Mutational Alterations in the Homotetrameric Chaperone SecB That Implicate the Structure as Dimer of Dimers*

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Variant forms of SecB with substitutions of aminocarly residues in the region from 74 to 80 were analyzed with respect to their ability to bind a physiological ligand, precursor galactose-binding protein, and to their oligomeric states. SecBL75Q and SecBE77K are tetramers with affinity for ligand indistinguishable from that of the wild-type SecB, and thus the export defect exhibited by strains producing these variants must result from an effect on interactions between SecB and other components. SecBF74I is tetrameric but binds ligand with a lower affinity. Substitutions at positions 76, 78, and 80 cause a shift in the equilibrium so that the SecB tetramer dissociates into dimers. We conclude that the tetramer is a dimer of dimers and that the residues Cys76, Val78, and Gln80 must be involved either directly or indirectly in forming the interface between dimers. These variant species are defective in binding ligand; however, because their oligomeric state is altered, no conclusion can be drawn concerning the direct role of these residues in ligand binding.

SecB is a chaperone from Escherichia coli involved in the facilitation of export of proteins from the cytoplasm to the outer membrane, which lies beyond the cytoplasmic membrane, and to the periplasm, the aqueous space between the two membranes. Like all chaperones it binds its ligands by virtue of their non-native state. The binding is highly selective for unstructured proteins; however, no motif among the ligands has been found that might serve as a recognition element for SecB (1, 2). Mutant strains of E. coli that produce altered species of SecB and are defective in protein export have been isolated by Kumamoto and co-workers (3). Analyses of complexes between SecB and a ligand, precursor maltose-binding protein, isolated by co-immunoprecipitation from these mutant strains led to the proposal that the aminocarly residues in the region from 74 to 80 at the even numbered positions, i.e. Phe74, Cys76, Val78, and Gln80, are involved in interaction with the ligand (3). Substitutions at the alternating positions, Leu75 and Glu77, had no effect on the efficiency of co-immunoprecipitation of SecB and ligand; thus, it seems that the effect on export observed in vivo is the result of a defective interaction with other components of the export pathway. Here we have further investigated the nature of the defects exhibited by the altered species of SecB and conclude that the region implicated in binding of the precursors is crucial in maintaining the quaternary structure of SecB. SecB is a tetramer of identical subunits, but the species with substitutions at the positions 76, 78, and 80 exist as dimers. We conclude that these residues must be involved either directly or indirectly in forming the interface of dimers that normally associate to form tetramers and that substitutions at these positions shift the equilibrium so that the tetramer dissociates.

EXPERIMENTAL PROCEDURES

Protein Purification—Mature galactose-binding protein and precursor galactose-binding protein were purified as described (4, 5). SecB was purified from strain BL21(DE3) pJW25 (6). SecBL75Q, SecBE77K, and SecBF74I were purified from strain CK2212 (BL21 (DE3) secB::Tn5 srl::Tn10 recA) containing two plasmids. One plasmid contains the secB variant gene under its natural promoter, and the second plasmid contains the secB variant gene under control of the T7 promoter.

SecB and the three SecB variants were purified as described (7) except the bacterial cultures were grown in M9 minimal medium (8) supplemented with 0.4% glycerol, 4 μg/ml thiamine, and appropriate antibiotics. The concentrations of the purified proteins were determined spectrophotometrically at 280 nm using coefficients of extinction as follows: SecB, 84,000 M<sup>-1</sup> cm<sup>-1</sup> for the tetramer, and denatured precursor and mature galactose-binding proteins, 37,410 M<sup>-1</sup> cm<sup>-1</sup>.

Preparation of Soluble Lysates—Cells were grown at 37 °C in M9 minimal medium supplemented with 0.2% glycerol, 0.4% maltose, and 4 μg/ml thiamine to an optical density at 560 nm of 0.8. The cells were harvested, harvested, converted into spheroplasts (9) collected by centrifugation, and suspended in 10 mM Hepes, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.6. The spheroplasts were disrupted by sonication 5 × 15 s in a cup horn sonicator (Tekmar), and the lysate was incubated on ice for 10 min after the addition of magnesium acetate to a final concentration of 20 mM and DNase I (10 μg/ml) to reduce the viscosity before centrifugation for 5 min at 200,000 g with a TL100.1 rotor in a Beckman TL101 centrifuge. The supernatant, denoted soluble lysate, at a concentration equivalent to 7.5 × 10<sup>9</sup> cells/ml was adjusted to 2 mM diithiothreitol, 300 mM KAc, 0.1 mM phenylmethylsulfonyl fluoride, mM EGTA.

