We have shown here that the cytosolic bacterial chaperone SecB is a structural dimer of dimers that undergoes a dynamic equilibrium between dimer and tetramer in the native state. We demonstrated this equilibrium by mixing two tetrameric species of SecB that can be distinguished by size. We showed that the homotetrameric species exchanged dimers, because when the mixture was analyzed both by size exclusion chromatography and native polyacrylamide gel electrophoresis a third hybrid tetrameric species was detected. Furthermore, treatment of SecB with 5,5′-dithiobis-(2-nitrobenzoic acid), which modifies the sulfhydryl group on cysteines, caused irreversible dissociation to a dimer indicating that cysteine must be involved in the stabilizing interactions at the dimer interface. It is clear that the two dimer-dimer interfaces of the SecB tetramer are differentially stable. Dissociation at one interface allows for a dynamic dimer-tetramer equilibrium. Because only dimers were exchanged it is clear that the other interface between dimers is significantly more stable, otherwise oligomers should have formed with a random distribution of monomers.

The protein SecB is a chaperone of Gram-negative bacteria that is dedicated to the localization of specific proteins to the periplasmic space or to the outer membrane of these organisms. It binds precursors of exported proteins, maintaining in a state that is competent for membrane translocation, and delivers them to SecA, the peripheral ATPase of the membrane translocase (reviewed in Ref. 1).

SecB is a homotetramer of molecular weight 68,600 (2). Homotetramers can be of two structural sorts. Either they are cyclic tetramers, which have 4-fold symmetry (C4), or they are motetramers can be of two structural sorts. Either they are random distribution of monomers.

produced by mutated secB genes gave evidence for the existence of a species that eluted at a position consistent with that expected for a dimer (3). In contrast, a study using differential scanning calorimetry concluded that SecB is likely to be a cyclic tetramer, because there was no evidence of dimer intermediates in the thermal unfolding of SecB to monomers (4). The previous studies (2, 3) indicating that SecB is a structural dimer of dimers required the use of mutationally altered species of SecB or extreme conditions of pH to cause dissociation into dimers. This is the first use of wild-type SecB in a demonstration that it is structurally a dimer of dimers. In addition, we show that under physiological conditions one dimer interface is considerably more stable than the other so that the homotetramer undergoes a dynamic dimer-tetramer equilibrium.

**Experimental Procedures**

Purification of SecB and Truncated SecB—SecB was purified as described (5). SecB142, a form of SecB that is truncated at amino acid 142 of a total of 155 amino acids, was purified from strain CK2212 (BL21(AD3) secB::Tn5, srl::Tn10 recA1) containing a plasmid carrying the secB142 gene under control of the T7 promoter (6). Cells were grown in M9 minimal medium (7) supplemented with 0.4% glucose, 4 μg/ml thiamine, and 100 μg/ml ampicillin at 30 °C. Induction and purification were as described for SecB (5). A version of SecB that is truncated at amino acid 141 was generated for some experiments from purified wild-type SecB by proteolysis with protease K as described (8). Whenever this form of truncated SecB was used, a solution containing an equivalent of protease K inactivated by phenylmethylsulfonyl fluoride was added to the sample containing the full-length SecB.

Exchange of Subunits between Wild-type SecB and Truncated SecB—Wild-type SecB and truncated SecB were dialyzed into 30 mM NaPO₄ buffer, pH 7.6, and mixed at room temperature so that the final concentration of each was 22 μM and then were subjected to native polyacrylamide gel electrophoresis with little or no delay. To observe exchange of monomers of SecB, 90 μM wild-type SecB, 90 μM truncated SecB, and a mixture of 45 μM of each species in 10 mM HEPES-KOH, pH 7.6, 10% glycerol, 50 mM NaCl, 10 mM Mg(OAc)₂, pH 7.6, were unfolded by dilution into 2 volumes of 9 M urea (ultrapure urea; Roche Molecular Biochemicals). In the control, two volumes of water were added instead of the urea. The samples were incubated at 24 °C for 30 min followed by 3 h at 0 °C. Refolding was initiated by rapid dilution of one volume of denatured SecB with 22 volumes of 10 mM HEPES-KOH, pH 7.6, 10% glycerol, 50 mM NaCl, 10 mM Mg(OAc)₂. All samples were then dialyzed against 50 mM NaPO₄, pH 7.6, before being applied to gels.

