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No Specific Recognition of Leader Peptide by SecB, a Chaperone Involved in Protein Export

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Most proteins destined for export from *Escherichia coli* are made as precursors containing amino-terminal leader sequences that are essential for export and that are removed during the process. The initial step in export of a subset of proteins, which includes maltose-binding protein, is binding of the precursor by the molecular chaperone SecB. This work shows directly that SecB binds with high affinity to unfolded maltose-binding protein but does not specifically recognize and bind the leader. Rather, the leader modulates folding to expose elements in the remainder of the polypeptide that are recognized by SecB.

PROTEINS DESTINED FOR EXPORT from the cytoplasm of *Escherichia coli* to the periplasm or outer membrane are made as precursors containing amino-terminal leader sequences that are essential for localization and that are removed during the process. The initial step during export of a subset of outer membrane and periplasmic proteins including the maltose-binding protein is recognition by SecB, an oligomeric cytosolic factor (monomer; M_r, 16,600) (*1–3*). Investigations carried out both in vivo and in vitro indicate that SecB binds to precursor maltose-binding protein at sites that are distinct from the leader (signal) sequence and blocks folding of the precursor, thus maintaining it in an export-competent state (*2–6*). An opposing view, that SecB specifically recognizes the leader sequence, has been presented on the basis of observations of a complex between SecB and precursor maltose-binding protein in vitro and the inability to demonstrate a similar complex between SecB and matured maltose-binding protein (*7*). The resolution of this issue is central to the understanding of initial events during protein localization in prokaryotes, and in eukaryotes as well, since it has been shown that SecB is functionally similar to the mammalian signal recognition particle (*7*).

In the investigations described here we have used purified proteins (*8*) so that the parameters of the system are well defined. Determination of the sequence of the first four amino acyl residues of the purified precursor and matured forms of maltose-binding protein established that each had the authentic amino terminus (*9*). The kinetics of the denaturant-induced, reversible unfolding of these polypeptides has been extensively characterized, allowing us to precisely control the rate of refolding (*6, 10, 11*). The free species of maltose-binding protein (M_r, ~40,000) were resolved from the oligomeric complexes of SecB and maltose-binding protein by gel-filtration chromatography. SecB formed a complex with precursor maltose-binding protein when refolding of the denatured precursor was initiated in the presence of an excess of SecB

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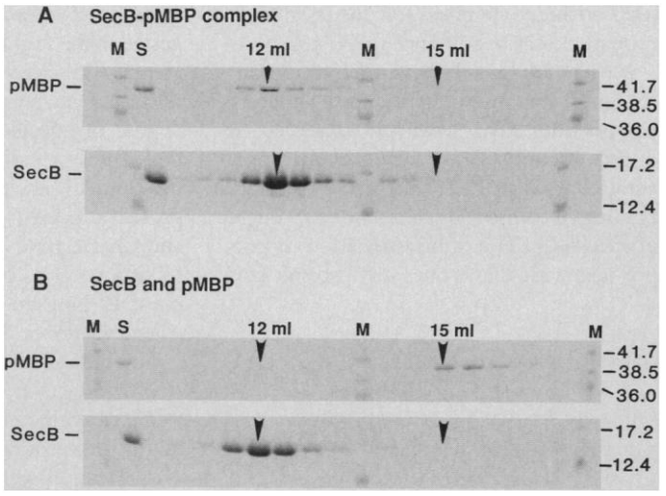

