

## Synthesis of Precursor Maltose-binding Protein with Proline in the +1 Position of the Cleavage Site Interferes with the Activity of *Escherichia coli* Signal Peptidase I *in Vivo*\*

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The residues occupying the -3 and -1 positions relative to the cleavage site of secretory precursor proteins are usually amino acids with small, neutral side chains that are thought to constitute the recognition site for the processing enzyme, signal peptidase. No restrictions have been established for residues positioned +1 to the cleavage site, although there have been several indications that mutant precursor proteins with a proline at +1 cannot be processed by *Escherichia coli* signal peptidase I (also called leader peptidase). A maltose-binding protein (MBP) species with proline at +1, designated MBP27-P, was translocated efficiently but not processed when expressed in *E. coli* cells. Unexpectedly, induced expression of MBP27-P was found to have an adverse effect on the processing kinetics of five different nonlipoprotein precursors analyzed, but not precursor Lpp (the major outer membrane lipoprotein) processed by a different enzyme, signal peptidase II. Cell growth also was inhibited following induction of MBP27-P synthesis. Substitutions in the MBP27-P signal peptide that blocked MBP translocation across the cytoplasmic membrane and, hence, access to the processing enzyme or that altered the signal peptidase I recognition site at position -1 restored both normal growth and processing of other precursors. Since overproduction of signal peptidase I also restored normal growth and processing to cells expressing unaltered MBP27-P, it was concluded that precursor MBP27-P interferes with the activity of the processing enzyme, probably by competing as a noncleavable substrate for the enzyme's active site. Thus, although signal peptidase I, like many other proteases, is unable to cleave an X-Pro bond, a proline at +1 does not prevent the enzyme from recognizing the normal processing site. When the RBP signal peptide was substituted for the MBP signal peptide of MBP27-P, the resultant hybrid protein was processed somewhat inefficiently at an alternate cleavage site and elicited a much reduced effect on cell growth and signal peptidase I activity. Although the MBP signal peptide also has an alternate cleavage site, the different properties of the RBP and MBP signal peptides with regard to the substitution of proline at +1 may be related to their respective secondary structures in the processing site region.

Most proteins translocated across the cytoplasmic membrane of bacterial cells and the rough endoplasmic reticulum membrane of eukaryotic cells are synthesized as precursor proteins with an amino-terminal extension termed the signal peptide. This structure is thought to have a primary role in initiating precursor translocation through the membrane and subsequently is removed during or immediately following the completion of translocation (1, 2). Although signal peptides share little primary sequence homology, they do exhibit three common structural features: a hydrophilic amino terminus with 1-3 basic residues followed by a 9-15-residue hydrophobic core and a more polar carboxyl terminus that immediately precedes the cleavage site (for review, see Ref. 3). The signal peptide is thought to insert into the membrane as a reverse hairpin structure, exposing the cleavage site on the external surface (4). The enzymes responsible for the endoproteolytic processing of precursor proteins are signal peptidases (5). Several eukaryotic signal peptidases have been characterized as integral membrane complexes of two to six polypeptides (6, 7). Two distinct signal peptidases have been identified and purified from *Escherichia coli* cells. Each is an essential, integral cytoplasmic membrane protein composed of a single polypeptide. Signal peptidase II removes the signal peptide from glyceride-modified lipoprotein precursors (8). Signal peptidase I (also called leader peptidase) removes the signal peptide from other exported proteins (9-11).

Eukaryotic precursor proteins can be correctly processed by signal peptidase I, and prokaryotic precursors, excluding lipoproteins, can be processed by eukaryotic signal peptidases (5). Comparative analyses of numerous eukaryotic and prokaryotic signal peptides have revealed that the residues at positions -3 and -1 relative to the cleavage site are strongly conserved; amino acids with small, neutral side chains, most commonly alanine, predominate at these two positions. Based on these frequency analyses, residues -3 and -1 have been proposed to constitute a recognition site for the processing enzyme (12-14). Several studies have provided strong experimental support for this model (15-19). In addition, a  $\beta$ -turn initiating 4-6 residues upstream of the cleavage site is thought to be required for proper alignment of this site as it emerges from the membrane, with respect to the signal peptidase active site (12, 16).

Although there is a very strict limitation on residues that can occupy the -1 position of precursor cleavage sites, no such limitation has been established for the +1 position, *i.e.* the first residue of the mature protein. Indeed, all 20 amino acids are encountered in this position in eukaryotic precursors, and only glutamine, isoleucine, leucine, methionine, and proline have not been found to occupy +1 in prokaryotic precursor proteins, in what is a significantly smaller data base

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(14, 20). The possibility that proline is necessarily excluded from the +1 position of bacterial precursor proteins was of interest for three reasons. First, it is very uncommon for a protease to cleave an X-Pro bond (21, 22). Second, Pluckthun and Knowles (17) reported that mutant  $\beta$ -lactamase species with proline at position +1 are not processed and, furthermore, are highly toxic to *E. coli* cells for an unknown reason. Third, Yamane *et al.* (23) reported that a pro-OmpA-Lpp<sup>1</sup> hybrid protein with proline at +1 was not processed during *in vitro* translocation into *E. coli* membrane vesicles.

*In vivo* processing of the *E. coli* MBP has provided an excellent system in which to investigate cleavage site structural requirements (18, 19). In this study, a mutant MBP species with proline at the +1 position, designated MBP27-P, has been constructed and found to exhibit some interesting properties. Not only is pre-MBP27-P not processed at either the normal site or an upstream alternate site previously identified (19), induced synthesis of this protein is inhibitory to cell growth and causes a pleiotropic defect in processing of all nonlipoprotein precursors examined. The data strongly indicate that pre-MBP27-P specifically interferes with the activity of signal peptidase I.

