Two Regions of Mature Periplasmic Maltose-Binding Protein of *Escherichia coli* Involved in Secretion

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Six mutations in *malE*, the structural gene for the periplasmic maltose-binding protein (MBP) from *Escherichia coli*, prevent growth on maltose as a carbon source, as well as release of the mutant proteins by the cold osmotic-shock procedure. These mutations correspond to insertion of an oligonucleotide linker, concomitant with a deletion. One of the mutations (*malE127*) affects the N-terminal extension (the signal peptide), whereas the five others lie within the mature protein. As expected, the export of protein MalE127 is blocked at an early stage. This protein is neither processed to maturity nor sensitive to protease K in spheroplasts. In contrast, in the five other mutants, the signal peptide is cleaved and the protein is accessible to protease K added to spheroplasts. This indicates that the five mutant proteins are, at least in part, exported through the inner membrane. We propose that the corresponding mutations define two regions of the mature protein (between residues 18 and 42 and between residues 280 and 306), which are important for release of the protein from the inner membrane into the periplasm. We discuss the results in terms of possible conformational changes at this late step of export to the periplasm.

Instructions for the cellular localization of proteins are contained within their structural genes. It is already well established that bacterial proteins, which are exported through the cytoplasmic membrane, are synthesized with an N-terminal extension, the signal peptide, which is cleaved upon export (21, 26, 28). For periplasmic proteins, such as the maltose-binding protein (MBP) of *Escherichia coli*, export requires at least three steps: initiation of transfer, translocation through the inner membrane, and release into the periplasm. In a normal situation, these steps are parts of a continuous process; some of them can be detected with radioactive labeling. Under some conditions (mutations, inhibitors, low temperatures, etc.), intermediate forms are observed, which may, in favorable cases, represent true intermediates of the export pathway (for recent reviews, see references 5 and 34).

The best-defined part of the structural gene involved in export corresponds to the signal peptide (for recent reviews, see references 4, 31, and 34). The signal peptide of the periplasmic MBP, the product of the *malE* gene, presents characteristics which are common to all signal peptides from this bacterium (6, 7, 37). Three regions can be distinguished: the extreme amino-terminal sequence is composed of eight residues, three of which are basic; it is followed by a stretch of hydrophobic and neutral residues (the hydrophobic core); the last six residues before the cleavage site are believed to contain residues from the recognition sequence of the processing enzyme. In particular, the sequence Ala-Leu-Ala, at positions -3 to -1 relative to the site of proteolytic cleavage, is similar to the consensus (Ala-X-Ala) for recognition by signal peptidase I (16, 17, 35). Despite intense efforts, the exact roles played by the signal peptide in export are still not clear. The signal peptide appears to be involved in maintaining the precursor in an export competent conformation and to promote attachment of the precursor to the inner membrane (34). These effects may require additional factors. Later in the process, cleavage of the signal peptide is required to release the translocated protein from the inner membrane; cleavage is not required for translocation itself (11, 17).

Much less information is available on the regions in the mature protein important for export and on the role of these regions in the export process. A priori, the sequence of the transferred protein has to satisfy at least two conditions. On the one hand, it has to be export compatible: for example, it should not include highly hydrophobic membrane anchor sequences (12). On the other hand, it may also play an active role in export. For periplasmic proteins such as MBP, it was already shown that the C-terminal 25 to 35 residues are needed for release into the periplasm but are not required for translocation (22). It was also shown that mutations in the mature part of the protein could reverse, at least to some extent, the effect of mutations of the signal peptide affecting initiation of export (for reviews, see references 10 and 36): three amino acids in the mature portion of the protein (Lys-1, Gly-19 and Tyr-283) have been identified by this criterion. On the basis of in vivo competition experiments between wild-type and export-defective proteins, it has also been proposed that amino acid residues 89 to 189 contain an internal export sequence (1, 2). In addition, it has been suggested that MBP is competent for export only before it folds into its final stable tertiary conformation (33). All this points to a role for the mature part of the protein in export, but the exact functions and the nature of the sequences involved remain to be elucidated.

