

SecB-Mediated Protein Export Need Not Occur via Kinetic Partitioning

Beena Krishnan¹, S. Rajendra Kulothungan¹, Ashish K. Patra², Jayant B. Udgaonkar² and Raghavan Varadarajan^{1,3*}

¹Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

²National Center for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bangalore 560 065, India

³Chemical Biology Unit, Jawaharlal Nehru Center for Advanced Scientific Research, Jakkur PO, Bangalore 560 004, India

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In *Escherichia coli*, the cytosolic chaperone SecB is responsible for the selective entry of a subset of precursor proteins into the Sec pathway. *In vitro*, SecB binds to a variety of unfolded substrates without apparent sequence specificity, but not native proteins. Selectivity has therefore been suggested to occur by kinetic partitioning of substrates between protein folding and SecB association. Evidence for kinetic partitioning is based on earlier observations that SecB blocks the refolding of the precursor form of maltose-binding protein (preMBP)⁵ and slow-folding maltose-binding protein (MBP) mutants, but not faster-folding mature wild-type MBP. In order to quantitatively validate the kinetic partitioning model, we have independently measured each of the rate constants involved in the interaction of SecB with refolding preMBP (a physiological substrate of SecB) and mature MBP. The measured rate constants correctly predict substrate folding kinetics over a wide range of SecB, MBP, and preMBP concentrations. Analysis of the data reveals that, for many substrates, kinetic partitioning is unlikely to be responsible for SecB-mediated protein export. Instead, the ability of SecB-bound substrates to continue folding while bound to SecB and their ability to interact with other components of the secretory machinery such as SecA may be key opposing determinants that inhibit and promote protein export, respectively.

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Introduction

In *Escherichia coli*, export of the majority of proteins from the cytoplasm to the periplasm occurs via the Sec pathway. Export of a subset of periplasmic proteins mediated by the Sec pathway is aided by the

tetrameric cytoplasmic chaperone SecB. SecB performs a dual function in the export process; it binds to newly synthesized precursor proteins, thus preventing their premature aggregation and/or folding, and subsequently targets them to the translocation machinery on the membrane.^{1,2} SecB(−) cells have recently been shown to exhibit a cold-sensitive phenotype.³ There have been a number of *in vitro* studies on the interaction of SecB with both its physiological and its model substrates.^{4–9} These studies demonstrated that SecB binds to diverse unfolded/partially folded proteins with little sequence specificity, but not to their corresponding native states. The crystal structure of SecB from *E. coli*¹⁰ and *Haemophilus influenzae*¹¹ suggests that unfolded proteins bind to a surface-exposed groove on SecB.

Insights into the mechanism of substrate selectivity have been primarily inferred from studies of SecB with one of its natural ligands, maltose-binding protein (MBP). MBP of *E. coli* is a 370-residue two-domain periplasmic protein required for maltose and maltodextrin transport. It is a well-characterized

*Corresponding author. Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India. E-mail address: varadar@mbu.iisc.ernet.in.

Present address: B. Krishnan, Department of Biochemistry and Molecular Biology, University of Massachusetts-Amherst, Amherst, MA 01003, USA.

Abbreviations used: preMBP, precursor form of maltose-binding protein; MBP, maltose-binding protein; WT, wild type; BPTI, bovine pancreatic trypsin inhibitor; ANS, 1-anilino-8-naphthalene-sulfonate; FRET, fluorescence resonance energy transfer; MBP_u, unfolded MBP; preRBP, precursor form of ribose-binding protein; GdnHCl, guanidine hydrochloride.

protein with respect to its structure,¹² binding properties, folding thermodynamics, and kinetics.^{13–15} The precursor form of maltose-binding protein (preMBP) is synthesized in the cell with a 26-residue signal peptide at the N-terminus. preMBP—not MBP—is the physiological substrate of SecB. The binding motif for SecB recognition has been found to lie in the mature region of the protein.¹⁶

At room temperature, SecB slows down but does not block the folding of MBP, while it completely blocks the folding of preMBP and slow-folding mutants of MBP.⁸ At room temperature, preMBP folds approximately 5-fold slower than wild-type (WT) MBP. At a reduced temperature of 5 °C, the folding rate of MBP has been reported to be similar to that of preMBP. Under these conditions, MBP folding is also blocked by SecB. On the basis of the above studies, the binding selectivity of SecB has been explained by kinetic partitioning between folding of the polypeptide and its association with SecB.^{8,17–19} The kinetic partitioning model is conceptually elegant and accounts qualitatively for much experimental data. However, this model has not been quantitatively validated. In addition, there are some observations that are at odds with this model. Measurement of the binding kinetics of unfolded bovine pancreatic trypsin inhibitor (BPTI) and barnase to SecB^{7,20} indicates that these proteins bind SecB in a diffusion-controlled reaction. Given the rapid binding kinetics in these cases, at physiological concentrations of SecB, binding is likely to occur more rapidly than folding for many proteins, including MBP. However, as noted above, SecB does not block MBP refolding. In addition, the refolding rate constant of prePhoE is considerably faster (refolding occurs within 10 s) than that of either MBP or preMBP, yet it is a physiological substrate of SecB and forms a stable complex with SecB *in vitro*.²¹ Effects of SecB on the folding kinetics of the small protein barstar were also found to be inconsistent with kinetic partitioning.²² Other experiments using an *in vitro* protein synthesis system demonstrate that SecB can be isolated in a complex with the nascent chain of precursor proteins^{23,24} and before folding occurs. Furthermore, in SecB(–) cells, cotranslational—but not posttranslational—translocation of MBP is defective, suggesting that SecB plays an important role early in the export process before the entire polypeptide is synthesized and, therefore, the folding rate of the polypeptide may not be important.²⁵

In the present study, we have carried out detailed kinetic analyses of the binding of both MBP and preMBP to SecB and have estimated all the rate constants involved in binding and folding in order to quantitatively validate the kinetic partitioning model. We were able to quantitatively reproduce the observed folding kinetics of both MBP and preMBP at multiple substrate and SecB concentrations using the measured rate constants. We could also explain why SecB is able to block the refolding of preMBP, and not MBP. The data demonstrate that kinetic partitioning between folding and SecB binding is unlikely to be responsible for SecB selectivity. Instead, the ability of

SecB-bound substrates to continue folding while bound to SecB and their ability to interact with other components of the secretory machinery such as SecA may be the key opposing determinants that inhibit and promote protein export, respectively.

Results

Model of preMBP/MBP refolding in the presence of SecB

Two general simplified kinetic schemes for protein folding in the presence of SecB can be considered. These are depicted as Schemes 1 and 2, respectively. Scheme 1 is similar to the kinetic partitioning model proposed by Hardy and Randall.^{8,19} Kinetic partitioning, as the name suggests, emphasizes kinetic competition between folding and chaperone binding (i.e., the relative rates of $k_f[I]$ and $k_{on}[I][S]$).⁸ Scheme 1, in addition, incorporates the rate $k_{off}[IS]$. In Scheme 1, if the binding and dissociation of the intermediate (I) to SecB (S) are assumed to be considerably faster than the transition between I and the native/native-like state N*,^{15,26} preequilibrium will be achieved between I and IS prior to the rate-controlling transition of I to N*. Based on the preequilibrium assumption and also assuming that refolding in the presence and in the absence of SecB is described by a single exponential, the observed rate constant of folding k_{app} is given by:

$$k_{app} = \frac{k_f}{[I_0]} \times \frac{-([S_0] - [I_0] + K_d) \pm \sqrt{([S_0] - [I_0] + K_d)^2 + 4[I_0]K_d}}{2} \quad (1)$$

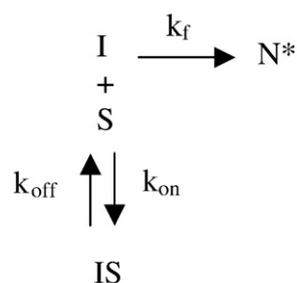
Here, $[I_0]$ and $[S_0]$ are the total concentrations of MBP/preMBP and SecB, respectively, and $K_d = k_{off}/k_{on}$.

Another possible mechanism (Scheme 2) includes an additional step of MBP folding with a rate constant of k'_f while it is bound to SecB.

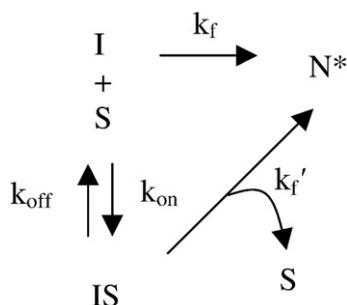
Similar to Scheme 1, assuming a preequilibrium to be established, k_{app} can be further written as:

$$k_{app} = k_f + (k'_f - k_f) \times \frac{([S_0] + [I_0] + K_d) \pm \sqrt{([S_0] + [I_0] + K_d)^2 - 4[I_0][S_0]}}{2[I_0]} \quad (2)$$

Scheme 2 contains an additional pathway relative to Scheme 1 wherein the substrate is able to fold with a



Scheme 1. Substrate Folding only in the free state.



