Mapping of the Binding Frame for the Chaperone SecB within a Natural Ligand, Galactose-binding Protein*

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The chaperone SecB selectively binds polypeptides that are in a non-native state; however, the details of the interaction between SecB and its ligands are unknown. As a step in elucidation of the molecular mechanism of binding, we have mapped the region of a physiologic ligand (galactose-binding protein) that is in contact with SecB. The binding frame comprises 160 aminoacyl residues and is located in the central portion of the primary sequence. Comparison to the binding frame within maltose-binding protein, which is similarly long and positioned around the center of that polypeptide, reveals no similarity in sequence or in folding motif. The results are consistent with the proposal that the selectivity in binding exhibited by SecB is based on the simultaneous occupancy of multiple binding sites, each of which demonstrates low specificity, by flexible stretches of polypeptide that are only accessible in non-native proteins.

SecB is a molecular chaperone in Escherichia coli dedicated to facilitation of the export of newly synthesized proteins from the cytoplasm to their final destination in the periplasmic space or in the outer membrane (Kumamoto and Beckwith, 1985; Collier, 1993). For the polypeptides to be transferred through the cytoplasmic membrane, they must be in a competent state that can be described as neither folded into a stable structure nor aggregated. Binding by SecB to polypeptides either during or shortly after completion of their synthesis maintains them in this competent state (Randall and Hardy, 1986; Kumamoto and Gannon, 1988; Kumamoto, 1989; Liu et al., 1989; Weiss and Bassford, 1990). As is true for all molecular chaperones studied, SecB binds its ligands with high selectivity for the non-native state, but with low specificity (Randall and Hardy, 1995). The binding of several ligands to SecB has been characterized, and it is known that, even though the binding affinity is high, the ligand is in rapid equilibrium between the free and bound state (Khisty and Randall, 1995; Randall and Hardy, 1995). It is of great interest to understand the structural basis of the interactions that provide the energy for the observed high affinity in the absence of any consensus in sequence among the ligands. To this end, we previously mapped the regions that are directly bound to SecB within one physiologic ligand, maltose-binding protein. A binding frame was identified that consisted of multiple contiguous sites positioned around the center of the primary sequence and covering 170 residues (Topping and Randall, 1994). Here, we have determined the binding frame within a second physiologic ligand, galactose-binding protein. Although there is no obvious sequence similarity between the two ligands, the binding frame for SecB within each polypeptide is approximately the same length, and each is poised around the center of the primary sequence.

EXPERIMENTAL PROCEDURES

Materials—Guaniidine chloride (ultrapure) was purchased from Schwarz/Mann, and HEPES and proteinase K were from Sigma.

Purification of Proteins—The mature form of galactose-binding protein, the product of the mgb gene, was purified from E. coli strain NM303 (F“ mgf503 lacZ redA1) harboring plasmid pSF5 (Careaga and Falke, 1992), which contains the gene for galactose-binding protein under its natural promoter. Cells were grown for 3 h at 35 °C in tryptone broth containing 50 μg/ml ampicillin to maintain the plasmid and in 1.0 mM α-D-fucose to induce maximal expression of galactose-binding protein. Galactose-binding protein was purified from proteins that were released from cells by a standard procedure for osmotic shock (Heppel, 1971; Kellermann and Ferenc, 1982) by ion-exchange chromatography using a Q-Sepharose column (Pharmacia Biotech Inc.) and a linear gradient from 0 to 0.1 M NaCl. The fractions that contained galactose-binding protein were pooled, and the protein was concentrated using an Amicon ultrafiltration cell with a YM-10 membrane. A portion of the protein was removed and stored frozen in its native state. The remainder was denatured, and residual bound galactose and Ca2+ were removed by the addition of guanidinium chloride (GdmCl)1 to 3 M followed by extensive dialysis. Each dialysis was carried out against 100 volumes of buffer for >8 h at 4 °C as follows: twice against 3 M GdmCl, 150 mM potassium acetate, 10 mM HEPES, pH 7.6, and then twice against 1 M GdmCl, 150 mM potassium acetate, 10 mM HEPES, pH 7.6. The protein was stored at -70 °C in the final dialysis buffer. The galactose-binding protein obtained was >99% pure as determined by SDS-polyacrylamide gel electrophoresis (Randall and Hardy, 1977). Typically, 15 mg of galactose-binding protein was obtained from 1 liter of culture. SecB was purified as described (Randall et al., 1990).