In the present paper, we provide information pertinent to the role of the mature part of the periplasmic maltose-binding protein of *E. coli* in the last step of the export: release into the periplasm. We show that instructions for this step are encoded in two distinct regions internal to the mature protein and discuss possible implications of this result.

**MATERIALS AND METHODS**

Media, bacterial strains, and growth conditions. Minimal medium M63 and indicator media have been described (29).
All strains used in this study are isogenic derivatives of *E. coli* K-12 strain pop3325 (F− ΔlacU169 araD139 rpsL150 thi fliB3501 deoC7 ptsF25 relA1 malT(Con)I). The malT(Con)I allele confers constitutive expression on the maltose operons. PMPD92 was constructed by introducing the *metA* allele into pop3325 by transduction to rifampin resistance and screening for a Met− phenotype by using bacteriophage P1 *vir* grown on MM131 (9). The *malE* mutations were crossed by P1 *vir* transduction into strain PMPD92. Met− transductants were selected and screened for a Mal− phenotype on MacConkey maltose plates.

All strains were grown overnight at 37°C in M63B1 medium containing 0.2% glycerol and diluted 1:50 into the same medium containing 0.2% glycerol and 0.2% maltose. Cultures growing exponentially were labeled at a cell density of 3 × 10⁶ cells per ml.

**Pulse-chase experiments.** The cells were labeled with 50 μCi of [35S]methionine per ml for 15 s. A chase with unlabeled methionine was performed by addition of an excess of nonradioactive methionine (final concentration, 0.05%) for 45 s. The pulse-labeling and pulse-chase-labeling experiments were terminated by mixing the sample with an equal volume of ice-cold 10% trichloroacetic acid. In experiments in which export was blocked, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 8 μM. 5 min before the cells were labeled with 50 μCi of [35S]methionine per ml for 1 min. CCCP is an uncoupler that dissipates proton motive force and blocks export (32).

**Localization of proteins.** Cultures (1.5 ml) were labeled with 40 μCi of [35S]methionine for 40 s. The samples were chilled by being mixed with 1.5 ml of a crushed-ice solution containing 200 μg of chloramphenicol per ml and 200 mM sodium azide. The cells were washed with 0.5 ml of 50 mM Tris hydrochloride (pH 8)−15 mM TAME (tosyl arginine methyl ester) and suspended in 0.3 ml of 0.1 M Tris hydrochloride (pH 8)−0.5 M sucrose−5 mM EDTA−15 mM TAME. Lysozyme was added to a final concentration of 160 μg/ml, and then 0.3 ml of ice-cold water was added. After incubation for 30 min on ice, the spheroplasts were divided into three portions of 0.2 ml and subjected to three parallel treatments.

(i) **Protease K treatment in lyed cells.** The spheroplasts were centrifuged, and the supernatant was kept as the periplasmic fraction. The spheroplasts were lysed by suspension in 0.8 ml of 50 mM Tris hydrochloride (pH 8)−2.5 mM EDTA. After the spheroplasts were lysed, the periplasmic fraction was added back. The samples were then treated with protease K at a final concentration of 50 μg/ml after addition of MgSO₄ to a final concentration of 20 mM. The suspensions were incubated on ice for 20 min, and phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM.

(ii) **Proteinase K treatment in intact spheroplasts.** MgSO₄ was added to a final concentration of 20 mM, and the spheroplasts were treated with protease K as described above for lysed spheroplasts.