#### MATERIALS AND METHODS

**Bacterial Strains and Plasmids**—*E. coli* K12 strain BAR1091, a derivative of MC4100 (24), was used as the host strain for plasmids encoding wild-type MBP, or mutant MBP or RBP-MBP proteins. This strain harbors *malE* $\Delta$ 312, an in-frame, nonpolar deletion that removes DNA sequences encoding residues 15–185 of pre-MBP and was described previously (25). Strain GBG25 is MC4100 *malE*27-P. This *malE* mutation substitutes proline for lysine at position 27 in pre-MBP (+1 relative to the signal peptidase I cleavage site) and was recombined from plasmid pGG25 (see below) into the chromosome using a strategy previously described (26). Strain MM52 is an MC4100 derivative harboring a temperature-sensitive *secA* allele (27).

Plasmid pJF2 is a derivative of pBR322 carrying the *malE*<sup>+</sup> gene under regulatory control of the *lacUV5* promoter-operator and the phage M13 intergenic region. Plasmid pJF8 is a derivative of pJF2 with an amber mutation in codon 23 of the MBP signal peptidase coding region. Plasmid pJF13 is also a pJF2 derivative harboring *malE*24-D (previously designated *malE*24-I; Ref. 18). Plasmid pSMS41 encoding RBP-MBP was described previously (28). An intermediate in the construction of plasmid pSMS41 was plasmid pSMS40 having a *NarI* restriction site located at the junction between the coding regions for the RBP signal peptide and MBP mature moiety. Fortuitously for this study, this *NarI* site resulted in the substitution of proline for lysine in the +1 position of the hybrid protein (RBP-MBP26-P) and no other changes in the amino acid sequence. Likewise, plasmid pDNC187 (28), has a *NarI* site in the analogous position in an intact *lacUV5-rbsB* gene (RBP26-P). Plasmid pJW4 encodes MBP $\Delta$ 116, a proteinase K-sensitive MBP species deleted for residues 168–176 of the mature moiety (29). To introduce the *malE* $\Delta$ 116 mutation into plasmids encoding MBP species with processing site alterations, the small *EcoRI*-*BglII* fragment of the latter was ligated to the large *EcoRI*-*BglII* fragment from pJW4. Plasmids packaged in M13 particles were prepared using M13KO7 as the helper phage (30). Plasmid pTD101 is a derivative of pBR322 carrying the *lepAB* operon (31).

**Reagents**—Minimal medium M63 supplemented with carbon source (0.2%) and thiamine (2  $\mu$ g/ml), maltose tetrazolium indicator agar, and tryptone yeast agar were prepared as described previously (32). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50  $\mu$ g/ml, respectively. To induce *malE* gene expression from plasmids, IPTG was used on agar plates and in liquid media at 1 and 5 mM, respectively. Induction of *malE*

or *lamB* gene expression from the chromosome was carried out by the addition of 0.2% maltose. Expression of the *oppA* gene was induced by the addition of 50  $\mu$ g/ml leucine. [<sup>35</sup>S]Methionine (1154 Ci/mmol) was obtained from Du Pont-New England Nuclear. Rabbit anti-MBP, anti-OmpA, and anti-RBP sera have been described previously (28, 33, 34). Rabbit anti-Bla serum was provided by Vytas Bankaitis (University of Illinois, Urbana, IL) and anti-LamB serum by Tom Silhavy (Princeton University), and anti-Lpp sera were obtained from Paul Ray (Wellcome Research Laboratories, Research Triangle Park, NC) and Henry Wu (Uniformed Services University, Bethesda, MD). Purified anti-EF-G rabbit IgG was provided by P.-C. Tai (Georgia State University, Atlanta).

**Oligonucleotide-directed Mutagenesis of *malE***—To introduce mutations into the *malE* gene, the oligonucleotide-directed mutagenesis method of Zoller and Smith (35) was used as described previously (19). Single-stranded templates containing uracil were prepared from cells of *E. coli* strain CJ236 (*ung dut*; Ref. 30). The oligonucleotide used to convert codon 27 of the *malE* gene from AAA (lysine) to CCA (proline) was 5'-TCTCGCCCCAATCGAAGAAGG-3' and is complementary to the packaged, single-stranded DNA of plasmid pJF2. This plasmid encoding MBP27-P (see below) was designated pGG25. A single oligonucleotide, 5'-TCGGCTCTCNACCAATCGAAGAAGG-3', was used to construct plasmids encoding MBP26-H,27-P and MBP26-N,27-P, using single-stranded pJF8 DNA as template. Note that N indicates that an equal mixture of all four nucleotides was employed for the step in the synthesis of the mutagenic primer corresponding to the first position of codon 26. Since the primer also repaired amber codon 23 of plasmid pJF8, mutagenized plasmids conveniently were identified by their ability to confer a Mal<sup>+</sup> phenotype to BAR1091 cells. Those with a histidine codon (CAC) or an aspartic acid codon (GAC) substituted for the alanine codon (GCC) at position 26 of the *malE* gene subsequently were identified by DNA sequencing (36). The oligonucleotide used to convert codon 26 of *malE* from GCC (alanine) to CCC (proline) (MBP26-P) was 5'-TCGGCTCTCCCCAAAATCGAAGAAGG-3', and once again single-stranded pJF8 DNA was used as the template. The *malE*15-K mutation was introduced into pGG25 (MBP15-K,27-P) by oligonucleotide-directed mutagenesis, as described previously (28). Mutagenic primers were prepared with an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis, as described by Hutchinson *et al.* (36). Mutagenized plasmids were transformed into competent cells of strain BAR1091 and subsequently reintroduced into BAR1091 cells by phage M13-mediated transduction. All mutations were confirmed by DNA sequencing, also as described previously (36).

