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Determination of the binding frame within a physiological ligand for the chaperone SecB

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Abstract

The hallmark of the class of proteins called chaperones is the amazing ability to bind tightly to a wide array of polypeptide ligands that have no consensus in sequence; chaperones recognize non-native structure. As a step in the elucidation of the molecular mechanism of such remarkable binding, we have characterized complexes between the bacterial chaperone SecB and a series of ligands related to maltose-binding protein. SecB interacts at multiple sites on its polypeptide ligand. The entire binding region covers approximately half of the primary sequence of maltose-binding protein and comprises contiguous sites positioned around the center of the sequence.

Keywords: binding; chaperone; maltose-binding protein; protein export; SecB

SecB, a tetrameric molecular chaperone in *Escherichia coli*, binds precursors destined for export and maintains them in a loosely folded, unaggregated state that is competent for translocation across the cytoplasmic membrane (Randall & Hardy, 1986; Kumamoto & Gannon, 1988; Liu et al., 1989; Weiss & Bassford, 1990). Although the natural ligands of SecB are synthesized as precursors containing amino-terminal leader peptides, these leaders are not specifically recognized and bound by SecB (Gannon et al., 1989; Lecker et al., 1989; Liu et al., 1989; Weiss & Bassford, 1990; De Cock et al., 1992). The leader modulates the folding to expose elements in the remainder of the polypeptide that are bound (Park et al., 1988). There is no consensus in sequence among the ligands. Existing evidence supports the idea that selectivity in binding is governed in part by a kinetic partitioning between folding of the polypeptide and association with SecB (Hardy & Randall, 1991). It has been shown that non-native maltose-binding protein without a leader can bind SecB if its folding is slowed down either by decreasing the temperature (in vitro) or by single amino-acyl substitutions that decrease the rate of folding (both in vitro and in vivo) (Liu et al., 1988; Randall et al., 1990). Thus, SecB can bind tightly to a site that is entirely contained within the mature species. Here we characterize the binding interactions in complexes between SecB and a series of related ligands: the precursor and mature forms of wild-type maltose-binding protein and a precursor and a mature form of slow-folding variants of maltose-binding protein. In all cases, SecB binds to the middle region of the polypeptide ligand in what appears to be the same binding frame.

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Results

We determined what portions of mature maltose-binding protein are bound directly to SecB by subjecting the complex to proteolytic digestion and analyzing the fragments of the maltose-binding protein that were protected from degradation and remained bound. The complex was formed by first unfolding maltose-binding protein in 2 M guanidinium chloride (GuHCl) and then rapidly diluting the denaturant in the presence of SecB (Randall et al., 1990) or by diluting the denaturant first and adding the SecB within 5 s. In one instance, the unfolded maltose-binding protein would undergo the transition to the collapsed state in the presence of SecB and in the other, which may more closely mimic the interaction as it would occur in vivo, collapse would precede addition of SecB. The results obtained for the 2 cases were indistinguishable. The SecB tetramer was present in excess so that all of the maltose-binding protein would be bound. The amount of proteinase K added (0.015 mg/mL) and the time of incubation on ice (10 min) were determined such that all of the maltose-binding protein was cleaved and the SecB remained intact. After incubation of the complex with proteinase K, a portion of the sample was reserved for analysis of the total peptides present and the remainder was applied to a Superose 12 sizing column to separate free peptides from those bound to SecB. The peptides in each of the 3 sets—total, bound, and free—were resolved by reversed-phase HPLC (Fig. 1) followed by electrophoresis of each fraction (Fig. 2) using a peptide gel system. The sequence of the first 5 or 6 amino-acyl residues was determined for all material recovered in sufficient quantity to allow analysis (28 of the 46 bands; see Tables 1, 2). This information, in combination with molecular weights calculated from the positions of migration on the peptide gel, allowed us to position the peptides within the sequence of maltose-binding protein. It is