The concentrations of the proteins were determined by absorbance at 280 nm using extinction coefficients for the SecB monomer and galactose-binding protein of 11,900 and 37,700 M-1 cm-1, respectively. The extinction coefficients were determined by measuring the absorbance at 280 nm of protein preparations that were also submitted for determination of amino acid composition.

Proteolysis of the Complex between SecB and Galactose-binding Protein—Complexes between SecB and galactose-binding protein were formed one of two ways as follows. 1) Denatured galactose-binding protein in 1 M GdmCl, 150 mM potassium acetate, 10 mM HEPES, pH 7.6, was diluted into a solution containing SecB; or 2) denatured galactose-binding protein was diluted into a solution without SecB, and SecB was added within 10 s. In both cases, the solutions were held on ice, and the final concentrations were as follows: 37 μM SecB tetramer, 18 μM galactose-binding protein, 50 mM GdmCl, 100 mM NaCl, 7.5 mM potassium acetate, 50 mM Tris-HCl, 1.5 mM HEPES, pH 7.0. After incubation on ice for 10 min, proteinase K was added to 0.01 mg/ml. After an additional incubation for 10 min on ice, phenylmethylsulfonyl fluoride was added.

* The abbreviations used are: GdmCl, guanidinium chloride; HPLC, high performance liquid chromatography.

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was added to 1 mM to stop proteolysis.

Formation of a Complex between SecB and Proteolyzed Galactose-binding Protein—Denatured galactose-binding protein in 1 M GdmCl, 150 mM potassium acetate, 10 mM HEPES, pH 7.6, was diluted into a solution containing proteinase K at 0.005 mg/ml. After a 5-min incubation on ice, phenylmethylsulfonyl fluoride was added to 1 mM to stop proteolysis; subsequently, the SecB tetramer was added to a final concentration of 37 μM, and the mixture was incubated for 10 min on ice before further analysis. The final concentrations of the salts were as given above in “Proteolysis of the Complex between SecB and Galactose-binding Protein.”

Isolation and Analysis of Fragments of Galactose-binding Protein in Complex with SecB—The proteolyzed mixtures were analyzed as described previously (Topping and Randall, 1994) with minor modifications. One-third of the proteolyzed sample was used for analysis of the complete mixture of peptides generated from galactose-binding protein (termed total). The remaining two-thirds of the proteolyzed sample was subjected to high performance liquid chromatography (HPLC) on a TSK3000 SW size-exclusion column (TosoHaas) to separate the bound and free peptides. Chromatography was carried out at 10 °C in 100 mM NaCl, 50 mM Tris-HCl, pH 7.0, at a flow rate of 1 ml/min. The complex comprising SecB with fragments of galactose-binding protein bound eluted between 13.5 and 16.0 min. These fractions were pooled, and the peptides they contained are referred to as “bound peptides.” Analysis of a portion of each fraction by gel electrophoresis indicated that free peptides were eluted between 19 and 26 min. However, to ensure
complete recovery of all peptides, the fractions from 16.5 to 30 min were pooled, and the peptides therein are referred to as “free peptides.” The total, bound, and free peptides were processed for analysis by reversed-phase HPLC using a Vydac C4 column at room temperature. To expose all peptides to the same conditions, the unfractonated sample was diluted 7.5-fold with the buffer used for chromatography on the TSK3000 SW column to mimic passage through the column. The following additions were made to the total and to the bound peptide samples at room temperature to disrupt the complex between SecB and peptides and to precipitate SecB: solid GdmCl to 3 M, acetonitrile to 20%, and trifluoroacetic acid to give a pH of 2.0. The pool of free peptides was lyophilized and suspended in 18% acetonitrile, 0.1% trifluoroacetic acid. Each of the three samples (total, bound, and free) was clarified by centrifugation (10,000 × g for 15 min) before application to the C4 column. After sample application and washing the salts through with 18% acetonitrile in water containing 0.1% trifluoroacetic acid, a gradient of acetonitrile in water containing 0.1% trifluoroacetic acid was developed from 18 to 54% over 70 ml at a flow rate of 1 ml/min. Fractions of 1.5 ml were collected and taken to dryness in a SpeedVac apparatus (Savant Instruments, Inc.).

