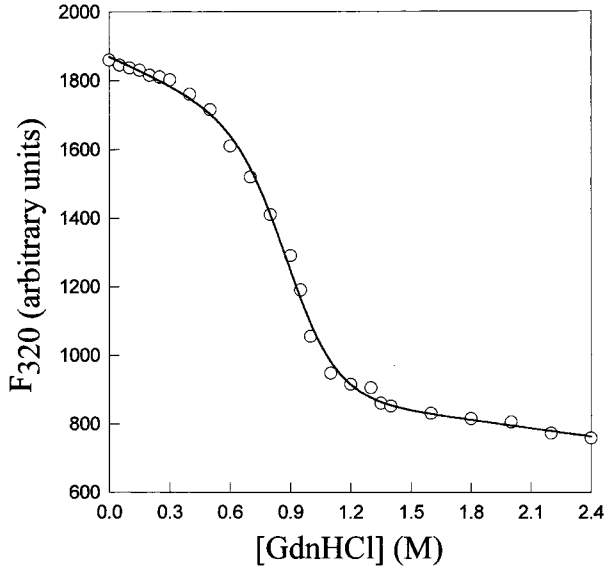

FIGURE 1: GdnHCl denaturation curves of 2 μM CAM-V73A barstar at 25 °C and pH 7. GdnHCl-induced denaturation was followed by monitoring the fluorescence emission at 320 nm on excitation at 287 nm. The solid line through the data is a nonlinear least-squares fit of the data to a two-state unfolding model (*24*) with values for $\Delta G°$, C_m, and m of 4.2 kcal mol^{-1}, 0.89 M, and 4.7 kcal M^{-1} mol^{-1}, respectively.

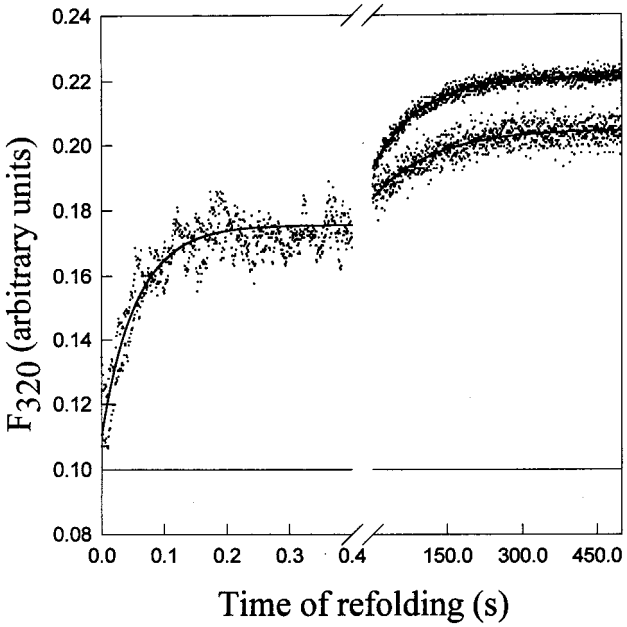

FIGURE 2: Refolding kinetics of 15 μM barstar in the presence (lower trace) and absence (upper trace) of 40 μM SecB measured by stopped-flow fluorescence in 50 mM potassium phosphate and 1 M GdnHCl at pH 7 and 25 °C. The folding of barstar was monitored by the change in fluorescence at 320 nm after excitation at 287 nm. The fluorescence contribution of SecB was appropriately subtracted from the refolding traces for both the fast and slow phases. The solid horizontal line represents the fluorescence of 15 μM barstar in 6 M GdnHCl.

CAM-V73A barstar (0.5 μM) and SecB (5 μM) in 1 M GdnHCl are identical to the spectrum of a mixture of CAM-V73A barstar and SecB containing the same final concentrations of each molecule as the mixtures characterized by the individual spectra (data not shown). Thus, SecB does not interact with the unfolded state of barstar.