Scheme 2. Substrate Folding in free and chaperone bound states.

rate constant k_f' into a native-like state while bound to the chaperone. At a late stage in folding, this native-like state (N^*) dissociates irreversibly from the chaperone and continues folding to the native state in a spectroscopically silent process. Derivations for Eqs. (1) and (2) are provided in Supplementary Information. k_{app} is calculated by using the (+) sign in front of the square root in Eq. (1) and by using the (-) sign in Eq. (2).

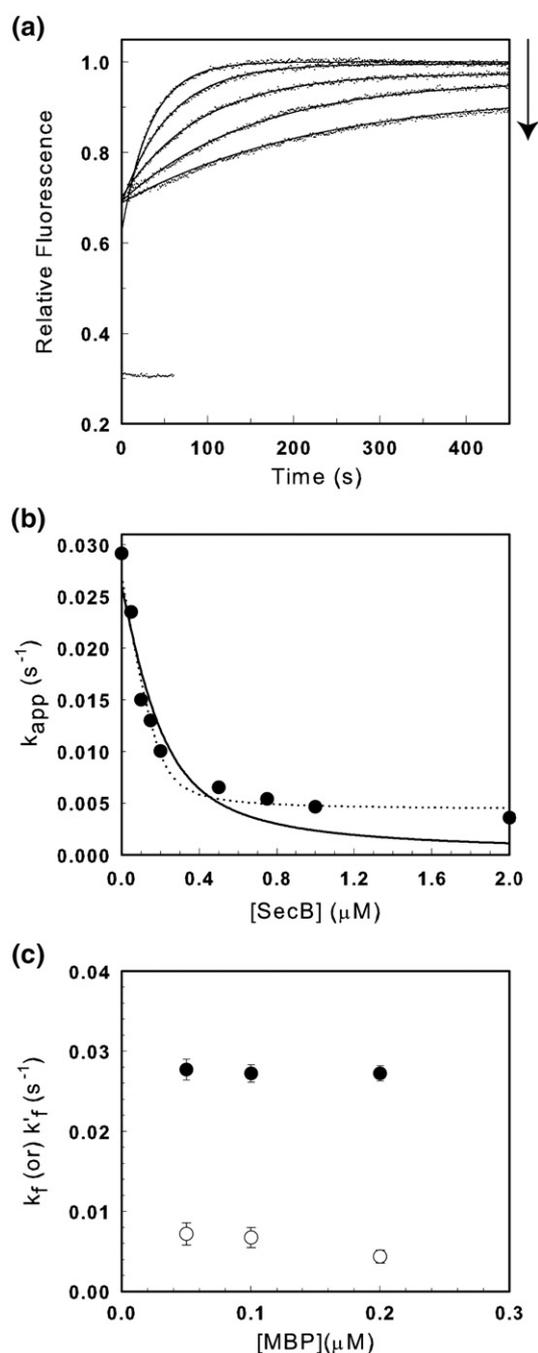
Effect of SecB on the refolding kinetics of MBP and preMBP

preMBP and MBP contain eight tryptophan residues. Refolding of the protein was accompanied by an increase in tryptophan fluorescence intensity. SecB contains only a single tryptophan residue, W36, and hence the fluorescence contribution of SecB is much smaller than that of an equimolar amount of MBP/preMBP. Intrinsic tryptophan fluorescence was therefore used as a probe to monitor

the effect of SecB on the refolding kinetics of MBP and preMBP. In order to further minimize the spectroscopic contributions of SecB fluorescence, we used the W36Y mutant of SecB in preliminary experiments. However, this mutant was found to be appreciably less active than WT SecB in our hands and was therefore not used further. Due to difficulty in isolating WT preMBP, a mutant of preMBP carrying an alanine-to-glutamate substitution at position 14 in the signal peptide (A14E preMBP) was used as substitute for authentic preMBP.^{8,27}

The refolding of MBP is a complex multiexponential process involving three distinct kinetic phases. The major amplitude changes take place in the burst

Fig. 1. Refolding kinetics of MBP in the presence of SecB monitored by tryptophan fluorescence. (a) Refolding of 0.2 μM MBP in the absence (upper trace) and in the presence of 0.2, 0.5, 1.0, and 2.0 μM SecB (upper trace to lower trace, respectively) in CGH10 buffer containing 150 mM NaCl and 0.05 M GdnHCl (pH 7.2) at 25 $^\circ\text{C}$. Prior to refolding, the protein was denatured in the above buffer containing 2 M GdnHCl. Unless otherwise mentioned, identical unfolding and refolding buffers were used in all subsequent experiments. The solid line represents a fit to a single-exponential function for the kinetic traces. Fluorescence contribution from SecB was appropriately subtracted, and the intensity of kinetic traces was represented relative to the refolded proteins in the absence of SecB, with the fluorescence intensity of completely refolded protein in the absence of SecB taken as 1.0. The lowest trace represents the unfolded baseline. (b) Each kinetic trace in (a) was fitted to a single exponential to obtain an apparent rate constant (filled circles). The lines represent a nonlinear least squares fit of the data to either Eq. (1) (solid line) or Eq. (2) (dotted line). (c) Kinetic parameters for MBP refolding in the presence of SecB were analyzed according to Scheme 2. Represented in the figure are the experimentally measured rate constants for folding of unbound MBP k_f (\bullet) and the rate constant for folding of MBP when present as a complex with SecB k_f' (\circ), obtained from fitting the data in (b). The direction of the arrow (\downarrow) in (a) and all subsequent figures indicate the direction of increasing SecB concentration.



phase (~52%) and in the slow phase (33%). The fast phase accounts for the remaining amplitude. Refolding of preMBP involves two phases: a burst phase and a single slow-folding phase.¹⁵ Refolding of proteins in the presence of increasing concentrations of SecB did not affect the amplitude of the burst phase, but resulted in an increase in the rate of the fast phase. This implies that SecB binding occurs subsequent to the formation of the initial collapsed intermediates of MBP and preMBP. This is also consistent with a recent single-molecule study of MBP interaction with SecB.⁹ The apparent rate constant k_{app} of the fast phase in the presence of SecB increased linearly as a function of SecB concentration. This

suggests that the fast phase represents SecB binding to the proteins. The association rate constant for SecB binding to MBP/preMBP was further determined using the fast phase data (described later). In all cases, it has been assumed that the SecB tetramer can bind a single molecule of refolding MBP/preMBP substrate. This binding stoichiometry is known from earlier isothermal titration calorimetry studies.²⁸

In order to investigate the effect of SecB on the slow-phase kinetics of MBP/preMBP refolding, manual mixing experiments were performed (Figs. 1 and 2). As previously observed,⁸ the refolding rate of MBP slows down in the presence of increasing concentrations of SecB. It finally reaches a limiting