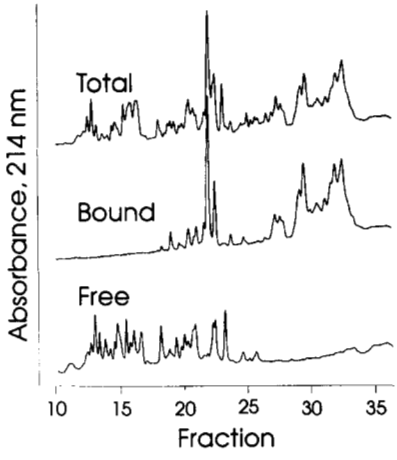

Fig. 1. Analysis of total, bound, and free peptides by HPLC. The chromatograms shown are profiles of peptides generated by proteolysis of a complex between SecB and wild-type mature maltose-binding protein as described in the Materials and methods. Only the portion of the chromatograms that contain peptides is shown. Fractions are 1.5 mL.

striking that the peptides recovered as bound were all derived from the middle region of maltose-binding protein (Fig. 3). As a set, the peptides define a binding frame that covers approximately half of the linear sequence. The peptides that were recovered as free, i.e., not bound to SecB, were derived from 5 regions, 4 of which lie within the binding frame. Each peptide derived from a region within the binding frame was closely related to, but smaller than, peptides recovered bound to SecB. The decrease in length might have decreased the affinity, resulting in dissociation during fractionation on the column. The other region, which lies outside the binding frame, was represented among the free peptides by 2 fragments with amino-terminal residues at positions 11 and 13. We do not believe that those peptides are present because they were protected from proteolysis by association with SecB, but rather because the region of maltose-binding protein that they represent is inherently more resistant to degradation by proteinase K than are other regions. This interpretation is supported by the observation that when non-native maltose-binding protein was incubated with proteinase K under the same conditions as used for the complex (10 min on ice) but in the absence of SecB, proteolysis was nearly complete, yet the peptides from positions 11 and 13 were among the few peptides recovered (usually 5–8 peptides).

The portion of maltose-binding protein from residue 260 to the end was not represented among the peptides sequenced. It is likely that those regions were not directly in contact with SecB or were only loosely associated so that they were completely degraded. We demonstrated that peptides derived from the unrepresented regions would have been detected in our analysis had they been present, by decreasing the time of incubation of non-native maltose-binding protein with the protease to 1 min so that the extent of proteolysis was similar to that seen with the complex. After resolution by HPLC and electrophoresis, sequence was determined for all peptides present in sufficient quantities (11 of the 35 ranging in size from 750 to 17,000 Da). In contrast to the population of peptides recovered as bound to SecB (Fig. 4, upper panel), the population of peptides derived from free maltose-binding protein contains representatives from all regions of the polypeptide (Fig. 4, lower panel). Comparison of the relative recovery of peptides from along the sequence shows that those from the amino- and carboxyl-terminal regions are present in higher amounts than those derived from the middle region. This observation not only indicates that we can detect peptides from the ends but also reinforces the interpretation that peptides derived from the middle region and recovered in the analysis were present because they were protected from proteolysis and not because they were inherently resistant to degradation.

Earlier work led to the conclusion that in vivo the leader peptide present in precursor maltose-binding protein plays an essential but indirect role in binding to SecB by retarding folding of the polypeptide and exposing binding sites that lie within the mature region (Liu et al., 1989). If this is true, the binding frame for precursor maltose-binding protein should be the same as that described for the mature protein. Analysis of a complex between the precursor form and SecB demonstrated that this is the case. All 3 sets of peptides—total, bound, and free—generated by proteolysis of the complex showed patterns very similar to the patterns of the corresponding peptides from the complex with the mature protein. Figure 5 shows the absorbance profile from the reversed-phase chromatography of the bound peptides (compare to Fig. 1, Bound), and Figure 6 shows the peptides present in the total digest as displayed by gel electrophoresis (compare to Fig. 2, Total). All peptides present in each of the patterns from mature were also present in the analysis of the precursor. The only observable difference was the presence in the total digest of precursor in complex with SecB of 2 bands (Fig. 6; indicated by arrows) that were not detected among the peptides derived from the complex with mature protein. We were not able to determine whether this material came from the leader or the mature region because only the first 3 cycles of sequencing yielded any amino acids, and in each cycle 3 different residues were equally present. Nonetheless it is likely that the peptides were derived from the leader because they appeared in the 4 analyses of precursor species and were not present in the 5 analyses of the complex formed with mature species. These peptides were recovered in the free fraction but were not recovered bound to SecB. It is possible that they were bound originally but do not bind tightly or that they are inherently resistant to proteolysis as was discussed for the peptides recovered in the free fraction derived from the complex with mature maltose-binding protein. Clearly even if they were bound, interaction with the region of the polypeptide that they represent does not change the overall binding frame.