Purification of DTNB-treated SecB—Cysteine residues on SecB were modified by treatment with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB1) or Ellman’s reagent (Sigma). DTNB (1.6 mM) was added to SecB (40 μM) in 0.1 M sodium phosphate, pH 8, 1 mM EDTA in a total volume of 0.6 ml and incubated at 23 °C for 2 h. After treatment SecB could be resolved into two peaks by high performance liquid chromatography using a TSK G3000SW size exclusion column (60 cm × 7.5 mm; Tosoh...
SecB Undergoes a Dynamic Dimer-Tetramer Equilibrium

RESULTS

Evidence for a Dynamic Dimer-Tetramer Equilibrium—Full-length SecB can be distinguished from versions truncated at the C terminus that are missing either 14 aminoacyl residues, generated by mild proteolysis, or 13 aminoacyl residues, produced as the product of a modified secB gene, on the basis of the reduction in size. Previous analysis has shown that the truncated SecB exists as a tetramer and is active in binding ligand although with a lower apparent affinity (2, 8). Analysis of the proteins in their native tetrameric state shows that when subjected to size exclusion chromatography separately, full-length SecB elutes at a position well ahead of that of the truncated species (Fig. 1, upper panel, compare dotted and dashed lines). When the two species of SecB are mixed before application to the column, a species is detected that elutes at a position between that of the pure homotetrameric species of SecB (Fig. 1, upper panel) and truncated monomers, indicating the existence of a dynamic equilibrium by which subunits of the tetramers were exchanging (Fig. 1, lower panel).

The exchange of subunits was also evident when equimolar mixtures of wild-type SecB and truncated SecB were subjected to nondenaturing polyacrylamide gel electrophoresis (Fig. 2). When applied separately the two species of SecB were well

FIG. 1. Exchange of subunits shown by size exclusion chromatography. Full-length SecB and truncated SecB were mixed and incubated at room temperature for 1 h before injection onto a TSK G3000SW column equilibrated in 10 mM HEPES-KOH, pH 7.5, 300 mM KOAc, 5 mM Mg(OAc)$_2$, 1 mM EGTA. The concentration of the protein was determined by the BCA protein assay (Pierce) using bovine serum albumin as a standard.

Identification of Cysteine Residues Modified by MIANS—MIANS-SecB was generated as described (8). MIANS-SecB or SecB was precipitated with trichloroacetic acid and suspended in 0.1 M ammonium bicarbonate and 7.2 M urea. V8 protease digestion the sample was diluted to contain 1 mg/ml SecB, 50 mM ammonium bicarbonate, 2 M urea. V8 protease was added to 40 g/ml, and the samples were incubated at 37 °C for 5 h. The proteolytically generated fragments were separated by reversed phase high performance chromatography on a Jupiter (Phenomenex) C4 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid (Amicon, and dialyzed against 10 mM HEPES-KOH, pH 7.5, 300 mM KOAc, 5 mM Mg(OAc)$_2$, 1 mM EGTA. The concentration of the protein was determined by the BCA protein assay (Pierce) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis—Denaturing polyacrylamide (15%) gel electrophoresis was performed as described (9). For native polyacrylamide (9%) gel electrophoresis the procedure was the same with the exception that SDS was not included in any solutions, and samples were not boiled.

Molar Mass Determination—High performance liquid size exclusion chromatography was performed on a TSK G3000SW column in 10 mM HEPES-KOH, pH 7.5, 300 mM KOAc, 5 mM Mg(OAc)$_2$, 1 mM EGTA. The elution profiles were monitored by multiangle laser light scattering at 690 nm and differential refractometry (DAWN-EOS and Optilab instruments, respectively; Wyatt Technology Corp., Santa Barbara, CA). The molar mass was determined using a specific refractive index increment of 0.195 and the Debye plotting formalism of the Astra software supplied by Wyatt Technology Corp.

FIG. 2. Native polyacrylamide gel electrophoresis of full-length and truncated species of SecB. Lanes were loaded as follows: lane 1, full-length SecB; lane 2, both full-length and truncated SecB in the same well with minimal mixing; lane 3, truncated SecB; lane 4, both full-length and truncated SecB mixed and incubated at 24 °C for 17 min; lane 5, both species of SecB mixed and incubated on ice overnight; lane 6, full-length SecB refolded from 6 M urea; lane 7, both full-length and truncated SecB mixed in 6 M urea and refolded together; lane 8, truncated SecB refolded from 6 M urea; lane 9, full-length and truncated SecB refolded separately from 6 M urea and then mixed.

Haas) equilibrated in 10 mM HEPES-KOH, pH 7.5, 300 mM KOAc, 5 mM Mg(OAc)$_2$. To optimize resolution multiple chromatography runs were performed using a 200-µl sample volume. Fractions of 0.5 ml were collected for each run, and the 4 fractions containing the SecB in the later eluting peak were pooled, concentrated using a Centricon10 (Amicon), and dialyzed against 10 mM HEPES-KOH, pH 7.5, 300 mM KOAc, 5 mM Mg(OAc)$_2$, 1 mM EGTA. The concentration of the protein was determined by the BCA protein assay (Pierce) using bovine serum albumin as a standard.

