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ARTICLE *in* BIOCHEMISTRY · JANUARY 1992

Impact Factor: 3.02 · DOI: 10.1021/bi00115a006 · Source: PubMed

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Use of Site-Directed Mutagenesis To Define the Limits of Sequence Variation Tolerated for Processing of the M13 Procoat Protein by the *Escherichia coli* Leader Peptidase†

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Received June 6, 1991; Revised Manuscript Received August 14, 1991

ABSTRACT: Leader peptidase cleaves the leader sequence from the amino terminus of newly made membrane and secreted proteins after they have translocated across the membrane. Analysis of a large number of leader sequences has shown that there is a characteristic pattern of small apolar residues at −1 and −3 (with respect to the cleavage site) and a helix-breaking residue adjacent to the central apolar core in the region −4 to −6. The conserved sequence pattern of small amino acids at −1 and −3 around the cleavage site most likely represents the substrate specificity of leader peptidase. We have tested this by generating 60 different mutations in the +1 to −6 domain of the M13 procoat protein. These mutants were analyzed for in vivo and in vitro processing, as well as for protein insertion into the cytoplasmic membrane. We find that in vivo leader peptidase was able to process procoat with an alanine, a serine, a glycine, or a proline residue at −1 and with a serine, a glycine, a threonine, a valine, or a leucine residue at −3. All other alterations at these sites were not processed, in accordance with predictions based on the conserved features of leader peptides. Except for proline and threonine at +1, all other residues at this position were processed by leader peptidase. None of the mutations at −2, −4, or −5 of procoat (apart from proline at −4) completely abolished leader peptidase cleavage in vivo although there were large effects on the kinetics of processing. Intriguingly, leader peptidase could not cleave, in vivo, eight of the nine mutants that replaced the proline at −6, including a glycine, which is also a helix breaker. This result suggests that the −6 residue can influence the substrate conformation around the cleavage site.

Many secreted and membrane proteins are synthesized in a precursor form with an amino-terminal extension peptide of 15–30 amino acid residues. These leader (signal) sequences function to initiate protein translocation across the membrane and are removed by a membrane-bound leader (signal) peptidase during or after protein export.

Despite extensive research over the last decade (Mollay et al., 1982; Lively & Walsh, 1983; Jackson & Blobel, 1980), only recently has signal peptidase been isolated from eukaryotic cells. Signal peptidase from dog pancreas consists of a complex of five polypeptide chains of molecular mass 25, 23/22, 21, 18, and 12 kDa (Evans et al., 1986). Whereas there are five proteins in canine pancreas, there are only two subunits in the signal peptidase from the chicken oviduct (Baker & Lively, 1987). In contrast, each of the two bacterial leader peptidases that have been isolated consists of only one polypeptide. Lipoprotein signal peptidase (Innis et al., 1984) processes several inner and outer membrane proteins, termed lipoproteins, while leader peptidase (Dalbey et al., 1986) processes the other membrane proteins and almost all periplasmic proteins (Wolfe et al., 1983; Dalbey & Wickner, 1985). This leader peptidase, whose active site faces the periplasmic side of the membrane (Wolfe et al., 1982), has been purified to homogeneity (Zwizinski & Wickner, 1980; Wolfe et al., 1982). In addition, its gene has been cloned (Date & Wickner, 1981) and sequenced (Wolfe et al., 1983), and the enzyme that it codes for has been overexpressed under inducible/repressible regulation. The physiological role of leader peptidase is to release periplasmic and outer membrane proteins from their leader peptide, which acts as a transient membrane anchor (Dalbey & Wickner, 1985).

The *Escherichia coli* leader peptidase, which has very wide substrate specificity, processes many preproteins (Watts et al., 1983; Dalbey & Wickner, 1985), including most bacterial exported proteins, yeast pre-acid phosphatase, honey bee prepromellitin, and human prehormones (preproinsulin, pregrowth hormone, preinterferon, etc.). These leader peptidase substrates do not have any sequence homology. Rather, they have a common pattern (von Heijne, 1983; Perlman & Halvorson, 1983) of small amino acids at residues −1 (Ala, Gly, or Ser) and −3 (Ala, Gly, Ser, Leu, Val, or Ile) and a helix-breaking residue around position −6 (with respect to the cleavage site). Earlier studies have shown that the region immediately preceding the leader peptidase cleavage site is important for processing: (i) Mutations that alter the helix-breaker proline to either a leucine or a serine residue of the precursor to β-lactamase block leader peptidase processing but not translocation (Koshland et al., 1982); (ii) various mutations that change the amino acid at −1, −3, or −6 of the M13 procoat protein prevent cleavage (Kuhn & Wickner, 1985); and (iii) leader peptidase can cleave small peptides, including the 16 amino acid peptide containing residues −9 to +5 of the procoat

† This work was supported by National Science Foundation Grant DCB-8718578, an American Cancer Society Junior Faculty award, a seed grant from the Office of Research and Graduate Studies, a Basil O'Conner starter grant from the March of Dimes, a pilot research grant from the American Cancer Society (Ohio State University), and a grant from the American Cancer Society (Ohio Division). A.K. would like to acknowledge that this work was supported by a grant (3.533–0.86) from the Swiss Science Foundation and a grant (749/1-1) from the Deutsche Forschungs-Gemeinschaft.