Fig. 1. Binding of precursor maltose-binding protein to SecB. A mixture of precursor maltose-binding protein (pMBP) and SecB was subjected to gel-filtration chromatography on a Pharmacia Superose 12 HR 10/30 column in 50 mM tris-HCl and 0.1 M NaCl (*p*H 7.6), at a flow rate of 0.1 ml/min. SDS–polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.5-ml fractions, starting after elution of 9.5 ml, was carried out. Only the regions of the Coomassie blue–stained gel containing SecB and pMBP are shown. Arrowheads indicate the positions of SecB-MBP complex (left) and free monomeric maltose-binding protein (right). Lanes S contain 20% of the quantity of sample applied to the column. Lanes M contain molecular weight markers (actin, maltose-binding protein, glyceraldehyde 3-phosphate dehydrogenase, myoglobin, and cytochrome c). (**A**) Precursor maltose-binding protein unfolded in 2 M guanidinium hydrochloride (GuHCl) was diluted into chromatography buffer containing an excess of SecB, and the mixture was applied to the column. (**B**) As in (A) except that the SecB was added after the unfolded precursor was allowed to refold by dilution into chromatography buffer and incubation on ice for 5 hours. The concentrations in the samples applied were: pMBP, 0.25 μM; SecB, 5 μM; and GuHCl, 0.1 M.

(Fig. 1A). No complex was detected when the precursor was allowed to refold before SecB was added (Fig. 1B). Since only unfolded precursor formed a stable complex even though the folded precursor carried a full-length leader peptide, we conclude, in agreement with previous investigators (*3, 5, 6*), that SecB interacts with elements that lie outside of the leader peptide in another portion of the precursor. One might argue that SecB does not, in fact, bind to the mature portion of the unfolded precursor, but that, in the folded state of the precursor, the leader is inaccessible for interaction. However, this is not the case, since SecB did form a complex with matured maltose-binding protein that was devoid of a leader, provided, as was also true of precursor, that refolding of the denatured protein was initiated in the presence of SecB (Fig. 2). Thus SecB does directly interact with elements within the mature protein.

Neither these experiments nor the previously published direct demonstration of binding of SecB to unfolded, matured maltose-binding protein (*6*) provides information about the affinity of SecB for matured maltose-binding protein relative to that for precursor maltose-binding protein. Here we show that SecB has similar affinities for the two forms of the protein. Refolding of precursor and matured maltose-binding protein was initiated in solutions in which the quantity of SecB was limiting and the ratio of precursor to matured maltose-binding protein present in the complex was determined (Fig. 3). If SecB were binding to matured maltose-binding protein at sites with very low affinity relative to the affinity for the leader, then the complex would have been enriched for precursor. This was not observed; in fact, the complex was enriched for matured maltose-binding protein. The amount of complex present at a molar ratio of SecB to total maltose-binding protein of 3:1 was 1.75 times greater than the amount of complex observed when the molar ratio was 1.5:1 (compare Fig. 3A and 3B). In both cases, the ratio of matured to precursor maltose-binding protein in the complex was 2.2:1 (*12*). This observation obviates the hypothesis that SecB did bind precursor maltose-binding protein with a much higher affinity than it bound matured maltose-binding protein and that there were two populations of precursor in the sample: one, completely bound, the other, inactive. Were this the case, the ratio of matured maltose-binding protein to precursor in the complex would have increased with the increasing amounts of SecB, in contrast to the observed result.

Information about the affinities is not easily obtained from the relative quantities of polypeptides in the position of uncomplexed matured maltose-binding protein because, although the matured species behaves as a monomer and is completely recovered, the uncomplexed precursor is spread over many fractions and is incompletely recovered (Fig. 3C). When the polypeptides in the mixture were allowed to refold before SecB was added, no complex was detected (*13*).

The results presented here and those presented previously (*6, 10, 11, 14*) are consistent with the hypothesis (*15*) that entrance into the export pathway is not achieved through specific recognition of the leader peptide by a signal recognition factor, but rather that the leader modulates folding to expose elements in another portion of the polypeptide that are recognized by export factors such as SecB. The features that define a binding site for SecB are unknown. SecB facilitates the export of several different proteins, thus it seems likely that the specificity for binding is low; perhaps any extended, flexible portion of a polypeptide chain or any hydrophobic area would provide a site for binding. Such sites might have a low affinity for SecB, so that stable interaction and blockage of folding would be achieved only by simultaneous binding at several different sites. If this were the case, then leader peptides might interact with SecB with the same affinity as any one of multiple internal binding sites. In this model, binding of the leader alone would not result in a stable complex. The data do not indicate that SecB does not bind the leader, but rather that, if the leader is bound, it is not bound in preference to internal sites; in other words the leader is not specifically recognized as a hallmark for export.

