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Published in:
Biochemistry

DOI:
[10.1021/bi00031a032](https://doi.org/10.1021/bi00031a032)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

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Citation for published version (APA):

Fekkes, P., Blaauwen, T. D., & Driessen, A. J. M. (1995). DIFFUSION-LIMITED INTERACTION BETWEEN UNFOLDED POLYPEPTIDES AND THE ESCHERICHIA-COLI CHAPERONE SECB. *Biochemistry*, 34(31), 10078-10085. <https://doi.org/10.1021/bi00031a032>

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Diffusion-Limited Interaction between Unfolded Polypeptides and the *Escherichia coli* Chaperone SecB[†]

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Received March 1, 1995; Revised Manuscript Received May 4, 1995[⊗]

ABSTRACT: SecB is a chaperone dedicated to protein translocation in *Escherichia coli*. SecB binds to a subset of precursor proteins, and targets them in a translocation-competent state to the SecA subunit of the *translocase*. The nature and kinetics of the interaction of SecB with polypeptides were studied by spectroscopic techniques using the reduced form of bovine pancreatic trypsin inhibitor (BPTI) as a model substrate. Binding of SecB to BPTI resulted in an increase in the fluorescence of the surface-exposed tryptophan residue 36 of SecB. SecB reversibly binds BPTI in stoichiometric amounts. Labeling of BPTI with the fluorophore acrylodan allowed the analysis of the binding reaction at nanomolar concentrations. High-affinity binding (K_D of 5.4 nM) of labeled BPTI to SecB resulted in a blue shift of the acrylodan emission maximum and an increase in the fluorescence quantum yield, suggesting that BPTI binds in an apolar environment. Stopped-flow acquisition of rate constants of complex formation between SecB and BPTI yielded a second-order binding rate constant of $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, and a dissociation rate constant of 48 s^{-1} . These data demonstrate that *in vitro*, the association of SecB with polypeptide substrates is limited by the rate of collision. *In vivo*, SecB binding is selective, and predominantly occurs with nascent polypeptides. Since these chains are not expected to fold into stable structures, SecB association may be governed by “more or less” specific interactions and be limited by the rate of chain elongation rather than the rate of folding.

Molecular chaperones are proteins that bind other proteins to assist them in correct folding (Bochkareva *et al.*, 1988), and complex formation (Ellis & Hemmingsen, 1989), to prevent aggregation (Hartl *et al.*, 1994), and to target them to the appropriate compartment (Kumamoto, 1991). In *Escherichia coli*, some of the precursor proteins designated to become secreted across the inner membrane are stabilized in a translocation-competent state by the chaperone SecB. SecB is a homotetrameric protein (Watanabe & Blobel, 1989) that forms a stoichiometric complex with precursor proteins (Lecker *et al.*, 1989). Unlike many other chaperones, it has never been shown that ATP influences the interaction between SecB and its substrates. *In vitro*, SecB is capable of binding to a variety of unfolded proteins, but not to native proteins (Hardy & Randall, 1991). *In vivo*, however, SecB is found in complex with only a subset of proteins, most of them precursors of outer membrane proteins (Kumamoto, 1989; Kumamoto & Francetić, 1993). SecB seems to act early in the translocation pathway as it is able to bind the precursor protein as a nascent chain while it emerges from the ribosome (Kumamoto & Francetić, 1993). This concept is reinforced by the observation that SecB is also needed for cotranslational translocation (Kumamoto & Gannon, 1988). SecB is the major chaperone in protein translocation since it is the only one that binds to SecA, the peripheral

ATPase subunit of the precursor protein *translocase* (Hartl *et al.*, 1990; Hoffschulte *et al.*, 1994). This interaction is enhanced in the presence of precursor proteins. At the membrane surface, SecB transfers the precursor protein to SecA, and translocation is initiated at the expense of ATP [for reviews, see Wickner *et al.* (1991) and Driessen (1994)].

Although SecB has an apparent preference for precursor proteins, the signal sequence itself is generally not involved in this interaction (Collier *et al.*, 1988; Gannon *et al.*, 1989; Randall *et al.*, 1991). In some cases, however, it can bind to SecB; e.g., the signal sequence of the outer membrane protein LamB is part of the binding site for SecB (Altman *et al.*, 1990). The signal sequence retards the folding of the mature domain (Park *et al.*, 1988), and it is believed that this retardation of folding allows SecB to discriminate between precursor proteins and other proteins in the cell, as formulated in the “kinetic partitioning model” (Hardy & Randall, 1991). In this model, cytosolic proteins would escape the stable interaction with SecB by folding more rapidly than precursor proteins, and the final distribution of the precursor protein among different pathways in the cell is determined by partitioning that is dependent on the rate of folding or aggregation relative to the rate of binding to the chaperone (Randall & Hardy, 1995).

In order to interact efficiently with its polypeptide substrates, SecB should be able to associate with precursor proteins at a rate that is faster than the folding rate. On the other hand, rates of association should be sufficiently slow to prevent stable interactions with cytosolic proteins. Although there have been speculations on the rate by which SecB associates with polypeptide substrates (Randall & Hardy, 1995), experimental kinetic data are lacking. There-

[†] This work was supported by a PIONIER grant of the Netherlands Organization for Scientific Research (N.W.O.).