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0006-2960/91/0430-11775$02.50/0

Table I

mutated codon	mutagenic primer[a]
+1	ATCGTCACCCTCNNNAGCGAAAGACAG
−1	GTCACCCTCAGCNNNGAAAGACAGCAT
−2	ACCCTCAGCAGCNNNAGACAGCATCGG
−3	CTCAGCAGCGAANNNCAGCATCGGAAC
−4	AGCAGCGAAAGANNNCATCGGAACGAG
−5	AGCGAAAGACAGNNNCGGAACGAGGGT
−6	GAAAGACAGCATNNNAACGAGGGTAGC

[a] Altered codons are underlined.

protein (Dierstein & Wickner, 1986) and the 5 amino acid peptide corresponding to positions −3 to +2 of the precursor to the maltose binding protein (Dev et al., 1990).

In this report we have directly tested whether the strongly conserved residues at −1, −3, and −6 of the leader sequence represent the substrate specificity of leader peptidase. Oligonucleotide-directed mutagenesis was used to generate a large number of mutant procoats with mutations in the region +1 through −6. Most of these proteins were characterized for their in vivo and in vitro processing, as well as for their protein translocation across the cytoplasmic membrane. Our results show that the specificity determinants for the processing of procoat are located at −1, −3, and −6. Small residues at −1 and −3 of procoat and only proline at −6 were cleaved by leader peptidase. All mutants with alterations at +1 (except for proline and threonine) were cleavable. In addition, because leader peptidase can cleave almost all of the procoat mutants at the −2, −4, and −5 positions, it has very broad substrate specificity. All mutations that we created at the −6 position (apart from glutamine, which was processed slowly) completely abolished processing by leader peptidase in vivo, including glycine and other good helix breakers.

Materials and Methods

Materials. "Translabel", a mixture of 85% [^{35}S]methionine and 15% [^{35}S]cysteine (1000 Ci/mmol), was from ICN. Proteinase K was from Boehringer Mannheim. Phenylmethanesulfonyl fluoride (PMSF) and [^{35}S]dATP were from Sigma and New England Nuclear, respectively.

Bacterial Strains and Plasmids. *E. coli* strains HJM114 (Δ*lacpro*), F′ (*lacpro*), MC1061 [Δ*lac*X74, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, *hsr*, *hsm*, *str*A], and JM103 [Δ(*lacpro*), *thi*, *str*A, *sup*E, *end*A, *sbc*B, *hsd*R, *tra*D36, *pro*AB, *lac*IqZ M15] were from our collection. The pT712 plasmid containing the procoat gene under the control of the T7 promoter was described in Bilgin et al. (1990).

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis (Zoller & Smith, 1983) was used to generate multiple mutations at residues +1, −1, −2, −3, −4, −5, and −6 within the procoat gene. Each oligonucleotide, which, except for the randomized codon, is complementary to the antisense procoat DNA, was synthesized on an Applied Biosystems model 380B instrument by The Ohio State University Biochemical Instrument Center (Table I). After annealing the mutagenic oligonucleotide and the universal primer to the single-stranded M13mp19 DNA containing the procoat gene, DNA polymerase I (large fragment) and T4 ligase were used to prepare the double-stranded DNA. To increase the efficiency of mutagenesis, the template DNA was prepared from phage grown on *E. coli* strain RZ1032 (*ung*$^-$*dut*$^-$). After transformation into *E. coli* JM103 (*ung*$^+$), the phage was isolated from single plaques and the mutation identified by dideoxynucleotide sequencing (Sanger et al., 1977). The replicative form of the M13mp19 DNA containing the mutation was digested with *Eco*R1 and *Sal*1, and the fragment was inserted either into the pJF119HE (Furste et al., 1986) or the pT712 plasmid (Pharmacia). Each of the plasmids were also sequenced to verify the mutation.

DNA Manipulations. DNA manipulations were performed as described by Maniatis et al., (1982). All cloning procedures used restriction enzymes from New England Biolabs or Bethesda Research Laboratories. T4 kinase and T4 DNA ligase were from Bethesda Research Laboratories and New England Biolabs, respectively. DNA polymerase 1 (Klenow fragment) was from Boehringer Mannheim. Transformations were carried out following the calcium chloride procedure (Cohen et al., 1973).