Kinetics of Barstar Refolding. The refolding of barstar both in the presence and in the absence of SecB was monitored by measuring the increase in fluorescence intensity that occurs during the folding of barstar. Refolding was initiated by diluting a solution of equilibrium unfolded barstar in 6 M GdnHCl into native buffer with or without SecB. The final concentration of denaturant was 1 M. As discussed above, the unfolded state of barstar consists of two species, U_S and U_F, which differ in the isomerization state of the Tyr47−Pro48 peptide bond. It has previously been shown that in 1 M GdnHCl the refolding of barstar has a fast and a slow phase (*13*, *19*).

The effects of SecB on the rate constants and amplitudes of the two phases have been investigated (Figure 2). Stopped-flow refolding experiments of barstar in the absence of SecB reconfirmed the presence of the two phases. The relative amplitudes of the fast and slow phases are 70 ± 5 and 30 ± 5%, respectively. The rate constants of the two phases are 18 and 0.009 s^{-1}, respectively. These values are identical to those obtained previously (*19*). In the presence of SecB, there is no change in either the amplitude or the rate constant of the fast phase, suggesting that SecB does not bind the unfolded state of barstar. We have therefore shown only a single fitted curve for the fast phase data.

The rate constant of the slow phase (0.009 s^{-1}) is also unaffected by the presence of SecB, but there is a decrease in the final amplitude of the slow phase, suggesting that binding of SecB to barstar occurs during the slow phase of barstar refolding. The concentration dependence of barstar binding to SecB was examined by carrying out manual mixing experiments at different SecB concentrations as described above. In these experiments, only the slow phase of refolding can be measured. The final fluorescence amplitude (relative to the difference in amplitude between folded and unfolded barstar in the absence of SecB) decreased with increasing SecB concentration and reached a plateau value of about 71% (Figure 3A). This is identical to the relative amplitude of the fast phase alone. The data suggest that SecB does not bind to any species of barstar formed during the fast phase of refolding. However, SecB is able to bind to I_N and possibly other intermediates formed between I_N and the native state. The data summarized in Figure 3B show that the rate constant of the slow phase is independent of SecB concentration.

Delayed Mixing Experiments. SecB did not interact with the denatured state or any intermediates of barstar formed before I_N, and did not markedly affect the rate constant of the slow phase of barstar refolding. We therefore investigated the interaction of SecB with late kinetic intermediates on the folding pathway of barstar by adding SecB after various times of barstar folding. Barstar was allowed to refold for a given time in 1 M GdnHCl. The barstar solution was added to a solution containing a 40-fold excess of SecB in 1 M GdnHCl, and the final amplitude after 300 s was recorded (Figure 4). The final amplitude in each case was compared to the amplitude of barstar refolding in the absence of SecB at a time equal to the time at which SecB was added. Interestingly, the two amplitudes were identical. This suggests that the fluorescence of barstar in the SecB−barstar complex is identical to the fluorescence of free barstar in the intermediate state that binds to SecB. The data also show that SecB does not bind to the native state of barstar as addition of SecB after 300 s of folding (when all the barstar

