Chaperones are a class of proteins that possess the remarkable ability to selectively bind polypeptides that are in a nonnative state. The selectivity of SecB, a molecular chaperone in Escherichia coli, for its ligands can be explained in part by a kinetic partitioning between folding of the polypeptide and association with SecB. It has clearly been established that kinetic partitioning can be poised to favor association with SecB by changing the rate constant for folding of the ligand. We now demonstrate that binding to SecB can be given a kinetic advantage over the pathway for folding by modulating the properties of the chaperone. By poising SecB to expose a hydrophobic patch, we were able to detect a complex between SecB and maltose-binding protein under conditions in which rapid folding of the polypeptide otherwise precludes formation of a kinetically stable complex. The data presented here are interpreted within the framework of a kinetic partitioning between binding to SecB and folding of the polypeptide. We propose that exposure of a hydrophobic patch on SecB increases the surface area for binding and thereby increases the rate constant for association. In this way association of SecB with the polypeptide ligand has a kinetic advantage over the pathway for folding.

The prokaryotic chaperone SecB facilitates export of a subset of proteins (1, 2) that are delivered to the periplasmic space or outer membrane of Escherichia coli. SecB, the sole chaperone dedicated to export, has affinity for SecA, the peripheral membrane ATPase of the export apparatus. SecB binds precursor polypeptides in the cytoplasm before they assume their native, stably folded structures (3–5) and maintains them in an export-competent state that is neither folded nor aggregated (2, 3, 6–10). Translocation of the polypeptide across the membrane is then initiated upon ATP hydrolysis by SecA (11).

In addition to being the only chaperone dedicated to protein localization, SecB also differs from other members of the chaperone family in that it is a tetramer and functions independently of nucleotide triphosphate hydrolysis. However, one fundamental feature common to all chaperones is the remarkable ability to selectively bind nonnative polypeptides. The ability of SecB to recognize proteins as nonnative is governed in part by a kinetic partitioning (see Fig. 1) (6, 12, 13). The formation of a complex between SecB and a protein that possesses nonnative structure will depend on the rate of folding of the polypeptide relative to its rate of association with SecB. Since SecB has no affinity for native, stably folded polypeptides (12, 14–17), only those proteins that fold slowly are favored to bind SecB and enter the export pathway. All physiologic ligands of SecB are synthesized as precursor species that contain amino-terminal stretches of aminoacyl residues designated leader or signal sequences. Investigations carried out both in vivo and in vitro indicate that the physiologic function of the leader in binding to SecB is to slow folding of the precursor by interfering with the folding reaction that lies on the pathway to the native state (18–21). Retardation of folding exerted by the leader sequence thus provides the cell with an exquisite mechanism whereby SecB can engage the polypeptide before it folds into its native form.

Studies of the interaction between purified proteins have shown that SecB can also bind the mature protein, which does not carry a leader, if folding is slowed by other means. For example, at 25 °C rapid folding of wild-type mature maltose-binding protein precludes formation of a detectable complex with SecB; however, if the folding reaction is slowed by decreasing the temperature from 25 to 5 °C, SecB binds the mature protein (12, 19). Furthermore, three species of maltose-binding protein have been shown to contain single aminoacyl substitutions in the mature portion of the protein (MalE Y283D, MalE A276G, and MalE W10A) that slow folding sufficiently so that SecB can bind even when the temperature of the folding reaction is 20–25 °C (12, 19, 21). It is clear from these examples that kinetic partitioning can be poised to favor association with SecB by changing the rate constant for folding of the polypeptide (k_f) (refer to Fig. 1). However, it is also possible that binding to SecB could be given a kinetic advantage over the pathway for folding by modulating the properties of SecB and thereby affecting the rate constants for association (k_on) and/or dissociation (k_off).

Binding studies with short peptide ligands indicate that the SecB tetramer contains multiple subsites for binding of flexible unstructured polypeptides carrying a net positive charge (22). Saturation of these subsites induces a conformational change in SecB, exposing a hydrophobic patch that is thought to serve as an additional binding site for polypeptide ligands (Ref. 22; see Fig. 6 and 7). Here we test the idea that binding to SecB could be given a kinetic advantage over the pathway for folding by exposing the putative hydrophobic binding site on SecB.