Size Exclusion Chromatography—All chromatography was carried out using a TSK G3000SW (TosoHaas) size exclusion chromatography column (7.5 mm inner diameter × 60 cm) equilibrated in 10 mM Hepes, 1 mM EGTA, 300 mM KAc, 2 mM diithiothreitol, pH 7.0. Samples of 200 μl (for soluble lysates this volume contained the equivalent of 1.5 × 10<sup>9</sup> cells) were injected, separation was carried out at 5 °C at 1 ml/min, and absorbance was monitored at 280 nm. Fluorescence was detected using an in-line fluorescence detector (Shimadzu) adjusted to its most sensi-
Oligomeric State of Chaperone SecB

tive detection level with excitation set at 290 nm and emission set at 340 nm. Fractions were collected as indicated and brought to 10% (w/v) with trichloroacetic acid, and the precipitated proteins collected by centrifugation, washed with acetone, and suspended in sample buffer for polyacrylamide gel electrophoresis.

Interaction of Pure SecB and SecB Variant Proteins with Galactose-binding Protein—Purified unfolded galactose-binding protein in 1.0 M GnHCl, 10 mM Heps, 1 mM EGTA, 300 mM KAc, pH 7.0, was diluted into a solution containing the pure SecB protein to achieve the desired concentrations of proteins in 0.16 M GnHCl, 10 mM Heps, 1 mM EGTA, 300 mM KAc, 2 mM dithiothreitol, pH 7.0. Immediately following mixing, the samples were analyzed by size exclusion chromatography using a TSK G3000SW column.

Distribution of SecB between Tetramer and Dimer—Soluble lysates were prepared from strains HK57 (MC4100 secB::Tn5 malT::Tn10 recaI) harboring a plasmid containing either the secB, secBC76Y, secBV78F or secBQ80R gene (3) as described above. Soluble lysates were injected onto the TSK G3000SW as described above and fractions of 1 ml were collected and processed for polyacrylamide gel electrophoresis followed by immunoblotting using an antibody to SecB.

Interaction of SecB and SecB Variant Proteins with Precursor Galactose-binding Protein in Lysates—Soluble lysates were prepared (as above except that the growth temperature was 35 °C) from the following strains: CK1953 (10), which carries the secB::Tn5 mutation and is secBQ80R::Tn10 (D3B) JW25 (6), which carries the wild-type secB on the chromosome as well as on the plasmid; and CK2212 with two plasmids secB::Tn5 and is secB::Tn10 recaI harboring a plasmid containing either the secB, secBC76Y, secBV78F, or secBQ80R gene (3) as described above. Soluble lysates were injected onto the TSK G3000SW as described above and fractions of 1 ml were collected and processed for polyacrylamide gel electrophoresis followed by immunoblotting using an antibody to SecB.

**Fig. 1. Complexes between wild-type SecB and precursor galactose-binding protein.** A, complex at a molar ratio of 3 μM SecB to 3 μM precursor galactose-binding protein. B, complex at a molar ratio of 6 μM SecB to 3 μM precursor galactose-binding protein. Upper panel, absorbance profiles of complexes of precursor galactose-binding protein and SecB resolved by size exclusion chromatography. HPLC of protein mixtures were carried out as described under “Experimental Procedures.” The dotted line represents the mixture of SecB and the unfolded precursor galactose-binding protein species at the 3:3 molar ratio, the dashed line represents these species at a 6:3 molar ratio, the solid line represents SecB only, and the dotted-dashed line represents unfolded precursor galactose-binding protein. In A and B, SDS-polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.35-ml fractions, starting after elution of 13 ml. Lane S contains 2% of the quantity of sample applied to the column. The positions of precursor galactose-binding protein (pGBP) and SecB are indicated.

electrophoresis was carried out on 14% polyacrylamide gels (12). Immunoblotting was performed as described (13) using antisera to SecB and to galactose-binding protein and the chromogenic dye 4-chloro-1-napthol for detection.