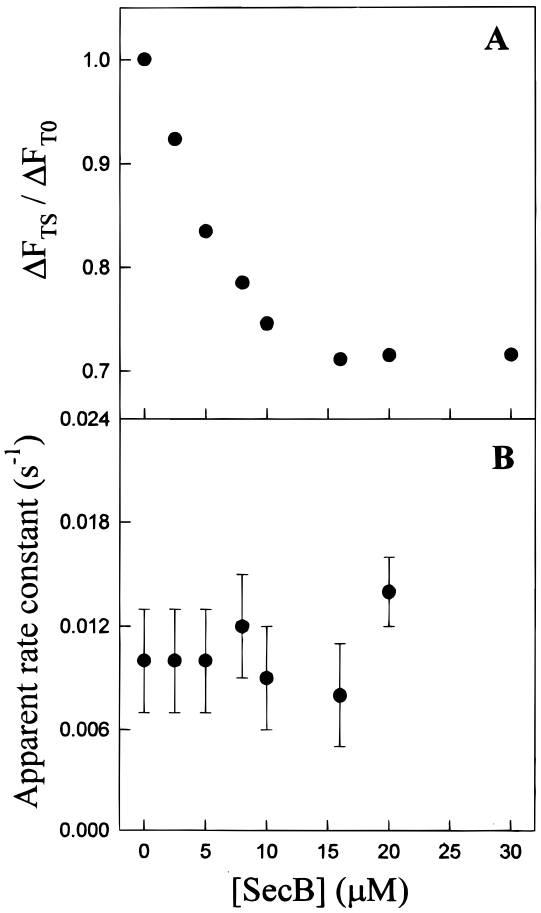

FIGURE 3: Effect of SecB concentration on the slow phase of the refolding kinetics of 0.5 μM barstar in 1 M GdnHCl at pH 7.0. (A) Ratio of final fluorescence amplitude changes upon folding in the presence (ΔF_{TS}) and absence (ΔF_{T0}) of SecB. This ratio is plotted as a function of SecB concentration. The fluorescence contribution of SecB was subtracted from the kinetic traces, and the final amplitudes were measured after 300 s. (B) Rate constants as a function of SecB concentration.

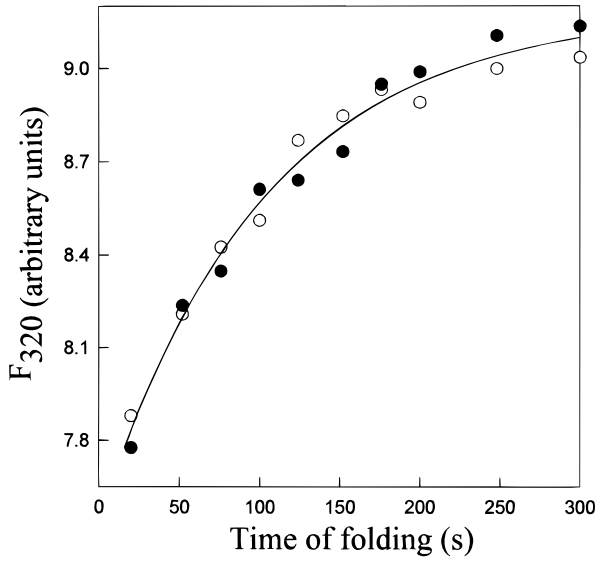

FIGURE 4: Effect of addition of SecB after variable times of barstar refolding. One hundred twenty microliters of 0.5 μM barstar in buffer containing 1 M GdnHCl was allowed to a refold for variable amounts of time. The solution was then transferred into a fluorescence cuvette containing 880 μL of 20 μM SecB in the same buffer and the flouresence emission monitered at 320 nm for 300 s. The final fluorescence intensity was corrected for the SecB contribution and plotted as a function of the total SecB concentration (○). Also shown (●) is the fluorescence intensity of a solution of 0.5 μM barstar refolding in the absence of SecB. This fluorescence was measured at a time equal to the time at which barstar was allowed to refold before SecB addition in the previous experiment.

is in the native state) does not result in any change in fluorescence.

DISCUSSION

The secretory pathway chaperone SecB has been shown to maintain nascent polypeptides in a translocation competent state. Little is known about the conformation of this translocation competent state. The selective entry of polypeptide chains into the export pathway has been explained by the kinetic partitioning of the nascent unfolded polypeptide chain between folding and binding to SecB. The primary evidence for kinetic partitioning comes from experimental studies of the interactions of SecB with MBP and its precursor preMBP. Studies of the interaction of SecB with MBP mutants have shown that the extent of blockage of MBP folding effected by SecB is larger for slow-folding mutants than for the wild type protein (*27*, *28*). While it is known that SecB can bind to a collapsed state of MBP, it is not known whether binding precedes or follows the collapse (*28*). Although the data for the interaction of MBP with SecB are qualitatively consistent with kinetic partitioning, no quantitative fits of the experimental data to a specific kinetic scheme have been performed to date. This is probably because MBP is a large two-domain protein, and the kinetics of folding of MBP in the absence of SecB are complicated and not completely characterized. It is therefore important to carry out kinetic studies of SecB ligand binding in simpler systems.