EXPERIMENTAL PROCEDURES

Materials—The sources of chemicals were: Amylose resin, New England Biolabs; HEPES, Proteinase K, PMSF, and 1-anilino-naphthalene-8-sulfonate (ANS), Sigma; 2-4'-maleimidylalaninino-naphthalene-6-sulfonic acid (MNANS), and 1-anilino-naphthalene-8-sulfonate (ANS), Sigma; 2-4'-maleimidylalaninino-naphthalene-6-sulfonic acid, sodium salt; GuHCl, guanidinium chloride; Lys, a lysine polymer comprising 10 residues; poly-L-lysine, a homopolymer of L-lysine of molecular weight 42,000; SecB141, a form of SecB from which 14 aminoacyl residues have been removed from the C terminus by cleavage with protease K at Leu-141; MIANS-SecB, a chemically modified species of SecB that was alkylated with the sulf-hydryl-specific reagent MIANS.
fonic acid, sodium salt (MIANS), Molecular Probes, Inc.; GuHCl (ultrapure), ICN Biomedicals, Inc.

**Preparation of SecB141—Proteinase K** was prepared from a stock frozen in small portions at 2.5 mg/ml in 10 mM HEPES, pH 7.6, that was thawed and diluted immediately before use. SecB was digested at 2.8 mg/ml with 0.04 mg/ml proteinase K as follows: 27 μl of proteinase K at 0.05 mg/ml in 10 mM HEPES, pH 7.6–7.7, was added to 11 μl of SecB at 9.6 mg/ml in 10 mM Tris, 150 mM KC₂H₃O₂, pH 7.8, and incubated on ice for 20 min. The digestion was stopped by adding 38 μl of 1 mM PMSF. The PMSF, stored as a stock at 0.1 M in ethanol, was diluted into 10 mM HEPES, pH 7.6, on ice just before use. After adding the protease inhibitor, the concentration of the KC₂H₃O₂ was 21 mM. Since it has been observed that SecB loses structure during prolonged exposure to low ionic strength (26), we also prepared proteinase K-cleaved SecB that was restored to high ionic strength by including KC₂H₃O₂ in the 1 mM PMSF stock used to terminate digestion. We demonstrate here that the activity of proteolyzed SecB stored in low salt (21 mM KC₂H₃O₂) is comparable to that of proteolyzed SecB stored in high salt (150 mM KC₂H₃O₂).

**Chemical Modification of SecB with 2-(4'-Maleimidomethyl)anilinophenol-6-sulfonic acid (MIANS-SecB)—** SecB (36 μm monomer, each monomer contains 4 cysteine residues) was incubated with MIANS (150 μM) in 10 mM HEPES, pH 7.6, for 22 h at 4 °C in the dark. The alkylation of SecB with MIANS was terminated by adding 0.75 mM dithiothreitol.

**Titration Calorimetry**—Calorimetric titrations to obtain dissociation constants were carried out using the OMEGA titration calorimeter from MicroCal, Inc. (Northampton, MA) and the Origin software supplied with the instrument. The system has been described in detail in Wise-man et al. (28). The SecB tetramer (unmodified, SecB141, or MIANS-SecB) was held in the cell at 5 μM in 1.345 ml of 10 mM HEPES, pH 7.8, 150 mM KC₂H₃O₂, 0.1 μM GuHCl, 0.45 mM EGTA, and was titrated with galactose-binding protein that was unfolded in 10 mM HEPES, pH 7.8, 150 mM KC₂H₃O₂, and 1.0 mM GuHCl and diluted into the buffer for calorimetry immediately before loading the syringe for injection. Complete binding isotherms were generated at 7 °C by a sequence of 17 injections, each of 18 μl spaced at 11 min intervals. The best fit of the data for a model of one binding site was obtained using a least squares deconvolution algorithm based on the Marquardt method. The absolute binding constants determined in these experiments are subject to some uncertainty because the ligand can refold during the course of the titration. Therefore we report relative affinities that are valid since the identical titration schemes were used.

**RESULTS**

**Potentiation of SecB to Block Folding of Maltose-binding Protein at 21 °C**—It has clearly been established that kinetic partitioning can be poised to favor association with SecB by changing the rate constant for folding of the ligand ($k_f$) (12, 19, 21). Here we demonstrate that binding to SecB can be given a kinetic advantage over the pathway for folding by modulating the properties of SecB. Ligands such as short basic peptides or polymers of L-lysine, which have lower affinity for SecB than do the physiologically relevant ligands, induce SecB to undergo a conformational change that exposes a hydrophobic site proposed to function in ligand binding (22). We asked whether exposure of this hydrophobic patch on SecB induced by incubation with polymers of L-lysine would allow us to detect a complex between SecB and maltose-binding protein under conditions in which rapid folding of the polypeptide would otherwise prevent formation of a kinetically stable complex. We reasoned that exposure of this hydrophobic patch might serve to increase the binding area on SecB and therefore increase the rate constant for association such that binding to SecB would have a kinetic advantage over the pathway for folding. The interaction between SecB and maltose-binding protein can be monitored in vitro by assessing the ability of SecB to block the
Fig. 2. **Potentiation of SecB using poly-L-lysine.** The interaction between SecB and wild-type mature maltose-binding protein (MBP) was assessed at 21 °C by monitoring the ability of SecB to block the unfolding of denatured maltose-binding protein. The ability of SecB to block unfolding was compared in the presence of poly-L-lysine and absence of poly-L-lysine. When poly-L-lysine was present, it was added to the solution containing SecB before the addition of the maltose-binding protein. In the absence of poly-L-lysine, SecB was stored in high salt (150 mM KC$_2$H$_3$O$_2$), and in the presence of poly-L-lysine, SecB was stored in low salt (21 mM KC$_2$H$_3$O$_2$).