**Radio-labeling of Proteins, Immune Precipitation, SDS-PAGE, Autoradiography, and Protein Localization**—Cultures were grown to mid-log phase in glycerol minimal medium, supplemented with ampicillin when required. At the specified times, cells were labeled with [<sup>35</sup>S] methionine for 15 s, followed by a chase with excess cold methionine for 1 min (37). The chase was extended for various periods when specified. Proteins were immunoprecipitated from solubilized cell extracts and resolved by SDS-PAGE and autoradiography as described previously (33), with the exception that EF-G was resolved using a 7.5% polyacrylamide gel and precursor and mature Lpp species were resolved by SDS-PAGE in a phosphate buffer system, as described by Inouye *et al.* (38). Localization of protease-sensitive MBP species was performed by analysis of spheroplast association and proteinase K accessibility, as described previously (18). Quantitation of proteins was carried out using an AMBIS Radioanalytic Imaging System; calculation of the ratio of precursor to mature species included adjustments for additional methionines in precursor proteins.

#### RESULTS

**High Level Production of MBP27-P Inhibits Processing of Exported Proteins**—Plasmid pJF2 carries the *malE* gene under control of the *lacUV5* promoter (18). A derivative of pJF2, designated pGG25, was constructed by oligonucleotide site-directed mutagenesis (see "Materials and Methods") to encode pre-MBP with proline substituted for lysine at position 27 (designated MBP27-P), *i.e.* at position +1 relative to the normal site of cleavage by signal peptidase I (see Fig. 1). Cells of *E. coli* strain BAR1091 carrying pGG25 grew normally on maltose minimal agar but formed very small colonies when the growth medium was supplemented with IPTG to induce

<sup>1</sup> The abbreviations used are: OmpA, outer membrane protein A; OppA, oligopeptide-binding protein; MBP, maltose-binding protein; RBP, ribose-binding protein; Lpp, the major outer membrane lipoprotein; Bla, TEM  $\beta$ -lactamase; EF-G, elongation factor G; pre- (as prefix), precursor form of a protein; m (as prefix) mature form of a protein; IPTG, isopropylthiogalactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

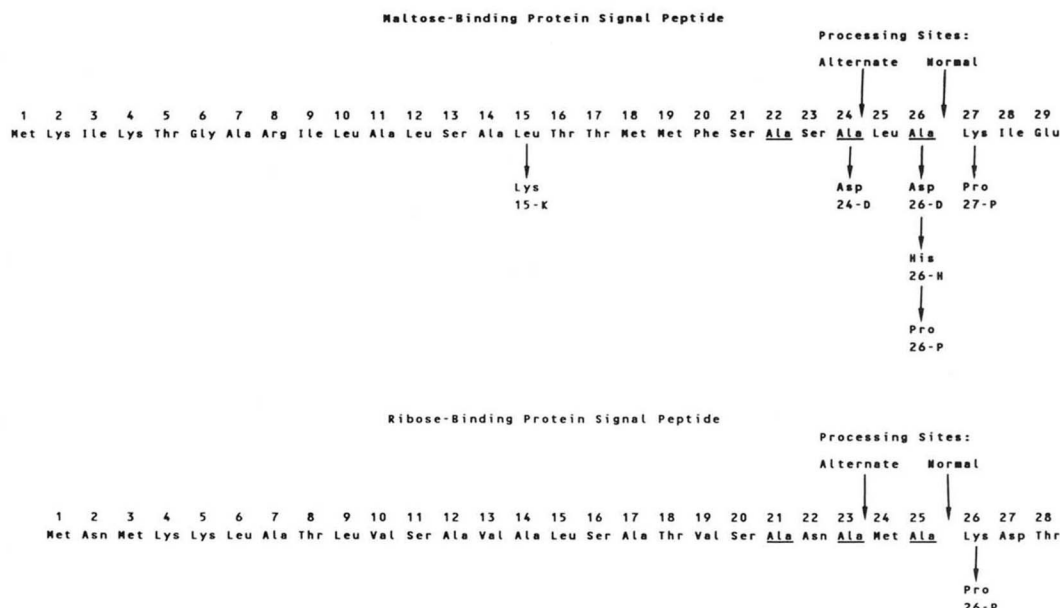


FIG. 1. **MBP and RBP signal peptides.** The amino-terminal 29 and 28 residues of pre-MBP and pre-RBP, respectively, are shown, including the entire signal peptides and processing sites. Ala residues comprising the consensus signal peptidase I recognition site for the primary and alternate cleavage sites for both precursor proteins are underlined. Amino acid substitutions discussed in the text are indicated by *arrows* and the corresponding allelic designations. Note that the substitution at position 15 of pre-MBP is not expected to affect processing *per se*, but results in an MBP species that is strongly translocation-defective (28). See text for further details.

high level MBP27-P expression. Likewise, the addition of IPTG to a mid-log phase liquid culture resulted in complete cessation of growth within less than one-half generation time. Examination by dark-field microscopy revealed no gross changes in cell morphology. The effect of MBP27-P induction was bacteriostatic, in that growth resumed following the removal of IPTG (data not shown).

In cells expressing MBP27-P, processing of pre-MBP to the mature protein was not detectable. Unexpectedly, IPTG-induced synthesis of MBP27-P was found to decrease the efficiency of processing of various periplasmic and outer membrane proteins, as shown in Fig. 2. Mid-log cells of *E. coli* strain BAR1091 harboring pGG25, either induced for MBP27-

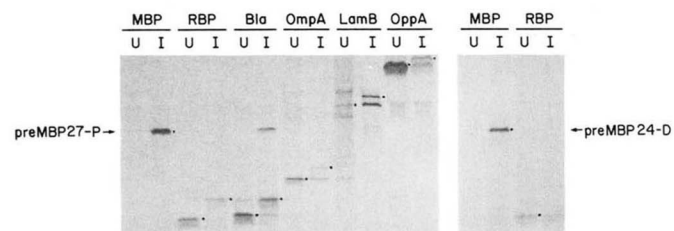


FIG. 2. **Effect of MBP27-P synthesis on maturation of other exported proteins.** Periplasmic and outer membrane proteins were immunoprecipitated individually from radiolabeled cellular extracts of strain BAR1091 cells carrying plasmid pGG25 (*malE27-P*, first panel) or pJF13 (*malE24-D*, second panel). IPTG was added to one-half of each culture 90 min prior to radiolabeling. Mid-log phase cells were pulse-radiolabeled with [<sup>35</sup>S]methionine for 15 s and chased in the presence of excess cold methionine for an additional 60 s. The chase was terminated by addition to one-third volume ice-cold 15% trichloroacetic acid. The cellular precipitates were solubilized and the protein indicated *above* each pair of lanes recovered by immunoprecipitation and then analyzed by SDS-PAGE and autoradiography. *U*, the immunoprecipitate was obtained from cells uninduced for expression of MBP; *I* the precipitate was from cells induced for MBP expression by the addition of IPTG. Note that the antiserum used to precipitate Bla also recognized MBP. Mature and precursor species, where appropriate, are indicated in each lane by a *dot*. See text for further details.

