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Analysis of Mutational Alterations in the Hydrophilic Segment of the Maltose-Binding Protein Signal Peptide

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Oligonucleotide-directed mutagenesis was employed to investigate the role of the hydrophilic segment of the *Escherichia coli* maltose-binding protein (MBP) signal peptide in the protein export process. The three basic residues residing at the amino terminus of the signal peptide were systematically substituted with neutral or acidic residues, decreasing the net charge in a stepwise fashion from +3 to −3. It was found that a net positive charge was not absolutely required for MBP export to the periplasm. However, export was most rapid and efficient when the signal peptide retained at least a single basic residue and a net charge of +1. The nature of the adjacent hydrophobic core helped to determine the effect of charge changes in the hydrophilic segment on MBP export, which suggested that these two regions of the signal peptide do not have totally distinct functions. Although the stepwise decrease in net charge of the signal peptide also resulted in a progressive decrease in the level of MBP synthesis, the data do not readily support a model in which MBP synthesis and export are obligately coupled events. The export defect resulting from alterations in the hydrophilic segment was partially suppressed in strains harboring certain *prl* alleles but not in strains harboring *prlA* alleles that are highly efficient suppressors of signal sequence mutations that alter the hydrophobic core.

The periplasmic maltose-binding protein (MBP) of *Escherichia coli* is synthesized with an amino-terminal signal peptide that is thought to be chiefly responsible for initiating the export of this protein from the cytoplasm. Although the MBP signal peptide exhibits little primary sequence homology with other signal peptides, it exhibits conserved features typical of signal peptides of both procaryotic and eucaryotic origin (28, 34, 39). It is 26 amino acids long and has three recognizable regions. The first eight residues constitute the hydrophilic segment. This region carries a net positive charge because of the presence of three basic residues. The hydrophilic segment is followed by the hydrophobic core, a region devoid of charged residues and predicted to assume an α-helical conformation (6). Both statistical studies (6, 28, 34, 39) and mutational analyses (3, 32) have demonstrated that the overall hydrophobicity of this region is a major determinant of signal peptide function. Finally, the six carboxyl-terminal residues of the MBP signal peptide (residues −1 to −6 relative to the cleavage site) represent the recognition sequence for the processing enzyme, signal peptidase I. This region is the most highly conserved one among various signal peptides, since the cleavage site must be recognized by the processing enzyme (38). Numerous alterations in this region that affect MBP processing but not translocation across the cytoplasmic membrane have been obtained (12; J. D. Fikes, G. A. Barkocy-Gallagher, D. G. Klapper, and P. J. Bassford, Jr., submitted for publication).

A specific role for the hydrophilic segment in protein export was first suggested in the loop model (19). The basic residues of the hydrophilic segment were proposed to initiate an ionic interaction between the signal peptide and the negatively charged inner face of the cytoplasmic membrane. Several studies have sought to experimentally determine the role of this region, particularly the basic residues, in the export process. Inouye et al. (20) and Vlasuk et al. (37) found that changing the net positive charge at the amino terminus of the lipoprotein (Lpp) signal peptide from +2 to −2 resulted in an accumulation of precursor Lpp as well as a decrease in overall Lpp synthesis. Hall et al. (16) observed that a mutation that removed one of the two basic residues from the LamB signal peptide caused a decrease in LamB synthesis but did not appear to otherwise affect LamB export to the outer membrane. Most recently, Iino et al. (18) also found that a net positive charge in the hydrophilic segment of the staphylokinase signal peptide was required for efficient export when the protein was expressed in *E. coli*, but a significant decrease in protein synthesis was not observed.

Genetic selections have been successfully used to generate mutational alterations that result in an export-defective MBP signal peptide (summarized in reference 4). However, even though one such selection was specifically designed to yield signal peptides exhibiting only a minor export defect, alterations in the hydrophilic segment that conferred an export defect were not obtained. Several mutations affecting the hydrophilic segment have been isolated. These mutations eliminated one of the three basic residues and were encountered among linked revertants that restored function to signal peptides with defective cores (3, 32). Thus, these studies failed to reveal any information on the role of the hydrophilic segment in MBP export.

In this study, the function of the hydrophilic segment of the MBP signal peptide was investigated. Through the use of oligonucleotide-directed mutagenesis, the basic residues in this region were systematically replaced with neutral or acidic residues, and the effect of these alterations on MBP synthesis and export was determined. In addition, export of

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these altered MBP species was analyzed in strains harboring certain *prlA* or *prlD* mutations that suppress mutations altering the hydrophobic core of the MBP signal peptide (2, 9, 10, 31).

MATERIALS AND METHODS

Bacterial strains and plasmids. Strain BAR1091, a derivative of strain MC4100 (8), has been described previously (30). It harbors the *malEΔ312* mutation, an inframe, nonpolar deletion that removes DNA sequences encoding residues 15 of the MBP signal peptide through 159 of the mature moiety. Isogenic derivatives of BAR1091 harboring various *prl* alleles were constructed by P1 transduction, using standard genetic techniques. Strain KI2100 (MC4100 *prlF1*) (23) was obtained from T. Silhavy, Princeton University, Princeton, N.J. Strain CJ236 (24) was obtained from T. Kunkel, National Institute of Environmental Health Sciences, Research Triangle Park, N.C. The *lacUV5 malE* plasmids pJF2 (13), pJW8, and pJW28 (40a) are described elsewhere.