**RESULTS**

We purified SecB and three of the altered species of SecB, SecBL75Q, SecBE77K, and SecBF74I, to homogeneity and examined their binding to a natural ligand, the precursor form of the periplasmic galactose-binding protein. SecB only binds proteins as ligands if they are in a non-native state (14, 15). Therefore, to assess interaction, the refolding of denatured precursor galactose-binding protein was initiated by dilution of the denaturant, guanidinium chloride, in the presence of the chaperone SecB. When the mixture was analyzed by size exclusion HPLC, the galactose-binding protein coeluted with SecB ahead of the position of free SecB (16 ml) and of free precursor galactose-binding protein (17.9 ml for unfolded and 20.8 ml for folded) as indicated by the absorbance profiles and analysis of the fractions by gel electrophoresis (Fig. 1). When applied in an equimolar mixture (Fig. 1A, dotted line, 3 μM each) not all the galactose-binding protein remained in complex during the fractionation, whereas at a 2-fold molar excess of SecB tetramer (Fig. 1B, dashed line, 6 μM SecB: β μM precursor galactose-binding protein) all the precursor galactose-binding protein was recovered in complex with SecB. Complexes were readily detected between the precursor and the altered SecB species, SecBL75Q (Fig. 2A), SecBE77K (Fig. 2B), and SecBF74I (Fig. 3). Comparison of the distribution of precursor galactose-binding protein chromatographed after mixing with the various species of SecB indicates that SecBF74I has a lower affinity for precursor galactose-binding protein than does the wild-type SecB because when applied as an equimolar...
lished (12) for the wild-type SecB (Table I).

The one titration done with SecBF74I at the same concentrations as those used for the wild-type SecB and for SecBL75Q indicated that the affinity was lower by at least 10-fold. An accurate value for the dissociation constant could not be determined because it would have required use of the proteins at 10-fold higher concentration (i.e. 1 mM for SecB). As has been shown previously for the wild-type SecB (12), SecBL75Q binds the mature form of galactose-binding protein with a higher affinity than it binds the precursor form. This was demonstrated by carrying out gel filtration chromatography on a solution containing a mixture of precursor and mature galactose-binding protein and a limiting quantity of SecB. When precursor and mature forms were present in equimolar quantities, the ratio of ligands recovered in complex with SecB, whether wild-type or SecBL75Q (Figs. 4 and 5), showed an enrichment for the mature form. In the case of SecBF74I binding was too weak to make a definitive conclusion concerning relative affinities (data not shown). These results are consistent with the published results of Kimsey et al. (3): SecBL75Q and SecBE77K bind ligand normally, whereas SecBF74I has a lower affinity.

The forms of SecB with substitutions at the three other positions in the region spanning aminoacyl residues 74–80, i.e. SecBC76Y, SecBV78F, and SecBQ80R, could not be purified. When strains harboring plasmids encoding these proteins were grown under conditions to produce large quantities of SecB, the proteins aggregated and were recovered in inclusion bodies. Thus it seemed likely that the region of SecB that had been altered was involved in folding of the polypeptide or its assembly into tetramers. Because the original characterization of the SecB in these strains as defective in ligand binding was based on the inability of SecB to be co-immunoprecipitated with the ligand maltose-binding protein it was important to determine whether these variants of SecB existed in a folded, tetrameric state at the level of expression used in the original study. To address this question the strains used in Kimsey et al. (3) were grown as described in that work, and a cellular lysate was prepared. The lysates were subjected to centrifugation to separate soluble protein from both the membrane and proteinaceous aggregates. Analysis of each fraction by gel electrophoresis and immunoblotting using an antiserum raised to SecB demonstrated that the soluble fraction and the pellet, usually approximately 50% in each. The soluble fraction from each of the strains was subjected to gel filtration chromatography, and the presence of SecB among the proteins eluted from the column was detected by SDS-gel electrophoresis and immunoblotting. The SecB in the lysate of a strain producing wild-type SecB was eluted at the same position as is the purified tetrameric protein (16 ml) (Fig. 6A). To address the question of stability of the wild-type tetramer, the purified SecB was applied to the column at increasingly lower concentrations in an attempt to approach the dimer-
tetramer equilibrium constant and to observe dissociation of the tetramer. At concentrations near the equilibrium constant the position of elution would reflect the weighted average molecular weight of all species present, and thus the elution position should increase as the equilibrium moves toward favoring the dimeric species. Even at the lowest concentration that could be detected using the intrinsic fluorescence of SecB (20 nM), the elution position did not change (data not shown). Therefore, the equilibrium constant for the wild-type SecB must be well below 20 nM. Analyses of lysates containing the species that remained soluble during purification, i.e., SecBL75Q, SecBE77K, and SecBF74I, indicated that these variant forms of SecB were also tetrameric (data not shown). In contrast, SecBV78F was distributed between two peaks, one eluting at 16 ml, the position of the tetrameric species, and a second peak eluting at 20 ml (Fig. 6B). Native galactose-binding protein, which has a molecular mass of 33 kDa, elutes at 20 ml, and thus it seems that the species of SecB at that position is a dimer. Almost all of the SecBC76Y in the lysate eluted between 20 and 21 ml, indicating that it is entirely dimeric (Fig. 6C). We were unsuccessful in expressing SecBQ80R in this strain in a quantity comparable with that of the other species, but what was detectable was distributed between dimeric and tetrameric species (Fig. 6D).