P synthesis with IPTG for 90 min or not induced, were pulse-radiolabeled with [<sup>35</sup>S]methionine for 15 s and chased with an excess of unlabeled methionine for an additional 60 s. The chase period was terminated by the addition of an aliquot of ice-cold trichloroacetic acid; specific envelope proteins were immunoprecipitated individually from solubilized cell extracts with the appropriate antisera, then analyzed by SDS-PAGE and autoradiography. Only unprocessed pre-MBP27-P could be discerned in extracts obtained from uninduced (*U*) or induced (*I*) cells (Fig. 2, first two lanes). The periplasmic proteins RBP, Bla, and OppA, and the outer membrane LamB and OmpA proteins were observed primarily in their processed mature forms in extracts prepared from uninduced cells. However, the ratio of precursor to mature form for each protein increased markedly in the immunoprecipitates obtained from cells induced for high level MBP27-P synthesis.

MBP24-D has aspartic acid substituted for alanine at position 24 of the MBP signal peptide (see Fig. 1). This alteration at -3 in the signal peptidase I recognition site has no apparent effect on pre-MBP translocation across the cytoplasmic membrane, but the protein is essentially unprocessed (18, 19). In contrast to MBP27-P, high level synthesis of MBP24-D did not noticeably alter processing of RBP (Fig. 2, second panel, last two lanes) or other envelope proteins (data not shown).

The consequence of MBP27-P accumulation in cells on RBP maturation was investigated further (Fig. 3). One min

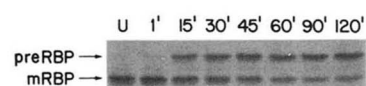
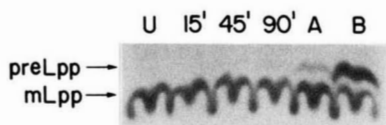


FIG. 3. **Effect over time of induced expression of MBP27-P synthesis on RBP maturation.** The experimental conditions were as described in the legend to Fig. 2, except RBP processing was examined by individual pulse-chase analyses at each of the specified times postinduction of MBP27-P synthesis. *U*, the immunoprecipitate was obtained from cells radiolabeled prior to induction with IPTG.

after the initiation of induced MBP27-P synthesis, a slight defect in RBP processing was detected. The ratio of pre-RBP to mature RBP detected in each sample increased throughout the induction period; by 90 min postinduction, the majority of RBP examined after a pulse-chase analysis was found in its precursor form. An identical experiment revealed that processing of RBP remained unaffected by synthesis of MBP24-D throughout 120 min postinduction (data not shown).

**Processing of Lipoprotein Is Unaffected by Induced Levels of MBP27-P**—The observed pleiotropic effect of MBP27-P synthesis on processing of exported proteins could result from either an inhibition of the processing step itself or from inhibition of an earlier step in the export pathway. Note that each of the proteins analyzed above is processed by signal peptidase I. If high level MBP27-P synthesis specifically affected this enzyme, then the maturation of lipoproteins would have remained unaltered since these are processed by a different enzyme, signal peptidase II (8). Lpp was immunoprecipitated from cells pulse-radiolabeled at various times postinduction of MBP27-P synthesis. Electrophoresis was carried out in a phosphate buffer system to specifically separate precursor and mature Lpp species (38). In contrast to RBP processing (Fig. 3), the efficiency of Lpp processing was unchanged through 45 min postinduction of MBP27-P synthesis (Fig. 4). A very small amount of pre-Lpp was detected by this assay 90 min postinduction, probably as an indirect result of the effect of MBP27-P synthesis on cell growth (see above). The level of labeled pre-Lpp increased markedly in cell extracts of strain MM52 (*secA<sup>ts</sup>*) shifted to the nonpermissive temperature (see Fig. 4, lanes A and B). Such cells are known to have a generalized defect in translocation of both lipoproteins and nonlipoproteins at the nonpermissive temperature (39, 40). Thus, high level synthesis of MBP27-P evidently did not inhibit protein translocation *per se*. It also was not solely blocking SecB function (29), since export of a SecB-independent protein, RBP (28), was strongly inhibited. These findings indicated that MBP27-P probably is specifically inhibiting processing of nonlipoproteins by signal peptidase I.

