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**Binding of a soluble factor of *Escherichia coli* to preproteins does not require ATP and appears to be the first step in protein export**  

*(translation/wheat germ system/*Escherichia coli* system/mutant maltose binding protein/inverted vesicles)*

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**ABSTRACT** We have constructed a mutant form of the maltose binding protein precursor, termed preMBP*, that, unlike its wild-type counterpart preMBP, retains translocation competence after synthesis. In a homologous *Escherichia coli* translation/translocation system, preMBP* was translocated either co- or posttranslationally with virtually 100% efficiency into inverted vesicles (INV) derived from the *E. coli* plasma membrane. Translation of preMBP* mRNA in a wheat germ system and subsequent incubation with INV yielded no translocation. However, addition of increasing amounts of an *E. coli* postribosomal supernatant (PRS) to the wheat germ extract stimulated preMBP* translocation with virtually 100% efficiency occurring at 100 μg of PRS per 50 μl of incubation mixture. The activity in the *E. coli* PRS appears to be identical to the previously described “export” factor. The soluble activity can bind to preMBP* posttranslationally and in the absence of ATP. Subsequent targeting to INV and/or translocation, however, requires ATP. Binding of the soluble activity to preMBP* thus appears to be the first step in a multistep translocation reaction.

In *Escherichia coli*, protein export or translocation can occur co- and posttranslationally (1). Although little is known concerning the actual mechanism of translocation, it does appear that the efficiency of translocation is greatly influenced by precursor structure. In fact, if the precursor assumes a stable folded structure prior to translocation, translocation competence is commonly lost (2, 3). In this context, several unf–antifolding factors, such as heat shock proteins (4–8), have been reported to stimulate protein translocation, although it is uncertain whether such unf–antifolding factors affect precursor conformation.

In this study, a mutant form of maltose binding protein precursor (preMBP), termed preMBP*, was constructed and, in contrast to wild-type preMBP, was found to retain posttranslational translocation competence in an *E. coli* cell-free translation/translocation system, suggesting that preMBP* assumes a quite different structure from that of wild-type preMBP (2, 6). As preMBP* can be translocated in the absence of any unf–antifolding factors, we have used this precursor to determine whether a specific membrane targeting factor, analogous to canine signal recognition particle, exists in *E. coli*.

In previous reports (9, 10) from this laboratory, it was shown that protein export in *E. coli* is stimulated by a cytosolic factor. Requirement for an *E. coli* cytosolic factor was demonstrated in two assay systems: first, in a homologous *E. coli* translation/translocation system that was highly subfractionated and thereby made dependent on a cytosolic factor (9) and subsequently in a heterologous wheat germ translation system (presumably lacking such a factor) supplemented with inverted vesicles (INV) derived from the plasma membrane of *E. coli* (10).

In this study, we report that a mutant form of preMBP, preMBP*, is efficiently translocated across INV in the heterologous system. We present evidence indicating that translocation is likely to be composed of a number of steps differing in the requirement for cytosolic factors and ATP.

**MATERIALS AND METHODS**

**Plasmid Construction**. Plasmid pBAR107 containing the structural gene for maltose binding protein (MBP), *maltE*, cloned into the *EcoRI/Sma* I site of the polylinker site of SP65 was a gift of B. A. Rasmussen (Princeton University). The plasmid was digested at the Nco I site and both sticky ends resulting from the digestion were filled in with Klenow fragment and ligated to each other with T4 DNA ligase. As a result, 4 base pairs were inserted into the Nco I site, shifting the reading frame and leading to the introduction of a termination codon. This plasmid, termed pBAR107N, encodes a protein that is identical to preMBP (containing 396 amino acid residues) up to Met-347 but then contains 7 residues of an unrelated sequence:

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TGA Met His Gly Lys Arg Pro Glu Arg *

**In Vitro Transcription**. Plasmids pBAR107 and pBAR107N were linearized with HindIII, and plasmid pLB8000 containing the structural gene for λ phage receptor, lambD, was linearized with *EcoRI* prior to transcription. Transcription with SP6 RNA polymerase was done as described (11).

**E. coli Cell Fractions**. An S-30 was prepared according to Müller and Blobel (12) from the *E. coli* strain MRE600. A membrane-free S-135 was obtained by centrifuging 175 μl of S-30 per tube in the A-100/18 rotor of the Beckman Airfuge at 4°C for 13.2 min at 30 psi (1 psi = 6.9 kPa) (135,000 × g) and collecting the top 125 μl. Postribosomal supernatant (PRS) was prepared by centrifuging S-30 at 4°C for 2.5 hr at 150,000 × g, in the T865 Sorvall rotor.

