β-Turn Formation in the Processing Region Is Important for Efficient Maturation of *Escherichia coli* Maltose-binding Protein by Signal Peptidase I in Vivo

(Received for publication, September 29, 1993, and in revised form, March 1, 1994)

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**Signal peptidase I (also called leader peptidase)** is the endopeptidase that removes the signal peptides of most secreted proteins during or after translocation in *Escherichia coli*. Precursor recognition is contingent in part on recognition of prepropeptide residues in the -3 and -1 positions relative to the cleavage site, and may also depend on the structure of the processing region. Most precursor processing regions include residues likely to form a β-turn. Mutations were introduced into the processing region of maltose-binding protein (MBP) that altered the prediction of β-turn formation in this region. MBP species with a decreased probability of β-turn formation were processed slowly or not at all, whereas MBP species with an increased probability of β-turn formation were processed efficiently. Mutations altering the probability of β-turn formation in the MBP processing region were also made in *cis* to a proline in the +1 position. Cleavage at the normal processing site is blocked by proline in the +1 position; this MBP species, MBP-27-P, inhibits processing of other proteins by signal peptidase I. Decreasing the probability of β-turn formation in the processing region of MBP-27-P eliminated the inhibition of signal peptidase I, and these MBP-27-P derivatives remained unprocessed, suggesting that the formation of a β-turn in the MBP processing region was necessary for recognition by signal peptidase I. Increasing the probability of β-turn formation in *cis* to proline at +1 in MBP did not alter recognition of the protein by the processing enzyme. The results presented here are consistent with the hypothesis that the efficiency of recognition and processing by signal peptidase I is increased by the formation of a β-turn in the processing region of the MBP signal peptide.

An N-terminal signal peptide is commonly present on proteins that traverse the cytoplasmic membrane of bacterial cells or the rough endoplasmic reticulum membrane of eukaryotic cells. Most of the time, the signal peptide is removed at the distal surface of the membrane after irreversible initiation of translocation. In *Escherichia coli*, signal peptidase II removes the signal peptides of lipoproteins and signal peptidase I (or leader peptidase) removes the signal peptides of other precursors (1). If a signal peptide is not removed, the protein usually remains membrane-bound but can be released into the periplasmic space in *E. coli* (2–5).

Signal peptides share little primary sequence homology but do have three common structural features (6, 7). The hydrophilic N-terminal region includes 1–3 positively charged residues, and the central core is composed of 9–15 hydrophobic residues likely to form an α-helix. These two structures are involved in the initiation of export and translocation of the precursor across the membrane (8). The signal peptide C-terminal processing region consists of 4–6 amino acids and is usually more polar than the hydrophobic core. Positions -3 and -1 relative to the cleavage site are important for recognition and processing by signal peptidase I (9–13).

In addition, most precursor processing regions include residues likely to form a β-turn (11). Some reports suggest that substitutions resulting in structural changes around the signal peptide cleavage site affect translocation (14, 15), but the majority of studies report that alterations in the processing region directly affect processing. Substitutions changing the proline residue in the processing region of M13 procoat or pre-β-lactamase decrease or eliminate processing but not export (4, 5, 10, 16, 17). Proline frequently occupies position -6, -5, or -4 relative to the cleavage site (13); proline residues break α-helical structures and are often found in β-turns (18). Alterations in the early mature region of a chimeric OmpA-staphylococcal nuclease A protein that decrease the probability of β-turn formation at the processing site decrease processing (19). Kendall and co-workers (20) found that substitution of leucine for the proline residue at position -5 in the PhoA signal peptide dramatically decreases processing. Subsequently, Laforet and Kendall (21) postulated that the structure of the processing region is not a determining factor for maturation, but that the relative hydrophobicity of the amino acids preceding the cleavage site is important.

The probability of β-turn formation in the MBP signal peptide processing region is strongest for the Ser-Ala-Ser-Ala sequence at positions -6 through -3 (see Fig. 1). Since the propensity for β-turn formation in this case does not rely strongly on a single residue as it does for proline-containing processing regions, MBP provides a good model to examine the effects of different substitutions near the processing site. In addition, the secretion and processing of MBP have been studied in detail (for review, see Ref. 22). In this study, changes designed to alter the probability of β-turn formation were introduced into the MBP signal peptide processing region. Alterations decreasing the probability of β-turn formation slowed or eliminated processing.