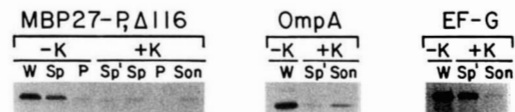
**Localization of Pre-MBP27-P**—In a previous study, MBP24-D was shown to be anchored to the periplasmic face of the cytoplasmic membrane; it was not released from cells by osmotic shock or conversion to spheroplasts but was accessible to proteinase K degradation in spheroplasts (18). Since wild-type MBP is proteinase K-resistant, the protease accessibility studies were performed with cells synthesizing MBP24-D, $\Delta$ 116 (18). The latter species is proteinase K-sensitive due to the deletion of 9 amino acid residues from the mature moiety. This deletion has no effect on the export properties of MBP with a wild-type signal peptide (41). In this study, the cellular location of pre-MBP27-P was investigated in a similar manner. A plasmid encoding MBP27-



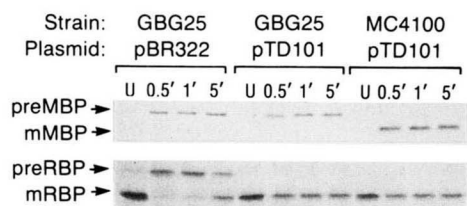
**FIG. 4. Effect over time of induced expression of MBP27-P synthesis on Lpp maturation.** The experimental conditions were as described in the legend to Fig. 3, except that Lpp was immunoprecipitated and SDS-PAGE was performed in a phosphate buffer system required to separate precursor and mature Lpp (38). A and B, immunoprecipitates were obtained from strain MM52 (*secA<sup>ts</sup>*) cells at the permissive temperature and after 2 h at the nonpermissive temperature (42 °C), respectively. These provided a reference marker for pre-Lpp. See text for further details.

P, $\Delta$ 116 was constructed as described under "Materials and Methods." As shown in Fig. 5, the great majority of MBP27-P, $\Delta$ 116 synthesized was accessible to proteinase K degradation in intact spheroplasts. Disruption of outer membranes during spheroplast preparation was demonstrated by susceptibility of OmpA to protease degradation, and the resistance of the cytoplasmic protein EF-G to degradation indicated that cytoplasmic membranes remained largely intact. Since MBP27-P, $\Delta$ 116 remained spheroplast-associated, this protein (as well as MBP27-P) likely was translocated normally to the periplasm but, like other uncleavable pre-MBP species, remained tethered to the cytoplasmic membrane by its unprocessed signal peptide. This also further indicated that high level MBP27-P synthesis does not inhibit protein translocation. As an additional control, a plasmid encoding MBP15-K,27-P, $\Delta$ 116 was constructed. The substitution of Lys for Leu at position 15 of the MBP27-P, $\Delta$ 116 signal peptide was predicted to render this MBP species translocation defective (28) and, thus, resistant to proteinase K degradation in spheroplasts. Little of the MBP15-K,27-P, $\Delta$ 116 was accessible to protease digestion in intact spheroplasts (data not shown), confirming that this pre-MBP species was not translocated across the cytoplasmic membrane.

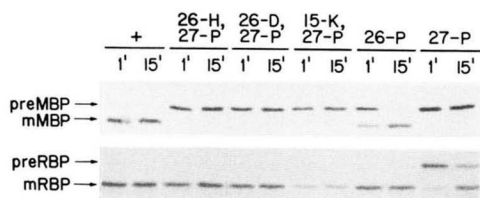
**Overproduction of Signal Peptidase I Restores Efficient RBP Processing in Cells Producing MBP27-P**—The *malE27-P* allele was recombined into the *E. coli* chromosome (see "Materials and Methods"), resulting in synthesis of MBP27-P under *malEp* promoter control. Thus, in cells of this strain, designated GBG25, high level synthesis of MBP27-P is induced by maltose. Strain GBG25 cells formed small colonies on maltose minimal agar (data not shown). Processing of MBP27-P and RBP in strain GBG25 was investigated by pulse-chase analysis prior to and at 90 min postinduction of MBP27-P synthesis. Induced expression of *malE27-P* (GBG25/pBR322; Fig. 6, lanes 1–4) strongly inhibited RBP processing. Plasmid pTD101 is a derivative of pBR322 constitutively expressing signal peptidase I from its own promoter; cells harboring this plasmid produce excess signal peptidase I (31). Strain GBG25 cells harboring pTD101 formed colonies of normal size on maltose minimal agar (data not shown). Induction of MBP27-P synthesis in GBG25/pTD101 cells had no noticeable effect on the processing kinetics of RBP (Fig. 6, lanes 5–8). Precursor RBP was not detectable after 30 s of chase. The processing kinetics were indistinguishable from those in cells of strain MC4100/pTD101 expressing wild-type MBP (Fig. 6, lanes 9–12). Note that MBP27-P remained unprocessed in cells overproducing signal peptidase I. From these results, it was concluded that



**FIG. 5. Localization of MBP27-P, $\Delta$ 116.** Glycerol-grown cells induced with IPTG for 45 min were pulse-labeled with [<sup>35</sup>S]methionine for 15 s, chased in the presence of excess cold methionine for an additional 60 s, and then converted to spheroplasts. Spheroplasts were pelleted to separate spheroplast-associated (*Sp*) and periplasmic (*P*) proteins and then were subjected to proteinase K treatment (+K) or not treated (-K). Unfractionated spheroplasts (*Sp'*) and sonicated spheroplasts (*Son*) also were treated with proteinase K. MBP was immunoprecipitated from these solubilized samples and solubilized untreated whole cells (*W*). OmpA and EF-G were immunoprecipitated from untreated whole cells (*W*), proteinase K-treated unfractionated spheroplasts (*Sp'*), and sonicated spheroplasts (*Son*). Each precipitate was subsequently analyzed by SDS-PAGE and autoradiography. See text for further details.



**FIG. 6. Processing kinetics of MBP and RBP in cells over-producing signal peptidase I.** MBP- and RBP-processing kinetics were examined by pulse-chase analysis. Synthesis of chromosomally encoded MBP was induced by the addition of maltose to glycerol-grown, mid-log phase cultures 90 min prior to radiolabeling. Cellular proteins were radiolabeled with [<sup>35</sup>S]methionine for 15 s, followed by a cold methionine chase for the indicated time periods. U, immunoprecipitates were obtained from uninduced cells pulse-radiolabeled for 15 s followed by an additional 60-s chase period. Cells of strain GBG25 synthesize MBP27-P. Cells of strain MC4100 synthesize wild-type MBP. Signal peptidase I is overproduced 30-fold in cells harboring plasmid pTD101 versus vector pBR322 alone. See text for further details.