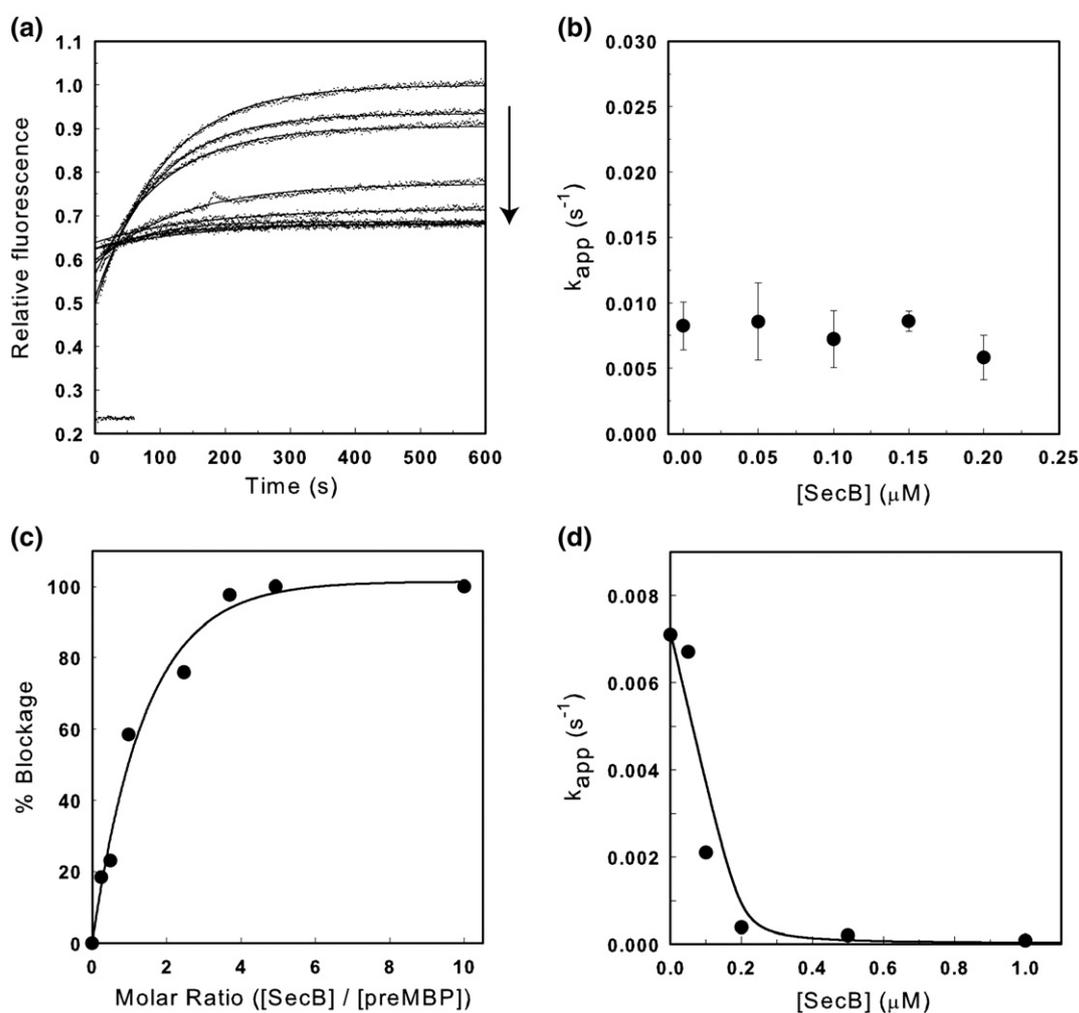


Fig. 2. Refolding kinetics of preMBP in the presence of SecB monitored by tryptophan fluorescence. (a) Refolding of 0.2 μM preMBP (unfolded in 2 M GdnHCl) was monitored in the presence of up to 2 μM SecB in CGH10 buffer containing 150 mM NaCl and 0.05 M GdnHCl (pH 7.2) at 25 °C. The traces represent the presence of 0.0, 0.1, 0.2, 0.5, 1.0, and 2.0 μM SecB (upper trace to lower trace, respectively). The fluorescence of free SecB was subtracted from all kinetics traces where the SecB:MBP molar ratio was greater than 1. In such cases, the free SecB concentration was calculated while assuming that 0.2 μM SecB was present as a complex with preMBP. The data were normalized as described in Fig. 1a. (b) Apparent rate constant of refolding of preMBP (k_{app}) as a function of SecB concentration. At SecB:preMBP molar ratios greater than 1, k_{app} could not be accurately measured, as most of the preMBP is complexed to SecB. (c) Blockage of the folding of 0.2 μM preMBP by SecB at 25 °C. The extent of blockage (% blockage) was calculated from the concentration of the complex formed at the end of 10 min at each SecB concentration. (d) Refolding of preMBP in the presence of SecB was monitored on longer timescales extending to hours to monitor the complete refolding of preMBP (see also Supplemental Fig. 2). Data were fitted to a single exponential to obtain k_{app} . The solid line represents a nonlinear least squares fit of the data to Eq. (1) corresponding to Scheme 1.

value (Fig. 1a and b). Slowdown of the refolding kinetics of MBP by SecB was observed for MBP concentrations ranging from 0.05 to 0.5 μM . SecB concentrations were varied over a 10-fold molar range at each MBP concentration. In each case, the apparent refolding rate constant (k_{app}) reaches a saturating value of about $0.007(\pm 0.002) \text{ s}^{-1}$ once a 2-fold molar excess of SecB is present. It should be noted that SecB slows down but does not block the refolding of MBP (Fig. 1a and Supplemental Fig. 1). The activity of refolded MBP was assayed by its ability to bind maltose as described previously,²⁶ and the refolded protein had activity identical to that of the native protein. k_{app} values for MBP in the presence of SecB were fitted to Eqs. (1) and (2) (Fig. 1b). The dotted line in Fig. 1b shows that the observed dependence of k_{app} on SecB concentration in the case of MBP is much better accounted for by Scheme 2 than by Scheme 1, especially at higher concentrations of SecB. Similar results were observed when refolding experiments were carried out at MBP concentrations varying from 0.05 to 0.2 μM (data not shown), suggesting that the interaction of MBP with SecB cannot be described by Scheme 1.

In contrast, the apparent rate constant for refolding of preMBP was independent of SecB, and there was a decrease in the amplitude of the refolding phase in the presence of increasing SecB concentrations (Fig. 2a and b). At a 4-fold or greater molar excess of SecB, there was no time-dependent change in tryptophan fluorescence, and traces perfectly overlaid on each other after appropriate subtraction of the fluorescence contribution of free SecB. Increasing SecB concentration resulted in a reduced final yield of the refolded preMBP, indicating that SecB blocks the refolding of preMBP (Fig. 2c).

Thus, at substoichiometric SecB:preMBP ratios, some preMBP is complexed to SecB, and the remaining excess of preMBP that is free in solution expectedly folds with the same rate constant as in the absence of SecB. When SecB is present in molar excess, preMBP does not fold and is rapidly trapped as a stable complex with SecB within the dead time of manual mixing ($\sim 10 \text{ s}$). The burst-phase change in these experiments is due to the fact that binding of preMBP to SecB results in an increase in fluorescence intensity, relative to unfolded preMBP. The preMBP—SecB complex does not dissociate on the timescales of $\sim 1000 \text{ s}$ examined here (Supplemental Fig. 2b). To determine whether preMBP bound by SecB is eventually released, we also monitored the refolding process for longer times extending to several hours (Supplemental Fig. 2). At these longer timescales, it was observed that SecB does indeed release preMBP, and all of the starting protein is obtained in native form because the final fluorescence intensity of the sample is that expected for a mixture of free SecB and free native preMBP. The refolded preMBP was found to be active, as assayed by the maltose-binding assay described above for MBP. The apparent refolding rates obtained from the longer timescale kinetic traces were observed to decrease with increasing SecB concentrations (Fig. 2d). Data in Fig. 2d were also fitted to Eqs. (1) and (2). The solid line shows the fit to Eq. (1) (Scheme 1). The fit to Eq. (2) was identical with the fit to Eq. (1) and yielded an identical K_{d} value. The value of k_{f}' was $-0.0001 \pm 0.001 \text{ s}^{-1}$. Since the value of k_{f}' was not well determined and also did not improve the quality of the fit, we did not show the fit to Scheme 2.

Data from manual mixing experiments (Figs. 1b and 2d) are therefore all qualitatively consistent

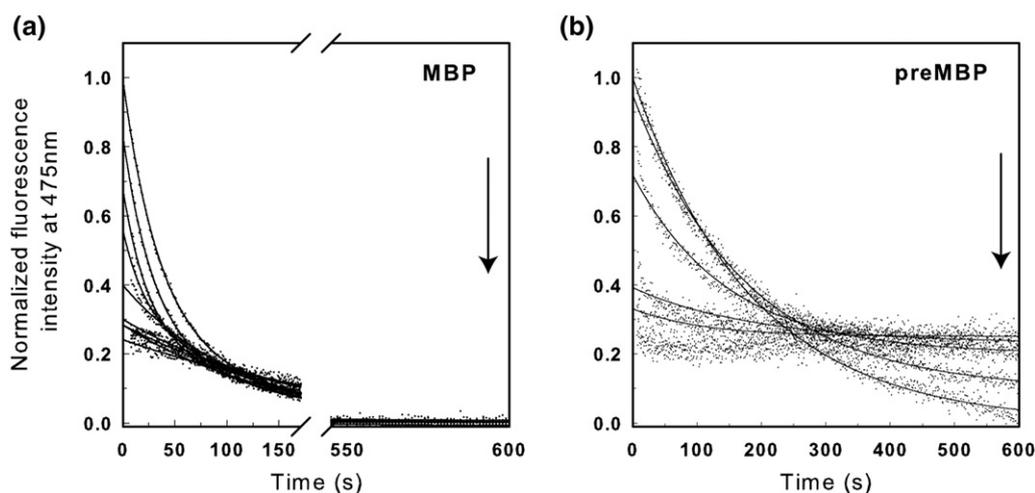


Fig. 3. Refolding kinetics of MBP (a) and preMBP (b) as monitored by ANS fluorescence in the presence and in the absence of SecB. Refolding kinetics, initiated by a 40-fold dilution of the unfolded protein (final concentration of MBP/preMBP, 0.2 μM ; final concentration of GdnHCl, 0.05 M) was monitored by the time-dependent decrease in fluorescence energy transfer from tryptophans on MBP/preMBP to bound ANS. The traces from top to bottom represent refolding in the absence of SecB and refolding in the presence of increasing amounts (0.05, 0.1, 0.15, 0.2, 0.5, 1.0, and 2.0) of SecB. Refolding was monitored by the change in ANS fluorescence at 475 nm after excitation at 280 nm. The fluorescence contribution of ANS bound to SecB was appropriately subtracted from the refolding traces prior to analysis. Solid lines represent single-exponential and biexponential fits to the traces for MBP and preMBP, respectively.

with preMBP folding according to [Scheme 1](#) with $K_d=0.4\pm 2$ nM, and with MBP refolding according to [Scheme 2](#) with $K_d=20\pm 10$ nM and $k_f'\sim 0.0067$ (± 0.002) s^{-1} . The k_f' value obtained from the fit to Eq. (2) (dotted line in [Fig. 1b](#)) is identical with the value measured by direct experiments at saturating SecB concentrations. Similar analyses were carried out at MBP concentrations of 0.05 and 0.1 μ M, and the fitted k_f' values shown in [Fig. 1c](#) are independent of concentration within experimental error. Derivation of Eqs. (1) and (2) involved various assumptions. Hence, to further validate our proposal, we have independently measured each of the individual rate constants in the proposed schemes, as described in later sections. Using these measured

rate constants, we show that the observed kinetics can be quantitatively described by [Scheme 1](#) for preMBP/SecB and by [Scheme 2](#) for MBP/SecB.