**E. coli Cell-Free Translation/Translocation**. In *vitro* transcript (500 ng) was translated in 25 μl of *E. coli* translation mixture as described (12) in the absence or presence of high salt washed INV (0.5 A280 unit/ml), prepared as described (12), at 37°C for 20 min. For posttranslational translocation assays, PRS was prepared from the translation mixture by centrifugation for 30 min at 4°C in the A-100/18 rotor of the Beckman Airfuge at 30 psi (135,000 × g). The translocation reaction mixture (25 μl) contained 23 μl of PRS, 5 mM ATP, 8 mM creatine phosphate, 40 μg of creatine phosphokine.

Abbreviations: INV, inverted vesicles derived from the *E. coli* plasma membrane; MBP, maltose binding protein; MBP*, mutant maltose binding protein; preMBP, precursor MBP; PRS, postribosomal supernatant.

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per ml, and, where indicated, 0.5 A209 unit of high salt washed INV per ml, and was incubated at 25°C for 60 min.

Membrane Sedimentation and Protease Protection Experiments. After translocation, the mixture was centrifuged at 4°C through a 100-μl cushion consisting of 0.5 M sucrose/40 mM triethanolamine, pH 7.5/140 mM KOAc/11 mM Mg(OAc)2/1 mM dithiothreitol in the A-100/18 rotor in the Beckman Airfuge at 30 psi (135,000 × g) for 15 min. The pellet and the supernatant were processed for NaDodSO4/PAGE. For protease protection experiments, the translocation mixture was incubated with proteasine K (300 μg/ml) on ice for 30 min. Digestion was stopped by adding 1/10th vol of 100 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide and the mixture was then processed for NaDodSO4/PAGE.

Wheat Germ Cell-Free Translocation. Translation in a wheat germ system was carried out essentially as described (13). In vitro transcript (500 ng) was translated in 25 μl of translation mixture.

Translocation of Wheat Germ Cell-Free Translocation Product Across INV. PRS was prepared from the wheat germ translation mixture by centrifugation in the A-100/18 rotor of the Beckman Airfuge at 30 psi (135,000 × g) for 30 min at 4°C. The translocation reaction mixture (50 μl) contained 23 μl of PRS, 5 mM ATP, 8 mM creatine phosphate, creatine phosphokinase (40 μg/ml), 25 μl of buffer A [10 mM triethanolamine, pH 7.5/60 mM KOAc/14 mM Mg(OAc)2/1 mM dithiothreitol] containing various amounts of E. coli PRS and 0.5 A209 unit of high salt washed INV. Incubations were for 50 min at 25°C.

ATP Depletion. The posttranslational incubation of wheat germ PRS with E. coli PRS (see Fig. 5, VI) or of wheat germ PRS, E. coli PRS, and INV (see Fig. 6) was supplemented with hexokinase (1 unit/μl) and 20 mM glucose instead of ATP/creatine phosphate/creatine phosphokinase.

Sucrose Gradient Centrifugation. A 0.5-ml sample was layered onto 11.5 ml of 5–20% (wt/wt) sucrose gradient in buffer A. The gradients were centrifuged for 16 hr at 40,000 rpm (202,000 × gsw) in an SW 40 Beckman rotor. Twenty-four fractions of 0.5 ml each were collected and aliquots were processed for activity (see Fig. 4) or for NaDodSO4/PAGE (see Fig. 5). Cytochrome c (bovine heart), 1.7 S; liver aldolase (rabbit muscle), 7.3 S; and catalase (bovine liver), 11.4 S, were centrifuged under identical conditions on a separate sucrose gradient.

NaDodSO4/PAGE. Samples, except pellets of membrane sedimentation experiments, were precipitated by adding an equal volume of 20% trichloroacetic acid. Precipitates were dissolved in a solution of 6.25% NaDodSO4/0.5 M Tris base (sample buffer), boiled in the presence of 100 mM dithiothreitol, and the polypeptides were analyzed by NaDodSO4/PAGE in 10–15% acrylamide gels. Membrane pellets were directly dissolved in sample buffer. Gels were dried and subjected to autoradiography (see Figs. 2–6), processed for fluorography (see Fig. 1), or stained with Coomassie blue (see Fig. 4.4).