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[⊗] Abstract published in *Advance ACS Abstracts*, July 15, 1995.

fore, we used bovine pancreatic trypsin inhibitor (BPTI)¹ labeled with an environmentally sensitive fluorophore, in conjunction with stopped-flow spectrofluorometry, to analyze the kinetics of polypeptide binding to SecB. Polypeptide binding to SecB appears to be much faster than polypeptide chain elongation and folding. The implications of the results on the function and specificity of SecB are discussed.

EXPERIMENTAL PROCEDURES

Materials. Acrylodan [6-acryloyl-2-(dimethylamino)-naphthalene] and DPX [*p*-xylylenebis(pyridinium bromide)] were purchased from Molecular Probes, Inc. (Eugene, OR). Bovine pancreatic trypsin inhibitor (BPTI, Pronase) and proteinase K were from Boehringer (Mannheim, Germany). Other chemicals were obtained from commercial sources. Inverted *E. coli* inner membrane vesicles were prepared from *Escherichia coli* D10 (Crooke *et al.*, 1988). ³⁵S-Labeled proOmpA was synthesized and immunopurified as described (Crooke & Wickner, 1987). SecA (Cabelli *et al.*, 1988) and unlabeled proOmpA (Crooke *et al.*, 1988) were purified as described.

Strains and Plasmids. All strains used were *E. coli* K12 derivatives. Cloning experiments were performed in DH5 α (Hanahan, 1983). Single-stranded DNA for site-directed mutagenesis was isolated from CJ236 (Kunkel *et al.*, 1987), and for sequencing from JM101 (Yanisch-Perron *et al.*, 1985). Overexpression of SecB proteins was done in BL21/DE3 (Studier & Moffat, 1986). The plasmid pAK330 is described by Kumamoto and Nault (1989), and pBluescript was purchased from Stratagene (La Jolla, CA).

Acrylodan Labeling of BPTI. A sample of 50 μ g/mL BPTI was denatured and reduced in 5.4 M GdnHCl, 100 μ M DTT, and 50 mM Tris-HCl, pH 7.6, at room temperature for 30 min. Acrylodan (20 mM in dimethylformamide) was added to a final concentration of 200 μ M, and the labeling reaction was allowed to proceed overnight at 4 °C. The reaction was terminated by the addition of 10 mM DTT, and after 15 min, 25 mM iodoacetamide was added to eliminate free thiol groups. The acrylodan-labeled reduced BPTI (AR-BPTI) was separated from excess reagents by gel filtration on a Sephadex G-10 M column (Pharmacia, Upsalla, Sweden). Fractions were analyzed by fluorescence spectroscopy, using an excitation wavelength of 391 nm. Free acrylodan possesses an emission maximum of 540 nm (Prendergast *et al.*, 1983) that was shifted to 505 nm for the bound acrylodan. The amount of acrylodan per BPTI was estimated from the ratio of the protein absorbance at 280 nm ($\epsilon = 7750 \text{ M}^{-1} \text{ cm}^{-1}$) to the acrylodan absorbance at 360 nm ($\epsilon = 12\,900 \text{ M}^{-1} \text{ cm}^{-1}$) (Prendergast *et al.*, 1983).

Reduced BPTI (R-BPTI) was made by denaturing and reducing 390 μ g/mL BPTI in 5.4 M GdnHCl, 10 mM DTT, and 50 mM Tris-HCl, pH 7.6. After 30 min, 25 mM iodoacetamide was added and the R-BPTI was separated from an excess of reagents by gel filtration on a Sephadex G-10 M column.

Mutagenesis and Protein Purification. The plasmid pAK330 (Kumamoto & Nault, 1989) was digested with *Bam*HI and *Msc*I, and the 580 bp fragment on which *secB* is located was ligated into pBluescriptII KS⁺ digested with *Bam*HI and *Eco*RV to yield plasmid pET204. Trp-36 of the *secB* gene in pET204 was replaced by phenylalanine or tyrosine via site-directed mutagenesis (Kunkel *et al.*, 1987). The W36F (5'-GAAAGATTTCACCAACCAGAAGTTAAGCT-TGA, changed nucleotides are indicated in boldface) and W36Y (5'-GAAAGATTACCAACCAGAAGTTAAGCT-TGA-3') primers were used to produce the plasmids pET205 and pET206, respectively. All mutations were confirmed by single-stranded DNA sequencing (Sanger *et al.*, 1977). The plasmids pET204, pET205, and pET206 were digested with *Fsp*I and *Kpn*I, and the *secB*-containing fragment was ligated into pBluescriptII KS⁻ digested with *Sma*I and *Kpn*I to obtain pET210, pET211, and pET212, respectively. In this way, the *secB* genes were cloned behind the T7 promoter. Overexpression was obtained in *E. coli* BL21/DE3 after induction of the T7 RNA polymerase by adding 1 mM IPTG. Purification of wild-type, W36Y, and W36F SecB was performed as described by Weiss *et al.* (1988). At the DEAE-Sephadex chromatographic step, W36Y SecB was found to elute at a slightly higher ionic strength as compared to the wild-type or W36F SecB. SDS-PAGE (Laemmli, 1970) and subsequent Coomassie Brilliant Blue staining were done to verify the purity of the isolated proteins. To determine the concentration of the SecB proteins, amino acid analysis was done by Eurosequence (Groningen, The Netherlands).