The ability of variants of maltose-binding protein to form stable complexes with SecB under conditions in which the wild-type mature species does not has been attributed to a decrease in their rates of folding rather than to any effect of the amino-acyl changes on the true affinity for SecB (Liu et al., 1989). Analyses of complexes formed with 2 slow-folding variants, the mature form of a species with a tyrosine substituted for aspartate at position 283 (MalE Y283D; Liu et al., 1988) and the precursor form of a variant that has alanine substituted for glycine at position 276 (MalE A276G; Chun et al., 1993), show that SecB binds each of these species with binding frames indistinguishable from that characteristic of the wild-type mature and precursor species (see Figs. 1 and 5 for a comparison of the bound-peptide patterns). It is of particular interest that both of the substitutions, which affect the ability to form complexes, lie outside of

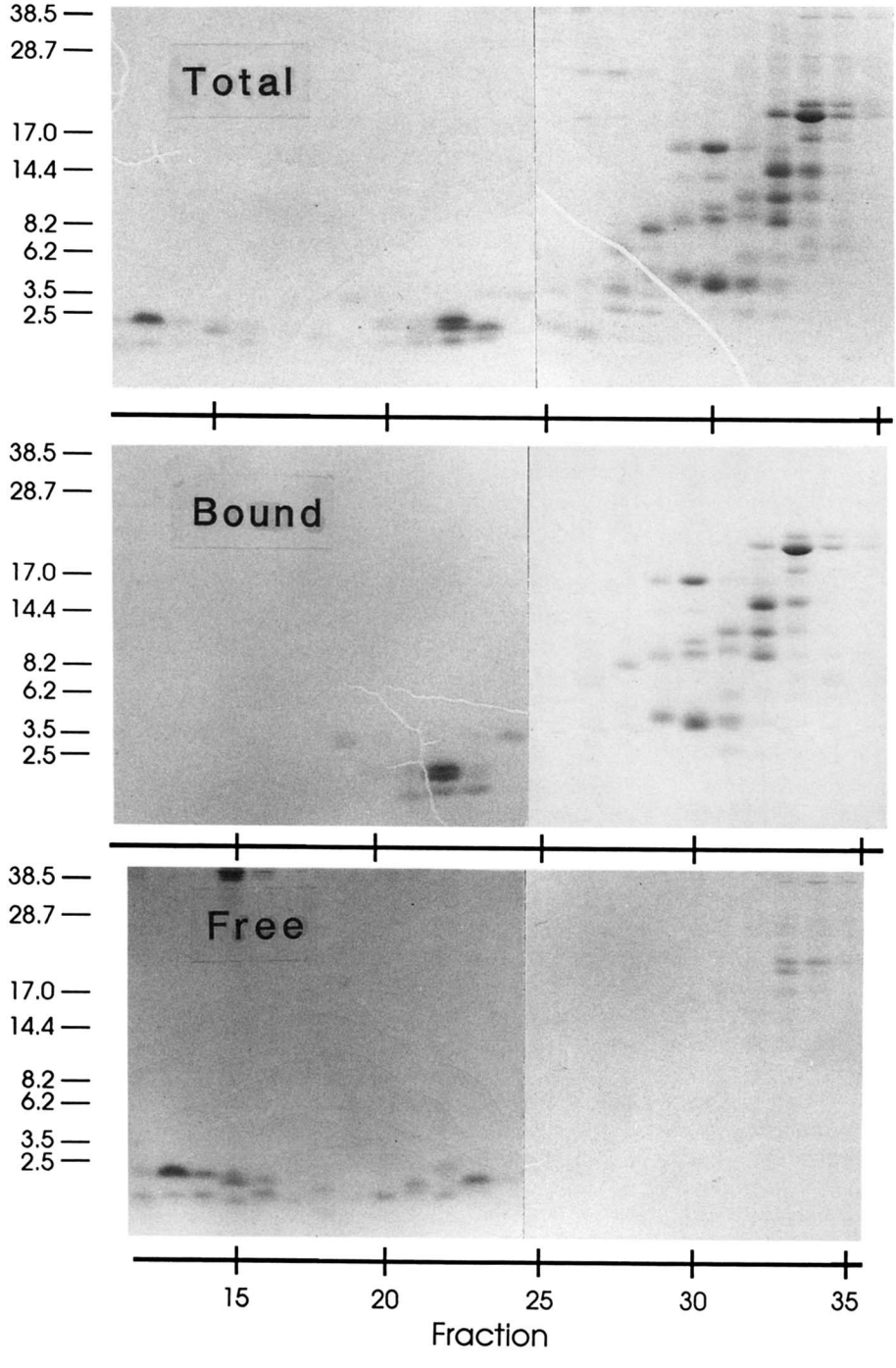

Fig. 2. Total, bound, and free peptides displayed by PAGE. Fractions of 1.5 mL from the HPLC chromatography are shown. Molecular weight markers were mature maltose-binding protein (38.5 kDa), carbonic anhydrase (28.7 kDa), myoglobin (17.0 kDa), myoglobin fragments generated by cyanogen bromide cleavage (14.4 kDa, 8.2 kDa, 6.2 kDa, 2.5 kDa; Sigma), and glucagon (3.5 kDa).

the binding frame itself. This observation strongly reinforces the interpretation that the differential ability of the wild type and the variants to bind SecB is the result of their altered rates of folding and not of changes in binding energy.

Discussion

A model for interaction of SecB and ligands based on studies of peptide binding (Randall, 1992) proposes that the tetramer of SecB has multiple sites that bind flexible, positively charged stretches of polypeptide approximately 15 amino-acyl residues in length. Saturation of these binding sites induces a conformational change and a hydrophobic site is exposed. This exposed hydrophobic site is proposed to interact with the hydrophobic regions of the non-native