Refolding monitored by 1-anilino-8-naphthalene-sulfonate fluorescence

The intermediates of the folding pathways of MBP and preMBP are known to bind 1-anilino-8-naphthalene-sulfonate (ANS).¹⁵ In order to preclude any effect of ANS on the refolding of MBP, its binding to the late kinetic intermediates was investigated by delayed addition. The fluorescence intensity at the time of ANS addition was calculated from the fitted data and compared to the corresponding fluorescence

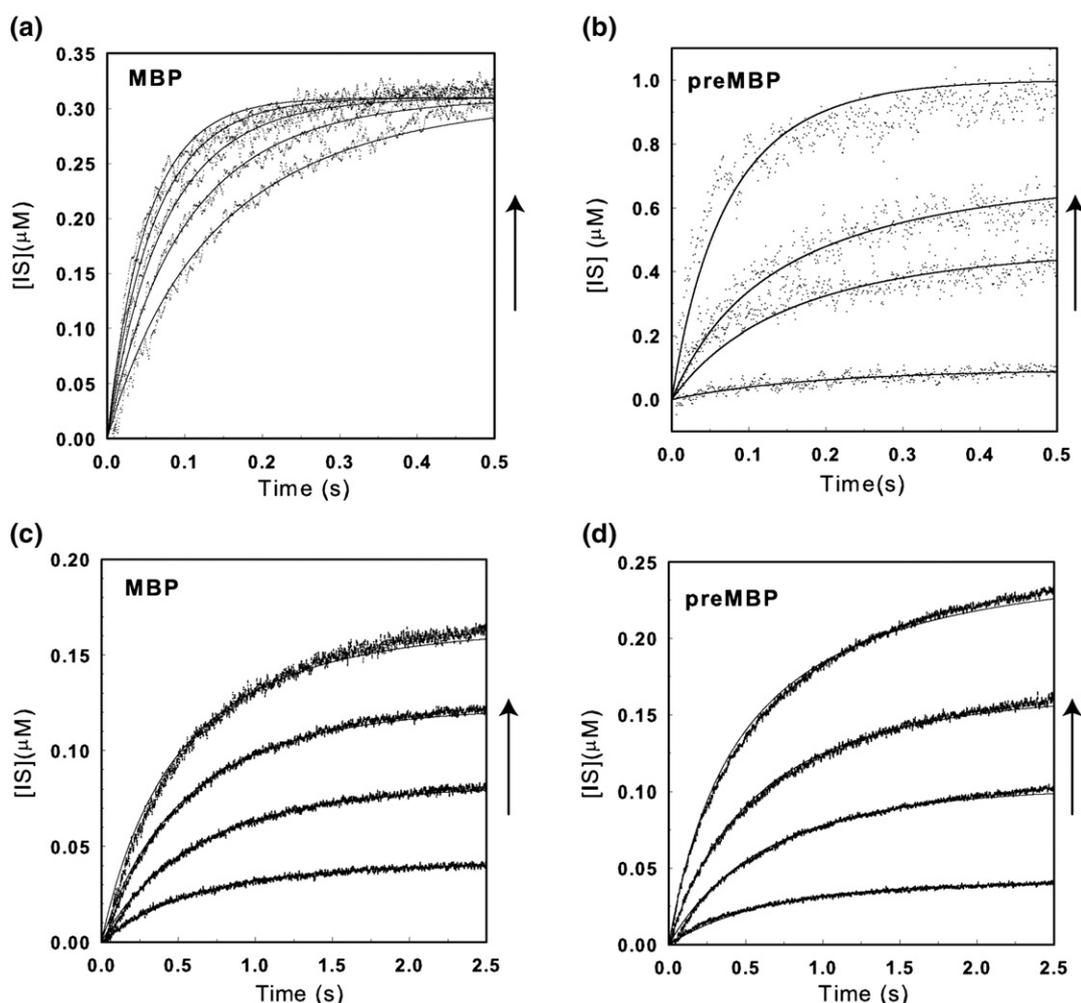


Fig. 4. Determination of k_{on} for the binding of SecB to refolding MBP/preMBP. The rate of increase in tryptophan fluorescence intensity monitored at 340 nm after excitation at 280 nm occurring in the first 0.5 s of the reaction was used to estimate k_{on} . [IS] values were estimated from fluorescence intensities as described in [Materials and Methods](#). Solid lines represent fits to a bimolecular binding model (Eq. 5). (a) MBP (0.3 μ M) was refolded in the presence of 0.075, 1.0, 1.5, 2.0, and 2.5 μ M SecB (kinetic traces from bottom to top). (b) preMBP (1 μ M) was refolded in the presence of 0.1, 0.5, 0.7, and 3.0 μ M SecB (kinetic traces from bottom to top). (c and d) Unfolded protein at a concentration of 0.3 μ M was refolded in the presence of increasing concentrations of acrylodan-labeled Q14C SecB. The rate of increase in acrylodan fluorescence intensity (monitored at 500 nm upon excitation at 280 nm) occurring in the first 3.0 s was used to estimate k_{on} . [IS] values were estimated from fluorescence intensities, and the data were fitted to a bimolecular rate equation. (c) Binding of the refolding intermediate(s) of MBP to 0.041, 0.082, 0.123, and 0.164 μ M acrylodan-labeled SecB (kinetic traces from bottom to top). (d) Binding of the early intermediate(s) of preMBP to 0.041, 0.103, 0.164, and 0.246 μ M acrylodan-labeled SecB (kinetic traces from bottom to top). Denaturation and refolding buffers were as described in [Fig. 1](#).

value when ANS was present in the refolding buffer at time $t=0$. The fluorescence intensities were in close agreement, and the rate constants of the two phases were found to be independent of the time of addition of ANS. This indicates that the presence of ANS does not affect the refolding process, and that ANS serves as an inert reporter.

The refolding of the proteins in the absence and in the presence of SecB was monitored by a decrease in fluorescence energy transfer from the tryptophan residues on the substrate protein to bound ANS. Both refolding and SecB binding lead to burial of hydrophobic surface and consequent expulsion of bound ANS from both SecB and refolding substrate.

In the case of MBP, there is a large biphasic decrease in ANS fluorescence as MBP refolds to its native state in the absence of SecB (apparent rate constants of 0.03 and 0.006 s^{-1} and relative amplitude changes of 0.7 and 0.3, respectively).¹⁵ In the presence of increasing concentrations of SecB, there is a progressive decrease in ANS fluorescence (Fig. 3a), indicating that the SecB-bound state of MBP contains a less-exposed hydrophobic surface than the unbound intermediate states (I). Binding of SecB to MBP results in an immediate expulsion of the ANS molecules bound to I and free SecB. This suggests that SecB competes with ANS for binding to hydrophobic surfaces on MBP folding intermediates. The amplitude of the burst phase (calculated with respect to the extrapolated $t=0$ s fluorescence for ANS binding to refolding MBP alone) was found to increase with an increase in SecB concentration and finally saturates. At substoichiometric concentrations of SecB, the data show a decrease in the amplitude of the faster phase ($k=0.03\text{ s}^{-1}$) and an increase in the amplitude of the slower phase (0.006 s^{-1}) with increasing SecB concentration. At higher SecB concentrations greater than a SecB:MBP molar ratio of 1:1, a single phase with a SecB-independent rate constant of 0.006 s^{-1} is sufficient to describe the refolding process.

We suggest that the saturating burst-phase fluorescence change at higher SecB concentrations corresponds to the fluorescence of ANS bound to the SecB-MBP complex. A further decrease in ANS fluorescence as a function of time is due to refolding of MBP while bound to SecB in the presence of excess SecB. A close agreement in the values of the apparent rate constant k_{app} obtained from tryptophan fluorescence and ANS binding data at higher SecB concentrations indicates that the saturating k_{app} value is the refolding rate constant (k_f') of MBP complexed to SecB.