**FIG. 7. RBP and MBP maturation in cells producing various MBP species.** The experimental conditions are the same as described in the legend to Fig. 6, except that plasmid-encoded MBP synthesis was induced with IPTG. Aliquots were removed at 1 and 15 min postlabeling and treated as described for Fig. 2. The MBP species being synthesized is designated above each corresponding pair of lanes. See text for further details.

synthesis of MBP27-P somehow interferes with the activity of signal peptidase I.

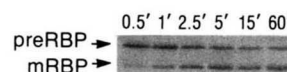
**Additional Alterations of the MBP Processing Site**—Specific substitutions at position 26 of pre-MBP, immediately preceding the normal processing site, block cleavage at this site but can allow inefficient processing at an alternate site 2 residues upstream (see Fig. 1). Specific substitutions at position 24 block processing at both cleavage sites. None of these MBP species with alterations at position 24 or 26 cause the pleiotropic, nonlipoprotein-processing defect characteristic of MBP27-P (19). This suggests that signal peptidase I does not recognize the former MBP species as substrates, but does recognize the latter, MBP27-P, even though it cannot be cleaved. If this is the case, then substitutions at position 26 of MBP27-P that prevent processing of otherwise wild-type MBP would be expected to relieve the inhibition of processing of nonlipoproteins. Derivatives of plasmid pGG25 encoding two different substitutions at position 26 of pre-MBP *in cis* to the proline at position 27 were constructed by oligonucleotide site-directed mutagenesis, as described under “Materials and Methods.” Processing of these MBP species, MBP26-H,27-P and MBP26-D,27-P, and RBP was analyzed 90 min postinduction of MBP synthesis (Fig. 7). A very small amount of mature MBP26-H,27-P was evident after a 15-min chase period, but processing of MBP26-D,27-P was not discerned. Synthesis of each species did not inhibit RBP processing, indicating that these mutant proteins did not interfere with signal peptidase I activity. Note that BAR1091 cells expressing these proteins were phenotypically Mal<sup>+</sup> (data not shown), confirming that the alterations surrounding the processing site were not adversely affecting MBP translocation across the cytoplasmic membrane.

In this same experiment, high level synthesis of MBP15-K,27-P was found to have no effect on RBP-processing efficiency (Fig. 7). This MBP species is strongly translocation-defective and therefore would not be expected to interact with signal peptidase I. A plasmid encoding an MBP species with proline substituted at position 26 also was constructed. Induced synthesis of MBP26-P had no effect on RBP processing (Fig. 7, last four lanes). Although MBP26-P was found predominantly in precursor form after 1 min of chase, maturation was nearly complete after 15 min of chase. The unaltered migration of the mature protein in a 7.5% SDS-polyacrylamide gel (data not shown) strongly suggests that MBP26-P processing occurred at the normal cleavage site (19). Processing of pre-Bla species with proline in the -1 position previously has been described (17).

**Pre-RBP Is Not Processed to Completion When MBP27-P Is Synthesized at Induced Levels**—To determine the extent of RBP maturation in the presence of high levels of MBP27-P, RBP processing over time was examined by pulse-chase analysis 45 min after induction of MBP27-P synthesis. Approximately 37% of the total RBP remained in precursor form after a 5-min chase period (Fig. 8). Approximately 31% of the protein was still in precursor form after a 60-min chase period and probably never was processed.

**An RBP-MBP Chimeric Protein with Proline at +1 Is Processed**—It was of interest to determine if synthesis of other exported proteins with proline substituted at the +1 position would inhibit the activity of signal peptidase I. Plasmid pSMS41 encodes an RBP-MBP hybrid protein (also under *lacUV5* promoter-operator control) in which the RBP signal peptide is fused to the mature MBP moiety precisely at the signal peptidase I cleavage site. The processing kinetics of RBP-MBP in BAR1091 cells are very similar to those of wild-type MBP (28). A related plasmid, pSMS40, encodes RBP-MBP26-P (see “Materials and Methods”), which has a proline at position +1 relative to the fusion cleavage site (note that the RBP signal peptide is 1 residue shorter than the MBP signal peptide).

Processing of RBP-MBP26-P and wild-type RBP in BAR1091 cells was analyzed prior to and 90 min after the addition of IPTG to mid-log phase cultures. Unlike MBP27-P, RBP-MBP26-P was processed to completion, although at a considerably slower rate than RBP-MBP. In addition, the resultant mature species migrated more slowly in a 7.5% SDS-polyacrylamide gel than the mature moiety derived from processing of RBP-MBP (Fig. 9, upper panel). In a previous study, the slower migration of mature MBP species indicated processing at the alternate site 2 residues upstream in the MBP signal peptide (19). An analogous alternate cleavage site is present in the RBP signal peptide (see “Discussion” and Fig. 1). RBP-MBP26-P probably was cleaved at this alternate site. Induction of RBP-MBP26-P synthesis clearly affected processing of wild-type RBP (Fig. 9). However, in contrast to the inhibition of RBP processing in BAR1091 cells synthesizing MBP27-P at induced levels (see Fig. 8), the inhibition of RBP processing was reduced markedly and RBP processing eventually proceeded to completion. Recently, processing of



**FIG. 8. RBP is not processed to completion in cells induced for MBP27-P synthesis.** The experimental conditions are the same as those described in the legend to Fig. 6, except RBP was immunoprecipitated from extracts prepared from cells pulse-radiolabeled 45 min following IPTG induction of MBP27-P synthesis. An aliquot was removed to ice-cold trichloroacetic acid at the chase times indicated above each lane.