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FIG. 1. Exchange of subunits shown by size exclusion chromatography. Full-length SecB and truncated SecB were mixed and incubated at room temperature for 1 h before injection onto a TSK G3000SW column equilibrated in 10 mM HEPES-KOH, 150 mM KOAc, pH 7.2. Upper panel, absorbance profiles of full-length SecB (dotted line), truncated SecB (dashed line), and the mixture (solid line). Lower panel, SDS polyacrylamide gel of trichloroacetic acid precipitates of 0.25-ml fractions collected during chromatography of the mixture. The lane labeled S represents 10% of the material loaded onto the column. One quarter of each fraction was loaded onto the gel. The positions of full-length SecB and truncated SecB are indicated.

FIG. 2. Native polyacrylamide gel electrophoresis of full-length and truncated species of SecB. Lanes were loaded as follows: lane 1, full-length SecB; lane 2, both full-length and truncated SecB in the same well with minimal mixing; lane 3, truncated SecB; lane 4, both full-length and truncated SecB mixed and incubated at 24 °C for 17 min; lane 5, both species of SecB mixed and incubated on ice overnight; lane 6, full-length SecB refolded from 6 M urea; lane 7, both full-length and truncated SecB mixed in 6 M urea and refolded together; lane 8, truncated SecB refolded from 6 M urea; lane 9, full-length and truncated SecB refolded separately from 6 M urea and then mixed.
resolved (Fig. 2, compare lanes 1 and 6 with 3 and 8), whereas three stained bands were observed when the mixture was analyzed. Two of the bands corresponded to the homotetrameric full-length and the homotetrameric truncated species of SecB, and the third band was observed midway between the two. The same ratio of the three species was seen whether the proteins were mixed when loading into the sample well (lane 2) or incubated together either for 17 min or overnight before loading (lane 4 and 5, respectively). The presence of only three bands strongly indicates that the two species of SecB exchanged only dimers and thus that SecB is a dimer of dimers. If it were instead a cyclic tetramer that exchanged monomers, or indeed a dimer of dimers in which the two different dimer interfaces were of near equal stability, one would expect to see in this experiment three intermediate bands between the homotetrameric species, representing the 3:1, 2:2, and 1:3 monomer compositions of the tetrameric species.

This expectation was realized when a mixture of full-length and truncated SecB species were denatured in 6 M urea and refolded in the same solution by dilution of the denaturant. Five bands representing all five possible tetrameric species were seen on the gel (Fig. 2, lane 7) indicating that the tetramers had assembled from monomers. This experiment therefore serves as a control for the spontaneous exchange of dimers from the native state, because it demonstrates that if monomers were exchanging we would have seen three intermediate species not just the one observed. When full-length and truncated SecB are refolded separately and then mixed, as expected only three bands were present indicating an exchange of dimers (Fig. 2, lane 9). We conclude that the tetramer dissociates as dimers from the native state, and the protein must be denatured to populate the monomer state.

An interesting and confirmatory feature of the gels shown in Fig. 2 is the appreciable diffuse staining between the visible bands of the lanes that contained mixtures of the two types of SecB, reflecting the continuous re-equilibration of the SecB species as they migrate through the gel. Material migrating beyond the position of the homotetrameric full-length SecB comprises tetramers of either two truncated dimers or hybrid tetramers of one truncated dimer and one full-length dimer. Whenever two full-length dimers derived from the dissociation of the hybrid tetramer form a tetramer, they migrate more slowly whereas two truncated dimers that form a tetramer migrate more rapidly than does the hybrid tetramer. This re-equilibration may account for the fact that the central band of the three, which would be expected to contain twice as much material as either the upper or lower band (assuming equal affinities for association of the different dimers), is relatively depleted in Fig. 2, lanes 2, 4, 5, and 9.
the normal position (16 ml) for the tetramer with only a small amount of the material eluting later at 17.5 ml (Fig. 3, dashed line). Prolonged incubation with the reagent resulted in modification of more than one cysteine per monomer (1.2 at 60 min and 1.6 at 130 min) with a concomitant increase in the later-eluting species (Fig. 3, dotted and solid lines). To establish the oligomeric state of the later-eluting species the proteins were analyzed using an on-line multirange static light-scatter detector that allows direct determination of molar mass. The intensity of light scattered is proportional to the product of the molar mass and the concentration of the particle under investigation. Thus it is apparent by simply comparing the profiles of light-scatter intensity (Fig. 4A, solid line) and protein concentration as determined by change in refractive index (Fig. 4A, dashed line) that the later peak has about half of the mass of the earlier peak, because for the same concentration the light scattered had half the intensity. To accurately determine the mass it was necessary to subject the later-eluting peak to chromatography a second time to remove any unresolved tetrameric species, because their presence would increase the weight average molecular mass (Fig. 4B). The molar mass calculated was 35,000, a value that is very near the expected molar mass of 34,300 for the dimeric form of SecB. The molar mass of unmodified SecB was also determined in the same manner to be 72,000. The accepted molar mass of the SecB tetramer is 68,600 (2).