Reagents. Minimal medium M63 supplemented with a carbon source (0.2%) and thiamine (2 μg/ml), maltose tetrazolium indicator agar, and TYE agar were prepared as described previously (25). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50 μg/ml, respectively. To induce *malE* genes under *lacUV5* promoter-operator control (30), isopropyl-β-D-thiogalactoside (IPTG) was used on agar plates and in liquid media at 1 and 5 mM, respectively. [^{35}S]methionine (Translabel) was obtained from ICN Biomedicals, Inc., Irvine, Calif. Rabbit anti-MBP serum and anti-OmpA serum have been described previously (1). Electrophoresis reagents and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. XAR film was obtained from Eastman Kodak Co., Rochester, N.Y.

Construction of plasmids. To construct derivatives of plasmids pJF2 (encoding the wild-type MBP signal peptide) and pJW28 (encoding the MBP R2 signal peptide [MBP-R2]) harboring mutations in the signal sequence-coding region, the in vitro oligonucleotide-directed mutagenesis procedure of Zoller and Smith (41) was used. Mutagenic primers, ranging in length from 15 to 26 nucleotides (depending on the mutation), were prepared with an Applied Biosystems 380A DNA synthesizer. Single-stranded DNA templates for mutagenesis were prepared from plasmids pJF2 and pJW28 (or mutant derivatives of these plasmids) by using an M13 helper phage, M13K07, as described elsewhere (36). Preparation of plasmid DNA from strain CJ236 (*ung dut*) resulted in the incorporation of uracil into the template DNA (24). After mutagenesis, heteroduplex DNA was transformed into competent cells of strain BAR1091 (*ung*$^+$ *dut*$^+$), which resulted in degradation of the DNA parental template strand. This allowed recovery of mutagenized plasmids at a sufficiently high frequency that a phenotypic screen to detect mutagenized plasmids was not required. Mutations were confirmed by DNA sequencing as described by Bankier et al. (5).

The following codon changes were made in the MBP signal peptide coding region. To construct pUZ3 and pTL1, the codon AAA (Lys at residue 4) of pJW28 or pJF2 was changed to ATA (Ile). For pUZ4 and pTL2, the codon AAA (Lys at residue 2) of pUZ3 or pTL1 was changed to ACA (Thr). For pTL3, the codon CGC (Arg at residue 8) of pTL2 was changed to CTC (Leu). For pUZ6 and pTL5, the codon ACA (Thr at residue 2) of pUZ4 and pTL3 was changed to GAA (Glu). For pUZ7 and pTL6, the codon ATA (Ile at residue 3) of pUZ6 or pTL5 was changed to GAA (Glu). For pUZ8 and pTL8, the codon ATA (Ile at residue 4) of pUZ7 and pTL6 was changed to GAA (Glu). Finally, to construct plasmid pTL7, the codon ATG (Met at residue 19) of pTL6 was changed to AGG (Arg).

Pulse-chase and cell fractionation experiments. Cells were grown in glycerol minimal medium at 30°C to mid-log phase and induced for MBP synthesis by the addition of IPTG for 45 min. Pulse-chase experiments were performed as previously described (31). The chase was terminated by precipitation of cellular proteins with an equal volume of ice-cold 10% trichloroacetic acid. For cellular fractionation experiments, a miniversion of the cold osmotic shock procedure of Neu and Heppel (26) was used as described previously (9). For quantitation of MBP synthesis, induced cells were radiolabeled with [^{35}S]methionine for 10 min, and the protein was precipitated as described above.

Immunoprecipitation, SDS-PAGE, and autoradiography. MBP and OmpA were immunoprecipitated from radiolabeled solubilized cell extracts by procedures described previously (31). Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, also as previously described (31). For quantitation of MBP synthesis, the corresponding precursor (pre-MBP) MBP, mature MBP (mMBP), and OmpA bands were excised from dried gels, rehydrated, and solubilized with 9:1 NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.)-water. The amount of radioactivity present in each band was then determined by scintillation counting in Scintiverse II (Fisher Scientific Co., Pittsburgh, Pa.). MBP counts were adjusted for the loss of methionine residues when pre-MBP is processed to mMBP (40a). It was assumed that the OmpA levels remained constant for cells of each strain tested, and determination of the amount of MBP synthesized was adjusted accordingly.

RESULTS

Oligonucleotide-directed mutagenesis of the hydrophilic segment of MBP-R2. The R2 signal peptide has a truncated hydrophobic core and one less basic residue than does the wild-type MBP signal peptide, yet it facilitates MBP export with virtually wild-type efficiency (3, 12). It was therefore decided to begin studies of the hydrophilic segment of MBP by systematically substituting the remaining charged residues of the R2 signal peptide with neutral, hydrophobic, or acidic residues. This was accomplished in vitro by oligonucleotide-direct mutagenesis. Plasmid pJW28 encodes MBP-R2 under regulatory control of the *lacUV5* promoter-operator (40a). This plasmid also harbors the intergenic region of phage M13. After M13 infection of cells harboring pJW28, plasmid DNA replication switches to the phage mode, and single-stranded plasmid DNA is packaged into phage particles that bud from the cell (36). This provided a convenient source of single-stranded template *malE* DNA for mutagenesis. Oligonucleotide-directed mutagenesis was performed as described in Materials and Methods; the mutational alterations generated in MBP-R2 are shown in Fig. 1.