A recent study (*26*) of the binding of SecB to reduced unfolded BPTI showed that SecB binding is rapid ($k_{on} = 5 \times 10^9$ M^{-1} s^{-1}) and of high affinity ($K_D = 5$ nM). Fersht and co-workers have examined the interaction of SecB with the protein barnase. Barnase is a small, 110-residue ribonuclease from *B. amyloliquefaciens*. It has been shown that SecB binds to unfolded barnase and catalyzes hydrogen exchange in barnase by binding to the unfolded state. Kinetic studies of the interaction of SecB with barnase mutants (*29*) have shown that SecB binds rapidly to the unfolded protein with a rate constant of approximately 10^8 M^{-1} s^{-1}. The kinetic data were fit to a model in which conversion of barnase from the unfolded to the folded state could occur both when it was bound to SecB and when it was free in solution. The rate constants for barnase refolding when it is bound to SecB ranged from 0.001 to 0.028 s^{-1} and depended upon the specific mutant and on the SecB concentration. In this system, unlike in the case of barstar, SecB does not block the refolding of barnase even at high ratios of chaperone to substrate.

SecB Does Not Bind to Unfolded Barstar. The evidence for this is twofold. First, studies of equilibrium binding of SecB to unfolded CAM-V73A barstar show no evidence for SecB binding. Second, stopped-flow studies of the interaction of SecB with barstar molecules under refolding conditions show that SecB does not bind to the unfolded molecules or early intermediates in the folding process. Binding only occurs to the late-folding intermediate I_N (Figure 2). The

lack of binding to unfolded barstar and early intermediates that is seen in stopped-flow studies could be due to either of two reasons. It is possible that the on rate for barstar binding is sufficiently slow that binding does not occur before 1 s at the SecB and barstar concentrations used in the experiment (low on and off rates, binding is kinetically controlled). Alternatively, binding may not occur because the affinity of SecB for the unfolded state is low (high on and off rates, but the off rate is considerably higher than the on rate, binding is under thermodynamic control). We favor the latter possibility because increasing SecB concentrations results in apparently saturable binding to barstar, and the rate constant for barstar refolding is independent of SecB concentration (Figure 3B). Also, previous kinetic measurements of the binding of SecB to two small proteins have shown that SecB recognizes unfolded BPTI and unfolded barnase at close to diffusion-controlled rates (*26*, *29*), so it is unlikely that the on rate for SecB binding to unfolded barstar is rate-limiting.

In earlier studies of SecB binding to barnase and reduced unfolded BPTI, it has been shown that SecB binds to the unfolded state of the protein. For barstar, this clearly does not occur. A possible explanation is that at pH 7 barnase and BPTI have charges of +5.9 and +5.8, respectively, while barstar has a negative charge of −5.9. SecB has a negative charge of −13.5. These charges were estimated from protein sequences using the Isoelectric program of the Wisconsin package (*30*). Earlier studies (*31*) have suggested that SecB has binding sites for both positively charged and hydrophobic ligands. Barstar is negatively charged at neutral pH. Hence, partial folding of barstar may be required to offset the unfavorable electrostatic interaction between barstar and SecB. Folding of barstar may result in partial screening of some of the negatively charged groups by hydrogen bonding or salt bridge formation. Alternatively, the formation of a hydrophobic patch in barstar may be a prerequisite for SecB binding. An earlier study of SecB binding to proOmpA (overall charge at pH 7 is −1.88) demonstrated that SecB was able to recognize a form of proOmpA with considerable secondary and tertiary structure (*32*). For negatively charged substrates, it is thus likely that formation of a hydrophobic patch is required for binding to SecB to compensate for the unfavorable electrostatic interaction between the two negatively charged proteins.