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* The abbreviations used are: MBP, maltose-binding protein (the prefix "pre" indicates the precursor form, whereas "m" indicates the mature form); RBP, ribose-binding protein; IPTG, isopropyl-1-thio-β-D-galacto-pyranoside; PAGE, polyacrylamide gel electrophoresis.

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cessing but did not affect translocation. In general, substitutions that increased the probability of β-turn formation did not alter processing efficiency.

MATERIALS AND METHODS

Strains and Plasmids—E. coli K12 strain BAR1091 (23) is a derivative of MC4100 (24) with a large, in-frame deletion in the MBP coding region. This strain was used as the host strain for plasmids encoding the MBP species. Strain CJ236 (dut ung; Ref. 25) was used to construct templates for oligonucleotide site-directed mutagenesis.

Plasmid pJF2 is a derivative of pBR322 carrying the M13 intragenic region (2). This plasmid carries the malE' gene under regulatory control of the lacUV5 promoter and all of the plasmids used in this study were derived from it. Plasmid pGG25 harbor the malE27-P gene encoding a MBP species with proline in the +1 position relative to the normal cleavage site (26). Plasmid pWF8 encodes an amber mutation at position −4 relative to the cleavage site (2). Plasmid pW4 carries the malEΔ116 allele (27). This gene encodes a proteinase K-sensitive MBP species deleted for residues 165–176 of the mature moiety. This change does not alter export of the protein. To introduce the malEΔ116 mutation into plasmids encoding MBP species with processing region alterations, the small EcoRI-BglII fragments of the latter were ligated to the large EcoRI-BglII fragment from pW4. Plasmids were packaged as M13 phage particles using M13K07 helper phage (25).

Reagents—Minimal medium M63 supplemented with 0.2% carbon source and thiamine was used for growth of E. coli and phage in agar wells on plates. IPTG, ligase, and proteinase K accessibility as described previously (2). Quantitation of preMBP and mMBP for these MBP species were described in Table 1. Mid-log phase cells induced for synthesis of MBP were pulse-labeled for 15 s with [35S]methionine, and aliquots were removed at specific times during the chase period for analysis of MBP processing. Wild-type preMBP was completely processed to the mature form within 2 min (see Fig. 3). By deleting residues −6 and −5, the probability of a β-turn occurring in the processing region was decreased and this MBP species, MBPA−5/6, was processed very slowly (Table I, Fig. 2). To further explore the role of residues −6 through −4 in maturation, the −5 alanine was reinserted (MBPAΔ−6) or substitutions were made in place of the serine residue at position −4 (MBPAΔ−5/6, −4). The probability of a β-turn forming in the processing region remained low for these MBP species (Table I). Reintroduction of the −5 alanine (MBPAΔ−6) did not restore efficient processing (Table I). The substitution of alanine at −4 in the shortened processing region (MBPAΔ−5/6−4A) slowed processing (Fig. 2). No processing was observed when leucine, isoleucine, or valine were substituted at the same position (Table I, Fig. 2) or when the −4 residue was deleted (MBPA−5/6−4A). Only the substitution of the α-helix breaker proline at the −4 position restored efficient processing (MBPA−5/6−4P; Table I, Fig. 2). These cells containing plasmids expressing each of these shortened MBP species were Mal+, indicating that the MBP species must have been translocated across the membrane to some extent. To confirm that these MBP species were rapidly translocated and remained tethered at the outer surface of the membrane, the locations of precursors MBPA−6, MBPA−5/6−4I, MBPAΔ−5/6−4A, and MBPA−5/6/4 in the cells were determined as described previously (2). MBP normally is protease-resistant; a deletion of 9 residues in the mature moiety of MBP renders the protein protease-sensitive without affecting secretion (27). The mutation encoding this deletion (Δ116) was introduced into plasmids encoding the aforementioned MBP species. The location of each MBP species was then determined by proteinase K accessibility in fractionated spheroplasts after a 1-min pulse-chase labeling. The precursor form of each of these MBP species was spheroplast-associated and digested by externally added proteinase K, indicating that they were rapidly translocated and located at the outer surface of the inner membrane (data not shown). Therefore, these deletions and substitutions decreasing the probability of β-turn formation in the MBP processing region affected maturation but did not detectably effect translocation.