In Vivo Assay for Processing of Procoat to Coat Protein. Cultures were grown to the mid-log phase under aeration in M9 minimal medium (Miller, 1972) supplemented with 0.5% fructose and 19 amino acids, except methionine. To induce synthesis of procoat, IPTG (1 mM final concentration) was added for 10 min to *E. coli* strain JM103. After 30 min, cells were labeled for 30 s with [^{35}S]methionine and chased with nonradioactive methionine for either 5 s or 1 min; the procoat was immunoprecipitated (Wolfe et al., 1982), loaded on a sodium dodecyl sulfate–polyacrylamide gel, and analyzed by fluorography (Ito et al., 1980).

In Vitro Assay for Processing of Procoat to Coat Protein. Cell-free synthesis of procoat labeled with [^{35}S]methionine was performed in an *E. coli* extract containing the pT712 plasmid encoding the procoat gene. In this system each reaction (30 μL) contained DNA, *trans*-[^{35}S]methionine (30 μCi), T7 RNA polymerase, Triton X-100 (0.4%), and S150-2 (Yamane et al., 1987). After 30 min at 37 °C, synthesis was stopped by diluting in ice-cold 50 mM Tris, pH 8.0, and 0.1% Triton X-100 (0.6 mL). To see if procoat was converted to the coat protein, an aliquot (10 μL) was treated with 2 μL of 1 mg/mL leader peptidase (final concentration 4 μM) and further incubated for 30 min at 37 °C.

Percent Processing. Fluorographs of gels containing either immunoprecipitated [^{35}S]methionine labeled procoat or in vitro synthesized [^{35}S]procoat were scanned using a Biomed Instrument Zeineh soft laser scanning densitometer. Since two of the three methionines are lost after leader peptide cleavage of the translation product of procoat, the following equation was used to quantitate the percent processing:

$$\text{percent processing} = \frac{(3 \times \text{coat protein}) \times 100}{(3 \times \text{coat protein}) + (\text{procoat protein})}$$

Protease-Mapping of Procoat Mutants. *E. coli* strain MC1061 bearing the pJF plasmid-encoding procoat was grown to the early log phase in M9 minimal media containing 0.5% fructose. Procoat was induced with IPTG and labeled with [^{35}S]methionine for 2 min. After pulse-labeling, cells were chilled on ice and converted into spheroplasts (Randall & Hardy, 1986). Aliquots were incubated on ice either with or without proteinase K (1 mg/mL). Where indicated, a portion of the cells was treated with Triton X-100 prior to the addition of proteinase K to lyse the cells. Subsequently, PMSF (5 mM final concentration) was added to inhibit the protease.

Results

Figure 1 shows the sequence of wild-type M13 procoat and the 60 mutations created at positions +1, −1, −2, −3, −4, −5, and −6 near the cleavage site. The mutations are designated by the one-letter code for each amino acid and are classified into three groups, depending on whether they are cleaved, slowly cleaved, or not cleaved in vivo. At each position the mutations are put into order according to decreasing hydro-

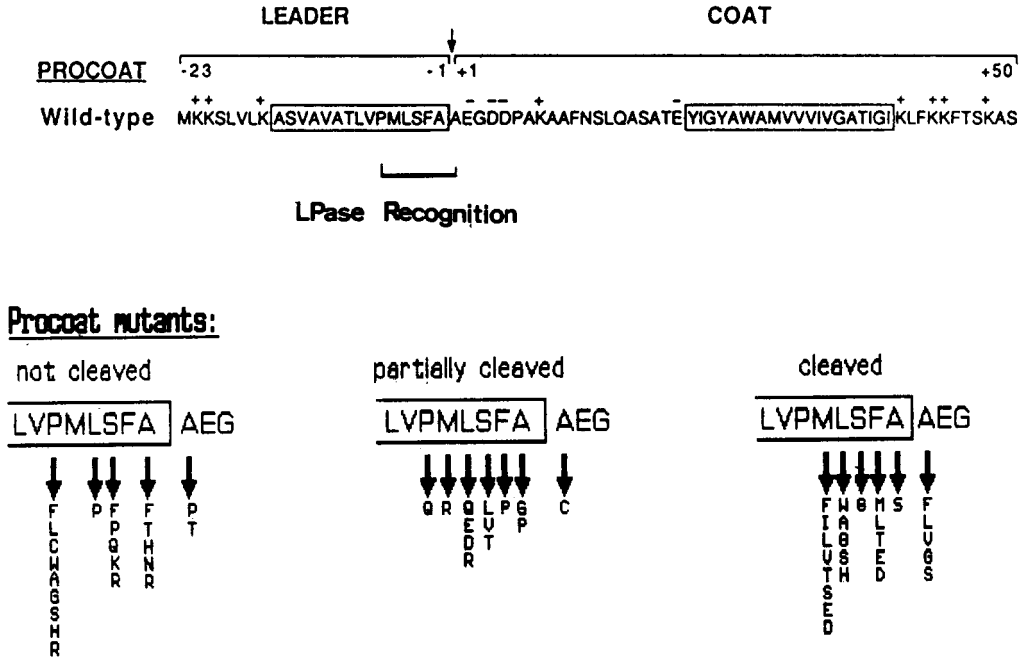

FIGURE 1: Recognition sequence of leader peptidase and summary of the in vivo cleavage results of procoat mutants. The partially cleaved class of mutants are those that show between 20 and 94% coat protein in the pulse–chase experiments. The arrow refers to the cleavage site. Amino acids are designated by the one-letter code, i.e., A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, and Y = Tyr.