In the presence of ANS, preMBP refolding is accompanied by a decrease in ANS fluorescence with a rate constant of 0.006 s^{-1} , identical with that observed with tryptophan fluorescence (Fig. 3b).¹⁵ In the presence of SecB, the extent of ANS binding to preMBP is reduced, indicating that SecB binds to hydrophobic regions of the preMBP intermediates. As with tryptophan fluorescence data, the apparent rate constant for the folding of preMBP monitored by ANS fluorescence does not change with increas-

ing SecB concentration. However, the amplitude of this phase decreases, and there is a corresponding increase in the magnitude of the burst phase as seen with MBP. Unlike the case for MBP, there was no time-dependent change in ANS fluorescence in the refolding of preMBP at higher SecB concentrations. This is consistent with the formation of a stable preMBP-SecB complex that does not dissociate on the timescale of ~ 1000 s.

Determination of the bimolecular rate constant k_{on} of SecB binding using tryptophan fluorescence

Refolding of MBP/preMBP as function of SecB concentration was monitored using stopped-flow mixing and tryptophan fluorescence. SecB affects the additional fast phase that is completed by 0.5 s in the refolding process. The apparent rate constant of this phase depends linearly on SecB concentration, suggesting that it represents SecB binding. At SecB:substrate molar ratios greater than 1, the refolding traces plateau at similar values after appropriate subtraction of the free SecB fluorescence. This saturation fluorescence represents the fluorescence value of the MBP-SecB complex (IS). [IS] is equal to the total MBP concentration ($0.3\text{ }\mu\text{M}$ in this case; Fig. 4a). From this, the value of [IS] at any time can be calculated as described in Data Analysis. Kinetic traces of preMBP refolding in the presence of SecB can be similarly analyzed (Fig. 4b). The data, when fitted to a bimolecular rate equation, gave average on-rate constant, k_{on} values of $6.13(\pm 1.47)\times 10^6$ and $1.03(\pm 0.23)\times 10^7\text{ M}^{-1}\text{ s}^{-1}$ for binding of SecB to preMBP and MBP, respectively. Measurement of k_{on} and k_{off} values of SecB binding to MBP and preMBP using acrylodan-labeled Q14C SecB was carried out under conditions where the off-rate from SecB, as well as the refolding rate, is small and can be neglected in comparison to the on-rate.

Determination of the k_{on} and k_{off} values of SecB binding to MBP and preMBP using acrylodan-labeled Q14C SecB

We designed a fluorescence resonance energy transfer (FRET)-based strategy to determine the binding and dissociation rate constants k_{on} and k_{off} using SecB labeled with an extrinsic fluorophore. Although SecB contains four Cys residues, these are all relatively buried and show slow labeling kinetics (data not shown). For the FRET-based experiments, we therefore introduced a solvent-accessible cysteine at position 14 in SecB (Q14C SecB). The exposed cysteine residue was labeled with a thiol-specific fluorophore acrylodan. The presence of one acrylodan per SecB monomer was confirmed by electrospray ionization mass spectrometry and 5,5'-dithiobis(2-nitrobenzoate) labeling. Acrylodan-labeled Q14C SecB showed identical structure (far-UV CD) and a tetrameric state (gel filtration) as WT SecB.

Determination of the binding rate constant k_{on}

Refolding of both preMBP and MBP in the presence of acrylodan-labeled Q14C SecB was monitored in a stopped-flow fluorimeter. Acrylodan fluorescence increases upon binding of preMBP/MBP due to energy transfer from tryptophan residues in preMBP/MBP to acrylodan. It saturates within about 3 s (Fig. 4c and d). The total fluorescence change was converted into the concentration of the complex formed as a function of time, as described in Data Analysis. The data, when fitted to a second-order rate equation, gave k_{on} values of $6.28 (\pm 0.88) \times 10^6$ and $6.01 (\pm 1.06) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for MBP and preMBP, respectively, similar to those obtained from tryptophan fluorescence measurements described above.

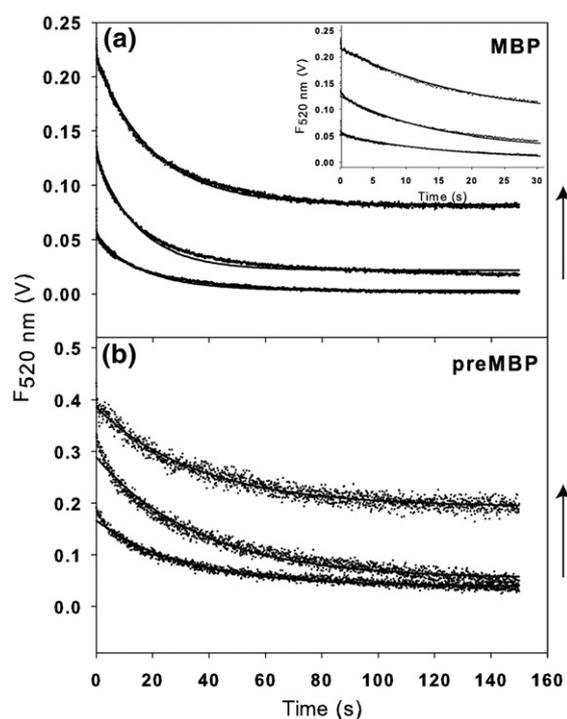


Fig. 5. Determination of k_{off} for the SecB–MBP complex (a) and the SecB–preMBP complex (b). Complexes were formed between $0.2 \mu\text{M}$ refolding protein (final concentration of GdnHCl, 0.05 M) and increasing concentrations of acrylodan-labeled SecB by rapid stopped-flow mixing. In each case, after allowing 3 s for complex formation, 10-fold or more molar excess of unlabeled WT SecB ($2 \mu\text{M}$) was added. The decrease in fluorescence energy transfer (excitation, 280 nm ; emission, 520 nm) at varying concentrations of labeled SecB ($0.05, 0.1, \text{ and } 0.2 \mu\text{M}$; kinetic traces from bottom to top) was measured at 100-ms intervals, and the data were fitted to a single-exponential function (solid lines) to estimate k_{off} . For the SecB–MBP complexes, data were also measured at 5-ms intervals for the initial 7 s [inset to (a)]. k_{off} was found to be independent of SecB concentration. k_{off} values were found to be $0.058 (\pm 0.008)$ and $0.029 (\pm 0.003) \text{ s}^{-1}$ for MBP and preMBP, respectively. These were similar to the values obtained in the manual mixing experiments (Supplemental Fig. 3), thus demonstrating that the k_{off} value is also independent of the time of addition of SecB.

Table 1. Binding parameters determined in the present study for the interaction of SecB with MBP and preMBP

Kinetic parameters	MBP	preMBP
$k_f (\text{s}^{-1})$	$0.03 (\pm 0.002)$	$0.0073 (\pm 0.0006)$
$k_{\text{on}} (\text{M}^{-1} \text{ s}^{-1})^{\text{a}}$	$1.03 (\pm 0.23) \times 10^7$	$6.13 (\pm 1.47) \times 10^6$
$k_{\text{on}} (\text{M}^{-1} \text{ s}^{-1})^{\text{b}}$	$6.28 (\pm 0.88) \times 10^6$	$6.01 (\pm 1.06) \times 10^6$
$k_{\text{off}} (\text{s}^{-1})^{\text{c}}$	$0.068 (\pm 0.003)$	$0.026 (\pm 0.001)$
$K_d (\text{nM})^{\text{d,e}}$	$8.9 (\pm 0.02)$	$4.3 (\pm 0.001)$
$k_f' (\text{s}^{-1})$	$0.007 (\pm 0.002)$	f

^a k_{on} determined from Trp fluorescence rapid kinetic measurements.

^b k_{on} determined using acrylodan-labeled SecB.

^c Average of k_{off} values determined by stopped-flow and manual mixing experiments.

^d K_d values are calculated by taking an average of k_{on} values determined in table footnotes a and b ($K_d = k_{\text{off}}/k_{\text{on}}$).

^e A K_d of 30 nM has been reported from isothermal titration calorimetry measurements performed with the unfolded mature and precursor form of the slow-folding mutant Y283D MBP near $6\text{--}7^\circ\text{C}$.²⁸

^f There was no experimental evidence to support folding of preMBP while bound to SecB, hence no k_f' value is reported for preMBP.