RESULTS

Effects of Deletions and Substitutions in the Processing Region of PreMBP—The processing region of the MBP signal peptide is 6 amino acids long (Fig. 1) and, based on Chou and Fasman analysis (18), includes residues that could form a β-turn (Table I). We wanted to determine the significance of residues in the processing region of the MBP signal peptide that are not part of the well established −3, −1 processing recognition site (9) but are potentially involved in the formation of a β-turn. Using oligonucleotide site-directed mutagenesis, derivatives of plasmid pJF2 were constructed encoding MBP species with deletions including the −6 to −4 residues of the processing region.

The kinetics of conversion from preMBP to mMBP for these MBP species are described in Table I. Mid-log phase cells induced for synthesis of MBP were pulse-labeled for 15 s with [35S]methionine, and aliquots were removed at specific times during the chase period for analysis of MBP processing. Wild-type preMBP was completely processed to the mature form within 2 min (see Fig. 3). By deleting residues −6 and −5, the probability of a β-turn occurring in the processing region was decreased and this MBP species, MBPA−5/6, was processed very slowly (Table I, Fig. 2). To further explore the role of residues −6 through −4 in maturation, the −5 alanine was reinserted (MBPAΔ−6) or substitutions were made in place of the serine residue at position −4 (MBPAΔ−5/6, −4). The probability of a β-turn forming in the processing region remained low for these MBP species (Table I). Reintroduction of the −5 alanine (MBPAΔ−6) did not restore efficient processing (Table I). The substitution of alanine at −4 in the shortened processing region (MBPAΔ−5/6−4A) slowed processing (Fig. 2). No processing was observed when leucine, isoleucine, or valine were substituted at the same position (Table I, Fig. 2) or when the −4 residue was deleted (MBPA−5/6−4A). Only the substitution of the α-helix breaker proline at the −4 position restored efficient processing (MBPA−5/6−4P; Table I, Fig. 2). These cells containing plasmids expressing each of these shortened MBP species were Mal+, indicating that the MBP species must have been translocated across the membrane to some extent. To confirm that these MBP species were rapidly translocated and remained tethered at the outer surface of the membrane, the locations of precursors MBPA−6, MBPA−5/6−4I, MBPAΔ−5/6−4A, and MBPA−5/6/4 in the cells were determined as described previously (2). MBP normally is protease-resistant; a deletion of 9 residues in the mature moiety of MBP renders the protein protease-sensitive without affecting secretion (27). The mutation encoding this deletion (Δ116) was introduced into plasmids encoding the aforementioned MBP species. The location of each MBP species was then determined by proteinase K accessibility in fractionated spheroplasts after a 1-min pulse-chase labeling. The precursor form of each of these MBP species was spheroplast-associated and digested by externally added proteinase K, indicating that they were rapidly translocated and located at the outer surface of the inner membrane (data not shown). Therefore, these deletions and substitutions decreasing the probability of β-turn formation in the MBP processing region affected maturation but did not detectably affect translocation.

Effects of Substitutions in the Processing Region of Full-
length PreMBP.—The decrease in processing of MBP species with shortened processing regions implied that the structure of the region could indeed be important for maturation but left open the possibility that the distance between the hydrophobic core and the cleavage site was a significant factor. We also investigated the processing of preMBP species with similar changes in the full-length processing region, also expressed from derivatives of plasmid pJF2 constructed by oligonucleotide site-directed mutagenesis. The probability of β-turn formation in the processing region and the kinetics of processing of preMBP species with substitutions at positions -6, -5, or -4 are indicated in Table II. Unlike the shortened MBP species described above, all but one of these preMBP species were processed completely within a 5-min chase period in pulse-chase labeling experiments (Figs. 3 and 4, data not shown).

The substitution of glycine, leucine, valine, or isoleucine at position -6 decreased the probability of a β-turn occurring in the processing region; concomitantly, processing of these preMBP species was slower than processing of wild-type MBP (Table II, Fig. 3). The substitution of alanine at position -6 also decreased the probability of a β-turn forming (Table II). Processing of preMBP-6A was complete within 2 min (Fig. 3) but was slightly slower than processing of wild-type preMBP when examined very soon after labeling (71% processed to mMBP-6A at 0 s of chase versus 85% mMBP (wild-type); data not shown). The remaining MBP species with substitutions at position -6, MBP-6P and MBP-6D, did not have a decreased probability of a β-turn occurring in the processing region; processing of these preMBP species was identical to that of wild-type preMBP (Table II, Fig. 3).