(iii) **Cell fractionation and control experiment in the absence of proteinase K.** The spheroplasts were either precipitated with 5% trichloroacetic acid or centrifuged to separate periplasmic proteins from spheroplast-associated proteins. In the latter case, the spheroplasts were stabilized with 20 mM MgSO₄ (final concentration) and centrifuged for 10 min. The supernatant was kept as the periplasmic fraction. The spheroplasts were washed once with 50 mM Tris hydrochloride (pH 8)−0.25 M sucrose and lysed in 50 mM Tris hydrochloride (pH 8)−2.5 mM EDTA.

**Immunoprecipitation and polyacrylamide gel electrophoresis.** All the samples were precipitated with 5% trichloroacetic acid (final concentration) and solubilized in 50 mM Tris hydrochloride (pH 8)−1% sodium dodecyl sulfate (SDS)−1 mM EDTA−1 mM phenylmethylsulfonyl fluoride. MBP was immunoprecipitated with rabbit anti-MBP serum and protein A (21). During the immunoprecipitation procedure, all the solutions contained 1 mM phenylmethylsulfonyl fluoride. Immune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (11% acrylamide) and autoradiography.

**RESULTS**

We described previously a set of MBP mutant proteins generated by random insertion of an oligonucleotide linker into *malE* and selection for stable mutant proteins which could be immunoprecipitated with an anti-MBP serum. In contrast to wild-type MBP and to the other 13 mutant proteins isolated, 6 mutant proteins could not be released from intact bacteria by cold osmotic shock (14). This procedure is taken as an operational criterion that the proteins are located in the periplasm (23, 24). Interestingly, the six mutant proteins were unable to function in the transport of maltose (14).

The nature of the six mutations is shown in Fig. 1. In all cases, a deletion of several base pairs took place in addition to the insertion of the linker. One insertion (*malE300*) changed the *malE* reading frame, resulting in an early termination of MBP.

**Processing.** To obtain more information on the cellular location of the six mutant proteins, we examined whether their signal peptides were processed. Since the processing enzyme, signal peptidase I, is located at the external face of the cytoplasmic membrane (38), processing points to at least a partial export of MBP for these mutants. Conversely, a defect in processing is a strong indication of a defect in export. There are, however, some exceptions, since mutations have been described which prevent signal peptide processing but not export (8, 17).

For the five mutants affected in the mature part of the protein, as well as for the wild type, immunoprecipitation after a 15-s pulse of radioactive [35S]methionine revealed two species of MBP on SDS-polyacrylamide gel electrophoresis (MalE302, MalE345, MalE312, and MalE364 [Fig. 2]; MalE300 [data not shown]). The slower-migrating bands disappeared after a chase of 45 s with an excess of nonradioactive methionine. These results suggest that the minor band corresponds to the precursor form (pMBP) and the major band corresponds to the mature form (mMBP) of the protein. They indicate that the signal peptides of these five mutant proteins are processed. In contrast, mutant MalE127 presented only one band, in which radioactivity continued to accumulate during the chase period. This band migrated with the same apparent molecular weight as pMBP, suggesting that no processing occurs (Fig. 2). The proposal that the signal peptide of mutant protein MalE127 is not processed is in good agreement with the fact that the *malE* mutation responsible for this mutant deletes amino acids 19 to 24 of pMBP, within the N-terminal extension and near the cleavage site (see Discussion).

In conclusion, the five mutations located within the mature part of the protein do not prevent in vivo cleavage of the signal peptide, whereas the one mutation located within the signal peptide prevents cleavage. The rate of maturation of
these mutant proteins seems comparable to that of wild-type MBP (16): only small amounts of mutant pMBP were detected after 15 s of labeling, and only the mature form was detected after a short chase period of 45 s.

**Accessibility to protease K.** To obtain independent evidence for localization, we examined the effect of the addition of protease K to the mutant proteins in spheroplasts. Sensitivity to protease shows that the protein, or at least part of it, has been exported through the cytoplasmic membrane. For each of the six mutants, cells were labeled for 40 s with [35S]methionine and spheroplasts were prepared and subjected to three parallel treatments before immunoprecipitation with an anti-MBP serum, SDS-polyacrylamide gel electrophoresis, and autoradiography: (i) treatment with protease K, (ii) no addition of protease K as a control for labeling and protein stability, and (iii) treatment with protease K after spheroplast lysis. This last condition served as a control for the sensitivity of the proteins to protease K.