Determination of the dissociation rate constant k_{off}

Displacement of the acrylodan-labeled Q14C SecB from a transiently formed complex between the labeled SecB and MBP/preMBP was carried out using a 10-fold excess of unlabeled WT SecB or unlabeled Q14C SecB. Dissociation was monitored by the decrease in acrylodan fluorescence as a function of time. Under these conditions, the dissociation of substrate from SecB is the rate-limiting step. Stopped-flow (Fig. 5) and manual mixing (Supplemental Fig. 3) yielded very similar k_{off} values of $0.058 (\pm 0.001) \text{ s}^{-1}$ and $0.078 (\pm 0.004) \text{ s}^{-1}$, respectively, for MBP, and $0.029 (\pm 0.001)$ and $0.023 (\pm 0.001) \text{ s}^{-1}$, respectively, for preMBP. The values were independent of the concentration of the SecB complex and the time of addition of unlabeled SecB. Importantly, the stopped-flow experiments showed that the entire dissociation was well accounted for by a single exponential process. Values of various parameters measured in the present study are summarized in Table 1.

Numerical analysis of the refolding kinetics of MBP and preMBP

To quantitatively explain the effect of SecB on the refolding kinetics of the proteins, we carried out computer simulations for kinetic Schemes 1 and 2, as described in Materials and Methods. Experimentally measured k_f, k_f', k_{on} , and k_{off} values were used in the simulation. We initially calculated theoretical refolding curves for MBP (according to Schemes 1 and 2) and preMBP (according to Scheme 1) in the presence of varying concentrations of SecB to obtain the concentrations of N*, I, S, and IS as a function of time. We did not simulate preMBP folding by Scheme 2 as there were no experimental data suggesting that

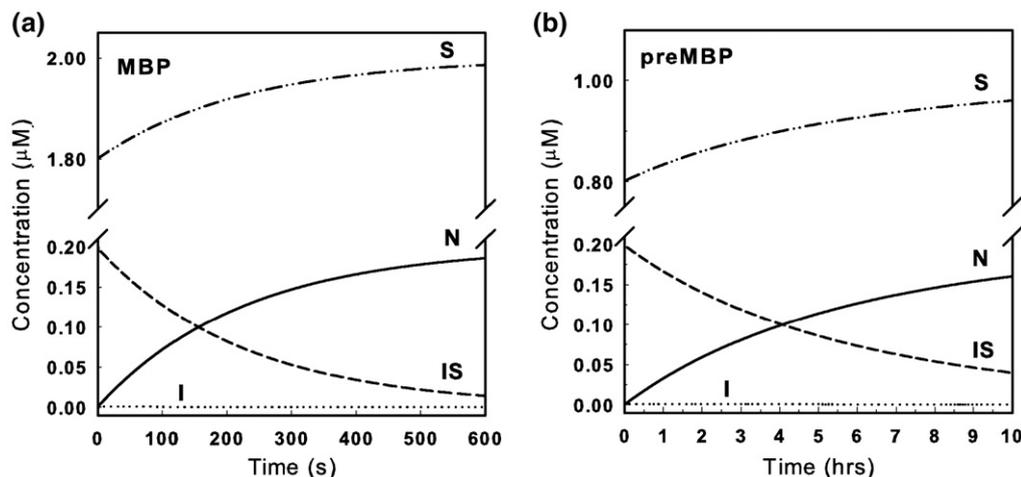


Fig. 6. Evolution of various species N (1; solid line), I (2; dotted line), IS (3; dashed line), and S (4; dot/dash line) obtained from the simulation data of the refolding of 0.2 μM MBP in the presence of 2 μM SecB from Scheme 2 (a) and for the refolding of 0.2 μM preMBP in the presence of 1.0 μM SecB from Scheme 1 (b). The values of the kinetic parameters k_f , k_f' , k_{on} , and k_{off} used in the MBP simulations were 0.03 s⁻¹, 0.0043 s⁻¹, 1.0×10^7 M⁻¹ s⁻¹, and 0.074 s⁻¹, respectively. The values of various parameters k_f , k_{on} , and k_{off} used in the case of preMBP refolding simulations were 0.007 s⁻¹, 7×10^6 M⁻¹ s⁻¹, and 0.026 s⁻¹, respectively.

preMBP could fold while bound to SecB, and hence no value of k_f' was available.

We next used the fluorescence intensity associated with each of these species—N*, I, IS, and S—obtained from experimental measurements and converted the simulated concentration data shown in Fig. 6 into fluorescence intensity data, as described in Data Analysis. The simulated and experimentally observed data were found to be in good agreement with MBP (Scheme 2) and preMBP (Scheme 1) (Fig. 7a and b), further validating the models considered for describing the interaction. The analysis clearly shows that MBP refolding in the presence of SecB cannot be accounted for by Scheme 1 (Fig. 7a, dashed lines). Refolding experiments were also carried out as a function of SecB concentration for MBP at concentrations of 0.05 and 0.1 μM, and for preMBP at a concentration of 0.1 μM. The close agreement between simulated and observed k_{app} values obtained with different MBP concentrations and SecB:MBP ratios is shown in Fig. 7c. Since no adjustable parameters are used in the simulation, this analysis strongly supports our proposed kinetic schemes for the interaction of SecB with MBP and preMBP.

Discussion

We have experimentally measured each of the rate constants involved in the interaction of SecB with refolding preMBP and MBP in order to quantitatively validate the kinetic partitioning model for SecB substrate selectivity. Refolding kinetics of preMBP and MBP in the absence and in the presence of SecB were measured at multiple substrate and SecB concentrations. The experimentally measured rate constants were used to quantitatively predict the observed refolding kinetics in all cases, without

any adjustable parameters. Determination of k_{on} and k_{off} allowed us to infer that the apparent dissociation constant K_d values characterizing SecB binding to unfolded MBP (MBPu) and preMBP were approximately 9 and 4 nM, respectively. This is in agreement with earlier reported K_d values with slow-folding MBP mutants and model-unfolded substrates.^{8,20,28} Furthermore, using our experimentally measured rate constants, we show below that, at physiological concentrations, the rate for SecB binding is faster than the refolding rate by orders of magnitude for both preMBP and MBP. This demonstrates that there exists no kinetic competition between folding and binding at physiological concentrations of SecB. Interestingly, our measured k_{on} value of about 10^7 M⁻¹ s⁻¹ for MBP and preMBP is considerably slower than the diffusion-controlled value of 10^9 M⁻¹ s⁻¹ previously measured for the binding of the model substrate, reduced carboxymethylated BPTI, to SecB.²⁰ This might be due to the fact that both SecB and MBP are negatively charged (~-14 and -7, respectively), while BPTI is positively charged (~+6), at neutral pH. The k_{off} values are also a hundredfold slower (~0.05 s⁻¹ for preMBP/MBP versus 50 s⁻¹ for BPTI), resulting in similar K_d values.

The *in vivo* concentration of SecB has been estimated to be ~4–40 μM,^{29,30} and 10% of intracellular SecB (0.4–4 μM) is estimated to be uncomplexed at any time. Under the approximately steady-state conditions prevailing inside a cell, the rate of binding of MBPu chains to SecB is given by $k_{on}[\text{MBPu}][\text{SecB}]$, whereas the corresponding rate of refolding is $k_f[\text{MBPu}]$. The ratio of these two rates is $k_{on}[\text{SecB}]/k_f$. It is assumed here that free SecB is in stoichiometric excess over MBPu. This must be true, as otherwise there would be no free SecB, given the high affinity of MBPu for SecB. Substituting the k_{on} and k_f values from Table 1 and using the [SecB] values indicated above, this ratio ranges from about

100 to 1000. Hence, the calculated binding rate is about 100- to 1000-fold faster than the refolding rate of the protein, implying that binding will always be preferred over folding for MBP and preMBP. Assuming a minimal free SecB concentration of 0.4 μM , substrate folding will only significantly outcompete binding for proteins that have folding rates that are 10-fold higher than binding rates (i.e., with rate constants $>40\text{ s}^{-1}$, corresponding to $t_{1/2} < 17\text{ ms}$). Exhaustive data for the folding rates of a large

number of *E. coli* proteins are not available. However, a recent study³¹ summarized k_f values for 36 two-state folding proteins and 25 multistate folding proteins from the literature. Approximately 30% of the former and 75% of the latter had k_f values less than 40 s^{-1} . Thus, the kinetic partitioning model, which only looks at relative rates of folding and binding, would predict that a large fraction of *E. coli* proteins would show SecB-mediated blockage of folding and is clearly not sufficient to account for the substrate selectivity of SecB. In practice, as exemplified by MBP, many proteins with folding rate constants substantially slower than 40 s^{-1} will not show SecB-mediated blockage, contrary to predictions of the kinetic partitioning model. This presumably occurs due to either high k_{off} values or nonzero k_f' values. This is true not only for cytosolic proteins but also for exported proteins. For example, the periplasmic protein ribose-binding protein does not depend on SecB for export³² and the precursor form of ribose-binding protein (preRBP) and ribose-binding protein fold with kinetics faster than that of preMBP but comparable to that of MBP ($k_f = 0.04\text{--}0.06\text{ s}^{-1}$).³³ The present analysis shows that the faster refolding rate of preRBP relative to that of preMBP is unlikely to account for its SecB-independent export. Explicit measurement of the individual rate constants involved in the interaction of SecB and preRBP should provide further insight into this issue.