In direct conflict with these findings, Watanabe and Blobel (*7*) concluded that SecB is a cytosolic signal recognition factor that specifically binds the leader sequence. They based this conclusion on their inability to detect binding of SecB to matured maltose-binding protein, whereas they observed a complex of SecB and precursor maltose-binding protein. The species of maltose-binding protein used in their study were the products of a cell-free system programmed with a mutated *malE* gene. Many extraneous components were present, thus making it difficult to compare their results with our demonstration of a complex generated with the purified proteins. Nevertheless, it may be relevant that the maltose-binding protein produced in their system was a truncated form. If, as discussed above, the stability of the complex is due to binding at multiple low-affinity sites, it may be that this mutant form of maltose-binding protein is missing one or more of the internal binding sites for SecB. To account for the difference in binding of the precursor and matured forms, which were products of the same mutated gene, we must assume that the leader does provide one of the low-affinity sites.

During export of maltose-binding protein, SecB maintains the precursor in a translocation-competent state by blocking

its folding. Thus SecB can be considered a molecular chaperone, a term used of proteins that, in order to prevent nonproductive interactions, bind to other polypeptides during a wide variety of processes involving folding and assembly (*16*). Other molecular chaperones involved in the export of bacterial proteins are the trigger factor and GroE (*17–19*). Molecular chaperones that function during protein localization in eukaryotic systems include members of the heat-shock protein classes hsp60 and hsp70 (*20*).

Most, perhaps all, molecular chaperones recognize and bind tightly to a variety of polypeptides that have no observed sequence identity. Thus they face the apparently contradictory requirements for stable binding of complex ligands and a low degree of specificity. The tight association of polypeptides with chaperones might result from binding to several low-affinity sites as suggested here for SecB. However, the low-affinity sites cannot be completely nonselective since chaperone proteins do exhibit a degree of specificity. Trigger factor binds the outer membrane protein precursor, proOmpA, but not the outer membrane protein precursor, proPhoE; whereas, SecB and GroEL bind both (*21*). In addition, the folding in vitro of precursor TEM β-lactamase is blocked by GroEL, but not by trigger factor or SecB (*19*).

The mammalian signal recognition particle, which acts in concert with the ribosome to retard synthesis of secretory polypeptides, might be considered a molecular chaperone if its function were to maintain the precursors in conformations compatible with membrane translocation. It has been shown that a signal recognition particle can substitute in vitro for trigger factor and stabilize proOmpA for membrane insertion (*17*). It is widely believed that the signal recognition particle specifically recognizes the signal sequence; however, trigger factor is known to bind to the mature moiety of OmpA (*17*). Signal recognition particle also interacts in vitro with precursor maltose-binding protein to arrest its synthesis. This arrest is released by addition of SecB, which presumably competes with the signal recognition particle for binding sites in the nascent polypeptide (*7*). In the light of these functional similarities it is probable that signal recognition particle, like SecB and trigger factor, interacts with polypeptides at sites additional to that in the signal sequence.