SecB Binds to Late Intermediates in Barstar Refolding. The maximal fluorescence decrease observed at the highest concentrations of SecB appears to correspond to complete blockage of the slow phase of barstar refolding. When barstar refolding was carried out in the presence of excess SecB, the final fluorescence amplitude change observed at the highest concentrations of SecB (relative to the difference in amplitude between folded and unfolded barstar in the absence of SecB) was 71%. If binding during the fast phase were to occur, then the final fluorescence amplitude would be expected to be less than 70%. Hence, the fast phase does not appear to be affected at all, even at high SecB concentrations. Stopped-flow fluorescence studies of barstar refolding in the presence of SecB confirmed that neither the amplitude nor the rate constant of the fast phase was affected by SecB. This suggests that SecB binds only to the I_N intermediate responsible for the slow phase of barstar refolding in 1 M GdnHCl at pH 7.0. It was not possible to measure the binding constant for binding of SecB to I_N because the system was not at equilibrium; SecB binding occurred in competition with barstar folding. However, the affinity of SecB for I_N is probably in the micromolar range because micromolar concentrations of SecB are required for blockage of barstar folding.

The fluorescence of barstar in the SecB−barstar complex is identical to the fluorescence of free barstar in the intermediate state that binds to SecB (Figure 4). This suggests that the environments of Trp residues in barstar bound to SecB and in I_N are similar. The structure of I_N differs from that of the native state in having a trans conformation about the Tyr47−Pro48 peptide bond (*11*). Unlike the native state, I_N binds the dye ANS, suggesting it has exposed hydrophobic pockets. This exposed hydrophobic surface may be important for binding to SecB since the native state, which does not bind ANS, also does not appear to bind to SecB.

SecB−Barstar Binding Kinetics Are Not Explained by Kinetic Partitioning. Two possible kinetic partitioning schemes for explaining SecB−barstar binding were considered. The rate expressions corresponding to each of the schemes are outlined in Experimental Procedures. In Scheme 1, the chaperone is predicted to decrease the apparent rate constant for formation of the native state. However, the final yield of the native state is unaffected (*25*). This is inconsistent with our data which show that the yield of the native state decreases with increasing SecB concentration. According to Scheme 2, an increase in chaperone concentration is predicted to result in a decrease in the yield and an increase in the apparent rate constant of formation of the native state. If we assume Scheme 2 is valid, from eq 7 one can estimate k_S by measuring the final yield of N_S as a function of S concentration. It is appropriate to use N_S (the concentration of native barstar formed during the slow phase) rather than the total barstar concentration because SecB binding only takes place during the slow phase of barstar refolding. The yield of N_S as a function of SecB concentration can be estimated from the data in Figure 4A as described in eq 9. From the above data, we can estimate that k_S equals 3×10^3 M^{-1} s^{-1}. The apparent rate constant for formation of N_S according to Scheme 2 is $k_f + k_S[S]$. When we substitute the values of k_f and k_S, the apparent rate constant for formation of N_S should increase from 0.009 to 0.06 (a change of about 600%) over the range of SecB concentrations used, whereas no change is observed experimentally (Figure 3B). Hence, Scheme 2 also cannot account for the observed kinetics.

A recent study of the interaction between GroEL and RNase T1 showed a similar lack of dependence of the apparent unfolding rate of RNase T1 on chaperone concentration (*33*). This was explained by a thermodynamic partitioning model. In these studies, since the rate constant for unfolding of N from I_N is not known and the system is not at thermodynamic equilibrium, we did not attempt to fit the observed kinetics to such a model. However, the available data suggest that the interaction between SecB and barstar, at least under the present experimental conditions, is controlled by thermodynamic rather than kinetic factors. SecB binds only to a late native-like intermediate, I_N, and the affinity of SecB for I_N detemines the extent of blockage of barstar refolding.

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