Fig. 3. **Ability of various species of SecB to block folding.** Blockage of folding of wild-type mature maltose-binding protein (MBP) was assessed at 5 °C in the presence of intact SecB (●) or SecB141 stored in high salt (150 mM KC$_2$H$_3$O$_2$, ▲) and low salt (21 mM KC$_2$H$_3$O$_2$, ◀). Poly-L-lysine potentiated both SecB141 and MIANS-SecB at 21 °C, where rapid folding of maltose-binding protein otherwise favors folding of the polypeptide over binding to SecB. Poly-L-lysine was shown to potentiate the activity of SecB141 to almost the same extent as intact SecB (Fig. 2, compare ● with ▲ and ◀). Thus, loss of 14 amino acids at the C terminus does not interfere with the ability of poly-L-lysine to potentiate SecB141. The defect caused by the MIANS modification could also be corrected by incubation with poly-L-lysine (Fig. 5). Whereas in the absence of poly-L-lysine MIANS-SecB at a 5-molar excess over maltose-binding protein decreased the rate of folding, inclusion of poly-L-lysine potentiated the MIANS-SecB so that the increase in fluorescence due to folding was completely eliminated. Consistent with the idea that potentiation is achieved by exposure of the hydrophobic patch, we demonstrated that SecB141 was able to undergo the conformational change exposing the patch, as detected by binding of ANS (Fig. 6). These findings suggest that exposure of the hydrophobic patch on SecB compensates for the loss of activity of SecB.

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DISCUSSION

A model rationalizing the ability of SecB to selectively bind nonnative proteins in the absence of any consensus in sequence or structure is illustrated in Fig. 7. The binding of a natural ligand to SecB involves interaction of regions of the polypeptide ligand with two different types of binding sites on SecB (Ref. 22; Fig. 7B). Simultaneous occupancy of multiple subsites of one type, which bind flexible stretches of ligand, causes a conformational change, exposing a second type of site, a hydrophobic patch that was detected by binding of the fluorescent probe ANS (Ref. 22; Fig. 7A). The binding of a natural, long polypeptide ligand would thus involve interaction with the sites occupied by the peptide ligands as well as with the hydrophobic patch. Exposure of the patch would be triggered by simultaneous occupancy of multiple subsites by flexible stretches of a nonnative polypeptide, which would be distinguished from a native, folded protein by the availability of several such stretches. High affinity binding selective for the nonnative state would result from these multiple interactions, each having low specificity. The association between SecB and its nonnative ligands, although being of high affinity ($K_d \sim 10^{-8} \text{ M}$) has been shown to be in an equilibrium in which both binding and release are rapid (13, 30). The unfolded protein continuously samples the free state and, with each cycle of dissociation, will partition between folding and rebinding. Thus, whether the polypeptide folds or forms a kinetically stable complex with SecB is based on a kinetic partitioning in which the relevant rate constants are the rate constant for folding and the rate constant for association with SecB.

Previous work in our laboratory using forms of maltose-binding protein that are altered in their folding properties revealed different effects of SecB on the folding of these polypeptides at 21 °C (21). While SecB only slightly decreased the rate of folding of wild-type maltose-binding protein, it drastically reduced the rate of folding of MalE A276G and completely blocked folding of MalE W10A. These differences in the effects of SecB can be explained in terms of kinetic partitioning and are attributed to differences in the rate constant for the folding reaction of the polypeptides (rate constants at 25 °C: wild type, 1.7 s$^{-1}$; MalE A276G, 0.24 s$^{-1}$; MalE W10A, 0.04 s$^{-1}$), since it is folding that is in competition with binding to SecB. Thus, the 7-fold decrease in the rate of folding of MalE A276G with respect to the rate of folding of wild-type maltose-binding protein allowed SecB to slow the appearance of the folded protein, whereas the 40-fold decrease in folding of MalE W10A poised partitioning to favor binding over folding to the extent that the observed effect was a blockage of folding. Here we have shown that poly-L-lysine can potentiate SecB to block folding of wild-type MalE at 21 °C. This potentiation can also be explained in terms of a kinetic partitioning. In this case, addition of poly-L-lysine does not alter the rate constant of folding of the polypeptide ligands, but rather we propose that it has its effect directly on SecB by increasing the rate constant for association. As shown previously (22), interaction of poly-L-lysine with SecB results in exposure of a hydrophobic patch, and if as proposed this patch is part of the ligand binding site, the rate constant for association would thereby increase, since the probability that productive collisions occur between SecB and maltose-binding protein is directly proportional to the reactive surface area of the two proteins.