Analysis of MBP-R2 derivatives with alterations in the hydrophilic segment. Since MBP must be secreted into the periplasm to facilitate the uptake and subsequent utilization of maltose, this characteristic provides an extremely sensitive assay for the export of functional MBP. The mutagenized derivatives of plasmid pJW28 were introduced into strain BAR1091, and the abilities of the various MBP species to facilitate maltose transport were determined by plating cells on maltose tetrazolium indicator agar. All of the plasmid-bearing strains exhibited a wild-type Mal$^+$ phenotype

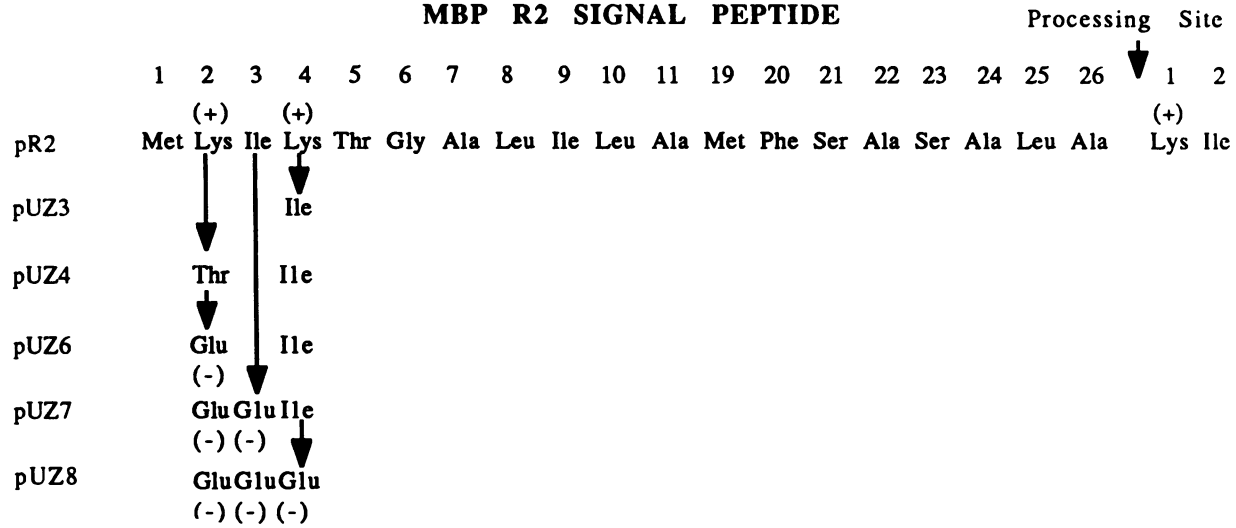

FIG. 1. Primary amino acid sequence of MBP-R2 derivatives with alterations in the hydrophilic segment. Plasmid pJW28, encoding MBP with the R2 signal peptide (top line), was subjected to oligonucleotide-directed mutagenesis (as described in Materials and Methods) in order to introduce various substitutions in the hydrophilic segment. Arrows indicate changes in primary amino acid sequence from the preceding construct.

with the exception of the strain harboring pUZ8 (−3), which yielded colonies displaying a strongly Mal$^-$ phenotype (data not shown). These same strains were tested for the ability to use maltose as a sole carbon source on maltose minimal agar. Only the strain harboring pUZ8 (−3) was unable to grow on this medium; growth of each of the remaining strains was indistinguishable from that of cells harboring the parental plasmid pJW28.

In previous studies, the processing of pre-MBP to mMBP has correlated well with MBP export (1–3, 9, 30–32). The rate of MBP processing was determined by pulse-chase analysis. Strains were pulse-labeled for 15 s with [^{35}S] methionine and then incubated for various periods in a chase solution containing unlabeled methionine. The chase periods were terminated at various times, and the labeled MBP species present at each chase point were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography (see Materials and Methods) (Fig. 2). As found previously (2, 12), the R2 signal peptide mediated very rapid MBP export; only a small amount of pre-MBP was detected at early time points. This also was the case for the MBP encoded by pUZ3 (+1). In contrast, the MBP species encoded by pUZ4 (0), pUZ6 (−1), pUZ7 (−2), and pUZ8 (−3) were processed with noticeably slower kinetics, with the ratio of pre-MBP to mMBP at later time points increasing as the charge of the hydrophilic segment decreased. In the case of cells harboring pUZ8 (−3), no mMBP could be discerned at the 10-min chase point, which correlated well with the Mal$^-$ phenotype of these cells.

To determine the intracellular location of the MBP species resulting from alterations in the hydrophilic segment, cells harboring pUZ7 (−2) were subjected to the osmotic shock procedure of Neu and Heppel (26). Cells in mid-log phase were incubated in the presence of [^{35}S]methionine for 10 min. MBP was immunoprecipitated from solubilized whole cells, shocked cells, and periplasmic fractions and analyzed by SDS-PAGE and autoradiography (Fig. 3). For the control cells harboring a plasmid encoding MBP-R2 (pJW8), the great majority of the mMBP was released by osmotic shock. For the control cells harboring plasmid pJW8, encoding MBP19-1, an MBP species that is strongly export-defective (4), the pre-MBP remained with the shocked cells. For cells carrying plasmid pUZ7 (−2), the pre-MBP clearly fractionated with the shocked cells, whereas the mMBP was released into the periplasmic fraction.