At this stage it is worth recalling that wild-type mMBP is resistant to proteases. For wild-type pMBP, treatment with proteases removes an amino-terminal domain corresponding approximately to the signal peptide but does not degrade the mature portion of the protein (13). Similarly, pMBP with signal sequence mutations usually yield the mature protease-resistant portion on protease treatment (33). The data shown in Fig. 3 confirm these results. Protease sensitivity was detected by the decrease or absence of the total amount of mutant protein immunoprecipitated. As expected, wild-type mMBP was resistant to protease K added to the cell lysates (Fig. 3, panel WT, lane S1), to the spheroplasts (panel WT, lane S), or to the periplasmic fluid (panel WT, lanes P and Sp). After treatment with protease K, mutant protein MalE127 was degraded to a form migrating like the wild-type MBP in lysed spheroplasts (Fig. 3, panel 127, lane S1). This is in agreement with the idea that MalE127 is a precursor species, which is processed to a form identical or similar to the mature wild type. MalE127 remained undegraded in intact spheroplasts (panel 127, lane Sp). In addition, in contrast to wild-type MBP, most of the MalE127 protein remained associated with spheroplasts (panel 127,
lanes P and S). These results are in agreement with the idea that MalE127 is not exported.

The five MBP carrying a mutation in the mature part of MBP were degraded by proteinase K in spheroplasts in the absence of lysis (Fig. 3, lanes Sp +): this confirmed their extracytoplasmic location. The extent of degradation varied among the five mutants: in particular, MalE364 was clearly less sensitive than the others. In all cases, this indicated that the conformation of the five mutant proteins was different from that of the wild type.

The integrity of the spheroplasts in the experiment was confirmed by the fact that neither the precursor form of wild-type MBP nor that of MalE127 was degraded by the addition of proteinase K to intact spheroplasts (Fig. 3, panels WT and 127, lane Sp).

**DISCUSSION**

We have examined six stable mutant MBP, which are deficient for maltose transport and are not released by osmotic shock. The mutations were due to insertion of an oligonucleotide linker concomitant with various deletions (Fig. 1). Five of the mutations lay in the part of the malE sequence corresponding to mMBP. One is internal to the signal peptide. We will discuss successively the results obtained with each of these two categories of mutants.

**Mutations in the mature protein.** The five mutant proteins affected in the mature part of the sequence are at least partially exported through the cytoplasmic membrane, since their signal peptide is processed and since they are sensitive to proteinase K in intact spheroplasts. The sensitivity to proteinase K also indicates that these proteins are not folded like wild-type mMBP, which is resistant to proteinase.

What is the status of these mutant proteins with respect to export? Several arguments suggest that these five mutants could be affected in a late step of export, whereby the protein is released from the inner membrane into the periplasm. Despite their sensitivity to proteinase, these proteins must be quite stable in vivo, since they can be detected in unlabeled form on a Coomassie blue-stained gel. This is compatible with the idea that they could be protected from degradation by being partially inserted in the membrane. In addition, cells harboring the five different mutated alleles on a multicopy plasmid lysed under inducing conditions, i.e., in the presence of maltose. Thus, the five mutant proteins, when overproduced, were toxic to the cell. Toxicity is usually observed for poorly exported hybrid proteins. Mutants blocked in a late stage of the secretion process have already been reported. Truncated forms of several periplasmic proteins (\(\beta\)-lactamase, MBP, and glycerol-phosphate phosphodiesterase) were processed but stayed associated with membranes and were sensitive to externally added proteases (6, 19, 22). For glycerol-phosphate phosphodiesterase, the accumulation of the truncated form appears to block a step common to export of different proteins. Recently, it has been shown that substitution at either of the two cysteines in \(\beta\)-lactamase prevented release from the membrane during secretion (18).

