Post-translational Export of Maltose-binding Protein in *Escherichia coli* Strains Harboring *malE* Signal Sequence Mutations and Either *prl* or *prl* Suppressor Alleles*

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We have studied the export kinetics of the maltose-binding protein (MBP) of *Escherichia coli*, the *malE* gene product, when it is synthesized with either a wild-type signal sequence or with a mutationally altered signal sequence that affects the efficiency of secretion to the periplasm. Our results confirm a very rapid export process for the wild-type protein and, in contrast, reveal a relatively slow post-translational mode of export for the altered precursor species. For each different signal sequence mutant, a fraction of the precursor MBP pool that is proportional to the strength of the export defect appears to never exit the cytoplasm. We have also analyzed MBP export in strains harboring *prl* mutations that suppress *malE* signal sequence mutations and are thought to somehow alter the specificity of the cell’s protein export machinery. The introduction of different *prl* alleles has no apparent effect on wild-type MBP export but increases both the amount of mutant MBP that is exported and the rate at which this is accomplished. In fact, the presence of two different *prl* alleles in the same strain can act synergistically in suppressing MBP export defects. The inhibition of total protein synthesis with chloramphenicol can also increase the proportion of pMBP that is post-translationally exported in these strains. A model that describes the initial steps in MBP export is presented.

Studies with eukaryotic systems have begun to yield a fairly clear picture of the early steps in the protein export process. Shortly after the amino-terminal signal peptide emerges from the ribosome, further translation of the nascent exported protein is selectively arrested by its interaction with a cytosolic ribonucleoprotein complex termed the signal recognition particle (1, 2). Subsequent interaction of this translation-arrested ribosome–signal recognition particle complex with a membrane receptor, the docking protein, on the surface of the rough endoplasmic reticulum releases the translation block and ensures that further synthesis of the nascent chain is directly coupled to translocation across the membrane (3).

Certain aspects of the export of the periplasmic MBP of *Escherichia coli*, the *malE* product, are consistent with the eukaryotic model as it has been applied to prokaryotic cells (4). The MBP is initially synthesized as a precursor having a 26-residue cleavable signal peptide that is similar to those characterized for both prokaryotic and eukaryotic systems. The MBP has been shown to be synthesized on cytoplasmic membrane-bound polysomes (5, 6) and can be cotranslationally secreted and processed, but only after approximately 80% of the nascent chain has been translated (7). This latter finding indicates that, in *E. coli*, elongation and translocation are not necessarily tightly coupled events.

Much of the information concerning the mechanism of MBP synthesis and secretion has resulted from the study of mutants affecting these processes (recently reviewed in Ref. 8). Strains harboring five different *malE* point mutations that alter the MBP signal peptide accumulate export-defective pMBP in the cytoplasm (Refs. 9–11; see Fig. 1). Extragenic suppressor mutations have been obtained in at least four different loci (designated *prlA*, *prlB*, *prlC*, *prlD*) that restore, to varying extents, export of MBP and other proteins having defective signal peptides (11–14). Although these mutations do not appear to affect normal protein export, previous studies have indicated that the *prlA* and *prlD* genes encode components of the normal protein export pathway (11, 14). In addition, mutations have been obtained in several loci, designated *secA*, *B*, *C*, and *Y* (*prlA*), that adversely affect synthesis and/or export of a number of *E. coli* envelope proteins, including the MBP (15–18). To date, only the *SecA* protein has been characterized (19).

In this study, we have investigated in detail MBP export in *malE* signal sequence mutants harboring either *prl* or *prl* suppressor alleles. In these strains, MBP export is achieved at a rate considerably slower than that exhibited by the wild-type protein. A model incorporating our results and related observations is presented that attempts to describe the initial steps in MBP export to the periplasm.

**MATERIALS AND METHODS**

**Bacterial Strains**—We have chosen to refer to the relevant genotype of particular strains rather than assign specific strain designations. All of the strains are isogenic derivatives of the *E. coli* K12 strain MC4100: F’ ΔlacU169 araD139 rpsL150 thi fbiB5301 deoC7 ptsF25 relA1 (20). Strains harboring *malE* signal sequence mutations in both *prl* and *prlA* suppressor strain backgrounds have been described (11, 14). The construction of *malE prlD2* and *malE prlA*.

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1 The abbreviations used are: MBP, maltose-binding protein and product of the *malE* gene (the prefix ‘p’ indicates the precursor form of the protein, whereas ‘m’ indicates the mature form); TosArgMe, p-tosyl-L-arginine methyl ester; DNP, 2,4-dinitrophenol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
prlD2 mutants using the generalized transducing phage P1 was performed as previously described (14, 21).