Quantitation and Definition of Percentage Translocation. Quantitation was performed by direct analysis of the gels using an AMBIS Radioanalytic Imaging System (Automated Microbiology Systems, San Diego, CA).

The precursor of MBP* has seven methionine residues, three in the signal sequence and four in the mature portion. In the E. coli translation system, precursor as well as mature forms are protected from protease digestion, suggesting that some of the precursor form is translocated without being processed. Thus, the ratio of mature form to the sum of mature and precursor forms is an underestimate of the translocation efficiency. The absolute amount of precursor and mature forms after protease digestion is not, however, a proper estimate of translocation as the total amount of chains protected from protease digestion is variable. In addition, membrane proteins are expected to have some protease accessible portions after integration into INV. However, the ratio of precursor form to mature form after protease digestion is reproducible. We estimate, therefore, that the amount of translocated precursor is equivalent to that of mature form (before protease digestion) corrected by this ratio.

In case of preMBP* synthesized in a wheat germ system, the mobility difference between the product initiated at Met-18 or 19 in the signal sequence (pre*MBP*) and the mature form (MBP*) is too small to be quantitated, but by membrane sedimentation each band can be quantitated, as almost all of the pre*MBP* remains in the supernatant fraction and all of the mature form is in the pellet fraction (compare Fig. 2 and Fig. 3). Taking these factors into consideration, we calculate percentage translocation as follows:

\[ \text{translocated precursor (cpm)} = \text{mature form (cpm)} \times \frac{[\text{protected precursor (cpm)}]}{[\text{protected mature (cpm)}]} \]

\[ \% \text{translocation} = \left( \frac{[\text{mature form (cpm)}] \times 7/4 + [\text{translocated precursor (cpm)}]}{[\text{mature form (cpm)}] \times 7/4 + \text{precursor (cpm)}} \right) \times 100. \]

RESULTS

The behavior of preMBP and preMBP* in an E. coli translation/translocation system in either a cotranslational or a posttranslational mode is illustrated in Fig. 1. When assessed by cosedimentation with INV (lanes 8–10) or by proteasine K resistance (lane 11), preMBP* was translocated with >90% efficiency in both co- and posttranslational assay systems. In contrast, wild-type preMBP was translocated only cotranslationally and only with ~30% efficiency, as assessed by cosedimentation with INV (lanes 2–4). Interestingly, there was a small amount of mature MBP generated in the posttranslational incubation (Fig. 1 Lower, compare lanes 1 and 2). However, in the centrifugation assay, the mature MBP chains remained in the supernatant (lane 3) rather than sedimenting with INV (lane 4), indicating that these chains were not translocated. One possible explanation of this result is that a small number of preMBP molecules remained in a partially translocation competent state so that their aminoterminal portion gained access to the luminal disposed signal peptidase activity of INV but that folding of the remainder of the chain prevented its complete translocation across the INV membrane. In the case of preMBP, proteinase K resistance cannot be used as a criterion for assessing translocation efficiency because the mature portion of preMBP assumes a proteinase K-resistant conformation (lanes 5 and 6) as reported by others (2, 6). In contrast, posttranslational incubation of preMBP* for 1 hr at 25°C does not yield folding into a proteinase K-resistant conformation.

Fig. 1. Co- or posttranslational translocation of preMBP or preMBP* in an E. coli translation/translocation system. p, Precursor form; m, mature form.
Based on previous results (10), we expected that preMBP* would be translocated into INV after synthesis in a wheat germ cell-free system, provided that the translocation reaction is supplemented with an E. coli PRS. It should be noted that translation of preMBP* mRNA in the wheat germ system yielded two major products, a slower moving polypeptide representing full-length preMBP* (designated p) and a faster moving polypeptide (designated i) resulting from initiation at a form furthest downstream methionine, presumably Met-18 or -19 of the 26-amino acid signal sequence. We therefore termed the product resulting from initiation at Met-18 or -19 pre*MBP*. Posttranslational incubation of a wheat germ PRS containing newly synthesized pre*MBP* and preMBP* with INV yielded translocation only of preMBP*, not of pre*MBP*, and only in the presence of E. coli PRS (Fig. 2 Upper). A similar dependence on an E. coli PRS was shown for the integration of the integral membrane protein preLamB into INV (Fig. 2 Lower) (14).