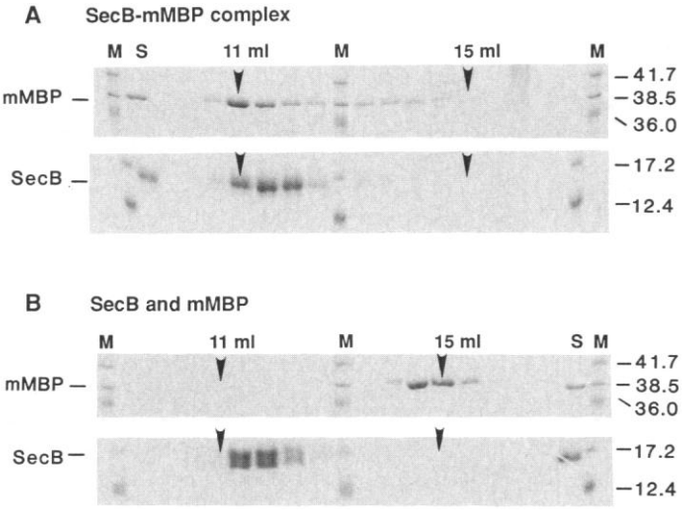

Fig. 2. Binding of matured maltose-binding protein to SecB. The experiment is similar to that described in Fig. 1. Arrowheads indicate the positions of SecB-MBP complex (left) and free monomeric maltose-binding protein (right). (**A**) Matured maltose-binding protein (mMBP) unfolded in 2 M GuHCl was diluted into chromatography buffer containing an excess of SecB. (**B**) As in (A) except that SecB was added after the unfolded maltose-binding protein was diluted into buffer and allowed to refold. The concentrations in the samples applied were: mMBP, 0.28 μM; SecB, 2.6 μM; and GuHCl, 0.06 M. Lanes S contain 15% of the quantity of sample applied to the column. Lanes M are markers as in Fig. 1.

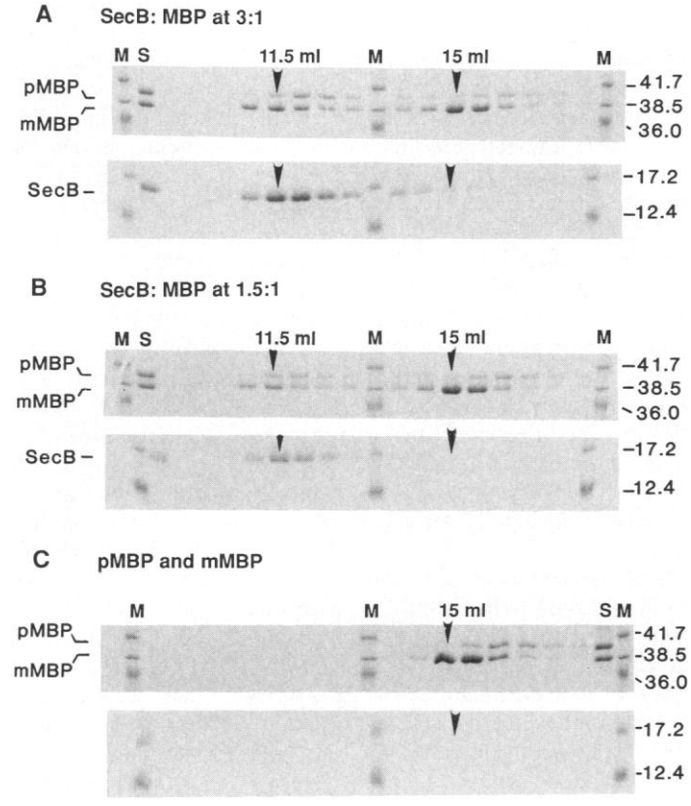

Fig. 3. Relative affinities of SecB for precursor (pMBP) and matured maltose-binding protein (mMBP). The experiment is similar to that described in Fig. 1. Approximately equimolar mixtures of unfolded precursor and matured maltose-binding protein were diluted into chromatography buffer containing (**A**) 1.5 μM SecB; (**B**) 0.75 μM SecB; or (**C**) no SecB and were subjected to gel-filtration chromatography. Final concentrations in the samples applied were: pMBP, 0.25 μM; mMBP, 0.25 μM; and GuHCl, 0.1 M. The concentrations of SecB are limiting for complex formations under these conditions. Arrowheads indicate the positions of SecB-MBP complex (left) and free monomeric maltose-binding protein (right). Lanes S contain 15% of the quantity of sample applied to the column. Lanes M are markers as in Fig. 1.