Polyacrylamide Gel Electrophoresis of Peptides and Determination of Amino Acid Sequence—The fragments of galactose-binding protein present in each fraction eluted from the HPLC C4 column were resolved using the peptide gel system described previously (Schägger and von Jagow, 1987; Topping and Randall, 1994). When the gel was run with the intention of determining the sequence of amino acid residues, 0.1 mM merthiolate was added to the running buffer, and the peptides were transferred to an Immobilon P 3Q polyvinylidene difluoride membrane (Millipore Corp.). The Coomassie Blue-stained bands were excised, and the sequence of the first 6–8 aminoacyl residues was determined using an Applied Biosystems 475A sequencing system with pulsed liquid update.

RESULTS

Galactose-binding protein is one of numerous proteins in E. coli that depend on SecB for efficient export to the periplasm (Powers and Randall, 1995). As was shown previously for the interaction of SecB with another of its natural ligands, periplasmic maltose-binding protein, SecB forms a complex with galactose-binding protein only if the protein is presented to SecB in a non-native state. Analysis by size-exclusion HPLC of a mixture of SecB and galactose-binding protein that was folded into its native state showed no detectable complex. The two proteins were well resolved from each other, with SecB and folded galactose-binding protein eluting at the positions characteristic of the free proteins, 14.5 min for SecB and 20 min for galactose-binding protein (Fig. 1, compare A, C, and D). However, if refolding of denatured galactose-binding protein was...
initiated by dilution of the denaturant, guanidinium chloride, in the presence of a 2-fold molar excess of tetrameric SecB, a complex of SecB and galactose-binding protein was formed. All of the galactose-binding protein coeluted with SecB at 13.5 min just ahead of free SecB (Fig. 1B) (the proteins present in each fraction were identified by SDS-gel electrophoresis (data not shown)). The approach we took to determine which portions of galactose-binding protein were directly bound to SecB was to subject complexes to proteolytic digestion and to determine which fragments of galactose-binding protein were protected from degradation and also remained bound to SecB. The same final results were obtained whether the complexes were formed as described above by diluting the denaturant to initiate folding of galactose-binding protein in the presence of SecB or by diluting the denaturant to allow galactose-binding protein to undergo an initial collapse followed within 10 s by the addition of SecB. In each case, the SecB tetramer was present in a 2-fold molar excess so that all of the galactose-binding protein would be in complex. The conditions of proteolysis were chosen such that SecB remained intact and all of the galactose-binding protein was fragmented. The pattern of peptides generated by digestion of galactose-binding protein in complex with SecB was compared with the pattern generated by proteolysis of denatured galactose-binding protein in the absence of SecB. The proteolyzed samples were subjected to reversed-phase HPLC (Fig. 2), and the peptides in each fraction were displayed by electrophoresis (Fig. 3) using a system designed to resolve small peptides (Schägger and von Jagow, 1987; Topping and Randall, 1994). It is clear that, while the peptide patterns are closely related, digestion of the complex results in the appearance of many peptides that are not present when galactose-binding protein is digested alone (Fig. 3). It should be recalled that, under the conditions of proteolysis used, SecB is not degraded, and furthermore, it is removed from the sample by precipitation before analysis of the peptides. Therefore, all of the peptides present should be derived from galactose-binding protein as was shown to be the case by direct determination of the aminoacyl sequence of the peptides as described below. The peptides that were unique to the samples derived from the digested complex represent regions of galactose-binding protein that were in direct contact with SecB and thus protected from proteolysis. To demonstrate that these peptides remained bound to SecB, the proteolyzed mixture was subjected to size-exclusion HPLC. After proteolysis, the complex eluted at 14.5 min, the position of free SecB; however, analysis by gel electrophoresis showed that those fractions containing SecB (eluting from 13.5 to 16 min) also contained fragments of galactose-binding protein. These fractions were pooled, and the peptides therein are referred to as bound, whereas peptides in the pool of fractions eluting between 16.5 and 30 min are referred to as free.