phobicity: F, M, I, L, V, C, W, A, T, G, S, P, Y, H, Q, N, E, K, D, and R. In this study, both in vivo and in vitro assays for leader peptidase processing were used to characterize the mutants. With protease-mapping experiments, we verified that those unprocessed mutants were translocated across the membrane.

Requirements of Small Residues at the −1 and −3 Positions. To determine whether leader peptidase requires small residues at positions −1 and −3 (relative to the cleavage site) in its substrates for processing, we isolated mutations at these positions using degenerate oligonucleotide (Table I). The specific mutation was identified by dideoxy sequencing of the single-stranded phage DNA containing the procoat gene. After subcloning the gene into the expression plasmid, we sequenced the gene again, as described by Chen and Seeburg (1985). The plasmids coding for the procoat mutants were designated OMXY or OLXY (for Oligonucleotide, mutation in Mature or Leader region, and position X of amino acid Y). To test if the procoat mutants were cleaved by leader peptidase, cells expressing these mutants were pulse labeled with [^{35}S]-methionine for 30 s, chased with cold methionine for 5 s or 1 min, then immunoprecipitated with antibody to procoat. Aliquots were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. The percent processing was determined after a 1-min chase by quantifying the procoat and coat bands by densitometry and then correcting for the number of methionines (one methionine in coat, three in procoat). Table II summarizes the results for mutations at −1. Procoat mutants with either serine or glycine at −1 were almost completely cleaved, whereas mutants with either Asn, Thr, His, Ile, Arg, or Phe at −1 were not. The proline mutant at −1 was partially cleaved. While at longer chase points procoat proteins with serine, glycine, or proline at −1 were completely cleaved, the other mutants exhibited very little processing (unpublished data). The −3 specificity of leader peptidase was examined in procoat by making nine mutations at this position (Table II). Only procoat with a small amino acid (serine or glycine) was efficiently cleaved in vivo; for the bigger amino acid side chains of threonine, valine, and leucine, cleavage was retarded;

Table II: In Vivo and in Vitro Processing of Procoat with Alterations at −1, −3, and −6

substitutions[a]	percent processed[a] in vivo	in vitro
	−1 Position	
F	0	nd
A (wt)	100	75
T	4	0
G	82	80
S	95	82
P	43	81
H	0	0
N	0	0
R	0	0
	−3 Position	
L	27	87
V	71	77
T	39	75
G	100	78
S (wt)	100	75
P	0	0
Q	0	nd
K	0	0
R	0	nd
	−6 Position	
F	0	50
L	0	0
C	0	87
W	0	65
A	0	nd
G	0	40
P (wt)	100	75
S	0	76
H	10	80
Q	56	40
R	0	0

[a] wt wild-type; nd, not done.

it did not occur with glutamine, arginine, or lysine residues, or the helix-breaker proline. Typical results from this type of analysis for the −1 and −3 mutants are shown in Figure 2A.

In parallel experiments, we tested whether the mutant procoats were substrates for leader peptidase in vitro. Most

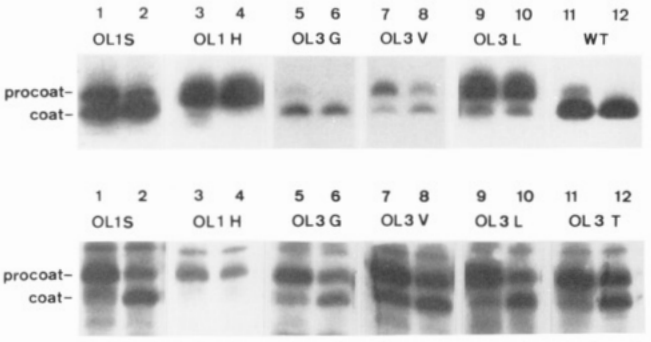

FIGURE 2: (A, top) In vivo processing of procoat mutants at −1 and −3. *E. coli* JM103 expressing procoat mutants were grown in M9 media to the mid-log phase and induced with 1 mM IPTG for 10 min. Cells were pulse labeled with ^{35}S translabel for 30 s and then chased with unlabeled methionine for 5 s (lanes 1, 3, 5, 7, 9, and 11) or 1 min (2, 4, 6, 8, 10, and 12). Samples were immunoprecipitated with antiserum to procoat and then analyzed by gel electrophoresis and fluorography, as described under Materials and Methods. (B, bottom) In vitro processing of procoat mutants at −1 and −3. Procoat mutants were synthesized in a cell-free system and then an aliquot (10 μL) was incubated at 37 °C with (lanes 2, 4, 6, 8, 10, and 12) or without (lanes 1, 3, 5, 7, 9, and 11) 2 μL of purified leader peptidase (1 mg/mL).