Translocation Assay. *In vitro* translocation of ³⁵S-labeled proOmpA (in 50 μ L) was performed as described (Hartl *et al.*, 1990) with 0.5 μ g of SecA, 2 mM ATP, 10 mM phosphocreatine, and 2.5 μ g of creatine kinase in buffer B [50 mM HEPES-KOH (pH 7.6), 30 mM KCl, 2 mM DTT, and 0.5 mg/mL bovine serum albumin]. When indicated, 0.5 μ g of SecB was added. ³⁵S-Labeled proOmpA was diluted 40-fold from a solution of 8 M urea and 50 mM Tris-HCl, pH 7.6. When indicated, the mixture was preincubated for 30 min at 37 °C, followed by the addition of urea-treated *E. coli* D10 inverted inner membrane vesicles at a final protein concentration of 44 μ g/mL. Reactions were incubated for 30 min at 37 °C, and stopped by cooling on ice. Samples were treated with proteinase K (1 mg/mL) for 30 min on ice, and terminated by the addition of PMSF (2 mM, final concentration). Samples were precipitated with 6.7% (w/v) trichloroacetic acid and analyzed by SDS-PAGE and autoradiography.

Steady-State Fluorescence Measurements. All steady-state fluorescence measurements were performed at 25 °C using the SLM Aminco 4800C spectrofluorometer. Tyrosine and tryptophan emission spectra were recorded (scan rate of 1 nm/s) between 300 and 420 nm, using excitation wavelengths of 280 and 297 nm and a slitwidth of 2 nm. Acrylodan fluorescence was recorded between 400 and 600 nm, using an excitation wavelength of 391 nm and a slitwidth of 4 nm. Slow-time-based Trp fluorescence measurements were conducted at excitation and emission wavelengths of 300 nm (slitwidth of 2 nm) and 344 nm (slitwidth of 4 nm), respectively. For AR-BPTI, excitation and emission were at 391 nm (± 4 nm) and 480 nm (± 8 nm), respectively. AR-BPTI fluorescence was corrected for the time-dependent bleaching of the acrylodan group.

¹ Abbreviations: acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; DPX, *p*-xylylenebis(pyridinium bromide); BPTI, bovine pancreatic trypsin inhibitor; R-BPTI, reduced BPTI; AR-BPTI, acrylodan-labeled reduced BPTI; GdnHCl, guanidiniumhydrochloride; DTT, dithiothreitol; IPTG, isopropyl 1-thio- β -D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride; k_{obs} , pseudo-first-order rate constant; k_{ass} , second-order binding rate constant; k_{diss} , first-order dissociation rate constant.

Quenching studies of the Trp fluorescence with the water-soluble quencher DPX were performed with excitation and emission wavelengths of 297 and 344 nm, respectively. Quenching constants were obtained by fitting the data to the Stern–Volmer equation:

$$\frac{F}{F_0} = 1 + K_Q[Q] \quad (1)$$

where F_0 is the initial fluorescence, F the fluorescence in the presence of quencher, K_Q the Stern–Volmer constant, and $[Q]$ the concentration of the quencher.

Stopped-Flow Fluorescence Measurements. Stopped-flow fluorescence measurements were performed on the SLM Aminco 4800C spectrofluorometer with the Milliflow stopped-flow reactor at a temperature of 25 °C. The estimated dead-time of the stopped-flow reactor is 10 ms. Excitation was at 380 nm using a slitwidth of 4 nm. Emission was measured with a high-pass filter with a cutoff of 400 nm. Data were fitted to the first-order reaction $A \rightarrow C$ or to the successive first-order reaction $A \rightarrow B \rightarrow C$, where A is the start situation, B an intermediate, and C the end product. The concentrations of A, B, and C will change in time according to

$$[A]_t = A_0 e^{-k_1 t} \quad (2)$$

$$[B]_t = \frac{k_1 A_0 (e^{-k_1 t} - e^{-k_2 t})}{k_2 - k_1} \quad (3)$$

$$[C]_t = A_0 \left[1 - \left(1 + \frac{k_1}{k_2 - k_1} \right) e^{-k_1 t} + \frac{k_1 e^{-k_2 t}}{k_2 - k_1} \right] \quad (4)$$

in which A_0 is the concentration of A at $t = 0$, and $[A]_t$, $[B]_t$, and $[C]_t$ are the concentrations of A, B, and C at time t , respectively. k_1 is the reaction rate of the conversion of A to B, and k_2 that of B to C. The total fluorescence will change in time according to

$$F_t = f_a[A]_t + f_b[B]_t + f_c[C]_t \quad (5)$$

where F_t is the fluorescence at time t , and f_a , f_b , and f_c are the fluorescence factors for A, B, and C, respectively.