The SecB dimer generated by DTNB modification did not redistribute to a mixture of tetramer and dimer when reapplied to the column. We conclude that this dimer can not reassociate with another dimer to form a tetramer. It should be noted that even with longer incubation with DTNB, monomers were not generated. This observation reinforces the conclusion drawn from the demonstration of the spontaneous dissociation; SecB is a dimer of dimers.

Because a tetramer exhibiting dihedral symmetry has two different interfaces between dimers it was of interest to determine whether the interface that was disrupted by chemical modification was the same interface as that involved in spontaneous dissociation. If the interface dissociating spontaneously were different from that modified by DTNB then one would expect the dimers generated by the modification to be able to exchange monomers by spontaneous dissociation at the other, unmodified interface. This was not the case as demonstrated using non-denaturing polyacrylamide gel electrophoresis of mixtures of the dimeric forms of wild-type SecB and truncated SecB, each generated by treatment with DTNB. No hybrid heterodimeric species were detected (data not shown).

We previously reported that modification of cysteine by the fluorescent reagent MIANS reduced the apparent affinity of SecB for ligands (8) but did not result in a change of the oligomeric state as assessed by size exclusion chromatography (data not shown). However, treatment of the MIANS-modified SecB with DTNB did not result in generation of dimers (Fig. 5, dotted line) as DTNB modification does for SecB (Figs. 3 and 5, solid line). Therefore it seems that although modification of cysteine by MIANS does not itself generate dimers it does protect the crucial cysteine from reaction with DTNB. There are four cysteinyl residues in SecB, at positions 76, 97, 102 and 113, that are candidates for modification. Mass spectrometry of peptide mixtures derived from proteolytic digestion of MIANS-modified SecB revealed the presence of a peptide with a mass corresponding to amino acid 78 to 112 carrying one MIANS adduct. Thus it is likely that either Cys-97 or Cys-102 lies at the dimer interface. However, because both cysteine residues Cys-97 and Cys-102 lie within the same fragment we could not determine which was modified. Unambiguous identification of the cysteine modified was further complicated by the fact that at least 2 different cysteine residues were modified both by MIANS and by DTNB. This is indicated by the kinetics of modification by DTNB, as well as by mass spectrometry of MIANS-modified SecB, before proteolytic digestion. When reaction with MIANS was terminated at a molar ratio of 1.6 reducible per monomer, mass spectrometry revealed a mixture of polypeptides carrying 0, 1, or 2 MIANS moieties (data not shown).

We assessed numerous other reagents that modify aminocycl side chains (bromoethylamine and 4,4'-dithiodipyridine (Fig. 5, dashed line), which modify cysteines and succinic anhydride, acetic anhydride, and citraconic anhydride, which modify lysines (data not shown)); none of the modifications resulted in dissociation of the SecB tetramer as assessed by size exclusion chromatography.
A homotetramer might be either a cyclic tetramer, in which the interactions at the interface of monomers are all identical (Fig. 6A), or it could be a dimer of dimers in which there are two different interacting interfaces (Fig. 6B). The two sorts of tetramers may be distinguished by the manner in which the subunits dissociate. For cyclic tetramers, because all of the interacting sites are identical, the probability that any one monomer dissociates is equal. Therefore either all four monomers would dissociate simultaneously, or if intermediate states were populated there would be dimers and trimers, as well as monomers. In contrast, because there are two different interactions stabilizing the two dimer interfaces in a dimer of dimers (Fig. 6B) one interface may be significantly more stable than the other, resulting in dissociation to dimers.

We have used two different approaches to investigate the oligomeric state of SecB. In the first approach we showed that SecB in the native state is in a dynamic equilibrium between dimer and tetramer. Two tetrameric species of SecB, one full-length and the other truncated, were mixed. A third tetrameric species containing one wild-type dimer and one dimer of the truncated species formed spontaneously indicating the existence of a dimer-tetramer equilibrium. To detect monomer exchange, the tetramers had to be fully denatured in 6 M urea and then refolded with both species of SecB present. We conclude that SecB is structurally a dimer of dimers and that one of the two interfaces is significantly more stable than the other. If both interfaces were of approximately equal energy of stabilization dissociation at each would occur with equal probability leading to formation of tetramers with all possible combination of subunits as was seen with monomer exchange. In the second approach, tetrameric wild-type SecB was induced to dissociate irreversibly by treatment with DTNB, which forms adducts with cysteine residues. The only species observed after prolonged treatment were dimers. Thus there must be at least one cysteine at the interface of the dimers. Because the SecB dimer created by modification of cysteine did not exchange monomers, we conclude that the interface destabilized by DTNB modification is the same interface that dissociates spontaneously.

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