Analyses of the bound and free sets of peptides by reversed-phase HPLC show that the set of bound peptides does account for the peptides that are unique to the complex (compare Figs. 2B and 4 and Figs. 3 and 5). The pattern of the peptides recovered free from SecB is similar to that obtained when galactose-binding protein in the presence of a 2-fold molar excess of tetrameric SecB, a complex of SecB and galactose-binding protein was digested alone. This information, together with molecular weights estimated from the position of migration on peptide gels, allowed us to position the peptides within the sequence of galactose-binding protein (Fig. 6 and Table I). The peptides recovered in the bound fraction cover approximately half of the primary sequence and are poised around the center of the sequence. The set of peptides recovered as free contains representatives of all regions of the sequence. Comparison of the relative recovery of material from the bound fraction shows that the region of low recovery among the population of free peptides from aminoacyl residue 120 to residue 200 corresponds to the region represented by the peptides recovered as free (see Table I for details).

The shortest of the peptides recovered as bound after proteolysis of the complex that had been formed between SecB and full-length galactose-binding protein had a M, of ~6000 (Table 1). To determine whether SecB could bind tightly to fragments of this length that were presented directly to SecB, free galactose-binding protein was proteolyzed first, and then SecB was added to the heterogeneous mixture of peptides. Conditions of proteolysis were chosen (protease K at 0.005 mg/ml, 5 min on ice) so that a wide range of peptide lengths would be present. SecB was added in a 2-fold molar excess calculated based on the amount of intact galactose-binding protein initially present to ensure maximal binding of the fragments, and the mixture
| M_r  | HPLC fraction | Position of amino termini of peptides within sequence of GBP<sup>a</sup> | Proteolyzed complex | Free<sup>d</sup> | Proteolyzed GBP, total |
|------|---------------|-------------------------------------------------------------|----------------------|-----------------|-----------------------|
|      |               | Total<sup>b</sup> | Bound<sup>c</sup> |                      |                       |
| 1300 | 16 p          | 217, 222         | p                    | 56, 67           |
| 1500 | 26, 27 p      | 277, 291, 281, 283 | p                    | 84, 285          |
| 1600 | 29, 30 217, 221, 223 | 217, 221, 223 | p                    | 217, 223         |
| 1700 | 12 p          | 281, 283         | p                    | 223             |
| 1800 | 5, 6 p        | 122, 262, 266    | p                    |                 |
| 1800 | 23 p          | 191, 217, 223    | p                    |                 |
| 2100 | 11 p          | 9, 11, 13, 143   | p                    |                 |
| 2200 | 14, 15 p      | 9, 11, 13, 143   | p                    |                 |
| 2200 | 34, 35 217, 223 | 9, 11, 13, 143  | p                    |                 |
| 2500 | 25 9, 11, 13, 143 | 9, 11, 13, 143  | p                    |                 |
| 2500 | 24 p          | 11              | p                    |                 |
| 2500 | 27 p          | 11, 217, 223     | p                    | 11, 217, 223    |
| 2500 | 10, 11 270, 272, 273, 277, 285 | 270, 272, 273, 277, 285 | p |                 |
| 2600 | 16 p          | 8, 217, 223     | p                    |                 |
| 2600 | 19 p          | 8, 256, 266     | p                    |                 |
| 2700 | 13             | 64, 84, 266     | p                    |                 |
| 2900 | 28 1          | 84, 128         | p                    |                 |