Media and Chemicals—M63 minimal salts medium was prepared as outlined by Miller (21). Maltose, glycerol, sucrose, sodium azide, EDTA, magnesium chloride, and trichloracetic acid were obtained as reagent grade chemicals from Fisher Scientific. Lysozyme, phenylmethylsulfonyl fluoride, proteinase K, chloramphenicol, TosArgMe, Tris base, and L-methionine were obtained from Sigma, and DNP was purchased from Eastman Kodak. Translation grade [35S]methionylmethylsulfonyl fluoride, proteinase K, chloramphenicol, and TosArgMe were formed as previously described (14, 21).

Tris base, and L-methionine were obtained from Sigma, and DNP was kindly provided by Paul Ray of the Wellcome Research Laboratories, Research Triangle Park, NC.

Pulse-Chase Experiments—Vigorously aerated 2.8-ml cultures were grown to midlogarithmic phase (A600 of 0.35) in glycerol minimal medium at 30 °C using a New Brunswick gyratory water bath shaker model G7B. The cells were then induced for the production of MBP by the addition to the cultures of 0.2% maltose (final concentration). Forty-five min postinduction, the cells were pulse radiolabeled for 10 s with 50 μCi of [35S]methionine. The pulse was terminated and the chase initiated by the addition to the culture of 2.8 ml of prewarmed solution A (maltose minimal medium supplemented with 0.8% unlabeled glycerol and 0.2% chloramphenicol) in a Sorvall Speed Vac concentrator. The cells were resuspended, sonicated with a Branson 12 1/2 water bath sonicator, and centrifuged at 10,000 rpm, 15 min. Aliquots (20 μl) of the clarified supernatant of these samples was used as the antigen extract in an immune precipitation procedure identical to one previously described (24).

Pulse-chase experiments addressing the effect of chloramphenicol on MBP maturation were also performed according to this protocol, except that 0.49 ml of 1 mg/ml chloramphenicol (in sterile distilled water) was added after 1.5 min of chase.

Cell Fractionation and Proteinase K Accessibility Experiments—Vigorously aerated 4-ml cultures were grown to midlogarithmic phase in glycerol minimal medium at 30 °C as described above, and the cells were induced for the synthesis of MBP with 0.2% maltose (final concentration) 45 min prior to labeling. The cells were pulse labeled for 10 s with 200 μCi of [35S]methionine and then chased by the addition to the culture of 4 ml of prewarmed solution A. One-min and thirty-min chase periods were terminated by adding 4 ml of culture to an equal volume of prewarmed solution B: 290 μg/ml chloramphenicol, 200 mM sodium azide, 10 mM DNP, 3.5% ethanol. The cells were then immediately placed in an ice water bath for a period of 1 min. As previously described, this termination procedure is as effective as using 5% trichloracetic acid (final concentration) and does not disrupt the integrity of the cell (24). Each cell pellet was resuspended in 2 ml of 10 mM Tris buffer (pH 8.0), and having removed 0.5 ml for s whole cell control, subjected to a mini version of the osmotic shock procedure of Neu and Heppel (25), exactly as described previously (11), except that the periplasmic fraction was precipitated with 5% trichloracetic acid (final concentration) rather than lyophilized. The shocked cell portion was further resolved into membrane and soluble (cytoplasmic) fractions. The shocked cell pellet was resuspended in 1.5 ml of 10 mM Tris (pH 8.0), 0.5 mM EDTA, and sonicated with a microtip for three 20-s bursts using a Fisher Sonic Dismembrator model 300 set at 10% duty. The unbroken cells were removed with a low speed spin (1,000 × g for 10 min), and the supernatant was centrifuged at 100,000 × g for 90 min to pellet the membranes. The soluble fraction (supernatant) was precipitated with 5% trichloracetic acid (final concentration). The acid precipitates of both the soluble and shockate fractions were pelleted and washed with acetone as described above. All three fractions (membrane, cytoplasmic, and periplasmic) were resuspended in 150 μl of 10 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS, and sonicated as described above. A portion (20 μl) of the clarified supernatant of the whole cell control and of each fraction was then subjected to immune precipitation by anti-MBP immunoglobulin coupled to Sepharose CL-4B.

Proteinase K accessibility experiments were performed as previously described (7, 24).