Because of the difficulties in purifying these proteins, we assessed the ability of the variant forms of SecB to form complexes when unfolded precursor galactose-binding protein ligand was added to the lysates to give a final concentration of 1 μM precursor. Precursor galactose-binding protein eluted from the size exclusion column in the same position whether it was applied as pure protein (Fig. 7) or after mixing with a lysate prepared from a secBnull strain (Fig. 7), indicating that in the absence of SecB there are not other components that affect the elution. However, addition of the precursor to a lysate that contained the SecB tetramer at 2 μM (Fig. 7) or at 1 μM (data not shown) resulted in the appearance of a complex as demonstrated by the coelution of the precursor and SecB. In contrast when precursor galactose-binding protein was added to a lysate containing SecBV78F (at a concentration of 2 μM expressed as tetramer) only a small amount of the precursor galactose-binding protein coeluted with tetrameric SecB, indicating weak interaction (Fig. 7). No interaction was observed between SecBC76Y and precursor galactose-binding protein when lysates were used that contained SecBC76Y at 1 μM (expressed as tetramer), which was the highest concentration we were able to achieve (Fig. 7). Attempts to concentrate the lysate resulted in loss of the SecBC76Y through aggregation.

SecBC76Y does not form any detectable tetramer. It is recovered predominantly in the position of dimer with a significant amount of the protein recovered as higher order species, probably nonspecific aggregates, that elute from the gel filtration column over a wide range from the void volume to the

FIG. 6. Distribution of SecB between tetramer and dimer. Total lysates from strains expressing SecB (A) SecBV78F (B), SecBC76Y (C), and SecBQ80R (D) were separated into pellet (lane P) and soluble lysate (lane S). The soluble lysate was applied to a TSK G3000SW column, and the material that eluted between 11 and 25 ml was subjected to polyacrylamide gel electrophoresis and detected by immunoblotting with antisera directed against SecB. Portions of the unfraccionated soluble lysate and pellet were also subjected to gel electrophoresis. For SecBQ80R the material loaded was nine times that used for the other lysates.
position of dimer. In contrast, SecBV78F eluted at the position of tetramer as well as dimer. When a radiolabeled lysate of cells expressing SecBV78F was fractionated (Fig. 8A) and the SecBV78F that eluted in the tetrameric position was reapplied to the column, the SecB redistributed between tetramer and dimer (Fig. 8B). In addition when the protein that had eluted at the position of dimer was mixed with nonradioactive lysate so that the concentration of SecB was close to that in the original lysate, again the radioactive SecBV78F redistributed between tetramer and dimer (Fig. 8C). Therefore, the observed distribution reflects a shift in the equilibrium between dimers and tetramers relative to that of the wild type and not a subpopulation of SecBV78F that cannot form tetramers.