In the case of preMBP, the observed refolding rate is approximately 5-fold slower than that for MBP. The on-rate and off-rate constants, and hence the apparent dissociation constant of 4 nM, for SecB binding to collapsed preMBP are not very different from those observed for MBP ($K_d \sim 8\text{ nM}$). MBP in the presence of excess SecB is able to refold to the native form on a timescale of a few minutes; in

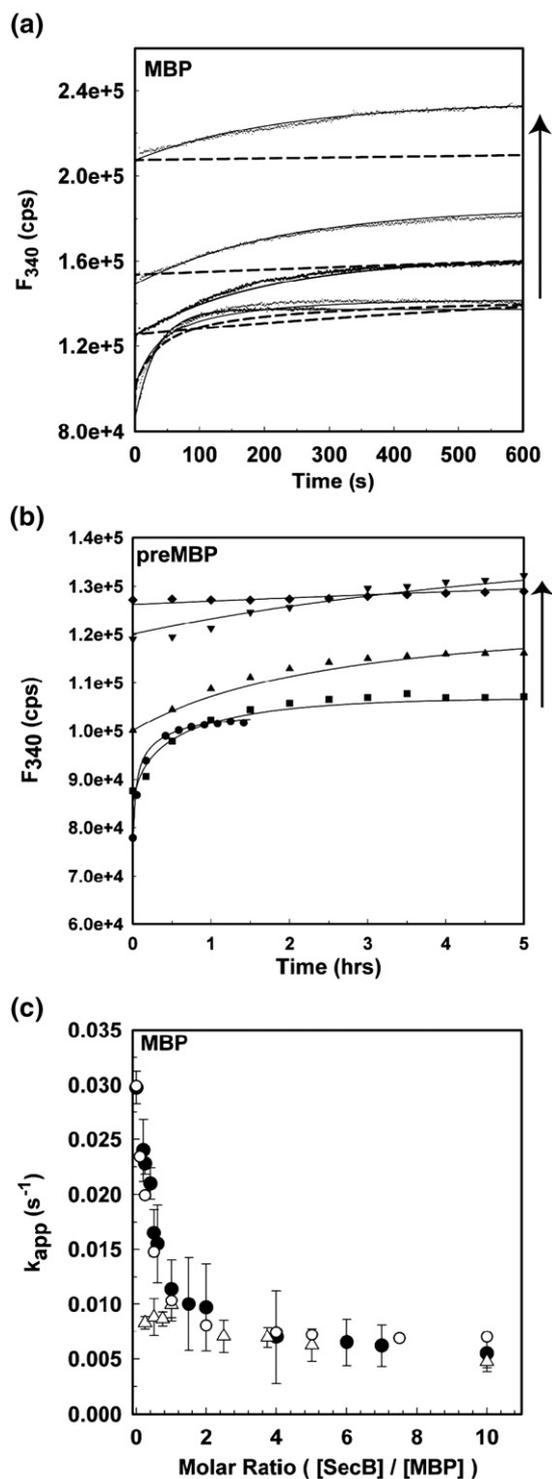


Fig. 7. Comparison of raw experimental data with simulated data for the refolding of 0.2 μM proteins in the absence and in the presence of SecB. Concentrations of various species as a function of time generated from the simulations were used to convert the simulated data into fluorescence intensity data, as described in Data Analysis. (a) The traces represent refolding of MBP in the absence (lowest trace) and in the presence of 0.2, 0.5, 1.0, and 2.0 μM SecB (lower traces to upper traces). Solid lines represent the simulated data from Scheme 2 overlaid onto raw experimental fluorescence data (dots). Dashed lines represent the fluorescence data generated following Scheme 1. (b) Experimental data for the refolding of 0.2 μM preMBP in the presence of 0.1 μM (\bullet), 0.2 μM (\blacksquare), 0.5 μM (\blacktriangle), 1.0 μM (\blacktriangledown), and 4.4 μM (\blacklozenge) SecB. The lines represent the simulated data generated following Scheme 1 overlaid onto raw experimental fluorescence data (symbols). (c) Comparison of the apparent rate constant k_{app} obtained from the single-exponential fits to the kinetic traces for the refolding of MBP in the presence of SecB monitored by ANS binding (Δ) and tryptophan fluorescence (\bullet), and from the simulated data (\circ). Error bars in the k_{app} measurement represent standard deviations from the measured k_{app} at a fixed SecB:MBP molar ratio at different final MBP concentrations ranging from 0.05 to 0.2 μM . ANS-monitored rate constants represent the average of two independent experiments.

contrast, complete refolding of preMBP occurs within a few hours in the presence of excess SecB. This difference is primarily due to the ability of MBP to refold even when bound to SecB, rather than the faster refolding rate of free MBP relative to that for preMBP, as proposed previously.⁸ Importantly, at stoichiometric excess of SecB, virtually all refolding chains of MBP/preMBP are transiently bound to SecB. We cannot rule out preMBP refolding when bound to SecB, albeit with a rate much slower than that for MBP bound to SecB. However, such a low folding rate constant could not be independently measured in the current study and is not required to explain the observed kinetics. An earlier study⁷ indicated that the model substrate barnase may also refold while bound to SecB.

preMBP, MBP at low temperature, and all the slow-folding mutants of MBP for which SecB has been shown to block folding have thermodynamic stabilities lower than that of MBP.^{14,15,34} We have previously suggested that signal peptides may have a role in stabilizing the unfolded state of pre-proteins.¹⁵ The differences in the unfolded state's structure and stability presumably result in a difference in the kind of interaction that preMBP and MBP have when bound to SecB. The higher thermodynamic stability of the mature protein over its precursor form favors the folding of the mature protein to its stable native form in comparison to its association with SecB. This kind of preference to fold over binding will be lowered for the less stable precursor protein.

SecB seems to bind MBP transiently, while it forms a more stable complex with preMBP. Consistent with this is the estimated half-life of ~2 min for the SecB-MBP ($\Delta 2-26$) complex *in vivo*.¹⁷ The present analysis demonstrates that the ability of SecB to block substrate refolding does not depend only on the relative k_{on} and k_f values. Instead, the ability of substrates to fold while tethered to SecB (nonzero k_f' , as well as the value of k_{off}) also plays an important role. High k_f' and k_{off} values promote transient binding to SecB. Based on our data, we also speculate that SecB can bind transiently to various proteins *in vivo*, including several proteins not destined for export. This idea is also substantiated by the suggested role of SecB as a generalized chaperone in *E. coli*.^{35,36} In addition to the kinetic factors described above, the association half-life of the SecB-substrate complex is likely to be further dictated by the affinity and kinetics of interaction with SecA, the next important protein in the secretory pathway.

Materials and Methods

Proteins and buffers

The MBP-deficient strain POP6590 harboring the plasmid pMAL-P2-MBP was used as a source of MBP and preMBP.³⁷ The SecB expression plasmid pJW25 in strain BL21(DE3) was the source of SecB. Mutations A14EpreMBP and Q14C SecB were generated by mutagenesis using the Stratagene

Quik Change™ site-directed mutagenesis protocol. preMBP was purified by amylose affinity chromatography using 10 mM maltose for elution.³⁸ For preMBP, an extinction coefficient of $66,350 \text{ M}^{-1} \text{ cm}^{-1}$ was used to determine protein concentrations.¹⁵ MBP was purified using an osmotic shock procedure.³⁹ SecB and its mutants were purified as described previously.²² All SecB concentrations (both unlabeled and acrylodan-labeled) mentioned are the tetrameric concentrations of the protein. The molar extinction of unlabeled tetrameric SecB at 280 nm was $47,600 \text{ M}^{-1} \text{ cm}^{-1}$.

All refolding experiments were carried out in CGH10 buffer (10 mM citrate, 10 mM glycine, and 10 mM Hepes; pH 7.2) containing 150 mM sodium chloride. The substrate protein preMBP or MBP was freshly unfolded in the above buffer containing 2 M guanidine hydrochloride (GdnHCl; ultra-pure GdnHCl; USB) for at least 2 h at room temperature prior to measurement. The concentration of GdnHCl was determined by refractive index measurements.

Acrylodan labeling of SecB

Acrylodan (4 mM in dimethylformamide; Molecular Probes, Inc.) was used at a final concentration of 5-fold molar excess over the protein (Q14C SecB) concentration. After 30-min incubation in the dark at room temperature, excess reagent was removed by desalting the protein on a PD10 (Amersham Biosciences) column in CGH10 buffer containing 150 mM sodium chloride (pH 7.2).