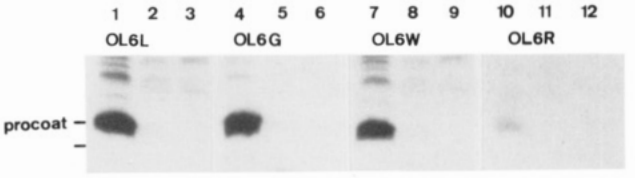

FIGURE 3: Protease mapping of procoat mutants with an alteration at the −6 position. *E. coli* JM103 expressing procoat OL6L, OL6G, OL6W, or OL6R was grown at 37 °C to the exponential phase and incubated with IPTG (1 mM) for 10 min. Cells (1 mL) were pulse labeled with ^{35}S translabel for 2 min and then chilled on ice. After collecting the cells by centrifugation, the cells were resuspended in 0.25 mL of EDTA buffer (0.1 M Tris-acetate, pH 8.2, 0.5 M sucrose, 5 mM EDTA) and treated with 20 μL of lysozyme (2 mg/mL) and 0.25 mL of ice-cold water. After a 5-min incubation, $MgSO_4$ was added to a final concentration of 18 mM and the cells were pelleted by centrifugation and resuspended in $MgSO_4$ buffer (50 mM Tris-acetate, pH 8.2, 0.25 M sucrose, 10 mM $MgSO_4$). Aliquots of these cells (150 μL) were incubated at 0 °C with either no addition (lanes 1, 4, 7, and 10), with proteinase K for 60 min (lanes 2, 5, 8, and 11), or with proteinase K after the cells were lysed by the addition of 2% Triton X-100 (lanes 3, 6, 9, and 12). After the addition of 5 mM phenylmethanesulfonyl fluoride (PMSF), samples were immunoprecipitated with antiserum to procoat and then analyzed by SDS–PAGE and fluorography.

of the mutants were synthesized in a cell-free transcription/translation system and incubated with leader peptidase for 30 min at 37 °C. As can be seen in Table II, the in vitro data correlate well with the in vivo results. For instance, alterations at position −1 with proline, glycine, and serine were processed in the detergent extracts, while, even at very high levels of purified leader peptidase, no processing was found with other substitutions. A similar correlation was found in the −3 position, except in the case of leucine, which was readily cleaved in the in vitro assay. Figure 2B shows some representative results obtained for the −1 and −3 mutants in this assay.

Requirement of a Proline Residue at −6. A characteristic feature of bacterial leader peptides is a helix-destabilizing residue such as proline or glycine between −4 and −6 of the peptide. To determine whether the helix-breaking proline at −6 of the procoat leader peptide is essential for cleavage or insertion, we created 10 mutants. Almost all of the substitutions at this position severely affected in vivo processing (Table II), including glycine, a good helix breaker. However, most of the mutants were cleaved in vitro (Table II). Possibly, the proline is required for translocation of the protein across the membrane but not for processing. To test this idea, protease-mapping studies were performed. Cells were pulse labeled for 2 min with [^{35}S]methionine, converted to spheroplasts, and incubated at 0 °C for 60 min with or without proteinase K. Aliquots were then immunoprecipitated with antibody to procoat and outer membrane protein A and subjected to SDS–polyacrylamide electrophoresis and fluorography. In these experiments, we had less than 20% lysis, as judged by the accessibility of cytoplasmic proteins. Each of the nonprocessed mutants tested was accessible to proteinase K, indicating that they were translocated across the plasma membrane (Figure 3).

In contrast to the in vivo processing results, most of the −6 mutant procoats were processed by leader peptidase in detergent extracts, indicating a difference between the two assay systems. This apparent discrepancy was resolved in a leader peptidase titration study (Figure 4). While mutant procoats synthesized in vitro were processed to the mature form at very high levels of leader peptidase, they were not cleaved at low levels, where the wild-type procoat is processed. Since increasing the concentration of leader peptidase drives processing, these data indicate that the mutations at −6 change the transmembrane arrangement of procoat and disturb the binding of procoat to leader peptidase.