RESULTS

Tryptophan Residue 36 of SecB Reports the Binding of Precursor Proteins. The intrinsic tryptophan fluorescence of proteins has been used to obtain qualitative information on the folding state of a protein when it forms a complex with a molecular chaperone. Measurements of the interaction between SecB and precursor proteins that contain one or more Trp residues may be complicated by the presence of a single Trp residue at position 36 in SecB. Therefore, we replaced it for a tyrosine (W36Y SecB) or phenylalanine (W36F SecB). Mutant SecB proteins were overexpressed and purified from an *E. coli* strain expressing normal levels of wild-type SecB. Both mutants showed a greatly reduced Trp fluorescence (excitation at 280 nm, *i.e.*, excitation of both Tyr and Trp; inset, Figure 1), although some contaminating Trp background fluorescence appeared to be present in W36F SecB (excitation at 297 nm, *i.e.*, excitation of Trp alone; Figure 1). This contamination likely arises from remaining wild-type SecB, as the W36F SecB was not well

FIGURE 1: Fluorescence spectra of the purified wild-type and mutant SecB proteins. Intrinsic tryptophan fluorescence spectra of wild-type (—), W36F (---), and W36Y (···) SecB using an excitation wavelength of 297 nm. Solutions were prepared at a final concentration of 80 μ g/mL protein. Inset: Fluorescence spectra of wild-type (—), W36F (---), and W36Y (···) SecB recorded with an excitation wavelength of 280 nm.

FIGURE 2: SecB-dependency of the translocation of proOmpA into urea-treated inner membrane vesicles. Translocation reactions were assayed as described under Experimental Procedures with the following modifications or additions: lane 1, no SecA, SecB, or ATP added; lanes 2 and 5, no SecB added; lanes 3 and 6, wild-type SecB; and lanes 4 and 7, W36Y SecB. In lanes 1–4, the 35 S-labeled proOmpA was immediately translocated after dilution from urea. In lanes 5–7, the proOmpA was first preincubated for 30 min at 37 °C prior to the initiation of the translocation reaction.

resolved from the wild-type SecB in the chromatographic steps (data not shown). Further experiments were, therefore, conducted with W36Y SecB.

In order to determine if W36Y SecB has the same binding and targeting properties as SecB, the *in vitro* translocation of urea-denatured proOmpA, the precursor of the outer membrane protein OmpA, in the presence of SecB was examined. Translocation was more efficient in the presence of wild-type (Figure 2, lane 3) and W36Y (lane 4) SecB protein than in their absence (lane 2). Urea-denatured proOmpA becomes translocation-incompetent when it is first diluted into buffer and incubated for 30 min in the absence of SecB (lane 5) (Lecker *et al.*, 1989). The addition of wild-type or W36Y SecB after this 30 min preincubation time had little effect on the translocation efficiency (data not shown). In contrast, when wild-type (lane 6) and W36Y (lane 7) SecB were present during the preincubation period, a significant portion of the proOmpA remained translocation-competent. Similar results were obtained with W36F SecB

FIGURE 3: Tryptophan fluorescence of SecB/proOmpA complexes. Fluorescence spectra of proOmpA in complex with wild-type SecB (—), proOmpA in complex with W36Y SecB (---), and proOmpA in the absence of SecB (····). Spectra were recorded with an excitation wavelength of 297 nm, and corrected for background SecB fluorescence. The final proOmpA and SecB concentrations were 50 and 80 $\mu\text{g/mL}$, respectively.

(data not shown). These data demonstrate that the W36Y and W36F SecB proteins have retained their activity.

W36Y SecB allows the analysis of the binding to proOmpA in a spectroscopically clear background. ProOmpA is a 346 amino acid polypeptide with 5 tryptophanyl residues in the amino-terminal half (residues 30, 36, 78, 123, and 164) (Chen *et al.*, 1980). The fluorescence properties of these tryptophanyl residues respond to the folding state of proOmpA (Lecker *et al.*, 1990). Little difference was notable between the fluorescence emission spectra of proOmpA free in solution or bound to W36Y SecB (Figure 3). In contrast, when proOmpA is in complex with the wild-type SecB, a higher Trp fluorescence is observed when the signal is corrected for the Trp fluorescence of native SecB (Figure 3). This must imply that the fluorescence intensity of Trp-36 is increased when SecB binds to proOmpA.