Mass characterization by electrospray ionization mass spectrometry confirmed that only a single Cys was labeled per monomer. The molar extinction coefficients of a single acrylodan-labeled mutant SecB monomer at 280 and 360 nm were estimated to be 20,303 and $11,878 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

Manual mixing experiments

Manual mixing experiments were performed on a SPEX Fluoromax3 spectrofluorimeter. Folding was initiated by diluting unfolded protein in 2 M GdnHCl to the required final protein concentrations in the refolding buffer. Refolding in the presence of SecB was observed by varying the SecB concentration in the refolding buffer in a range from 0 to 10-fold molar excess over the final refolded protein concentrations. Changes in intrinsic tryptophan fluorescence during refolding were monitored at 340 nm, with excitation at 280 nm.

ANS binding experiments

Excitation and emission wavelengths of 280 and 475 nm, respectively, were used. ANS concentration was always in 100-fold molar excess over the final refolding protein concentration. The fluorescence of ANS bound to SecB was appropriately subtracted from the refolding traces. At higher concentrations of SecB when preMBP does not fold (as observed in the tryptophan fluorescence measurement), the fluorescence contribution resulting from ANS bound to SecB was calculated by assuming that only free SecB binds ANS and that all preMBP are complexed.

Rapid kinetic experiments

Rapid mixing fluorescence experiments were carried out on a Biologic SFM-3 or SFM-4 stopped-flow module using a

FC-15 cuvette with a path length of 1.5 mm and a dead time of 5 ms at 25 °C. For tryptophan fluorescence measurements, the excitation wavelength was set at 280 or 295 nm with a bandwidth of 1.5 nm, and emission intensity was measured using a 320-nm bandpass filter with a bandwidth of 5 nm. For acrylodan fluorescence measurements, the excitation wavelength used was 280 nm with a slit width of 5 or 10 nm, and emission was monitored using a 500-nm bandpass filter or a 520-nm cutoff filter. Concentrations of acrylodan-labeled SecB greater than 0.3 μM could not be used because the fluorescence intensity then exceeded the linear range of the instrument.

Data analysis

Kinetic studies

Changes in signal intensity as a function of time in all the refolding experiments were fitted to the equation $[a_\infty + \sum a_i \exp(-k_i t)]$ or $a_0 + \sum a_i (1 - \exp(-k_i t))$, where a_∞ represents the amplitude achieved at equilibrium, a_0 represents the amplitude change occurring in the burst phase, and a_i represents the amplitude change of phase i occurring with an observed rate constant k_i .

Determination of k_{on} using tryptophan fluorescence data

In the case of MBP, k_{on} was determined by analyzing refolding traces only at SecB concentrations higher than 0.3 μM. Only under these conditions will the binding rate ($k_{on}[I][\text{SecB}_{(free)}]$) be appreciably greater than the refolding rate ($k_f[I]$). Using the saturating fluorescence intensity at $t=0.5$ s ($F(0.5)$) and the fluorescence intensity at time $t=0$ s ($F(0)$) corresponding to the fluorescence of 0.3 and 0.0 μM, respectively, the concentration of complex [IS] formed as a function of time was obtained using the following equation:

$$[\text{IS}] = \frac{F(t) - F(0)}{F(0.5) - F(0)} [I_0] \quad (3)$$

where $[I_0]$ is the total MBP concentration.

In the case of preMBP, the saturating fluorescence in each of the kinetic traces at substoichiometric concentrations of SecB corresponds to the fluorescence of the complex at a concentration equal to the concentration of SecB used. The concentration of the complex was calculated using Eq. (3), with $F(0.5)$ and $[I_0]$ corresponding to the saturating fluorescence for each trace and total SecB concentration, respectively.

Determination of k_{on} using acrylodan-labeled Q14C SecB

Refolding of the unfolded protein in the presence of acrylodan-labeled Q14C SecB results in an initial increase in acrylodan fluorescence, which saturates within 3 s, followed by a decrease in fluorescence at later times. The initial increase in acrylodan fluorescence reflects the binding process and was used to determine k_{on} .

The fluorescence change occurring in the first 3 s in the refolding experiments using acrylodan-labeled SecB was converted into the concentration of the complex $[\text{IS}^A]$ formed using Eq. (4):

$$[\text{IS}^A] = \frac{F(t) - F(0)}{F(3) - F(0)} [S_0] \quad (4)$$

where $[S_0]$ is the acrylodan-labeled Q14C SecB concentration.

The concentration of the complex ($[\text{IS}]$ or $[\text{IS}^A]$) formed as a function of time was fitted to a second-order rate equation (Eq. (5)) to obtain the bimolecular rate constant k_{on} :

$$k_{on} = \frac{1}{([I_0] - [S_0])t} \ln \left(\frac{[S_0][I_0] - [\text{IS}]}{[I_0][S_0] - [\text{IS}]} \right) \quad (5)$$

where $[I_0]$ and $[S_0]$ are the MBP/preMBP and SecB concentrations, respectively, and $[\text{IS}]$ is the concentration of the complex formed.

Determination of k_{off}

The off-rate constant k_{off} was measured by monitoring the decrease in FRET between acrylodan-labeled SecB and bound substrate as a function of time upon addition of at least a 10-fold excess of WT SecB. Varying concentrations of acrylodan-labeled SecB (0.05, 0.1, and 0.2 μM) and MBP/preMBP (0.2 μM) were rapidly mixed using stopped-flow mixing and incubated for 3 s to allow for complex formation. After this interval, an excess of WT SecB (2 μM) was added, and the decrease in energy transfer was measured for 150 s at 100-ms intervals. For MBP, the decrease in fluorescence intensity during the initial 7 s was also followed at 5-ms intervals. The data were fitted to a single-exponential equation. Similar experiments were also carried out using manual mixing. In these experiments, the approximate dead time for mixing was 10–15 s.

Numerical analysis

All computer simulations were performed on the International Mathematical and Statistical Library™ package. Ordinary differential equations based on the kinetic scheme were numerically solved using the IVPAG routine after input of the appropriate initial conditions (Supplementary Information).

In the case of MBP refolding, the slow phase of the refolding reaction involves the conversion of intermediate I into a near-native-like species N^* with a rate constant of 0.03 s^{-1} . N^* has secondary and tertiary structures that are nearly identical to those of the native state, and converts very slowly ($k=0.006 \text{ s}^{-1}$) to native protein N.¹⁵ Since N^* does not bind SecB and since the transition of N^* to N is invisible by tryptophan fluorescence, the scheme described for MBP refolding is analyzed without considering the N^* -to-N transition. In the case of preMBP, there is no evidence for a separate N^* -to-N transition, so N^* is identical with N.

A set of coupled differential equations for Schemes 1 and 2 can be written, respectively, as:

$$\begin{aligned} \frac{d[N^*]}{dt} &= k_f[I] \\ \frac{d[I]}{dt} &= -k_f[I] - k_{on}[I][S] + k_{off}[IS] \\ \frac{d[S]}{dt} &= -k_{on}[I][S] + k_{off}[IS] \\ \frac{d[IS]}{dt} &= k_{on}[I][S] - k_{off}[IS] \\ \frac{d[N^*]}{dt} &= k_f[I] + k'_f[IS] \\ \frac{d[I]}{dt} &= -k_f[I] - k_{on}[I][S] + k_{off}[IS] \\ \frac{d[S]}{dt} &= -k_{on}[I][S] + k_{off}[IS] + k'_f[IS] \\ \frac{d[IS]}{dt} &= k_{on}[I][S] - k_{off}[IS] - k'_f[IS] \end{aligned}$$

The total concentrations of MBP/preMBP ($[I_0]$) and SecB ($[S_0]$) are given by:

$$[I_0] = [I] + [IS] + [N^*] \text{ and } [S_0] = [S] + [IS]$$

Experimentally measured k_f , k_f' , k_{on} , and k_{off} values were used for the simulations. The concentrations of the various species involved in the reaction at time $t=0$ s were $[N^*]=0$, $[I]=[I_0]$, and $[IS]=0$.

Comparison of simulated data with experimental data

The concentration data obtained from the simulations were converted into fluorescence data using Eq. (6):

$$F(t) = \frac{(F_{N(0.2)}[N(t)] + (F_{IS(0.2)}[IS(t)] + (F_{I(0.2)}[I(t)] + (F_{S(0.2)}[S(t)]))}{0.2} \quad (6)$$

where $F_{N(0.2)}$, $F_{IS(0.2)}$, $F_{I(0.2)}$, and $F_{S(0.2)}$ represent a fluorescence intensity of 0.2 μ M for [N], [IS], [I], and [S], respectively. $[N(t)]$, $[IS(t)]$, $[I(t)]$, and $[S(t)]$ are obtained from the simulations.

Since SecB does not form a stable complex with MBP, it was assumed that the MBP–SecB and preMBP–SecB complexes have similar fluorescence intensities. The fluorescence contributions of all other species N^* , I, and S, except that of IS, were determined directly from the experiment. For MBP–SecB, $F_{IS(0.2)}$ was varied to within 15% of the value obtained from the preMBP data, if required, to obtain a better fit between the experimental data and the simulated data.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2008.10.094](https://doi.org/10.1016/j.jmb.2008.10.094)

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