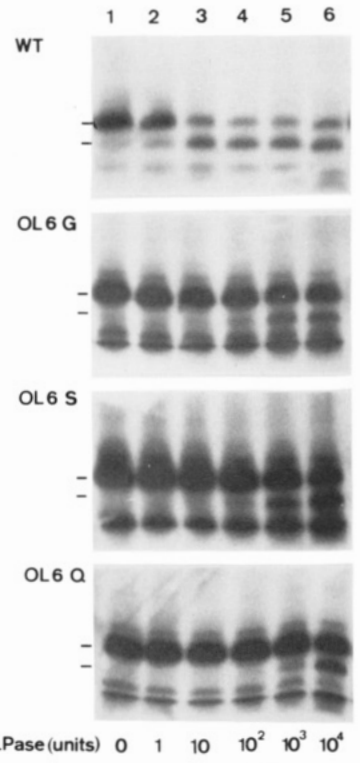

FIGURE 4: Processing of in vitro synthesized procoat mutants, with alterations at the −6 position, in detergent extracts with different amounts of leader peptidase. Cell-free synthesis of procoat labeled with [^{35}S]methionine was performed in an *E. coli* extract containing the pT712 plasmid encoding the procoat gene. In this system, each reaction (30 μL) contained DNA, [^{35}S]methionine (3 μL), T7 RNA polymerase, Triton X-100 (0.4%), and S150-2 (Yamane et al., 1987). After 30 min at 37 °C, synthesis was stopped by dilution in ice-cold 50 mM Tris, pH 8.0, 0.1% Triton X-100 (0.6 mL). Purified leader peptidase was added at the indicated concentration (1 unit = 0.4 nM), and the samples were incubated at 37 °C for 30 min.

Substitutions at +1 in the Mature Region. Sequence analysis of preproteins does not reveal any conserved residue

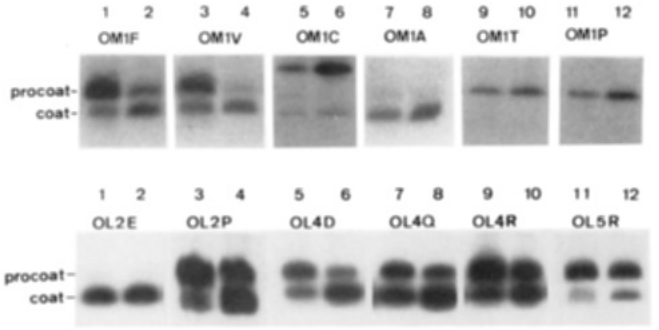

FIGURE 5: (A, top) Broad substrate specificity (except for proline, cysteine, and threonine) at the +1 position. *E. coli* JM103 bearing the plasmids coding for various procoat mutants were analyzed for their in vivo processing, as described in Figure 2. (B, bottom) The residues at −2, −4, and −5 affect the efficiency of processing. *E. coli* JM103 containing various plasmids that code for the procoat mutants were analyzed for their in vivo processing, as described in Figure 2. In both panels A and B, odd and even lanes correspond to the 5-s and 1-min chase points, respectively.

at this position. To test the +1 specificity of leader peptidase, we created eight mutations at this position within the procoat protein. Most of the +1 mutant procoats were processed in vivo, as well as in detergent extracts, except for those containing proline, threonine, or cysteine (Table III and Figure 5A). Protease-mapping studies confirmed that procoat with a proline or a threonine residue did translocate across the membrane (data not shown). With the cysteine mutation at +1, we observed a higher molecular mass band of 10 kDa. One likely possibility for this result is that the cysteine at +1 is being modified to a lipoprotein that cannot be cleaved by lipoprotein signal peptidase. Indeed, the addition of globomycin, an inhibitor of this fatty acid modification, results in the disappearance of the higher molecular mass species with the appearance of two bands, running at the expected position of the procoat and coat proteins (data not shown). This suggests that procoat with a cysteine at +1 is slowly cleaved by leader peptidase. It is unlikely that this larger species is a dimeric coat protein (resulting from disulfide formation) since the molecular mass is unchanged after adding a reducing agent and treating with iodoacetamide (data not shown).

Analysis of Mutations at −2, −4, and −5. We also directly tested whether there was any specificity for leader peptidase processing at the −2, −4, and −5 positions. Except for proline at −4, all of the 25 mutants created at these positions were processed (Table III). However, we found that there were two types of mutants (Figure 5B). In the first class, procoat was completely processed to the coat protein. Mutants in the second class of proteins, which showed delayed kinetics of processing, were poorer substrates. This indicates that while there are no critical specificity determinants for leader peptidase at these positions, certain residues are markedly preferred for processing.

Discussion

We report here a detailed study of the substrate specificity of leader peptidase, using the M13 procoat as a model substrate. This protein contains each of the conserved features found within the C-terminal domain of a leader sequence: a small apolar amino acid at positions −1 (alanine) and −3 (serine), with respect to the cleavage site, and a helix-destabilizing residue (proline) at −6. To determine the substrate specificity of leader peptidase, we created 60 mutations within procoat from positions +1 to −6 near the leader peptidase cleavage site. In addition to assaying the mutants for in vivo processing and protein translocation across the cytoplasmic membrane, we also analyzed most of them using an in vitro system.