Poly-L-lysine was effective not only in potentiating unmodified SecB to capture polypeptides that would otherwise fold too rapidly but also in restoring activity to two species of SecB that had been modified to decrease their ability to bind maltose-binding protein. Cleavage of 14 aminocyl residues from the C terminus of SecB to yield SecB141 caused a slight defect, whereas sulfhydryl modification (MIANS-SecB) caused a more...
The potentiation by poly-L-lysine. One possibility is that the hydrophobic site, and thus the probability of dissociation is increased in fluorescence intensity was 18 units. Triangles show the increase in ANS fluorescence with SecB141, where the maximal increase was 18 units. Fraction of response was calculated in both cases using 18 units as the maximal increase in ANS fluorescence.

FIG. 6. Effect of Lys₉₁₀ on ANS binding to SecB. The details of the assay are described under “Experimental Procedures.” Circles show the increase in ANS fluorescence with intact SecB, where the maximal increase in fluorescence intensity was 18 units. Triangles show the increase in ANS fluorescence with SecB141, where the maximal increase was 18 units. Fraction of response was calculated in both cases using 18 units as the maximal increase in ANS fluorescence.

FIG. 7. A model of the binding of peptide ligands and natural ligands to SecB. A, binding of a peptide ligand. B, binding of a natural ligand. As described in the text, simultaneous occupancy at multiple subsites on the SecB tetramer that bind flexible stretches of ligand induces a conformational change in the chaperone that exposes a hydrophobic surface. This hydrophobic patch is proposed to serve as an additional binding site for hydrophobic portions of the nonnative polypeptide.

severe defect in binding of maltose-binding protein. It was shown that when incubated with poly-L-lysine, SecB141 underwent a conformational change, exposing a hydrophobic patch, thus supporting the idea that potentiation is achieved by exposure of the patch. Poly-L-lysine also potentiated MIANS-SecB at 21 °C under conditions in which the ligand folds too rapidly to otherwise allow detection of a complex between unmodified SecB and maltose-binding protein.

The complex between SecB and poly-L-lysine has a dissociation constant in the range of micromolar (22), whereas the complex with maltose-binding protein has a $K_d$ in the range of $10^{-8}$ M. Since binding to all ligands by SecB is likely to be near collision-limited, as was shown to be the case for bovine pancreatic trypsin inhibitor (30), the difference in affinity between poly-L-lysine and maltose-binding protein is likely to reflect a difference in dissociation rate that can be rationalized by proposing that poly-L-lysine occupies the subsites for flexible stretches, whereas maltose-binding protein is also bound at the hydrophobic site, and thus the probability of dissociation is lower. There are at least two ways in which one can envision the potentiation by poly-L-lysine. One possibility is that the patch becomes exposed when poly-L-lysine binds, but upon dissociation, relaxation to the closed state is slow enough that maltose-binding protein can collide with SecB before the conformation returns to the prebound state. Alternatively, maltose-binding protein might bind the SecB-poly-L-lysine complex, initially via the exposed patch, if it were not occupied by the poly-L-lysine and once tethered, displace the poly-L-lysine from the subsites for flexible stretches in a stepwise manner. Whether the binding occurs when SecB is free or when poly-L-lysine is bound, we propose that exposure of a hydrophobic patch on SecB increases the surface area for binding and thereby increases the probability of productive collisions and thus the rate constant for association. In this way, association of SecB with the polypeptide ligand has a kinetic advantage over the pathway for folding. It seems unlikely that in the presence of poly-L-lysine, the dissociation rate constant would be affected to the same extent as is the association rate constant, since the binding of maltose-binding protein itself exposes the hydrophobic patch. However, even if the dissociation rate constant were lower, as long as dissociation is rapid enough to allow refolding to occur over the time course of the experiment, only the dissociation rate constant is crucial to the partitioning between folding and rebinding to SecB. In the unlikely event that the presence of poly-L-lysine has an extreme effect on $k_{off}$ resulting in an essentially irreversible interaction between maltose-binding protein and SecB, a blockage of folding would be observed.

Acknowledgments—We thank Traci B. Topping for purification of maltose-binding protein and critically reading the manuscript. We are grateful to Gerald D. Fasman for providing poly-L-lysine and Gerhard R. Munske for synthesis of Lys₉₁₀. We thank Simon J. S. Hardy for helpful discussions and critically reading the manuscript.

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