Polyacrylamide Gel Electrophoresis—Immune precipitates were resolved by SDS-PAGE and autoradiography (26). We used 16-cm 10% polyacrylamide gels stacked at 15 mA/gel and ran at 30 mA/gel. The use of an LKB 2202 Ultrascan laser densitometer interfaced with an Apple II computer allowed the relative quantitation of bands visualized on autoradiograms.

RESULTS

Rate of Maturation of Wild-type and Export-defective MBP—Strains representing malEl+ and each of the five different malEl signal sequence mutations (Fig. 1) were pulse labeled with [35S]methionine for 10 s and then incubated in the presence of excess unlabeled methionine for various lengths of time. The labeled MBP species at each chase point were analyzed by immune precipitation, SDS-PAGE, and autoradiography (see "Materials and Methods") (Fig. 2). The wild-type MBP was found predominantly in its mature form at the earliest chase point. At later chase points, pMBP was not detectable. In marked contrast, the appearance of mMBP was considerably delayed in each of the five mutants. For MBP exhibiting the most severe defect in export, the malEl18-1 product, the appearance of mMBP was barely discerned only after a 60-min chase period. Mature MBP appeared somewhat earlier in each of the other four mutant strains, with its time of appearance and the pMBP/mMBP ratio at the 60-min chase point correlating with the ability of these strains to utilize maltose as a carbon source (11, 27).

Table: Rate of maturation of wild-type and export-defective MBP

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (min)</th>
<th>pMBP/mMBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type MBP</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>malEl18-1 MBP</td>
<td>60</td>
<td>0.1</td>
</tr>
<tr>
<td>malEl16-1 MBP</td>
<td>60</td>
<td>0.05</td>
</tr>
<tr>
<td>malEl8-1 MBP</td>
<td>60</td>
<td>0.02</td>
</tr>
<tr>
<td>malEl6-1 MBP</td>
<td>60</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Rate of Maturation in prlD and prlA Strains—Mutations at the prlD locus have also been shown to suppress malEl signal sequence mutations (14). The prlD2 mutation was isolated as an extragenic suppressor mutation of the malEl14-
cells were pulse labeled each the presence of excess cold methionine. At the times indicated above mRNAs). The corresponding MRP was immune precipitated, and the precipitates were analyzed hv SDS-PAGE and autoradiography (see “Materials and Methods”). It seems clear that maturation, and hence translocation (see below), of export-defective MBP in either prl' or prl suppressor strains is a post-translational event, exhibiting a half-time of several min or more, depending on the exact malE and prl alleles involved. The effect of the inhibition of total protein synthesis on this post-translational mode of MBP export was investigated. In these experiments, cells were pulse labeled with [35S]methionine for 10 s, and 100 μg/ml chloramphenicol (final concentration) was added 1.5 min into the cold methionine chase period. The MBP species present at different time points during the chase period were identified. Similar results were obtained with a number of different strains; results for a malE18-1 prlA4 strain are presented in Fig. 5. It was found that little difference in the ratio of pMBP/ mMBP could be discerned after 2.5 min of chase when the immune precipitate derived from the chloramphenicol-treated cells was compared with the control. However, beginning with the 5-min chase point, a more rapid conversion of pMBP to

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1 allele. Maturation of MBP in various prlD2 strains was investigated (Fig. 4). As in the case of various prlA alleles, the prlD2 gene product did not appear to interfere with the maturation of wild-type MBP. Suppression of malE14-1 by prlD2 resulted in the increased production of mMBP, and the rate of maturation of export-defective MBP in this case was similar to that observed in prlA strains. The rate of MBP maturation in a malE14-1 strain harboring both the prlA4 and prlD2 alleles was also investigated (Fig. 4). The amount of mMBP that could be discerned at the 1-min chase point indicated that the degree of suppression achieved by the two different prl alleles in combination was significantly greater than that which might be expected by simple addition. By the 5-min chase point, only a minute amount of pMBP was detected. There appeared to be no further maturation of pMBP beyond this time point; a similar small quantity of pMBP was still detected after 60 min of chase.

**Effect of Chloramphenicol on prlA-mediated Suppression—** It seems clear that maturation, and hence translocation (see

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2 J. P. Ryan, unpublished data.
mMBP was apparent in the chloramphenicol-treated cells, with the greatest difference in the ratio of pMBP/mMBP observed after 30 min of chase.