MBP derivatives with alterations in the hydrophilic segment and a full-length hydrophobic core. Oligonucleotide-directed mutagenesis was used with plasmid pJF2, encoding the wild-type MBP (13), to systematically change the net charge of the hydrophilic segment from +3 to −3 as described above. The mutational alterations generated in the full-length MBP signal peptide are shown in Fig. 4. When tested on either maltose tetrazolium indicator agar or maltose minimal agar, each of the plasmid-bearing strains, including the strain harboring plasmid pTL8 (−3), exhibited a fully Mal$^+$ phenotype.

The rate of processing for these derivatives of the wild-type MBP was determined by pulse-chase analysis as described above (Fig. 5). Once again, the MBP species in which the net charge of the hydrophilic segment was less than +1 were processed with detectably slower kinetics. However, MBP species harboring changes in the hydrophilic segment adjacent to a full-length hydrophobic core were exported more efficiently than were their MBP-R2 counterparts. For MBP encoded by plasmids pTL5 (−1) and pTL6 (−2), pre-MBP could not be discerned at the 10-min chase point. Even for cells harboring plasmid pTL8 (−3), approximately 60% of the MBP synthesized had been matured after 10 min of chase.

Synthesis of MBP species with alterations in the hydrophilic segment at reduced levels. The relative levels of synthesis of each of the MBP species described above were determined by radiolabeling cells for 10 min with [^{35}S]methionine, followed by immunoprecipitation, SDS-PAGE, and autoradiography. As an internal standard for these experiments, OmpA protein was simultaneously immunoprecipitated from the solubilized cell extracts. After autoradiography, bands corresponding to MBP (both precusor and mature forms) and OmpA were excised from the gel and quantitated as described in Materials and Methods (Fig. 6). The results

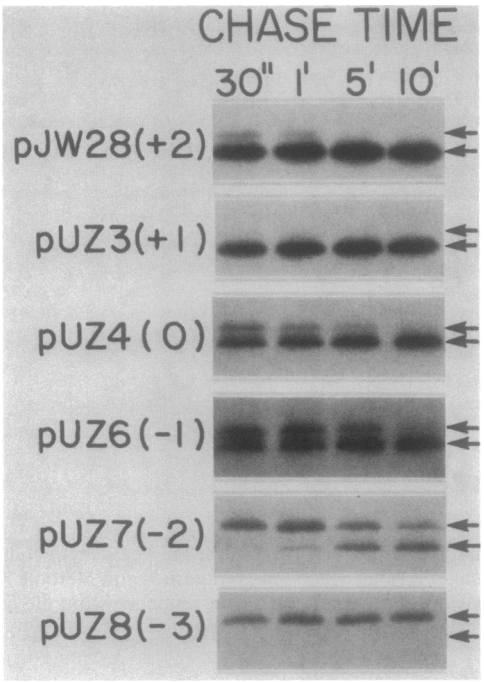

FIG. 2. Comparison of export kinetics for MBP-R2 and derivatives with alterations in the hydrophilic segment. Cells of strain BAR1091 harboring plasmid pJW28 (MBP-R2) or plasmids encoding various altered MBP-R2 species (see Fig. 1) were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine. At the indicated time points, equal portions were removed, the chase was terminated, and the MBP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Positions of pre-MBP and mMBP are indicated by arrows. The net charge of the hydrophilic segment of each mutant signal peptide is indicated at the left. Because of differences in the amounts of MBP synthesized by various mutants (see text), exposure times for the gels shown were not uniform.

clearly indicated that as the net charge of the MBP signal peptide decreased, the total amount of MBP synthesized relative to the amount of OmpA synthesized decreased as well. (Note that the pulse-chase experiments previously described indicated that mutational alterations in the hydrophilic segment had no effect on the rate of MBP turnover, which otherwise could have influenced these results. As shown in Fig. 2 and 5, the amount of total radiolabeled MBP precipitated at the 10-min chase point was not significantly different from the amount precipitated at the 30-s chase point.)

MBP19-1 is a strongly export-defective MBP species in which an Arg residue has been substituted for Met at position 19 in the hydrophobic core of a full-length signal peptide (4). This protein is synthesized at approximately the same level as wild-type MBP, regardless of whether the *malE* gene is located in the chromosome and transcribed from the *malE* promoter or is present on a multicopy plasmid under *lacUV5* promoter control (9, 30). Oligonucleotide-directed mutagenesis was used to introduce the *malE19-1* mutation into plasmid pTL6 (−2), thus placing the hydrophobic core alteration in *cis* to a hydrophilic segment having a net negative charge. Cells harboring this new construct, designated plasmid pTL7, were totally Mal$^-$, as demonstrated by their inability to grow on maltose minimal agar (data not shown). Cells harboring plasmids pJF2 (+3), pJW8 (+3, 19-1), pTL6 (−2), and pTL7 (−2, 19-1) were radiolabeled with [^{35}S]methionine for 10 min, and the relative levels of MBP synthesis were compared by immunoprecipitation, SDS-PAGE, and autoradiography (Fig. 7, lanes A through D). The MBP encoded by pTL7 (−2, 19-1) was synthesized at the same level as that encoded by pTL6 (−2). These results demonstrated that the absence or presence of an export-defective hydrophobic core did not influence the relative amounts of MBP synthesized by these cells. For cells harboring plasmid pTL7 (−2, 19-1), MBP was precipitated only in the precursor form, which correlated well with the Mal$^-$ phenotype of these cells.