| 3200 | 35 217, 223   | p*              | p                    |                 |
| 3300 | 19, 20 p      | 56, 64, 252     | p                    |                 |
| 3600 | 27 p          | 9, 11, 38, 41   | p                    |                 |
| 3600 | 29, 30 p      | 237             | p                    |                 |
| 4000 | 28 p          | 127, 245        | p                    |                 |
| 4000 | 18              | 64, 84         | p                    |                 |
| 4000 | 22, 23 p*      | 64, 84         | p                    |                 |
| 4100 | 34, 35 203    | p*              | p                    |                 |
| 4500 | 21 63, 64     | 64             | p                    |                 |
| 5400 | 33 217, 221, 223 | 217, 221, 223 | p                    |                 |
| 5500 | 18, 19 p      | 62, 67         | p                    |                 |
| 5500 | 16 p          | 74, 76, 223     | p                    |                 |
| 5700 | 26 p          | 120             | p*                   |                 |
| 6000 | 28 p          | 38             | p                    |                 |
| 6000 | 21 64        | 56             | p                    |                 |
| 6000 | 23, 24 64    | 64             | p                    |                 |
| 6000 | 20 p          | 64, 93         | 56, 64               |
| 6400 | 27 p          | 120             | p                    |                 |
| 8000 | 20 p          | 64, 67, 68     | 38                   |
| 8500 | 28 p          | 106            | p                    |                 |
| 8600 | 27 p          | 38             | p                    |                 |
| 9400 | 26 p          | 38             | p                    |                 |
| 10,000 | 22 p        | 64             | p                    |                 |
| 10,000 | 21 p        | 64, 84        | 64, 84               |
| 13,000 | 21 p        | 64, 67        | 64, 84               |
| 13,500 | 23 p        | 64             | p*                   |                 |
| 13,500 | 27 84, 90, 92 | 64, 84        | p                    |                 |
| 14,000 | 22 64, 67   | 64             | p                    |                 |
| 14,500 | 24 64       | 64             | p                    |                 |
| 14,500 | 26 64, 84, 90 | 64, 83, 92, 101 | p*               |                 |
| 15,000 | 24 56, 64  | 64             | p*                   |                 |
| 15,000 | 24 56, 64  | 64             | p*                   |                 |
| 15,000 | 27 64, 74, 76, 84 | 64, 74, 76, 84 | p*              |                 |
| 16,000 | 26, 27 64, 67, 68, 74, 76, 84 | 64, 67        | p                    |                 |
| 16,500 | 28–32 38, 64, 67 | 64, 67        | p                    |                 |
| 17,000 | 24 p*        | 64             | p                    |                 |
| 17,000 | 28–32 64, 67 | 64, 67        | p                    |                 |
| 18,000 | 26 p         | 56, 76         | p                    |                 |
| 19,000 | 28 38, 56, 64 | 56, 64        | p                    |                 |
| 19,000 | 27 56, 64   | 56, 64        | p                    |                 |
| 21,000 | 28, 29 38     | 38             | p                    |                 |
| 27,000 | 30 p         | 64             | p                    |                 |

<sup>a</sup> GBP, galactose-binding protein. Bands that were present at a given position but were not sequenced are represented by p; bands that were submitted for sequencing but were present in too low a quantity to determine the sequence are represented by p*. A blank space means that no peptide was detected in that position.