polypeptide ligand. The binding frame within the sequence of maltose-binding protein described here is consistent with this scheme of interaction. The data indicate that at least 3 stretches of the polypeptide ligand bind independently to SecB because nonoverlapping peptides of approximately 20–25 residues in length were recovered. The peptides

Table 1. *Peptides recovered bound to SecB*[a]

Position of N-terminus	Molecular weight	HPLC fraction
53	15,000	32
54	3,200	27
54	15,000	32
73	4,000	30
77	3,000	31
77	4,000	30
77	8,500	32
77	11,000	32
77	15,000	32
77	20,000	33
78	3,000	31
78	4,000	30
78	8,500	32
78	11,000	32
78	15,000	32
78	20,000	33
114	8,000	28
114	16,000	30
116	8,000	28
116	16,000	30
118	16,000	30
145	4,000	30
147	4,000	30
150	3,800	27
150	8,200	29
152	3,800	27
152	8,200	29
194	3,600	24
195	3,600	24
214	2,500	22
216	2,500	22
217	2,400	22
224	2,200	22
226	2,200	22
226	2,200	24
228	2,200	22

[a] These peptides are represented by closed bars in Figure 3.

Table 2. *Peptides recovered free from SecB*[a]

Position of N-terminus	Molecular weight	HPLC fraction
11	2,400	13
13	2,400	13
74	2,300	26
77	2,300	26
77	2,400	23
77	2,400	25
78	2,300	26
78	2,400	23
78	2,400	25
114	1,600	19
114	1,700	18
114	1,800	16
116	1,700	15
116	1,700	17
116	1,700	20
116	1,800	14
118	1,700	13
118	1,800	14
194	1,700	13
228	1,700	20

[a] These peptides are represented by open bars in Figure 3.

that start at positions 77 and 150 both contain linear sequences of at least 15 amino-acyl residues that have a net positive charge (for sequence of maltose-binding protein, see Duplay et al., 1984). The peptide beginning at 214 contains both positively and negatively charged amino-acyl residues arranged such that there is no continuous stretch of 15 that has a net positive charge. All 3 of the regions defined by the recovered peptides contain hydrophobic sequences between 8 and 10 residues in length. Thus, no striking pattern is apparent, but the distribution of amino-acyl residues within the binding frame is consistent with binding at multiple hydrophilic as well as hydrophobic sites.