Table III: In Vivo and in Vitro Processing of Procoat with an Alteration at +1, −2, −4, and −5

substitution[a,b]	percent processed[b] in vivo	in vitro
	+1 Position	
F	95	nd
L	100	76
V	95	77
C*	16	nd
A (wt)	100	75
T	10	0
G	100	72
S	100	77
P	0	0
	−2 Position	
M	98	70
L	100	74
T	100	61
F (wt)	100	75
P	81	55
E	100	73
D	100	nd
	−4 Position	
L (wt)	100	75
W	100	75
A	100	72
G	100	40
S	100	nd
P	12	0
H	100	nd
Q	89	60
E	65	nd
D	91	72
R	84	76
	−5 Position	
F	100	nd
M (wt)	100	75
I	100	nd
L	100	nd
V	100	nd
T	100	nd
S	100	nd
E	100	77
D	100	70
R	50	78

[a] An asterisk (*) indicates generation of a high molecular weight band. [b] wt, wild-type; nd, not done.

In these experiments, we found that the specificity determinants were located at −1 and −3 with respect to the cleavage site of the procoat protein. The small residues alanine, serine, glycine, and proline were processed at −1, whereas the larger residues, including asparagine, threonine, histidine, arginine, and phenylalanine were not. This is in excellent agreement with the extensive comparison studies of leader sequences by von Heijne (1983), and Perlman and Halvorson (1983). However, it was rather surprising that the rate of processing of the serine and glycine mutants was measurably slower than that of wild-type in vivo because some wild-type precursors have serine or glycine residues at the −1 position. At −3, serine, glycine, threonine, valine, and leucine, which are amino acids found at this position within leader peptides, were processed, although the kinetics of processing of the latter three were slow in vivo (Figure 2A and Table II). Other amino acids not commonly present at this position were not processed (Table II). The −2, −4, and −5 positions seem to be important for determining the efficiency of the processing. While all these mutants were processed, other than the proline mutant at −4, there were preferences for certain amino acids. There was also

broad specificity (except for proline, threonine, and cysteine) at the +1 position of procoat (Table III). Proline is never found in the +1 to −3 region of prokaryotic leader peptides (von Heijne, 1983). In addition, proline and threonine residues are known to affect the neighboring peptide architecture, which might explain their effect on the recognition sequence.

In addition to the conserved small apolar amino acids at −1 and −3, bacterial leader peptides have a conserved helix breaker at −4 to −6, usually proline or glycine. The proline residue at position −6 of procoat is absolutely essential for rapid in vivo processing by leader peptidase. The substitution of a glutamine for the proline residue slowed processing; histidine severely affected cleavage; and all other changes at this position were not processed at all, including glycine, a good helix breaker known to affect the protein conformation differently than proline (Chou & Fasman, 1974). These results reinforce the importance of the helix-breaking residue (Pugsley, 1989) and reemphasize the novel properties of proline residues in biology. We suspect that procoat is efficiently processed with a proline at −6 because proline is able to break the α-helical hydrophobic segment of the leader peptide and bring the cleavage site of procoat close to the active site of leader peptidase. This may also occur with glutamine and, to a lesser extent, with histidine residues, even though they are not good helix breakers. Clearly, however, procoat does not have an absolute requirement for a helix breaker at this position since many procoat proteins with mutational alterations at −6, even those with good helix formers (Chou & Fasman, 1974), were processed in detergent extracts with approximately 10^5 times the normal levels of leader peptidase. We believe it is the binding of procoat to leader peptidase that is impaired with mutational alterations at the −6 position since processing can be driven by increasing the concentration of leader peptidase (Figure 4). We should also note that unlike some of the −6 mutant procoats, each of the unprocessed mutants at the −1 and −3 positions were also not cleaved with high levels of pure leader peptidase in a detergent extract.

Recently, Laforet and Kendall (1991) showed that a proline was not required for the processing of an idealized leader peptide for alkaline phosphatase; cleavage occurred even when the proline at −6 was changed to an alanine. They suggested that proline is normally important for cleavage, not for its effect on protein conformation but rather for its hydrophobicity. Therefore, proline substitutions with other amino acids block processing because these alterations change the hydrophobicity of this region. However, our studies here show that neither alanine, glycine, nor tryptophan, which have comparable hydrophobicity (Eisenberg et al., 1984), can substitute for proline for processing in vivo. Moreover, procoat substituted with a glutamine at −6 was slowly processed in vivo, even though glutamine is significantly more polar than proline. Thus, we favor the idea that proline helps alter the conformation of the polypeptide chain surrounding the cleavage site.