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Inhibition of FKBP Rotamase Activity by Immunosuppressant FK506: Twisted Amide Surrogate

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The immunosuppressive agents cyclosporin A and FK506 inhibit the transcription of early T cell activation genes. The binding proteins for cyclosporin A and FK506, cyclophilin and FKBP, respectively, are peptidyl-prolyl-*cis-trans* isomerases, or rotamases. One proposed mechanism for rotamase catalysis by cyclophilin involves a tetrahedral adduct of an amide carbonyl and an enzyme-bound nucleophile. The potent FKBP rotamase inhibitor FK506 has a highly electrophilic carbonyl that is adjacent to an acyl-pipicolinyl (homoprolyl) amide bond. Such a functional group would be expected to form a stabilized, enzyme-bound tetrahedral adduct. Spectroscopic and chemical evidence reveals that the drug interacts noncovalently with its receptor, suggesting that the α-keto amide of FK506 serves as a surrogate for the twisted amide of a bound peptide substrate.

CYCLOSPORIN A **1** (CsA, FIG. 1) (*1*) is an immunosuppressive agent that has found widespread clinical use in organ transplantation. FK506 **2** (*2, 3*) is a structurally unrelated and potent immunosuppressant (*4*) shown to be effective in human organ recipients at doses significantly lower than those necessary with CsA (*5*). These compounds, especially FK506 (*6*), have potential for the treatment of autoimmune disorders (*7*) and for the prevention of graft rejection following organ transplantation.

Both CsA and FK506 suppress immune response by inhibiting the transcription of early T cell activation genes [interleukins-2, -3, and -4, granulocyte-macrophage colony stimulating factor, and interferon-γ (*8*)], apparently by controlling the synthesis of or modifying transcriptional regulators such as nuclear factor of activated T cells (NF-AT) (*9*). A possibly related finding is that the binding proteins for CsA and FK506, cyclophilin (*10*) and FKBP (immunophilins) (*11, 12*), respectively, are peptidyl-prolyl-*cis-trans*-isomerases (rotamases, Fig. 2), which are potently inhibited by their respective ligands. Furthermore, the inhibition is highly selective: FK506 does not inhibit the rotamase activity of cyclophilin, and CsA does not inhibit that of FKBP. This information, together with recent reports of related proteins implicated in signaling pathways (*13, 14*), led to the suggestion that the immunophilins are involved in signaling processes that lead to T cell activation (*11*); the biological importance of cyclophilin has been firmly established, in fact, by a study demonstrating that the protein mediates the effects of CsA on the lower eukaryotes *Neurospora crassa* and *Saccharomyces cerevisiae* (*15*).

Elucidation of the mechanism of catalysis by rotamases and the mode of binding and inhibition by immunosuppressive agents is prerequisite to understanding the role of the immunophilins in T cell activation. Herein, we report on a ^{13}C nuclear magnetic resonance (NMR)–based investigation of the complex between recombinant human FKBP (rFKBP) and FK506 that provides new insights into these issues.

The ability of the immunophilins to catalyze the interconversion of *cis* and *trans* proline amide rotamers implies that they recognize proline-containing epitopes. [In support of this suggestion, we note that the naturally occurring immunosuppressant FR900525 contains proline in place of pipicolinic acid but is otherwise identical to FK506 (*16*).] Recognition could involve either an enzyme-substrate tetrahedral adduct (Fig. 2B, tetrahedral adduct mechanism) or a twisted peptidyl-prolyl amide bond (Fig. 2C, twisted amide mechanism). Either mechanism would explain the catalytic properties of the immunophilins, as the loss of amide resonance should lower the barrier to rotation.

Early support for a tetrahedral hemithioorthoamide adduct mechanism in the case of cyclophilin came from the studies of Fischer *et al.*, who found that *p*-hydroxymercuribenzoic acid modifies an active-site cysteine residue with concommitant loss of rotamase activity (*17*) and observed an inverse secondary isotope effect ($k_H/k_D < 1$) with a deuterated substrate containing an [α,α-^{2}H]Gly-Pro fragment (*18*). However, the latter result has been challenged (*19*), and recent studies have shown that any of the four cysteines of cyclophilin can be mutated to alanine without affecting either affinity for CsA or rotamase activity (*20*).

The structures of FK506 **2** and a structurally related immunosuppressant rapamycin **4** (*21, 22*), which also binds FKBP (*11*) and

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