Suppression of the export defect of MBP species with alterations in the hydrophilic segment by extragenic *prl* alleles. Previous studies have shown that extragenic suppressor mutations designated *prlA* and *prlD* can restore, with various efficiencies, the export of proteins with defective signal peptides (2, 9, 10, 31). The effect of several of these *prl* alleles on the export of the MBP species encoded by pUZ7 (−2) was investigated by pulse-chase analysis (Fig. 8). For cells harboring one of two particularly strong *prlA* alleles, *prlA4* (9, 10) or *prlA402* (2), the export efficiency of the MBP encoded by plasmid pUZ7 (−2) was not improved. In fact, it appeared that the export of this MBP species actually was somewhat antagonized by the presence of the *prlA4* allele, since approximately 20% of the MBP had been processed by the 10-min chase point in *prlA4* cells, compared with approximately 50% processing in *prl*$^+$ cells. In contrast, MBP export was markedly improved in cells harboring the *prlD2* (31) allele, with approximately 80% maturation after 10 min of chase. A previous study had shown that the presence of both *prlA4* and *prlD2* in the same strain resulted in a synergistic suppression of *malE* signal sequence mutations (31). However, the effect on MBP export for *prlA4 prlD2* cells harboring plasmid pUZ7 (−2) was similar to that observed for *prlA*$^+$ *prlD2* cells. Consistent with these results was the finding that *prlD2* cells but not *prlA4* or *prlA402* cells harboring plasmid pUZ8 (−3) were able to utilize maltose for growth, although *prlD2*-mediated suppression of the MBP export defect could not be discerned by demonstration of pre-MBP processing (data not shown). Also, the presence of the *prlA402* allele did not improve export of the MBP species encoded by pTL7 (−2, 19-1) (Fig. 7, lane E). Greater than 80% of MBP19-1 with a normal hydrophilic segment was previously shown to be exported in *prlA402* cells (2).

Finally, the ability of the *prlF1* mutation to affect export of MBP species altered in the hydrophilic segment was investigated. This *prl* mutation was not obtained as a suppressor of signal sequence mutations; rather, it was obtained in a

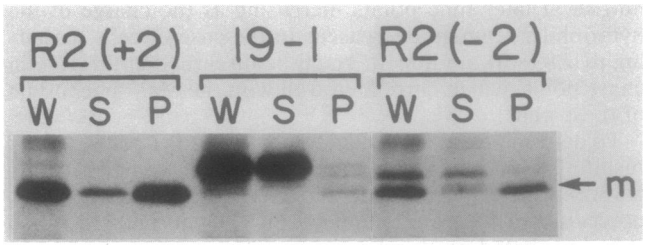

FIG. 3. Cold osmotic shock localization of MBP. Cells harboring plasmids pJW28 (MBP-R2), pJW8 (MBP19-1), or pUZ7 [MBP-R2(−2)] were radiolabeled with [^{35}S]methionine for 10 min. MBP was immunoprecipitated from solubilized whole-cell (W), shocked-cell (S), and periplasmic (P) fractions prepared as described in Materials and Methods. The arrow indicates the position of mMBP. See text for additional experimental details.

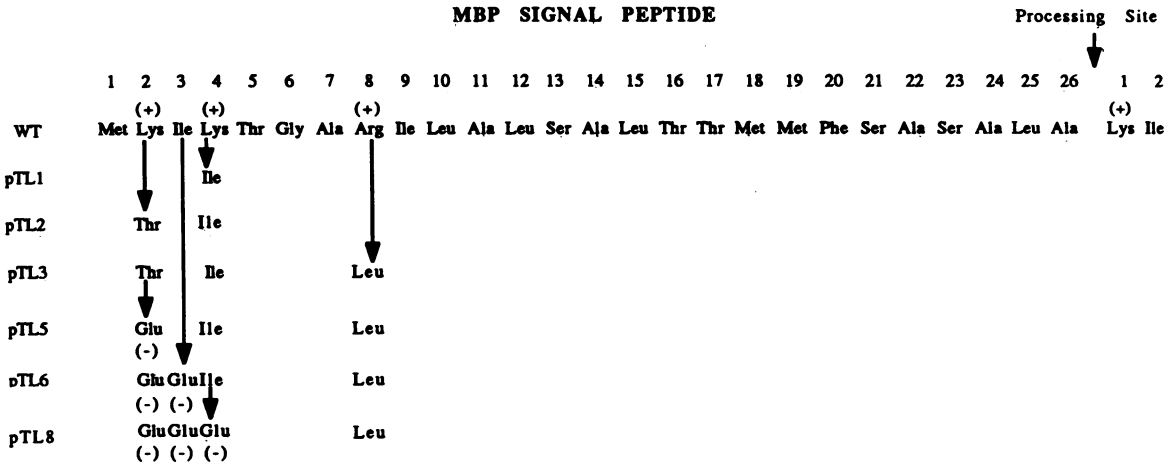

FIG. 4. Primary amino acid sequence of wild-type MBP signal peptide and derivatives with alterations in the hydrophilic segment. Plasmid pJF2, encoding the wild-type *malE* gene, was subjected to oligonucleotide-directed mutagenesis (as described in Materials and Methods) in order to introduce various substitutions in the hydrophilic segment. Arrows indicate changes in primary amino acid sequence from the preceding construct.

search for extragenic mutations that relieved the overproduction lethality resulting from synthesis of certain LamB-LacZ and MBP-LacZ hybrid proteins (23). Although an unambiguous effect was difficult to discern for most of the mutant proteins described above, it was found that *prlF1* cells harboring plasmid pUZ8 (−3) were able to utilize maltose for growth. In addition, some MBP processing was clearly detected (Fig. 9). It was interesting to find that, in this case, mMBP easily was discerned at the 30-s chase point, with little further change in the preMBP/mMBP ratio at later chase points. In contrast, for *prlA*- and *prlD*-mediated suppression of MBP export defects, kinetic studies invariably have shown that MBP export is achieved in a slow, post-translational manner, with little indication of improved MBP processing at early chase points (31; Fig. 8).