SecB Forms a Stoichiometric Complex with the Model Protein R-BPTI. The quantum yield of the Trp fluorescence enhancement of SecB upon precursor protein binding is too small to detect binding events close to the expected K_D , which is in the subnanomolar range. Moreover, the contribution of the five Trp residues of proOmpA to the fluorescence signal interferes with a proper interpretation of the binding studies, while proOmpA aggregates when it is present in excess over SecB (Lecker *et al.*, 1990; data not shown). Therefore, we used a model protein to study the kinetics of the interaction between SecB and polypeptide substrates. Bovine pancreatic trypsin inhibitor (BPTI) has proven to be a good substrate for SecB when it is present in its reduced form (Hardy & Randall, 1991). BPTI is a protein with a molecular mass of 6 kDa, and it is devoid of tryptophan residues. It contains three disulfide bonds that stabilize the tertiary structure. By blocking the reduced dithiols with iodoacetamide, the protein adopts a compact

FIGURE 4: Stoichiometry of the binding of reduced BPTI to SecB. The change in intrinsic Trp fluorescence of SecB (3.2 μM) was recorded in the presence of increasing amounts of R-BPTI. Inset: Quenching of the tryptophan fluorescence of SecB by DPX. The tryptophan fluorescence of SecB at 344 nm was determined in the presence of increasing concentrations of DPX for wild-type SecB (■), SecB in complex with R-BPTI (○), and SecB denatured in 5.4 M GdnHCl (▲).

conformation without any pronounced nativelike secondary structures (Gussakovskiy & Haas, 1992). This state is further referred to as R-BPTI. The change in the intrinsic Trp fluorescence of SecB was used to determine the stoichiometry of R-BPTI binding. The Trp fluorescence of SecB increased with the amount of R-BPTI added, reaching a maximum value of 1.5 times that of unliganded SecB (Figure 4). The Trp fluorescence level saturated at a concentration of R-BPTI that was approximately equal to that of the SecB monomer. These data suggest that R-BPTI binds in a stoichiometric fashion to the SecB monomers, which is in agreement with proteolysis studies (Randall, 1992). The increase in Trp fluorescence was accompanied by a small blue shift of the fluorescence emission maximum, *i.e.*, from 340 to 336 nm. This shift is indicative for a change in the polarity of the environment of Trp-36. The accessibility of Trp-36 for the hydrophilic quencher DPX was determined (inset, Figure 4), and it, as defined by the Stern–Volmer quenching constant, K_Q , slightly increased when SecB was denatured in 5.6 M GdnHCl solution, *i.e.*, K_Q values of 29 and 40 M^{-1} , respectively. The value for K_Q was not changed upon binding of R-BPTI to SecB. These results demonstrate that Trp-36 is exposed to an aqueous environment, and that the accessibility for DPX remains unchanged upon binding by R-BPTI.

SecB/R-BPTI Complexes Are Readily Exchangeable. The exchange of R-BPTI between wild-type and W36Y SecB was followed by changes in the Trp fluorescence. First, an equimolar complex of wild-type SecB was formed with R-BPTI. Complex formation was evident from the increase in Trp fluorescence (Table 1). When the concentration of total SecB was enhanced by a factor of 2 by adding an equimolar amount of W36Y SecB, the change in Trp fluorescence dropped within the mixing time (1–2 s; see

Table 1: Relative Changes in the Tryptophan Fluorescence of SecB upon Binding and Release of R-BPTI

sample	addition		ΔF_{344} (A.U.) ^a
	1	2	
wild-type SecB	R-BPTI		0.49 ± 0.06
	R-BPTI	W36Y SecB	0.21 ± 0.06
W36Y SecB	R-BPTI		0.04 ± 0.03
	R-BPTI	wild-type SecB	0.26 ± 0.05

^a The change in tryptophan fluorescence of wild-type SecB (excitation at 297 nm, emission at 344 nm) was followed after subsequent additions of R-BPTI and W36Y SecB to wild-type SecB or, in the reversed case, to W36Y SecB. Values were corrected for the background fluorescence of unliganded wild-type SecB. A.U. = arbitrary units.

below) by approximately 50%. This implies that there is a rapid redistribution of the R-BPTI between wild-type and W36Y SecB. The reversed experiment, *i.e.*, addition of SecB to a preformed complex of W36Y SecB and R-BPTI, is also shown in Table 1. In this case, the Trp fluorescence increased to the level observed for the reversed order of additions (Table 1) when corrected for the increase in Trp fluorescence due to the addition of SecB. These data demonstrate that the R-BPTI/SecB complex is readily dissociable and that the R-BPTI can be rapidly exchanged between SecB molecules.

AR-BPTI Associates with a Hydrophobic Polypeptide Binding Pocket of SecB. From the increase in intrinsic Trp fluorescence of SecB upon peptide binding, an estimate has been made of the affinity of SecB for R-BPTI (Hardy & Randall, 1991). The quantum yield of the Trp fluorescence of SecB is, however, too low to perform binding experiments at concentrations near to the assumed K_D of SecB for R-BPTI. Therefore, we used the environmentally sensitive fluorophore acrylodan to label R-BPTI. When excited at 390 nm, acrylodan emits fluorescence between 450 and 520 nm with a high quantum yield (Prendergast *et al.*, 1983). Acrylodan-labeled reduced BPTI (AR-BPTI) binds to SecB with a concomitant increase in acrylodan fluorescence, and a shift in the emission maximum from 505 to 487 nm (Figure 5). The blue shift and increase in fluorescence quantum yield are indicative for a transfer of the acrylodan to a more apolar environment upon binding to SecB (Prendergast *et al.*, 1983). The change in fluorescence could be measured at very low concentrations of both AR-BPTI and SecB. A fixed concentration of AR-BPTI of 0.7 nM was used wherein SecB was titrated. The acrylodan fluorescence increased with the amount of SecB added, reaching a maximum value of 3.35 times that of unliganded AR-BPTI (inset, Figure 6). Scatchard analysis (Scatchard, 1949) revealed that the affinity of SecB for AR-BPTI is very high, *i.e.*, a K_D of 5.4 nM (± 0.6 nM, $n = 2$) (Figure 6), and close to the value reported for R-BPTI (Hardy & Randall, 1991). These data also demonstrate that the binding of AR-BPTI to SecB is noncooperative. Competition experiments between AR-BPTI and R-BPTI indicated that both substrates bind with similar affinity to SecB (data not shown). W36Y SecB binds AR-BPTI with a slightly increased affinity, *i.e.*, a K_D of 3.1 nM (± 0.5 nM, $n = 2$). To ascertain that AR-BPTI and proOmpA bind to the same binding site on SecB, competition experiments were performed by titrating variable amounts of proOmpA into a solution containing preformed SecB/AR-