As indicated in Figure 3, both the amino- and carboxyl-terminal regions contain stretches of 15 amino-acyl residues that have a net positive charge and might serve as sites of interaction with SecB. However, peptides from these regions are not recovered among the bound peptides. It is possible that those regions are bound weakly and dissociate frequently, resulting in complete digestion by the protease. It is equally possible that SecB selectively binds to the middle of its polypeptide ligand. Perhaps the binding sites within the ligand must have a certain spacing that is optimal for simultaneous occupation of the corresponding sites in SecB, and in maltose-binding protein the sites in the middle are spaced the most favorably. A more intriguing possibility is that SecB selects the middle because the probability of multiple interactions leading to 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 first contact were near either end.

As discussed in the earlier proposal (Randall, 1992), the existence of 2 types of sites with different binding characteristics provides a mechanism for high selectivity even though each site independently exhibits broad specificity. SecB might interact weakly with any flexible loop on a native protein, but tight binding would occur only upon simultaneous occupation of multiple sites by different flexible stretches of a non-native polypeptide so that the hydrophobic site would also be accessible for binding. Exposure of the hydrophobic patch only after a ligand initiates interaction with SecB ensures that uncomplexed SecB will not aggregate.

There is a rapidly growing body of information pertinent to the recognition of non-native structure by chaperones. Chaperones can be divided in 2 broad classes. The members of one class, the chaperonins, are related to the bacterial GroEL, which is a complex of 2 rings of 7 identical subunits each of molecular weight 60,000. Image reconstruction of electron micrographs suggests that ligands bind within a central cavity of the cylindrical complex (Langer et al., 1992). Recognition of non-native structure by these large chaperones may differ fundamentally from recognition of ligands by the smaller chaperones such as SecB and the members of the hsp70 family. Knowledge concerning the binding to several of these chaperones is still too fragmentary to determine whether binding occurs by the mechanism

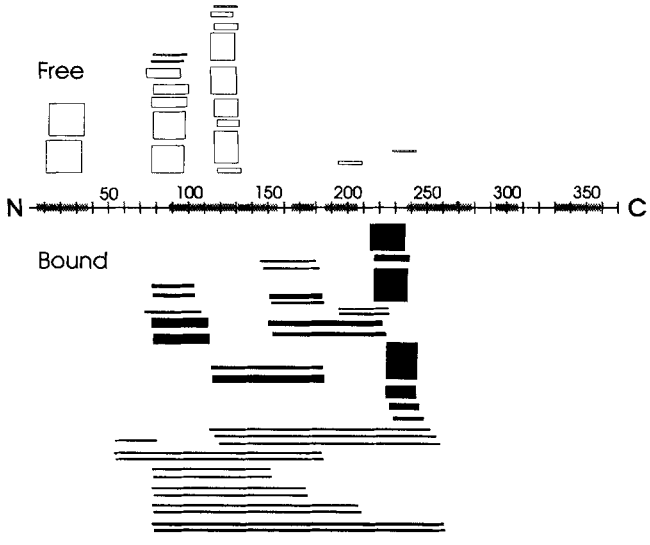

Fig. 3. Representation of the position of the peptides within the linear sequence of maltose-binding protein. Mature maltose-binding protein comprises 370 amino-acyl residues represented by the horizontal line at the center of the figure. All peptides that were sequenced are represented by bars placed at their position within the sequence of maltose-binding protein. The length of each peptide was calculated from its position of migration on peptide gels. The heights of the bars represent the relative recovery of the peptides estimated from the yield of amino acids obtained upon determination of the sequence. The closed bars represent those peptides that remained bound to SecB (see Table 1 for details); the open bars represent peptides that were recovered free from SecB (see Table 2 for details). The shaded areas on the horizontal line that represents the maltose-binding protein primary sequence indicate where positively charged stretches of 15 amino acids can be found.