A helix-breaking residue has also been shown to be important in the processing of eukaryotic preproteins. Nothwehr and Gordon (1989) introduced proline residues at various positions in the leader peptide of pre(Δpro)apoA-II and demonstrated that the site of cleavage is optimal at a distance of 4–5 residues from the proline. This is the typical distance between the end of the apolar core domain and the cleavage site of eukaryotic leader peptides. The reason for this optimal distance is not known. However, as pointed out by Gordon and colleagues, the proline may demarcate the boundary between the hydrophobic region of the membrane and the hydrophilic environment of the lumen of the ER. It is possible then that removing the helix breaker shifts the membrane-spanning region of the leader peptide and, therefore, prevents leader peptidase from interacting with the cleavage site of the preprotein on the lumenal surface of the membrane.

Fikes et al. (1990) recently reported the sequence requirements at −1 and −3 for efficient in vivo processing of the precursor to the *E. coli* maltose-binding protein (pre-MBP) by leader peptidase. In this study, they found that pre-MBP was rapidly processed at the correct site with alanine, glycine, serine, and cysteine at −1, in good agreement with our results. At −3, they observed rapid processing of the alanine, glycine, serine, cysteine, threonine, valine, isoleucine, leucine, and proline pre-MBP mutant. These results are different from ours in several respects. In our study, the in vivo kinetics of processing of the leucine mutant (at −3) were very slow, even though it was processed in vitro. In addition, the proline mutant at the −3 position of procoat was not processed in vivo, in contrast to MBP with proline at this position, which was processed nearly as well as wild-type. These results, showing that there is some variability in the sequence requirements of −1 and −3 for the various substrates, suggest that other factors, such as the substrate conformation, may also be important in processing by leader peptidase.

Our mutagenesis studies here prove that the −1, −3, and −6 positions of the procoat substrate are important for leader peptidase binding and catalysis. The −1 and −3 positions are the most critical. Although the helix-breaker proline at −6 is vital for in vivo processing, it is not absolutely required for leader peptidase processing in detergent extracts. Thus, in preprotein catalysis, the role of the helix breaker at −6 of procoat is secondary to that of the −1 and −3 residue. The information we have obtained here on the primary structure of the substrate is still not sufficient to account for all the specificity of leader peptidase. This protease must recognize some specific conformation of the substrates since it cleaves only precursor proteins. It will be important to further explore the nature of the substrate specificity at the level of secondary and tertiary structure. Determination of how this recognition takes place should deepen our understanding of how leader peptidase completes its difficult task.

Registry No. Pro, 147-85-3; leader peptidase, 65979-36-4.

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Determination of Peptide Regions on the Surface of the Eubacterial and Archaebacterial Ribosome by Limited Proteolytic Digestion†

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Received August 5, 1991; Revised Manuscript Received September 26, 1991

ABSTRACT: Limited proteolysis was used in combination with two-dimensional gel electrophoresis, blotting, and amino acid sequence analysis to investigate the surface of intact ribosomal subunits at the peptide and amino acid level. Surface sites of 14 ribosomal proteins from *Escherichia coli* 50S subunits were determined using proteases with different specificities. To assess the evolutionary conservation of ribosomal topography among eubacteria, large subunits from *Bacillus stearothermophilus* were also subjected to limited proteolysis. The results obtained indicate a conservation of the three-dimensional ribosomal structure at the peptide level. The data for the eubacterial ribosomes are in full agreement with the model of the 50S protein topography derived from immunological data. Furthermore, peptide surface regions of archaebacterial ribosomes have been investigated. The results presented in this work prove that limited proteolysis can successfully be applied to halophilic and thermophilic ribosomes from archaebacteria.

Ribosomes are an essential part of the protein biosynthetic pathway in all living cells. Despite this unique function, ribosomes isolated from species of different kingdoms vary considerably in size and number of ribosomal components (Wittmann, 1986). To understand how structurally diverse ribosomes maintain a common function in the various organisms, the primary structures of the ribosomal components have to be determined, their locations in the ribosome have to be identified, and the ribosomal topography has to be resolved at the molecular level. Therefore, data about the surface regions and interactions of the ribosomal components within the ribosome at the amino acid/nucleotide level have to be obtained. To date, the primary structures of about 500 r-proteins[1] (RIB databank, Max-Planck-Institut für Molekulare Genetik) and of more than one thousand rRNAs are known; still, the complete set of primary structures is only known for the *Escherichia coli* ribosome (Wittmann-Liebold, 1986; Noller et al., 1986).

Several approaches have been employed to localize the positions of the ribosomal components within the ribosome.

† This publication is dedicated to the memory of Dr. H. G. Wittmann. His encouragement and support during our work is deeply appreciated.
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[1] Abbreviations: r-proteins, ribosomal proteins; rRNA, ribosomal RNA; Lys-C, endoproteinase Lys-C; Glu-C, endoproteinase Glu-C; Eco, *Escherichia coli*; Bst, *Bacillus stearothermophilus*; Hma, *Halobacterium marismortui* (*Haloarcula marismortui*); 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; IEM, immunoelectron microscopy.

0006-2960/91/0430-11781$02.50/0