FIGURE 5: Acrylodan fluorescence emission spectra of AR-BPTI in the absence (a) and presence of an excess of SecB (b). AR-BPTI was present at a concentration of 35 nM. Excitation was at 391 nm.

FIGURE 6: Scatchard plot analysis of the binding of AR-BPTI to wild-type (○) and W36Y (●) SecB. AR-BPTI was present at a concentration of 0.7 nM, and the SecB concentration was varied between 0 and 100 nM. Excitation and emission were at 391 and 480 nm, respectively. Experiments were performed *in duplo*. Inset: Semilogarithmic representation of the binding of AR-BPTI to wild-type (○) and W36Y (●) SecB.

BPTI complex. AR-BPTI bound to SecB was nearly completely displaced by a stoichiometric amount of proOmpA, *i.e.*, 1 mol of proOmpA per 4 mol of SecB monomers (data not shown), demonstrating that AR-BPTI and proOmpA compete for the same binding site on SecB. Moreover, it implies that proOmpA binds with much higher affinity to SecB than AR-BPTI. Aggregation of proOmpA was observed when the protein was present in excess to SecB.

FIGURE 7: Stopped-flow analysis of the kinetics of association (A) and dissociation (C) of AR-BPTI and SecB. For the association rate, SecB and AR-BPTI were rapidly mixed in a one-to-one volume ratios to a final concentration of 225 and 26 nM, respectively. Changes in fluorescence were recorded in time, and fitted to a single exponential [solid line 1 in (A), with residual shown in (B)] as described under Experimental Procedures. The fluorescence of the uncomplexed AR-BPTI is represented by line 2 in (A). For the dissociation rate, the SecB/AR-BPTI complex was preformed at final concentrations indicated under (A), and rapidly mixed with an excess of R-BPTI (2 μ M). Data were fitted to a double-exponential decay [solid line 1 in (C), with residual shown in (D)] as described under Experimental Procedures. The fluorescence of the preformed complex is represented by line 2 in (C).

Fast Association and Dissociation of the SecB/AR-BPTI Complex. The high quantum yield of AR-BPTI allowed a stopped-flow analysis of the rates of association and dissociation. At a concentration of 225 nM SecB and 26 nM AR-BPTI, binary complex formation as revealed by the increase in acrylodan fluorescence is very fast. Although only 20% of the reaction could be followed, the reaction appeared to equilibrate with a pseudo-first-order rate constant (k_{obs}) of 137 s^{-1} (Figure 7A, line 1, and pretrigger signal, line 2; residuals shown in Figure 7B). With an assumed bimolecular reaction, k_{obs} would correspond to a second-order binding rate constant (k_{ass}) of $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. When the concentration of AR-BPTI was lowered, the signal to noise ratio dropped and thereby the precision of the measurement. At higher concentrations of AR-BPTI, the association reaction occurred too fast to measure. The dissociation rate was determined by following the displacement of 26 nM AR-BPTI bound to SecB (225 nM) by a 76-fold excess of R-BPTI (2 μ M). The time course of the dissociation reaction followed double-exponential kinetics (Figure 7C, line 1, and pretrigger signal, line 2; residuals shown in Figure 7D), with estimated first-order dissociation rate constants (k_{diss}) of 48 and 3.6 s^{-1} , respectively. The fast reaction most likely reflects the dissociation of AR-BPTI from SecB. The nature of the second slow phase, which amounts to less than 10% of the signal, is obscure, but may represent a rearrangement of the released BPTI from a partially folded to a more unfolded structure. With a k_{diss} of 48 s^{-1} , and a steady-state K_{D} of 5.4 nM (Figure 6), the calculated k_{ass} is on the order of $9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. This value is close to the k_{ass} that was

determined in a direct manner (Figure 7A), suggesting that the rate of AR-BPTI association to SecB is diffusion-limited.