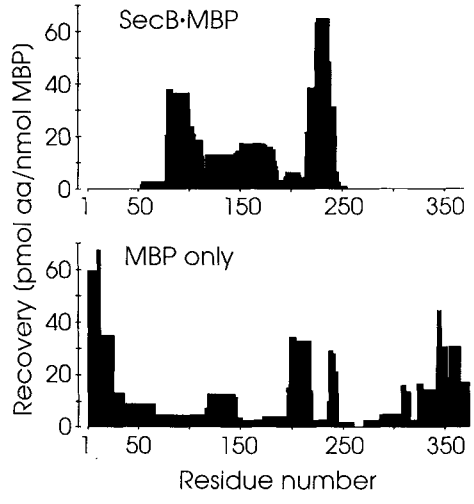

Fig. 4. Peptides generated by proteolysis of free or complexed maltose-binding protein. Maltose-binding protein was digested in complex with SecB (0.015 mg/mL proteinase K; 10 min, on ice) or after dilution from denaturant in the absence of SecB (0.015 mg/mL proteinase K, 8 μM maltose-binding protein, 0.1 M GuHCl, 50 mM Tris-HCl, pH 7.6, 0.1 M NaCl; 1 min, on ice). The sequence and length of each peptide was determined as in Figure 3. The recovery of amino acid residues at each position along the sequence was determined by summing the yield of every peptide that contained that amino-acyl residue.

proposed for SecB. Studies of the interaction of SecB with 2 ligands other than maltose-binding protein, the precursor of the outer membrane protein PhoE (Breukink et al., 1992) and a non-secretory polypeptide, a fragment of a tail fiber protein of phage T4 (MacIntyre et al., 1991), both led to the proposal that binding in the complexes is mediated by β-β interaction through the polypeptide backbone. DnaK, a member of the hsp70 family of chaperones, has been shown to bind a peptide ligand in an extended conformation (Landry et al., 1992). Two eukaryotic members of the hsp70 family have been investigated. BiP selectively interacts with peptides enriched in hydrophobic residues (Flynn et al., 1991), although hydrophilic peptides also bind (Flynn et al., 1989). A conformational change of Hsc70 was observed when decapeptide ligands (Park et al., 1993) were bound. Further studies will determine whether these observations reflect use of related mechanisms for recognition of non-native structure.

Materials and methods

Purification of proteins

Wild-type mature maltose-binding protein, mature MalE Y283D (Chun et al., 1993), and SecB (Randall et al., 1990) were purified as described. Precursor MalE A276G was purified from a SecA^{ts} strain of *E. coli*, MM52 (Oliver & Beckwith, 1981), carrying a deletion in the malE gene (Δ*malE*312; Fikes & Bassford, 1987) and harboring a plasmid that contains *malE A276G*. Growth and purification were as for wild-type precursor maltose-binding protein (Park et al., 1988).

Isolation of fragments of maltose-binding protein in complex with SecB

Maltose-binding protein (6.0 mg/mL) was unfolded in 2 M GuHCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, for 2 h at room temperature. A com-

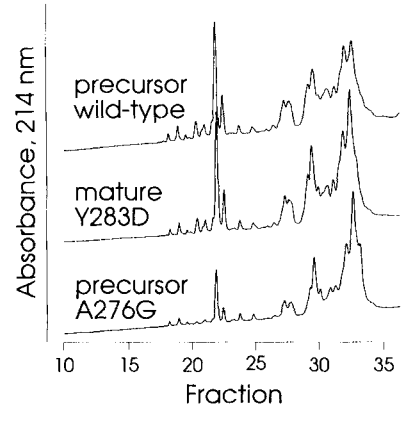

Fig. 5. Determination of the binding frame for variants of maltose-binding protein. Complexes were formed by diluting the denaturant from the unfolded wild-type precursor MalE, mature MalE Y283D, or precursor MalE A276G in the presence of SecB. Digestion, separation on a Superose 12 column, and HPLC were as described in the Materials and methods. The traces represent HPLC chromatograms of peptides isolated as bound.