Substitutions were introduced at positions -5 or -4 of MBP that increased the probability of a β-turn occurring in the processing region. With one exception, processing of these preMBP species was identical to processing of the wild-type protein, even when examined immediately post-labeling (Table II, Fig. 3).
terminal to the normal processing site (26). This protein is a less effective inhibitor of signal peptidase I than MBP-27-P. We postulated that processing of the chimeric protein was due to the higher probability of β-turn formation in the processing region of the RBP signal peptide relative to that of the MBP signal peptide. Therefore, increasing the probability of β-turn formation in the MBP-27-P processing region might result in processing at the alternate site.

To test this hypothesis, mutations increasing the probability of β-turn formation were introduced into the processing region of the MBP-27-P signal peptide; serine, proline, or threonine was substituted at position -5, or asparagine was substituted at position -4. The substitutions at position -5 changed the -3 residue of the alternate processing site, but each of these amino acids, in the normal -3 position permits efficient MBP cleavage (9). Because proline is a strong α-helix breaker (18), we also substituted proline at position -6 of MBP-27-P, even though this substitution does not change the prediction of β-turn formation. Only preMBP-27-P with an asparagine at position -4 was processed, and just 3.4% of the protein was converted to the mature form within 20 min post-labeling (Fig. 5). This mMBP species migrated slightly slower that wild-type mMBP in a 7.5% polyacrylamide gel, suggesting that it resulted from processing at the alternate site (9, 26). The MBP-27-P species with substitutions increasing the probability of β-turn formation in the processing region inhibited processing by signal peptidase I at least as well as unaltered MBP-27-P (Fig. 5, data not shown). Thus, increasing the probability of β-turn formation in cis to proline at +1 in MBP did not affect recognition of the precursor by signal peptidase I and did not result in processing of the precursor MBP species.

If the various changes decreasing the probability of β-turn formation decreased recognition of MBP by signal peptidase I, then processing of RBP by signal peptidase I should not be inhibited by MBP-27-P species with a lower probability of β-turn formation in the processing region. Deletion of the serine at position -6 or substitution of an alanine at -6 of MBP-27-P relieved the block of RBP processing exerted by the proline residue at position +1. Both preMBP-27-P species remained unprocessed (data not shown), so decreasing the probability of β-turn formation in MBP-27-P resulted in MBP species that were not effectively recognized by signal peptidase I.

DISCUSSION

Two major steps in the export of proteins across the inner membrane of E. coli are translocation across the lipid bilayer and removal of the N-terminal signal peptide. It has been proposed that the signal peptide forms an α-helix to cross the lipid bilayer (34). Consensus analysis of known signal peptides suggests that residues in the processing region form a β-turn ending the α-helix (11), but there is disagreement whether this proposed structure is important for processing, translocation, or even if it is at all significant (4, 5, 10, 14–17, 19, 21). Based on studies with an idealized PhoA signal peptide, Laforet and Kendall (21) have postulated that the hydrophobicity of the residues in the processing region is important, rather than the propensity for β-turn formation. However, Dalbey and co-workers (10) found no correlation between processing and hydrophobicity of residues in this region of M13 procoat. We found no consistent correlation between hydrophobicity and processing for the MBP species described here. The signal peptide processing region of MBP involves residues -6 through -1; the highest probability of β-turn formation in this region, based on Chou and Fasman analysis (18), involves residues -6 through -3. Although Chou and Fasman rules may not apply to the signal peptide during translocation, our data are consistent with the hypothesis that formation of a β-turn in the processing region of the MBP signal peptide affects recognition of the precursor by signal peptidase I but does not significantly affect translocation.

Changes in the MBP signal peptide that decreased the probability of β-turn formation in the processing region without altering the -3, -1 cleavage recognition site (9) slowed or eliminated processing. Substitutions at the -6 position that decreased the probability of β-turn formation consistently resulted in slowed kinetics of MBP processing. Deletions involving the -6 through -4 residues (MBPΔ-6, MBPΔ-5/6, and MBPΔ-4/5/6) also decreased the probability of β-turn formation in the processing region and decreased processing. Several substitutions were made at position -4 of MBPΔ-5/6, none of which restored a high probability of β-turn formation to the processing region. Of these, only proline restored efficient MBP processing (MBPΔ-5/6-4P), almost to wild-type levels.