DISCUSSION

For the first time, kinetic data on the interaction of the molecular chaperone SecB with a polypeptide substrate are presented. SecB is a chaperone dedicated to facilitate the export of precursor proteins in *E. coli* (Wickner *et al.*, 1991). To determine the polypeptide binding kinetics, bovine pancreas trypsin inhibitor (BPTI) was used as a substrate as this protein remains soluble in its unfolded, reduced state. BPTI binds with high affinity to SecB, and this binding is competed by precursor proteins (Hardy & Randall, 1991; this paper). Although polypeptide binding to SecB can be detected as an increase in the intrinsic tryptophan fluorescence of SecB, this method is not sensitive enough to measure the kinetics of binding. Therefore, BPTI was labeled with the environmentally sensitive fluorophore acrylodan, which possesses a much higher quantum yield. Direct measurements demonstrate that the association rate constant (k_{ass}) for the interaction of SecB with acrylodan-labeled BPTI is on the order of $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. Determination of the dissociation rate constant and the K_{D} provides an alternative means to estimate k_{ass} , producing a value of around $9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The experimentally determined value for k_{ass} is in the range of the collisional rate of molecules of this size (Janin & Chotia, 1990; Pontius, 1993), suggesting that the binding of SecB to polypeptide substrates is limited by diffusion.

The extremely fast association kinetics have important consequences for the functioning of SecB *in vivo*. The kinetic partitioning model of selective binding of nonnative proteins by SecB predicts that the pathway a polypeptide takes *in vivo* depends on the rate of folding and/or aggregation relative to the rate of its association with SecB (Hardy & Randall, 1991). Proteins that fold rapidly are precluded from binding, whereas the presence of a signal sequence which has been shown to retard folding of precursor proteins (Park *et al.*, 1988; Laminet & Plückthun, 1989) would favor association with SecB and direct the precursor protein into the pathway leading to export. This theory is partially based on the assumption that SecB interacts with precursor proteins with a k_{ass} of about $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Randall *et al.*, 1994). The k_{ass} value experimentally determined for unfolded BPTI is at least 10-fold higher. It is important to emphasize that due to technical limitation in the stopped-flow experiments the k_{obs} represents a minimal value, and could well be greater. With an assumed free cellular SecB concentration of 0.4 μ M (Hardy & Randall, 1991), the pseudo-first-order rate for association with SecB would be in the range of 400 s^{-1} rather than the suggested 40 s^{-1} . This is far in excess to estimates of the rate of cytosolic protein folding *in vitro* of 5–10 s^{-1} (Hardy & Randall, 1991). *In vivo*, the rate of protein folding is likely to be limited by the rate of translation. Our data show that the interaction of SecB with an unfolded polypeptide chain can occur on a millisecond time scale, while extrusion of a protein from the ribosome takes seconds to minutes (Freedman, 1992). It appears that SecB is optimized to collide nonspecifically with macromolecules at the highest possible rate (Pontius, 1993). In the cell, the size of SecB and the viscosity of the cytosol, densely packed with protein and other components, will govern the rate of association. K_{D} values *in vivo* are, therefore, likely to be poorer than observed *in vitro*.

In vivo, SecB binding is highly selective (Kumamoto, 1989). SecB appears to be mainly associated with nascent chains in the cell (Kumamoto & Francetić, 1993). Under the conditions tested, these were preLamB, preMBP (maltose binding protein), proOmpA, proOmpF, and an unidentified protein. Nascent polypeptides are not expected to adopt a stable structure; they also cannot retain an extended conformation. The consensus from refolding experiments is that early elements of folded structure, although they may be nativelylike, are in rapid equilibrium with the unfolded state. The formation of a stable structure requires the presence of a complete protein domain. Before this has happened, binding of SecB has likely occurred. In addition, the bacterial homolog of signal recognition particle (SRP) associates with nascent chains at a very early state by binding to their signal sequence (Luirink *et al.*, 1992). This may further prevent the stable folding of polypeptide domains bound to the ribosome. Therefore, we conclude that *in vivo*, the binding affinity for SecB is more likely to be decisive for a stable interaction rather than the rate of folding. Once a polypeptide has folded to its nativelylike structure, the site that is recognized by SecB may be buried and thus inaccessible for interaction. In this respect, the rate of unfolding and the lifetimes of folding intermediates may further facilitate this interaction *in vitro*. It is important to realize that SecB must be highly selective *in vivo* since cells are hardly hampered by the massive overproduction of SecB (Weiss *et al.*, 1988).

What elements of a nonnative protein determine this affinity, and why are these elements specific for precursor proteins? It has been proposed that SecB binds preferentially at sites exhibiting β -structure (MacIntyre *et al.*, 1991; Breukink *et al.*, 1992). Mapping of putative binding domains in precursor proteins either by genetic or by biochemical means has so far not provided any insight in the rules that dictate specificity. It has also been suggested that precursor proteins harbor at the most two binding regions (Gannon *et al.*, 1989; Altman *et al.*, 1990; De Cock *et al.*, 1992). In preMBP and preGBP (galactose binding protein), the binding region appears to be positioned in the middle of the primary sequence (Collier *et al.*, 1988; Randall & Hardy, 1995). This must imply that SecB will bind these nascent chains with high affinity only when a major portion of the protein has emerged from the ribosome. In this context, SecB appears to bind only very large nascent chains (Kumamoto & Francetić, 1993), while SRP associates with short polypeptide chains (Luirink *et al.*, 1992). This means that SRP- and SecB-dependent pathways either coexist for general protein export or represent different stages in the precursor protein targeting cascade.