**DISCUSSION**

SecB, which functions as a homotetramer (16, 17), is the product of the secB gene that encodes a polypeptide of 155 amino acids (18). At physiological ionic strength and pH, the SecB tetramer is stable. The equilibrium constant is such that even at 20 nM, which is well below the concentration in vivo, estimated to be 4 µM (2, 17), neither dimer nor monomer was detected when a solution was analyzed by size exclusion chromatography. Studies of the oligomeric state of SecB using electrospray ionization mass spectrometry optimized to study noncovalent association also detected a tetramer over a wide range of temperature and pH. The tetramer could be dissociated directly into monomers by heating at the atmosphere-vacuum interface of the mass spectrometer with no evidence of intermediate species. Thermal denaturation of the polypeptide probably occurs without first destroying specific interface contacts. In contrast, when disruption was effected by raising the pH to 11, a dimeric species was observed (16). The observation of dimeric intermediates during dissociation indicates that the monomers within the tetramer interact through two different interfaces because the stability of one interacting interface is more sensitive to high pH than is the other. If all interfaces were the same the dimeric state would not be favored; one would see monomers, dimers, and trimers as well. Thus it was concluded that the SecB tetramer is a dimer of dimers (16). This model for the structural organization is reinforced by the identification of single aminoacyl substitutions that shift the equilibrium to favor a dimeric form of SecB. The stretch of

**FIG. 7.** Complexes between SecB and precursor galactose-binding protein. Fractionation on a TSK G3000SW column was carried out on purified unfolded precursor galactose-binding protein (pGBP) as well as on soluble lysates and soluble lysates mixed with unfolded pGBP as indicated (as described under “Experimental Procedures” except that 5 mM magnesium acetate was included in the equilibration buffer). Portions of the fractions that eluted between 14.5 and 22.5 ml as well as the sample applied (S) were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using a mixture of antisera directed against SecB and galactose-binding protein. The positions of SecB (solid arrowheads) and pGBP (open arrowheads) are indicated. The asterisks mark the position of a dimeric form of SecB that is not disrupted during preparation of samples for gel electrophoresis.

**FIG. 8.** SecBV78F demonstrates an equilibrium between dimer and tetramer. A total lysate from a strain expressing secBV78F labeled with [35S]methionine was separated into pellet (lane P) and soluble lysate (lane S) fractions. A, the total soluble lysate was applied to a TSK G3000SW column (as described under “Experimental Procedures” except that 5 mM magnesium acetate was included in the equilibration buffer), and the eluted fractions were subjected to gel electrophoresis followed by detection with a PhosphorImager. Fractions that eluted at the position of the SecB tetramer (15–16 ml) (lane T) or at the position of dimer (20–21 ml) (lane D) were concentrated and rechromatographed as shown in rechromatographed tetramer (B) and rechromatographed dimer (C). Solid arrows indicate the position of SecBV78F. The positions and masses (in kDa) of molecular mass markers phosphorylase b (97.4), α-amylase (60.6), actin (41.7), glyceraldehyde 3-phosphate dehydrogenase (36.0), carbonic anhydrase (28.7), myoglobin (17.2), and cytochrome c (12.4) are indicated.
polypeptide spanning aminoacyl residues 76–80 must be involved in forming contacts at one of the two interfaces in the tetramer. These residues most likely are directly involved in contacts at the interface, but we cannot yet eliminate the possibility that the substitutions cause a conformational change that is propagated to the interface.

In light of the observation that the changes in the aminoacyl residues at positions 76, 78, and 80 alter the oligomeric state of SecB, the original conclusion (3) that they form the binding site for the ligand must be reassessed. Interaction between the SecB tetramer and its non-native polypeptide ligands, which is of high affinity but readily reversible, has been proposed to be the result of simultaneous occupancy of the subsites by different stretches of the polypeptide ligands (19, 20). The subsites might be contained entirely within monomeric units or involve interactions at one or both of the interfaces between dimers. Even if neither interface were part of the binding site, a dimeric form of SecB would be expected to have low affinity for its ligands because it could provide only half of the binding energy that would result from interaction with the tetrameric form. Thus we cannot definitively conclude that the residues that were altered in the variants are involved directly in forming the binding site. However, SecBF74I, which is substituted at the position adjacent to those that define the interface, is tetrameric at the concentrations used in this study, and yet it binds ligand weakly. Thus, the interface of dimers is a likely candidate for the site of ligand binding.

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