The efficiency of preMBP* translocation was proportional to the amount of E. coli PRS present during the translocation reaction (Fig. 3). In this experiment, we assessed the extent of translocation by cosedimentation of preMBP* or MBP* with INV (Fig. 3A Upper versus Middle) or by proteinase K resistance (Fig. 3A Lower). As in the homologous E. coli translocation system, the efficiency of translocation of preMBP* into INV from a wheat germ system was near 100% at the highest amount of E. coli PRS added (Fig. 3B).

We have previously characterized a soluble "export" factor from E. coli (9). Comparison of the previously published polypeptide profile of "export" factor with the analogous polypeptide staining pattern (Fig. 4A) and the peak of activity required for translocation of preMBP* (Fig. 4B Upper) or preLamB (Fig. 4B Lower) into INV from a wheat germ system indicated that the two activities are likely to be the same. However, the sedimentation rate of the export factor was previously estimated to be ∼12 S, based on internal markers such as the GroES protein and RNA polymerase (9). Using other marker proteins (such as cytochrome c, 1.7 S; albumin, 4.6 S; aldolase, 7.3 S; catalase, 11.4 S) in a parallel gradient, we now estimate that the export factor sediments as a broad peak at around 7 S.

If the export factor functions in signal sequence recognition and targeting to INV, one might be able to detect binding to preMBP* by sedimentation analysis in sucrose gradient centrifugation (Fig. 5). When synthesized in a wheat germ system, both pre*MBP* and preMBP* sedimented as mono-

**Fig. 2.** Synthesis of preMBP* and preLamB in a wheat germ system and posttranslational translocation into INV dependent on E. coli PRS. Where indicated, 100 µg of E. coli PRS in 25 µl of buffer was added per final 50 µl of translation mixture. p, Precursor form; i, intermediate form resulting from translation initiation at Met-18 or -19 of preMBP*; m, mature form.

**DISCUSSION**

In this report we describe a cell-free translation/translocation system in which the translocation of a model presecretory
protein preMBP* across *E. coli* inverted plasma membrane vesicle occurs posttranslationally and with high efficiency. In this system, translocation is strictly dependent on an *E. coli* cytosolic factor. We have observed that the cytosolic factor binds to the presecretory protein and that this binding does not require ATP. Subsequent targeting and/or translocation across INV is, however, ATP dependent. It appears that binding of the cytosolic factor to the preprotein may be the first step in a multistep translocation process.

The model presecretory protein that we used was a mutant of preMBP, termed preMBP*. PreMBP* differed from wild-type preMBP by an alteration in its carboxyl-terminal portion. Unlike wild-type preMBP, which is known to acquire a proteinase K-resistant conformation (comprising only the mature portion of preMBP) within minutes after synthesis (2, 6) (see also Fig. 1), mutant preMBP* remained sensitive to proteinase digestion (Figs. 1–3), presumably because an altered carboxyl-terminal end would no longer allow folding into a proteinase K-resistant conformation.

It has been demonstrated that acquisition of a proteinase K-resistant conformation of preMBP* correlated with a loss of

Fig. 5. Sedimentation analysis on sucrose gradients of either preMBP or preMBP*. The preproteins were synthesized, as indicated, either in a wheat germ system (W.G.) or in an *E. coli* system. In V and VI, synthesis in a wheat germ system was followed by posttranslational incubation of the wheat germ PRS (15 µl) with an *E. coli* PRS (125 µl) in 5 mM ATP/8 mM creatine phosphate/creatine phosphokinase (40 µg/ml) (V), or with 20 mM glucose/hexokinase (1 unit/µl) (VI) for 60 min at 25°C prior to sedimentation analysis. p and i are the same as in Fig. 2; 1.7 S, 4.6 S, and 7.3 S indicate positions of marker proteins sedimented in a parallel gradient.

Fig. 6. Posttranslational translocation of preMBP* into INV requires ATP. PreMBP* was synthesized in a wheat germ system and the wheat germ PRS was incubated in a translocation reaction mixture in the absence or presence of various components as indicated. Translocation was assessed either by sedimentation of INV, yielding a supernatant (s) and pellet (p) fraction, or by proteinase K, p, i, and m are the same as in Fig. 2.
translocation competence (2). Indeed, when mRNA for preMBP was translated in an *E. coli* cell-free translation system, only \( \approx 30\% \) of the chains were translocated when INV were present during translocation and none was translocated when INV were added posttranslationally (Fig. 1). Thus, it appeared that even in the cotranslational presence of INV, folding of preMBP into a translocation incompetent conformation might occur more rapidly than translocation (2).