DISCUSSION

Oligonucleotide-directed mutagenesis was used to investigate the role of the three basic residues residing in the amino-terminal hydrophilic segment of the MBP signal peptide in the protein export process. For maximal export kinetics, it appeared that retention of only a single basic residue at the amino terminus of either the full-length or the truncated R2 signal peptide was sufficient. This was not unexpected, since the average net charge exhibited by procaryotic signal peptides is +1.7, and a number of *E. coli* proteins have a signal peptide with only one basic residue at the amino terminus (34, 39). As the net charge was systematically changed from +1 to −3, both the rate and the efficiency of MBP export progressively decreased. However, even a signal peptide with a net charge of −3 and a full-length hydrophobic core (encoded by pTL8) could still facilitate the export, albeit with significantly slower kinetics, of approximately 60% of the MBP synthesized. For derivatives of the R2 signal peptide having a truncated hydrophobic core, decreases in net charge below 0 had a noticeably more detrimental effect on both the rate and the efficiency of MBP export such that a net charge of −3 resulted in a totally export-defective protein.

It appeared that the additional hydrophobicity provided by the full-length hydrophobic core compensated to a certain extent for adverse changes in the properties of the hydrophilic amino terminus. Although not as obvious because of the way the substitutions were constructed, it could well be that, in the case of the R2 signal peptide, the converse is also true. In this instance, the presence of the basic hydrophilic

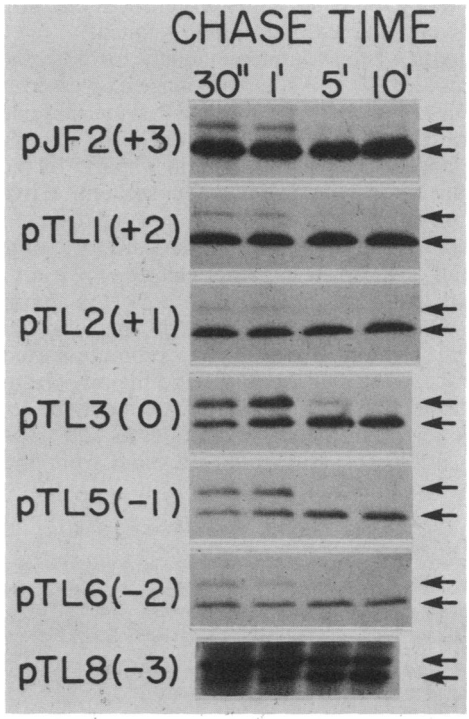

FIG. 5. Comparison of export kinetics for wild-type MBP and derivatives with alterations in the hydrophilic segment. Cells of strain BAR1091 harboring plasmid pJF2 (encoding wild-type MBP) or plasmids encoding various altered MBP species were pulse-radiolabeled, and the MBP was immunoprecipitated and analyzed as described in the legend to Fig. 2. The positions of pre-MBP and mMBP are indicated by arrows. The net charge of the hydrophilic segment of each mutant signal peptide is indicated at the left. Because of differences in the amounts of MBP synthesized by various mutants (see text), exposure times for the gels shown were not uniform.

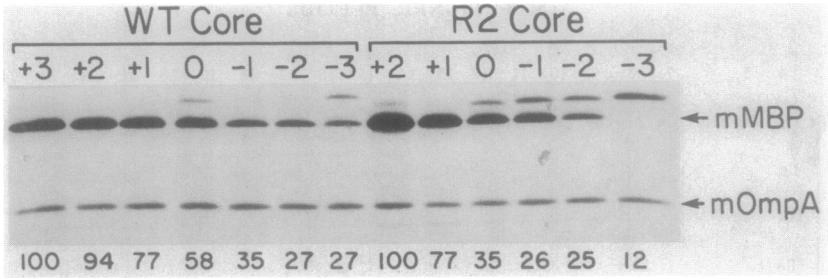

FIG. 6. Relative synthesis levels for MBP encoded by plasmid pJF2 (wild-type [WT] core), pJW28 (R2 core), and derivatives thereof. Mid-log-phase cell cultures at an identical optical density at 600 nm were radiolabeled for 10 min with [^{35}S]methionine and solubilized. The MBP and OmpA (as an internal standard) were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Bands were excised from dried gels, and radioactivity determined as described in Materials and Methods. Numbers at the bottom indicate relative levels of MBP synthesis, expressed as a percentage of MBP synthesis directed by pJF2 or pJW28. Note that MBP with an R2 signal peptide is synthesized at a somewhat higher level than is wild-type MBP.

segment may serve to compensate for the truncation of the core to promote near-normal export of the MBP. One might have expected to observe less efficient export of the R2 species encoded by pUZ4 (0) and pUZ6 (−1). However, with the substitution of Ile for Lys at position 4, the core was lengthened by two strongly hydrophobic residues, thereby probably making these MBP species less dependent on the nature of the hydrophilic segment. In the case of the MBP species encoded by pUZ8 (−3), the length of the hydrophobic core was identical to that of the parental R2 signal peptide (Fig. 1). One must conclude that the severity of the export defect exhibited by this protein was due primarily to the negatively charged hydrophilic segment.