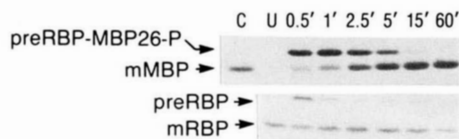


FIG. 9. **RBP-MBP26-P is processed.** The experimental conditions are the same as those described in the legend to Fig. 7. The immunoprecipitate shown in lane C is from cells synthesizing RBP-MBP (RBP was not precipitated). U, the immunoprecipitate was obtained from cells radiolabeled prior to induction of RBP-MBP26-P synthesis with IPTG. Note that mature MBP processed from precursor RBP-MBP26-P migrates slightly slower than mature MBP processed from RBP-MBP.

an RBP species with proline at +1, designated RBP26-P (see Fig. 1), has been examined. Like pre-RBP-MBP26-P, pre-RBP26-P also appeared to be slowly cleaved at the alternate processing site in the signal peptide (data not shown).

#### DISCUSSION

Protein export in *E. coli* follows a largely common pathway, and the removal of the signal peptide from the precursor protein at the outer surface of the inner membrane is one of the final steps in the process (for review, see Ref. 42). The efficiency of precursor protein processing often is used to assess the severity of defects in cellular protein export capabilities. For example, processing of virtually all precursor proteins is diminished significantly when cells synthesizing a temperature-sensitive SecA protein are shifted to the nonpermissive temperature (27) or following induction of synthesis of a LamB- $\beta$ -galactosidase fusion protein believed to physically obstruct function of the PrlA/SecY protein in the cytoplasmic membrane (43). In this study, induced synthesis of MBP27-P, a mutant MBP species with a proline substituted at position +1 of the mature moiety, was found to cause a pleiotropic defect in processing of precursor proteins. However, synthesis of MBP27-P seems to be interfering directly with the activity of the processing enzyme, signal peptidase I, rather than affecting an earlier step in the export pathway. The evidence for this can be summarized as follows.

1) As stated above, synthesis of MBP27-P inhibited processing of other precursor proteins. MBP27-P is one of a number of MBP species with alterations near the processing site that hinder or totally prevent pre-MBP cleavage by signal peptidase I (18, 19). Each of these MBP species is translocated normally, but the unprocessed molecules remain anchored to the periplasmic side of the cytoplasmic membrane by their signal peptide. Remarkably, only MBP27-P synthesis adversely affected the processing of other *E. coli* envelope proteins.

2) In contrast to processing of pre-RBP and various other precursor proteins by signal peptidase I, maturation of pre-Lpp was not affected by MBP27-P synthesis. Lpp utilizes much of the same export machinery as MBP and other nonlipoproteins but is processed by a different enzyme, signal peptidase II (8).

3) MBP27-P must be translocated across the cytoplasmic membrane to elicit a pleiotropic effect on precursor protein processing. When an alteration was introduced into the hydrophobic core of the MBP27-P signal peptide to block MBP translocation and, thus, access to the active site of signal peptidase I, normal processing of RBP was restored.

4) MBP27-P species with either aspartic acid or histidine substituted at the -1 position were found to have no effect on RBP processing. The same substitutions in otherwise wild-type pre-MBP previously were shown to block pre-MBP cleavage at the normal site, presumably by altering this site

so that it is no longer recognized by signal peptidase I (19). Since neither of these unprocessed precursors affected normal RBP processing, this is additional evidence that MBP27-P was not interfering with RBP export at a step in the pathway prior to processing by signal peptidase I. The ability of MBP27-P to interfere with signal peptidase I activity probably requires the processing enzyme to recognize the cleavage site of this mutant species, particularly the alanine residues at -3 and -1 in the signal peptide (12-14).

5) Cells harboring the multicopy plasmid pTD101 carrying the *lepB*<sup>+</sup> gene produce a 30-fold excess of signal peptidase I (31). In such cells, induced MBP27-P synthesis had no effect on RBP-processing kinetics. This result established a direct correlation between signal peptidase I availability and precursor processing in cells synthesizing MBP27-P. Moreover, signal peptidase I overproduction restored normal RBP-processing kinetics but did not concomitantly restore processing of MBP27-P. This also must be taken as further evidence that MBP27-P is interfering specifically with the activity of the processing enzyme and not adversely affecting some earlier step in the export pathway.

From this study, MBP27-P appears to be a very specific inhibitor of signal peptidase I activity *in vivo*, and is the only such specific inhibitor documented to date. Mutant Bla species with proline at +1 previously were shown to be translocated but not processed, as well as toxic to *E. coli* cells (17). It seems likely that these proteins also were interfering with signal peptidase I activity, although this has yet to be demonstrated. In addition, Zimmerman *et al.* (44) found that synthesis of a mutant M13 procoat slowed maturation of *E. coli* pro-OmpA. The procoat was not altered at the +1 position but contained several alterations in the early mature region and was inserted into the membrane in an energy-independent, posttranslational mode. It is not clear if the mutant procoat directly inhibited pro-OmpA processing or some earlier step in the export pathway. Finally, Wickner and colleagues (45) have described the inhibition of signal peptidase I activity *in vitro* by the signal peptide of M13 procoat.<sup>2</sup>

Signal peptidase I is an unusual enzyme. It specifically cleaves only precursor proteins, but the processing sites have relatively few sequence limitations compared with most protease cleavage sites (5). The enzyme is insensitive to all known protease inhibitors (46). Some progress has been made recently in the identification of residues within the signal peptidase I molecule that are important for enzyme function (47). However, the mode of action of this essential endoprotease remains elusive. Dev *et al.* (48) recently demonstrated that a nonapeptide corresponding to residues -7 to +2 of wild-type pre-MBP is efficiently cleaved *in vitro* at a rate close to that observed for pre-MBP maturation *in vivo*. It will be of interest to determine if chemically synthesized peptides corresponding to the cleavage site of pre-MBP27-P can inhibit signal peptidase I activity either *in vitro* or *in vivo*.