Localization of MBP in prlA4 Suppressor Strains—Earlier studies indicated that the pMBP that accumulates in strains harboring malE signal sequence mutations was primarily located in the cytoplasm (10). When prlA suppressor alleles were introduced into these strains, essentially all of the mMBP produced was found in the periplasm, as indicated by the finding that mMBP, but not pMBP, was released from cells by a cold osmotic shock (11). Since these studies made use of steady-state radiolabeling conditions, it was of interest to determine whether pulse-labeled pMBP and/or mMBP could be transiently localized to additional cellular compartments. Strains harboring the prlA4 suppressor allele and either the malE14-1 or malE18-1 mutation were pulse labeled with [35S]methionine for 10 s and then chased for 1 min and 30 min. The chase was terminated in a manner we have previously used that does not disrupt cellular integrity (11). Periplasmic, cytoplasmic, and whole membrane fractions were prepared as described under “Materials and Methods,” and the radiolabeled MBP species present in each fraction were identified (Fig. 6). The results indicated that, in every instance, the pMBP was largely confined to the cytoplasm, with a small amount of the total pMBP found in association with the membrane fraction. This membrane association, most evident at the 1-min time point for each strain, was unaffected by the salt concentration of the Tris buffer used during the fractionation procedure (results not shown). At each time point in both strains, the mMBP, allowing for some slight contamination of other fractions, was localized to the periplasm.

The possibility was considered that the soluble pMBP encountered in these strains was periplasmic but for some reason was not released from the cells by the cold osmotic shock procedure employed. If this were the case, then the pMBP in these strains should be rendered sensitive to externally added proteinase K following the conversion of cells to spheroplasts (7, 24, 28). In this experiment, cells were pulse labeled for 10 s with [35S]methionine, chased for 1, 5, 30, and 60 min with cold methionine, converted to spheroplasts, and incubated on ice with 55 μg/ml proteinase K (final concentration). After a 30-min incubation period, phenylmethylsulfonyl fluoride was added to inactivate the protease, and total protein was precipitated with trichloroacetic acid. The protein pellets were solubilized and the MBP analyzed by immune precipitation, SDS-PAGE, and autoradiography (Fig. 7).

As has been reported (7), mMBP was found to be resistant to proteinase K treatment under all circumstances. In addition, using radiolabeling conditions such that only pMBP was apparent, all of the precursor form in lysed spheroplasts was found to be degraded by proteinase K to a species which co-migrated with mMBP (data not shown). Consequently, any accessibility of pMBP to proteinase K was represented in the immune precipitate by a decrease in the amount of the precursor form and a reciprocal increase in the amount of the mature product. Thus, in Fig. 7, the discrepancies in the amount of mMBP discerned in the whole cell controls (lane a) and spheroplasts treated with proteinase K (lane c) were attributed to this degradation phenomenon. In both strains, and at every chase point, the majority of pMBP was not degraded, and the amount that was protected from proteinase K closely agreed with the fraction of pMBP found to be soluble and cytoplasmic in Fig. 6 (lane c). However, a small fraction of pMBP at any given chase point was accessible to the added protease. Since, as previously noted (24), cells synthesizing export-defective proteins are somewhat more osmotically fragile than normal cells, it seems likely that the
pMBP that was converted to mMBP by proteinase K in this experiment resulted from spheroplast lysis. Finally, we have found that the addition of uncouplers TosArgMe or DNP during the cold methionine chase period totally prevented further prlA-mediated maturation of pulse-labeled pMBP (data not shown). Since previous studies have demonstrated that such compounds interfere with export of E. coli envelope proteins at some step prior to the completion of translocation (29, 30), this further indicates that the pMBP detected in these malE prlA mutants had not been exported beyond the cytoplasmic membrane.

### DISCUSSION

In agreement with earlier studies (7, 14, 24, 31), we have found that the appearance of mMBP in the periplasm in a malE+ strain following its synthesis is a very rapid event. The export and processing of MBP in strains harboring different malE signal sequence mutations was found to be a much slower entirely post-translational process. For each of these malE mutants, there appeared to be two different categories of pMBP produced. First, some fraction of the pMBP produced was destined never to leave the cytoplasm. The amount of pMBP in this fraction was proportional to the strength of the export defect. Secondly, there was a certain fraction of pMBP that was slowly exported to the periplasm and processed. The rate at which this was achieved also correlated with the strength of the export defect. The existence of two categories of pMBP was best illustrated by studying the kinetics of export of the malE16-1 product in Fig. 2. After 15 min of chase, the pMBP/mMBP ratio was approximately 1:1; this ratio did not markedly change at later chase times.