Thus, as has been found previously for both the *E. coli* lipoprotein (20, 37) and staphylokinase expressed in *E. coli* (18), results obtained with the MBP signal peptide clearly demonstrate that a net positive charge at the amino terminus is not required to facilitate protein export. In fact, MBP export can still be achieved when the entire hydrophilic segment, residues 2 through 8, is deleted from the signal peptide (J. W. Puziss and P. J. Bassford, Jr., unpublished data). In all of these cases, a net charge of +1 at the amino terminus helps to promote protein export at the maximum rate and efficiency. As suggested by the loop model, the basic amino terminus may serve to promote an ionic interaction between the signal peptide and the cytoplasmic membrane. Although the hydrophilic segment may be considered a separate region of the signal peptide, its role in promoting export is not necessarily totally distinct from that of the hydrophobic core, as suggested by results indicating that these two regions can partially compensate for defects in the other. Ferenci and Silhavy (11) recently suggested that the signal peptide has multiple functions and that the different regions may have overlapping responsibilities. Furthermore, there may be a certain degree of redundancy built into the signal peptide. It was pointed out that for a number of signal peptides that have been genetically modified, a single alteration that totally abolishes function has not been found (11). Also, the MBP signal peptide has three basic residues at its amino terminus, whereas it would appear from this study that a single one would suffice.

In a recent study by Kaiser et al. (22), it was found that random sequences encoding peptides with an overall hydrophobic character could direct the export of the extracellular enzyme invertase in cells of *Saccharomyces cerevisiae*. A basic amino terminus was not encountered in many of the functional sequences that were determined, and in most cases translocation into the lumen of the rough endoplasmic

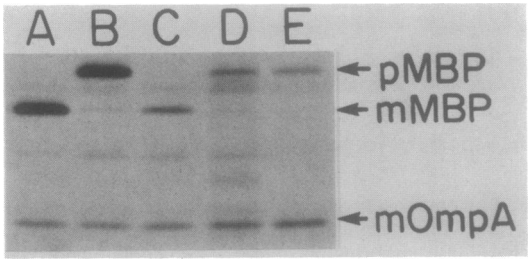

FIG. 7. Evidence that an export-incompetent hydrophobic core placed in *cis* to a negatively charged hydrophilic segment does not restore MBP synthesis to wild-type levels. Cells that had been radiolabeled for 10 min with [^{35}S]methionine were solubilized, and MBP and OmpA were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Shown are wild-type MBP (lane A), MBP19-1 (lane B), MBP (−2) (lane C), MBP (−2, 19-1) (lane D), and MBP (−2, 19-1) synthesized in cells harboring *prlA402* (lane E).

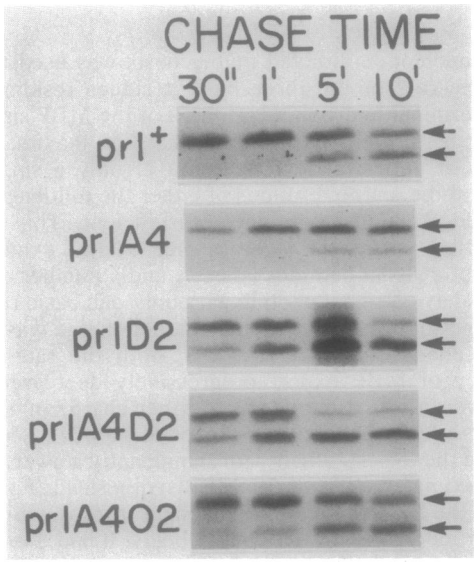

FIG. 8. Comparison of export kinetics of the MBP-R2 species encoded by pUZ7 (−2) in cells harboring various *prl* suppressor mutations. Cells were pulse-radiolabeled, and the MBP immunoprecipitated and analyzed as described in the legend to Fig. 2. Positions of pre-MBP and mMBP are indicated by arrows. The relevant *prl* alleles are indicated at the left.

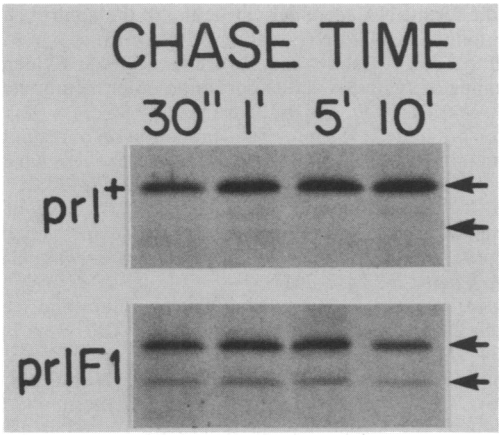

FIG. 9. Comparison of export kinetics of the MBP-R2 encoded by pUZ8 (−3) in *prl*$^+$ or *prlF1* cells. Cells were pulse-radiolabeled, and the MBP was immunoprecipitated and analyzed as described in the legend to Fig. 2. Positions of pre-MBP and mMBP are indicated by arrows.

reticulum occurred without signal peptide processing. No attempt was made to determine the kinetics of invertase export mediated by these randomly obtained signal peptides. From the extracellular activities and glycosylation analyses, it was apparent that in most cases these proteins were not secreted at rates and efficiencies comparable to those of wild-type invertase. Although it appears that a rudimentary hydrophobic core at the amino terminus may be sufficient to inefficiently facilitate some protein translocation, optimal translocation and efficient processing would seem to require a signal peptide that retains each of the three conserved regions found in virtually all natural signal peptides.