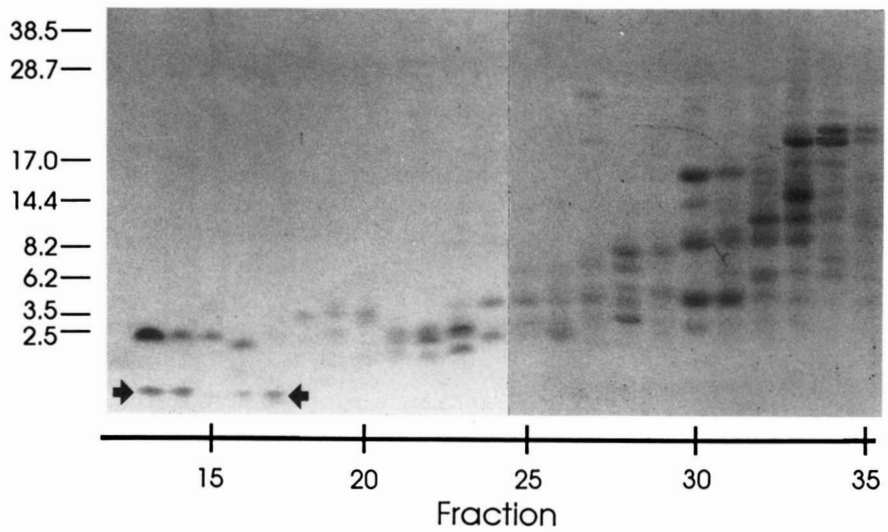

Fig. 6. Peptide pattern generated by proteolysis of precursor wild-type maltose-binding protein in complex with SecB. Following proteolysis, the unfractionated sample (total peptides) was subjected to HPLC chromatography and the peptides contained in the fractions were displayed by PAGE as in Figure 2. The bands mentioned in the text that are particular to precursor maltose-binding protein are indicated by arrows.

plex between SecB and maltose-binding protein was formed by diluting the denaturant from the maltose-binding protein on ice and adding SecB within 5 s. The final concentrations were: 16 μM SecB tetramer, 8 μM maltose-binding protein, 0.1 M GuHCl, 50 mM Tris-HCl, pH 7.6, 0.1 M NaCl. The complex was incubated with 0.015 mg/mL proteinase K on ice; after 10 min, 1 mM phenylmethylsulfonyl fluoride was added to stop digestion. One-third of the digested sample was used for analysis of the complete mixture of maltose-binding protein peptides generated (termed total). The remaining two-thirds of the digested sample was applied to a Superose 12 (Pharmacia) size-exclusion column (Randall et al., 1990) to separate the bound and free peptides. The fractions containing SecB in complex with fragments of maltose-binding protein (termed bound peptides) were pooled and processed for HPLC. The fragments of maltose-binding protein that were not in complex with the SecB (termed free peptides) eluted later from the column and were also processed for HPLC. In order to expose the peptides to identical conditions during preparation for HPLC, the total sample was diluted 7.5-fold with the Superose 12 column buffer to mimic passage through the column. The total and bound samples were treated as follows at room temperature. To disrupt the complex between SecB and the peptides, solid GuHCl was added to 3 M. After a 5-min incubation, acetonitrile was added to 20%, and after a further 5-min incubation, trifluoroacetic acid (TFA) was added to give a pH of 2 (0.2%). The majority of the SecB precipitated during a further 45-min incubation. The SecB precipitate was removed by centrifugation (12,100 × *g* for 15 min) leaving the peptides in the supernatant. The Superose 12 fractions containing the free peptides were taken to dryness in a SpeedVac (Savant). The dried fractions were suspended directly in 18% acetonitrile, 0.1% TFA and pooled, and the sample was clarified by centrifugation (10,000 × *g* for 15 min). Each sample (total, bound, and free peptides) was subjected to HPLC on a Vydac C-4 reversed-phase column. In each case, after the entire sample was applied to the column and the salts washed through, a gradient of acetonitrile from 18% to 54% in water containing 0.1% TFA was developed over 70 mL. Fractions of 1.5 mL were collected and taken to dryness in a SpeedVac.

Polyacrylamide gel electrophoresis and determination of protein sequence

PAGE for resolution of small polypeptides was performed as described (Schägger & von Jagow, 1987) except that the running buffer used at both the cathode and the anode contained 0.5 M Tricine and 0.5 M Tris. The gel system comprised stacking, spacer, and separation gels (15.5% acrylamide, 1% bisacrylamide). When the gel was run with the intention of sequencing the peptides, 0.1 mM thioglycolates were added to the running buffer and the peptides were transferred to a PVDF membrane (Immobilon P or Immobilon P^{SQ}, Millipore Corp.). Coomassie blue-stained bands were excised, and the sequence of aminoacyl residues was determined using an Applied Biosystems 475A sequencing system with pulsed liquid update.

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