We have also investigated the kinetics of MBP export in malE signal sequence mutants harboring various prlA and/or prlD suppressor alleles. It was found that prl-mediated MBP export increased both the fraction of MBP that was exported and the rate at which this was accomplished. However, MBP export in these strains was still demonstrably slower than that seen in a malE+ strain. A simple interpretation of these results is that the effect of the prl mutation was to shift some proportion of the pMBP in the nonexportable category to the slowly exportable category, the proportion shifted depending on the particular malE and prl alleles involved. For example, there was a small amount of pMBP produced by the malE14-1 prlA402 strain that was not matured even after 60 min of chase that represented the nonexportable pMBP fraction.

It had been reported that certain combinations of prlA and prlD alleles in the same strain resulted in cells exhibiting severe defects in both protein secretion and growth (14). It was suggested that the proper assembly of a complex involving both the PrlA and PrlD proteins is required for efficient export of E. coli proteins (14). Using a different combination of prlA and prlD alleles, we observed in this study a synergistic suppression of malE signal sequence mutations, further indicating that these two gene products directly interact with one another.

When total protein synthesis was inhibited with chloramphenicol following the completion of translation of pulse-labeled MBP, prlA-mediated MBP export was significantly enhanced. It has been suggested that there are a finite number of protein export sites within the cell (26). One possible explanation for our finding is that, under normal conditions, the mutant MBP in prlA strains inefficiently competes with other envelope proteins for export at these sites. However, since most envelope proteins are rapidly exported, the termination of total protein synthesis eventually clears these export sites, providing much easier access to prlA-mediated MBP export.

In this study, we utilized MBP processing as an indicator of export to the periplasm. It was important to rule out the possibility that the MBP exported in different malE signal sequence mutants was translocated across the cytoplasmic membrane at a rate comparable to the wild-type protein but was a poor substrate for the processing enzyme. In two different strains studied in detail, the great majority of pMBP that had been pulse labeled with [35S]methionine fractionated as a cytoplasmic protein by several criteria at each of the chase times tested. Since a significant proportion of the pMBP seen at the early chase times was eventually translocated and processed, this is good evidence that pMBP can exist as a soluble cytoplasmic species for some period of time without losing export competence. A slow post-translational mode of export is not unique to MBP; similar results have also been obtained in E. coli strains for mutant lipoprotein (32) and TEM β-lactamase (33).
We can propose a model of MBP export that takes into account our results presented here and related observations. As it emerges from the ribosome, the nascent pMBP interacts in the cytoplasm with a complex of proteins that may include both the PrlA and PrlD proteins. In addition to interacting with one another, evidence has suggested that the PrlA and PrlD proteins interact with the MBP signal peptide (11, 14). Assembly of this complex may also involve recognition of regions within the mature portion of the MBP (7, 24, 34, 35). In the case of the wild-type cell, formation of the complex is very efficient. Subsequently, this complex interacts with the cytoplasmic membrane, and translocation and processing of the MBP are rapidly effected. Membrane components involved in pMBP translocation may include the SecA and SecB proteins (15, 16, 19).

In strains producing MBP with an altered signal peptide, the efficiency of complex formation is reduced relative to the strength of the MBP export defect. In those instances when an export complex does not form, the pMBP is forever trapped in the cytoplasm. In those instances where a complex is formed, subsequent steps in protein export are still inefficient because of the presence of the altered signal peptide; hence the slow MBP export kinetics observed in these strains. Alteration of the PrlA and/or PrlD protein can improve both the efficiency of complex formation and the subsequent rate of pMBP translocation and processing. However, in both wild-type and mutant strains, the complex formed has a limited half-life and may disassemble prior to facilitating pMBP translocation. This could explain our result where the inhibition of total protein synthesis increased the proportion of previously synthesized pMBP that could be exported in malE prlA mutants by increasing the availability of the export machinery in the membrane during the period that the pMBP was still export competent. Also note that Chen et al. (36) have reported that prolonged incubation of wild-type precursor proteins rendered them inactive for translocation in an in vitro system.

In order to confirm this model, one would like to be able to demonstrate that newly synthesized pMBP is assembled into a complex of proteins prior to its translocation across the cytoplasmic membrane. Since intact pMBP is difficult to even detect in malE* strains, the isolation of such a complex may prove difficult. However, by using strains in which the kinetics of MBP export are significantly slower, it should prove easier to map out the precise route that this protein takes from its site of synthesis in the cytoplasm to the periplasm and to identify the various cellular components that interact with the pMBP while it is in transit.

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REFERENCES