For both the wild-type and R2 signal peptides, it was found that the total amount of MBP synthesized progressively decreased as the net charge of the hydrophilic segment decreased. Similar results have been reported for altered Lpp (20) and LamB (16) signal peptides. The resulting reduction in synthesis of Lpp and LamB has been interpreted as possible evidence for a mechanism that obligately couples protein export and translation (16, 20, 37), perhaps in a manner analogous to that described for the signal recognition particle in eucaryotic systems (reviewed in reference 40). On the other hand, a number of strongly export-defective MBP and LamB species have been described with alterations in the hydrophobic core of the signal peptide for which, in almost all cases, there is no significant diminution in the level of protein expression in comparison with expression of the fully export-competent wild-type protein (9). If synthesis and export are normally coupled processes, then the synthesis of these particular export-defective proteins, including MBP19-1, must be disconnected from such a coupling mechanism, possibly because the export-defective proteins do not cotranslationally enter the export pathway for a sufficient distance. To test the possibility that the synthesis of export-defective proteins with alterations in the hydrophilic segment is regulated by a putative coupling mechanism, a strong hydrophobic core alteration was placed in *cis* to a strong hydrophilic segment alteration [the MBP species encoded by plasmid pTL7 (−2, 19-1)]. Although the MBP was rendered totally export-defective by this additional signal peptide alteration, an increase in the level of expression of this mutant MBP species compared with the level in the corresponding −2 species was not observed. Furthermore, *prlD2*-mediated partial suppression of the export defect exhibited by the MBP species encoded by plasmid pUZ7 (−2) had no effect on the level of MBP expression. One might have predicted that, if a coupling mechanism were operating, partial suppression of the export defect would have partially restored MBP synthesis in this instance.

The mutations altering the hydrophilic segment of the signal peptide also induced changes very early in the coding region of the *malE* message. It is highly possible that the decreases in MBP synthesis observed in this study, as well as analogous decreases in expression of Lpp and LamB, are due to changes in mRNA secondary structure or translational initiation site accessibility. It is known that secondary structure of the message can play a role in determining the efficiency of translational initiation (15). Sequence comparisons between a number of *E. coli* ribosome-binding sites suggest that there are preferred sequences for translational initiation that extend into the coding region of the message (35). With this in mind, additional experiments to determine the cause of the decrease in synthesis of MBP species with altered hydrophilic segments are currently under way.

It was interesting in this study to find that the export defect resulting from alterations in the hydrophilic segment of the MBP signal peptide could be noticeably improved in a strain harboring the *prlD2* suppressor allele but not in strains harboring either the *prlA4* or *prlA402* suppressor allele. Iino and Sako (17) recently reported that *prlA4* does not suppress a hydrophilic segment mutation in the staphylokinase signal peptide. All of these suppressor mutations were originally isolated as extragenic suppressors of signal sequence mutations altering the hydrophobic core of either the LamB or MBP signal peptide. These mutations generally suppress all hydrophobic core mutations (both missense mutations and small, in-frame deletions) with some efficiency, and both *prlA4* and *prlA402* are considerably stronger suppressors of such mutations than is *prlD2* (31). The markedly different pattern of suppression observed with MBP species altered in the hydrophilic segment may be related to the nature of the *prl* gene products, both of which are known to be essential components of the protein export pathway. The PrlA (SecY) protein is an extremely hydrophobic, integral cytoplasmic membrane protein that may be responsible for mediating the actual protein translocation event (21). On the other hand, *prlD2* was recently shown (14) to be an allele of the *secA* gene that encodes a large, hydrophilic protein found in the cytoplasm (27, 33). Although a direct interaction between the signal peptide and these components of the export pathway has been suggested but not conclusively demonstrated (see references 14 and 29 for a detailed discussion of this point), it is tempting to speculate that SecA interacts more strongly with the hydrophilic segment of the signal and that PrlA interacts more strongly with the hydrophobic core.

Finally, there are two additional points that emerge from this analysis of mutational alterations in the hydrophilic segment of the MBP signal peptide. The first concerns the finding that the presence of the *prlF1* allele resulted in a noticeable improvement in the export of the MBP species encoded by plasmid pUZ8 (−3). Little is known concerning the *prlF1* gene product, and the mutation itself does not have an effect on normal protein export in *E. coli* (7, 23). The result reported here and a similar finding recently reported by Iino and Sako (17) suggest that the *prlF* product may indeed have a role in protein export. Second, this study provides an explanation of why mutational alterations were not encountered in the MBP signal peptide by the genetic selections that have previously been used. Beginning with

the wild-type *malE*$^+$ gene, construction of an MBP signal peptide that was sufficiently export-defective due to alterations in the hydrophilic segment (i.e., a net negative charge of −3) would have required at least three very specific substitution mutations. Such an event could not be expected to occur at a detectable frequency. Thus, the failure to obtain such mutations by genetic selection clearly should not have been taken as an indicator that the amino-terminal hydrophilic region does not have an important role in the protein export process.

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