The proline residue in MBPΔ-5/6-4P and the other amino acid substitutions described in this study could be interrupting the α-helical structure of the hydrophobic core other than by formation of a β-turn per se or could affect processing in an undetermined manner. However, we believe that our results are more consistent with the probability of β-turn formation in the region than with the α-helix breaking or other defined properties of the substituted amino acids. For example, even though glycine is described as a strong α-helix breaker (18), the substitution of glycine for serine at position -6 of MBP decreased the probability of β-turn formation and caused slower MBP processing. Glycine in place of proline in an idealized yeast signal peptide processing region or in M13 procoat does not support efficient processing, implying that glycine does not provide the necessary function in these circumstances, either (10, 35). Proline causes a kink in the peptide backbone and is commonly found in β-turns, so it is possible that a β-turn forms in MBPΔ-5/6-4P despite the low probability determined by the Chou and Fasman algorithm (18).

While there was a consistent correlation between the decreased probability of β-turn formation in the processing region and decreased processing of preMBP species, the magnitude of the change in processing did not always correlate with the magnitude of the decrease in β-turn forming probability. The
processing defects for the truncated MBP species were more dramatic than for MBP species with simple substitutions in the processing region, suggesting that the length of the processing region in MBP is important. This is an intriguing result, since MBP species with shortened hydrophobic cores are efficiently translocated and processed, and it has been implied that the hydrophobic core and processing region of MBP can overlap (36, 37). Most prokaryotic signal peptide processing regions that have been identified are 6 amino acids in length (38). Our data suggests that for MBP, 6 amino acids versus 4 in the processing region contributed additional flexibility or propensity to turn.

Plasmids were also constructed encoding MBP species with substitutions at position -6, -5, or -4 that increased the probability of β-turn formation. These full-length MBP species had processing kinetics that were unaltered or only slightly altered (MBP-5D) from the kinetics of wt preMBP processing, even though the predicted β-turns of several of these MBP species initiated at residue -7, one position beyond the designated processing region. Similarly, Nothwehr and Gordon (39) found that processing of a eukaryotic protein in an in vitro translocation system occurred when the predicted β-turn initiated within 1 residue of the processing region.

MBP27-P has a proline in the +1 position relative to the normal processing site, is not processed, and is a competitive inhibitor of signal peptidase I (26). Both RBP with a proline in the normal +1 position and an RBP-MBP chimeric precursor with proline at +1 position are processed, probably at an alternate site 2 residues upstream of the normal processing site (26). We hypothesized that processing of the latter two proteins resulted from the higher probability of β-turn formation in the RBP signal peptide processing region, which is due to an asparagine residue at position -4 relative to the normal cleavage site. In this study, we examined processing of MBP27-P derivatives with substitutions resulting in a high β-turn probability. Only MBP27-P with asparagine at position -4 was processed, probably at the alternate processing site. However, processing of the RBP and RBP-MBP chimeric proteins with proline at +1 is much more rapid than processing of MBP27-P-4N, and proceeds to completion (26). In addition, the MBP27-P derivatives with an increased probability of β-turn formation were effective inhibitors of signal peptidase I, unlike the RBP and chimeric proteins with proline in the +1 position. Therefore, increasing the probability of β-turn formation did not alter recognition of MBP27-P by signal peptidase I; processing did not occur at the alternate processing site.

It has been suggested that the presence of a β-turn in the processing region is important for protein translocation (14, 15). These reports are consistent with the loop model, which proposes that the signal peptide and early mature region insert into and through the membrane as a reverse hairpin structure (40). Perhaps the predicted β-turn early in the mature region of MBP plays this role. We found no evidence of a defect in trans-

location for MBP species with a decreased probability of β-turn formation, based on localization of unprocessed MBP species to the outer surface of the membrane and the Mal+ phenotype of the cells. However, it remains possible that we did not identify slight defects in translocation. The results of this study are consistent with the hypothesis that the β-turn in the processing region of the MBP signal peptide is important for the interaction of the protein with signal peptidase I, perhaps in presentation or binding of the processing site to the enzyme.

Acknowledgments—We thank Tom Silhavy for stimulating discussions and Ross Dalbey for critically reading the manuscript.

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