In contrast, preMBP* was translocated in a homologous *E. coli* system with near 100% efficiency, even when INV were added posttranslationally (Fig. 1). These results suggested that the translocation competence of most of the newly synthesized preMBP* molecules was not compromised by posttranslational folding. PreMBP* therefore appeared to be a suitable model secretory protein to study the mechanism of targeting and the possible requirement for cytosolic targeting factors, perhaps independently of nonspecific un-/antifolding factors, such as heat shock proteins (4, 5, 7, 8).

A frequently used approach to identify such cytosolic factors is to employ a wheat germ translation system, which lacks targeting as well as un-/antifolding factors (5, 7, 10, 16). As reported (10) for prepro-\( \alpha \) factor (a yeast presecretory protein) translocation of preMBP* across INV did not occur unless an *E. coli* PRS was included in the translocation reaction. The efficiency of translocation was observed to be proportional to the amount of added *E. coli* PRS, being virtually 100% at the highest concentration of PRS (Fig. 3).

The active fraction of the *E. coli* PRS cosedimented exactly with the previously reported export factor activity (compare figure 1 in ref. 9 with Fig. 4 in this report). Previously, however, export factor was reported to sediment at \( \approx 12 \) S, an estimate based on internal markers. Using a number of external proteins with known S values on a parallel sucrose gradient, we now estimate a sedimentation rate of 7 S for export factor (Fig. 4).

When preMBP* was synthesized in a wheat germ translation system, it sedimented as a monomeric species at \( \approx 2 \) S. However, when preMBP* synthesized in the wheat germ translation system was posttranslationally incubated with an *E. coli* PRS, it sedimented at \( \approx 5 \) S, at the same rate as preMBP* synthesized in the *E. coli* translation system (Fig. 5). These data suggested that synthesis of MBP* in an *E. coli* translation system was accompanied by an association of preMBP* with a soluble factor and that this association could occur posttranslationally when preMBP* was first synthesized in a wheat germ translation system in the absence of such a factor. The apparently complete shift of wheat germ synthesized preMBP* from 2 S to 5 S after incubation with *E. coli* PRS suggested that essentially all preMBP* molecules were able to associate posttranslationally with the *E. coli* soluble factor. In contrast, wild-type preMBP, even when synthesized in an *E. coli* translation system, was found to sediment at 2 S—i.e., as a monomeric unassociated species. Thus, apparently none of the preMBP molecules remained associated with the *E. coli* soluble factor even though this factor was present during translation in the *E. coli* translation system. It is likely that a soluble factor associated also with wild-type preMBP but that it was rapidly displaced by folding of the mature protein portion of preMBP. Those wild-type preMBP molecules that remained associated with soluble factor are likely to represent the fraction that was translocated during the cotranslational presence of INV. Taken together, the extent of association of soluble factor with preproteins (Fig. 5) correlates with the efficiency of translocation (Fig. 1). It therefore appears that association of preproteins with this factor, a reaction that in the case of preMBP* can occur even posttranslationally, is the first step in a multistep translocation process.

Interestingly, it appears that the soluble factor associates also with pre*MBP*, the product initiated at Met-18 or -19 in the signal sequence (Fig. 5), although this product cannot bind to INV (Figs. 3 and 6). We postulate that the soluble factor binding site of the preMBP* may be in the carboxy-terminal portion of the signal sequence, which overlaps with the signal peptidase recognition site, or some portion in the mature protein, and that the amino-terminal two-thirds of the signal sequence may be important for the targeting of precursor to INV—i.e., this portion may be recognized by a membrane-associated signal sequence receptor.

Finally, we have observed that association of preMBP* with the soluble factor does not require ATP. However, the subsequent targeting of the preMBP complex to INV and/or translocation of preMBP* does require ATP.

What is the nature of this soluble factor, how does it function, and is it related to any of the soluble factors reported by others (6, 17–19) to be required for protein export in *E. coli*? Elsewhere, we will describe the purification and detailed characterization of this soluble factor and show that it contains the product of the *secB* gene [described by Kumamoto and Beckwith (20)] and that it functions as the *E. coli* equivalent of a signal recognition particle (unpublished results).

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