These five mutants can be organized into three groups. Three mutations (malE302, malE345, and malE364) are clustered between residues 18 and 42 of the mature MBP sequence. These three deletions overlap at residues 32 and 33. Mutation malE312 defines a second region between residues 280 and 306. Finally, malE300 encodes a truncated form of the protein missing the last 20 C-terminal residues. It is striking that two regions described here include two of the three sites identified so far by mutations suppressing the effects of signal peptide mutation: site 19 (Gly to Val) (3) and site 283 (Tyr to Asp) (10). This strongly suggests that these regions play a critical role in the export. However, at present, it is not possible to exclude other explanations such as complete export of an insoluble protein which stays adsorbed on the outer face of the inner membrane.

How could an alteration in the mature portion of the MBP prevent the release of the protein into the periplasm? Conformational changes have been suggested to play a specific role during the release of the protein into the target compartment. Studies of \(\beta\)-lactamase export suggested that an intermediate, which would be protease sensitive and bound to the outer face of the inner membrane, may exist in the normal export of this periplasmic protein (30). The two cysteine

![Figure 3](http://jb.asm.org/DownloadedFromJ.Bacteriol/vol-170/issue-6/figure3.png)

**FIG. 3.** Localization and proteinase K sensitivity experiments. Strains were labeled for 40 s with \(\text{[35S]}\)methionine. Spheroplast-associated proteins (lanes S) and periplasmic proteins (lanes P) or proteins released after the spheroplasts were washed (lanes P') (see Materials and Methods) were separated for MalE127 and wild-type MBP. Unfractionated spheroplasts (lanes Sp) or lyzed unfractionated spheroplasts (lanes SI) were subjected to treatment with proteinase K (+) or not treated.
residues of β-lactamase were proposed to be involved in the conformational change during the release of the protein into the periplasm (18). For the carboxyterminal nonsense mutants (19, 22, 25), the lack of the C-terminal sequence might prevent the protein from achieving the conformation required for its release into the periplasm. The two regions of the mature protein described here appear to correspond to sites important for conformational changes of the protein during export. In this respect, it would appear logical that if a conformational change was involved in the insertion of a protein from the cytoplasm into the membrane, another change would be required for the protein to emerge on the other side (34). Finally, it is relevant to indicate that folding into the active form and protease resistance may not be required for release into the periplasm. Three linker insertion mutant proteins described previously (MalE120, MalE183, and MalE220) are released by osmotic shock but are inactive for transport; one of them (MalE220) is partially degraded in vivo (14). Further studies of the relation between sequence, conformation, and export will certainly benefit from the elucidation of the structure of the protein and of its mode of folding.

**Mutation in the signal peptide.** Mutant MalE127 is affected in the signal peptide. The protein is neither exported nor processed. The strain is completely unable to grow on maltose, which is not the case for most MalE signal peptide mutants (36). This may be due to two additive effects. On the one hand, the mutation corresponds to the introduction of an Arg residue (instead of Met) at position 19 of the signal peptide. It was already shown that such a mutation results in a tight block in export (36). This is enough to prevent in vivo processing of the signal peptide. In addition, there is also an Arg at position 21, which is -3 with respect to the normal cleavage site of the signal peptide. Introduction of a charged residue at this position is also known to be sufficient, by itself, to prevent processing by signal peptidase, but not export (16). It is also worth noting that the mature portion of MalE127 is resistant to proteinase K, as is wild-type MBP (13, 33). MalE127 is also able to bind amylase (6a, 14). This confirms that export and processing of the signal peptide are not required for pMBP to fold in a conformation similar to that of wild-type mMBP, i.e., to be able to bind substrate and to be proteinase K resistant (15, 20, 33).

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**LITERATURE CITED**