Proline is unique in that it is actually an imino acid. Its *R*-group is covalently bonded back to the  $\alpha$ -amino group, forming a cyclic structure that is incorporated into the peptide chain. This significantly limits the available conformations of a protein in the region of a proline. In addition, the peptide amine is not readily available to accept a proton in general acid-base catalysis, a common mechanism for protease cleav-

<sup>2</sup> It is worthwhile to note that MBP27-P is not processed by purified signal peptidase I *in vitro*. However, the significance of this observation is diminished greatly by the finding that certain other MBP species with alterations in the processing site region that are cleaved efficiently *in vivo* also are not processed *in vitro* (T. Talarico, G. Barkocy-Gallagher, P. J. Bassford, Jr., and P. H. Ray, manuscript in preparation).

age. It is very uncommon for a protease to cleave an X-Pro bond, although some retroviral proteases can do so (22). This study indicates that *E. coli* signal peptidase I is unable to cleave before a proline but still must recognize the normal processing site of the precursor proteins. Since most other amino acids can occupy the +1 position of precursor proteins (14, 20), it seems unlikely that proline at +1 would sterically block recognition of the cleavage site by the processing enzyme. The end result is that pre-MBP27-P interferes with the activity of signal peptidase I, probably by competing as a noncleavable substrate for the enzyme's active site.

When the RBP signal peptide was substituted for the MBP signal peptide in pre-MBP27-P, the resultant RBP-MBP26-P hybrid protein inhibited processing by signal peptidase I to a much lesser extent than MBP27-P and was itself processed slowly at an alternate site in the signal peptide. The most likely alternate processing recognition site in the RBP signal peptide is 2 residues upstream of the normal processing site and is comparable with the alternate site previously demonstrated in the MBP signal peptide (see Fig. 1). RBP-MBP26-P alternate processing may be related to secondary structure. The probability of a  $\beta$ -turn at position -6 relative to the normal cleavage site is much higher for RBP-MBP26-P than for MBP27-P (data not shown), and a  $\beta$ -turn 4-6 residues upstream of the cleavage site is thought to be a requirement for precursor processing (12, 16). The finding that alternate site processing was undetectable for MBP27-P and MBP26-D,27-P and only barely detectable for MBP26-H,27-P (see Fig. 7), whereas both MBP26-D and MBP26-H are alternately processed to a significant extent (19), indicates that the proline residue at +1 of pre-MBP prevents processing at both the normal and alternate sites. The proline at +1 may be affecting the secondary structure of the alternate cleavage site in the MBP signal peptide indirectly. Duffaud and Inouye (49) previously demonstrated that mutational alterations in the adjacent mature region predicted to alter the secondary structure at the cleavage site strongly decreased the efficiency of processing of an OmpA-staphylococcal nuclease hybrid protein expressed in *E. coli*. However, the efficacy of alternate site processing of MBP species with alterations at -1 could not be correlated with predicted structural changes to the processing site region (19). It is also interesting to note that the substitution of proline at position +2 had no effect on processing at the normal site, arguing against a structural significance for proline at +1 affecting alternate site processing (19).

Although some inhibition of signal peptidase I activity was detected as early as 1 min following induction of MBP27-P, as shown by the effect on RBP processing, the complete inhibition of RBP processing was never achieved (see Fig. 3). This was despite the fact that plasmid-encoded MBP27-P is produced in fairly large amounts from the *lacUV5* promoter (18) and signal peptidase I is a very minor cellular protein (50). These findings suggest that the interaction between the altered cleavage site of MBP27-P and the processing enzyme is transient and most efficient for a relatively short period immediately after translocation, when maturation of wild-type pre-MBP would normally occur. In other words, it is suggested that newly translocated MBP27-P is most efficient at interfering with signal peptidase I activity, whereas "old" MBP27-P that accumulates over time in the cytoplasmic membrane contributes minimally to the inhibitory effect. In this same regard, during the period that signal peptidase activity was made limiting by MBP27-P, most newly synthesized pre-RBP was processed over time, but some fraction of the molecules were never cleaved (Fig. 8). These molecules

may have assumed a conformation incompatible with processing or, for some other reason, their cleavage sites were rendered permanently inaccessible to the processing enzyme. For the same reason, pre-MBP27-P probably loses its ability to interact with signal peptidase I at some time after translocation.

Induction of MBP27-P synthesis led to a rapid cessation of cell growth, even though precursor protein processing was only partially inhibited. The essential nature of signal peptidase I has been established previously (9-11), and the precursor forms of periplasmic and outer membrane proteins that accumulate in cells made limiting for this enzyme remain anchored to the outer surface of the cytoplasmic membrane by their unprocessed signal peptides (10). Rapid growth arrest may result from the general accumulation of these precursor proteins in the cytoplasmic membrane or from the specific failure to efficiently release from the cytoplasmic membrane key proteins required for cell division or some other essential function. IPTG-resistant, Mal<sup>+</sup> mutants that continue to synthesize MBP27-P at induced levels currently are being analyzed. Certain of these mutants may produce an altered signal peptidase I that either can process MBP27-P at the primary or alternate site or is resistant to its inhibitory effects.

Statistical analyses have described eukaryotic signal peptidase recognition sequences as slightly different than those identified for bacterial precursors (51-53), although eukaryotic signal peptidases and prokaryotic signal peptidase I seem to process both types of signal peptides efficiently (5). A current data base of 583 eukaryotic precursor proteins includes three with proline residing in the +1 position (54).<sup>3</sup> This would indicate that eukaryotic signal peptidases can cleave an X-Pro bond. On the other hand, Nothwehr *et al.* (55), investigating the *in vitro* processing of a mutant human preproapolipoprotein A-II in which cleavage occurs almost equally at two sites 2 residues apart, found that substitution of a proline at +1 relative to the downstream processing site shifted all processing to the alternate site with no loss of efficiency. In addition, a study of related precursors among different eukaryotic species showed that a mutation resulting in the substitution of proline in the +1 position apparently resulted in a shift to processing at an alternate site (56). These studies, coupled with the general lack of proteolytic cleavage of X-Pro bonds cited above and the similarities in cleavage sites between prokaryotic and eukaryotic precursor proteins, strongly suggest that eukaryotic signal peptidases do not cleave X-Pro bonds and that processing sites having a proline in the +1 position have been identified incorrectly.

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