<sup>b</sup> These results are from two separate experiments. The positions of the amino termini obtained from one experiment are given in Roman type, and those from the other in italic type. When the same amino termini were obtained from the two separate experiments, the result is given in boldface type.

<sup>c</sup> Peptides recovered bound to SecB are represented by closed boxes in Fig. 6.

<sup>d</sup> Peptides recovered free from SecB are represented by open boxes in Fig. 6.
was subjected to size-exclusion HPLC. Each pool of peptides (total, bound, and free) was analyzed by reversed-phase chromatography (Fig. 8), and the peptides in each fraction were resolved by gel electrophoresis (Fig. 9). It is clear that fragments having $M_r$ values of <6200 were recovered quantitatively in the free pool of peptides, and only those fragments with a minimal length of between 55 and 80 residues were bound with sufficiently high affinity to be isolated in complex with SecB. There were peptides among those isolated as free that are longer than the length defined as minimal for binding.

It may be that these fragments were long enough to acquire structure that they rapidly aggregated and thereby were not able to interact with SecB.

**DISCUSSION**

Binding that involves recognition of non-native structure is the hallmark of the class of proteins termed chaperones. We are investigating complexes between SecB and its ligands in an attempt to understand the molecular basis of such interaction. The defining characteristic of the binding is that it occurs with low specificity, but when the ligand is a long polypeptide, the affinity is high (dissociation constants are in the range of 1–100 nM (Randall, 1992)). A model based on studies with synthetic peptide ligands attributes the high affinity to multiple binding sites on SecB. In the case of maltose-binding protein, the smallest peptides found bound after column chromatography were 25 residues long.

The binding frame for SecB within gelactose-binding protein and that within maltose-binding protein are not only similar in length (150 residues for galactose-binding protein and 170 residues for maltose-binding protein), but both are similarly positioned in the center of the primary sequence of each polypeptide. Comparisons of the two polypeptides with respect to aminoacyl sequence and to structure provide no clue as to why the binding frame covers the central portion of each polypeptide. Sequence analysis using the COMPARE program with the SIMPLIFY algorithm showed no significant sequence similarity within the binding frames. Furthermore, a comparison of...
the distribution of charges along the polypeptides did not reveal any common pattern (Randall and Hardy, 1995). The two proteins do have similar tertiary structures. Each contains two domains that comprise central cores of β-sheets flanked by α-helices. Thus, one might think that SecB recognizes a specific array of secondary structure in an intermediate along the folding pathway. However, the proteins display different connectivities between the elements of secondary structure. The stretches of amino acids covered by the binding frames, although located similarly along the linear sequences, are disposed differently among the elements of secondary structure (Fig. 10). Thus, it is not clear why SecB binds to the middle portion of the polypeptides. As suggested previously (Topping and Randall, 1994), the binding frame might lie at the center of the polypeptide ligand simply because the probability of multiple interactions resulting in tight binding would be higher if potential binding sites existed on each side of the first site of contact, a situation that would not occur if the initial contact were at either end.

The large size of the binding frame, covering ~50% of each of the physiologic ligands studied to date, may be crucial to the function of SecB as a chaperone. SecB facilitates export of polypeptides through the cytoplasmic membrane into the periplasm by binding the polypeptides before they can either fold into their thermodynamically stable state or aggregate. Since both folding and aggregation are rapid, the rate at which SecB binds its ligands must be high. As discussed previously (Randall and Hardy, 1995), SecB could have a rate constant of association that is $10^8 \text{ M}^{-1} \text{s}^{-1}$ or even higher since the target...
for collisions with SecB that lead to binding covers as much as 50% of the surface of the ligand. The ability to bind non-native ligands rapidly and selectively with high affinity is common among chaperones. It is likely that chaperones other than SecB also make use of multiple binding sites, each having a low specificity, and have large overall binding frames on the ligands to achieve rapid and high affinity binding in the absence of consensus in sequence among the polypeptides bound.

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