SecB does not bind pre- β -lactamase, both *in vivo* and *in vitro* (Lamiet *et al.*, 1991). It has been suggested that the lack of interaction could be due to a rapid transition of unfolded pre- β -lactamase into an intermediate form that is not recognized by SecB (Zahn *et al.*, 1994). Alternatively, it was proposed that pre- β -lactamase simply lacks a structural element that can be recognized by SecB. The latter seems more likely considering the extremely fast kinetics that allow SecB to bind to nonnative proteins.

SecB senses polypeptide binding through a conformational change that results in an increase in the intrinsic tryptophan fluorescence (Randall & Hardy, 1991; this paper). The Trp fluorescence of nonliganded SecB is rather low, but increases

50% upon polypeptide binding. In the case of the proOmpA/SecB complex, a significant contribution of Trp-36 of SecB to the total fluorescence signal is evident (Figure 3). Our data suggest that Trp-36 of SecB is surface-exposed, both in the liganded and in the nonliganded state. The increase in Trp fluorescence is likely the result of a relieve of quenching. In the nonliganded form, Trp-36 may be quenched by ionized amino acid side chains located in the vicinity. Mutational analysis demonstrates that Trp-36 of SecB is not essential for binding. Although the intrinsic Trp fluorescence was insensitive as a probe of polypeptide binding in kinetic experiments, it allowed us to determine the number of BPTI molecules bound per SecB homotetramer. The SecB homotetramer binds four BPTI molecules, indicating that it harbors four polypeptide binding sites. Proteolysis studies have led to the same conclusion (Randall, 1992). Both at high and at low SecB concentration, it appears that there is no cooperativity in the binding of BPTI between the monomers of the SecB homotetramer. In contrast to BPTI, which is a small protein, the large precursor proteins bind to the SecB homotetramer in stoichiometric amounts (Lecker *et al.*, 1990). It may contact the SecB homotetramer at its four binding sites simultaneously, giving rise to very tight binding.

Binding of AR-BPTI to SecB causes a shift in the emission maximum of the acrylodan fluorescence. This is indicative for a change from a polar to an apolar environment. It has been shown that SecB exposes a hydrophobic region upon the low-affinity binding of small peptides carrying a positive charge (Randall, 1992). This region becomes exposed only after occupation of all of the sites on SecB. In the case of AR-BPTI, that binds with high affinity, the increase in fluorescence is noncooperative. Binding of AR-BPTI always results in an increase in fluorescence. The lack of cooperativity suggests that this hydrophobic binding site on SecB might be different from the domain described by Randall (1992). Binding of a substrate to SecB must at least partially involve hydrophobic interaction.

SecB targets precursor proteins to SecA, the peripheral ATP binding subunit of the *translocase*. It binds with high affinity to SecA both in solution and at the membrane surface (Hartl *et al.*, 1990; Hoffschulte *et al.*, 1994). This interaction is stimulated by precursor proteins. *PrfA/PrfG* mutants of *E. coli* suppress certain signal sequence defects, and even allow the translocation of signal sequence-less proteins (Derman *et al.*, 1993; Flower *et al.*, 1994). These proteins are translocated in a SecB-dependent manner only, even though they are translocated SecB-independently as a full-size precursor protein. This suggests that the targeting function of SecB can compensate for the lack of a signal sequence, and gives strong support to the view that SecB functions as a coupling factor to allow efficient targeting and transfer of precursor proteins from the ribosome to the *translocase* (Kumamoto & Francetić, 1993). Moreover, it indicates that SecB has the ability to interact with other proteins not belonging to the subset of SecB-requiring precursor proteins.

A kinetic analysis of the interaction of an acrylodan-labeled peptide with the *E. coli* DnaK revealed a k_{ass} of $9400 \text{ M}^{-1} \text{ s}^{-1}$ (Schmid *et al.*, 1994). This rate is 10^5 times lower than the k_{ass} determined for the binding of SecB to AR-BPTI. DnaK transiently sequesters unfolded segments of proteins when they emerge from the ribosome and promote their

correct folding (Hartl *et al.*, 1994). The number of SecB (Watanabe & Blobel, 1989) and DnaK (Neidhard & Van Bogelen, 1987) molecules in growing cells is about 1200 (tetramers) and 2500 (dimers), respectively. Since these values are in the same order, one would expect that polypeptides are first bound by SecB before DnaK can interact. Obviously, this view is too simple, and other factors such as specificity and/or co-chaperones have to be implicated. It will be important to mimic the *in vivo* conditions of polypeptide binding more closely, for instance, by defining the selectivity and timing of SecB binding to nascent chains in cell-free extracts.

ACKNOWLEDGMENT

We thank Carol Kumamoto, Tufts University, Boston, for supplying us with pAK330, Jeroen van der Wolk for assistance in the *in vitro* protein translocation reactions, and Juke Lolkema for advise in the numerical data analysis.

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BI9504740