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MINI-REVIEW

Signal Peptide Mutants of *Escherichia coli*

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Abstract

Numerous secretory proteins of the Gram-negative bacteria *E. coli* are synthesized as precursor proteins which require an amino terminal extension known as the signal peptide for translocation across the cytoplasmic membrane. Following translocation, the signal peptide is proteolytically cleaved from the precursor to produce the mature exported protein. Signal peptides do not exhibit sequence homology, but invariably share common structural features: (1) The basic amino acid residues positioned at the amino terminus of the signal peptide are probably involved in precursor protein binding to the cytoplasmic membrane surface. (2) A stretch of 10 to 15 nonpolar amino acid residues form a hydrophobic core in the signal peptide which can insert into the lipid bilayer. (3) Small residues capable of β-turn formation are located at the cleavage site in the carboxyl terminus of the signal peptide. (4) Charge characteristics of the amino terminal region of the mature protein can also influence precursor protein export. A variety of mutations in each of the structurally distinct regions of the signal peptide have been constructed *via* site-directed mutagenesis or isolated through genetic selection. These mutants have shed considerable light on the structure and function of the signal peptide and are reviewed here.

Key Words: Signal peptide; loop model; mutation; *Escherichia coli.*

Introduction

A variety of mechanisms exist for the secretion of cytoplasmically synthesized proteins in Gram-negative bacteria (Pugsley, 1988). The majority of exported proteins employ a method requiring synthesis of a precursor protein containing an amino terminal extension variously known as the signal or leader peptide. The signal peptide encodes information required for

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the translocation of newly synthesized export-competent protein across the cytoplasmic membane. Following translocation, cleavage by a specific inner membrane peptidase will release the mature protein to the periplasm. Information encoded in the mature protein will specify final localization to the periplasm or outer membrane (Nikaido and Wu, 1984; Yamaguchi *et al.*, 1988).

A large number of signal peptide-containing bacterial proteins have now been identified (Watson, 1984). They vary in length, but are generally about 20 residues long. There is no significant sequence homology between them. Instead, signal peptides share a basic structural motif (Inouye and Halegona, 1980). The amino terminus is hydrophilic and contains one to three basic amino acid residues. This region is followed by a central core of 8 to 15 hydrophobic residues which generally have a predicted α-helix or β-sheet secondary structure (Bedouelle and Hofnung, 1981). The carboxyl terminus tends to include polar residues and contains the peptidase cleavage site. Recent experiments have emphasized that regions within the mature protein also play a role in secretion.

Models of Signal Peptide Function

Several models of protein secretion have been proposed. The signal peptide is envisioned to play a somewhat different role in each. In the signal hypothesis (Blobel and Dobberstein, 1975), the signal peptide provides a recognition site for entry of a nascent polypeptide into the export pathway. In this hypothesis the role of the signal peptide was conceptually proposed to be required for protein secretion; however, the structural basis of signal peptide function was not discussed. The functional and structural relationship of the signal peptide was first discussed by Inouye *et al.* (1977) on the basis of the unique features of the signal peptide of the *E. coli* major outer membrane lipoprotein. Because of the positively charged amino terminal region and the extremely hydrophobic core region of the signal peptide, they proposed that the basic region interacts with the acidic surface of the cytoplasmic membrane followed by insertion of the hydrophobic core region in the lipid bilayer by forming a loop. This model, designated the "loop model" (Inouye and Halegoua, 1980), is now widely accepted (see, for example, Coleman *et al.*, 1985 and Battenburg *et al.*, 1988).

The trigger hypothesis proposed by Wickner (1979) postulates that the signal peptide serves to maintain the precursor protein in a folded state which is compatible with membrane translocation. However, this hypothesis has not yet been vigorously tested, and it is not known at present how this hypothesis can be generally applied to secretory proteins.

Signal Peptide Mutant Isolation

In this review we attempt to assemble and describe all existing *E. coli* signal peptide mutants. The isolation and phenotypic characterization of mutations in the various regions of the signal peptide has provided significant insight into the structure and function of its component parts. The use of signal peptide mutants to isolate extragenic lesions in components of the cellular export machinery has led to the identification and purification of at least one protein essential to the export process. Consequently, the translocation process can now be investigated at the molecular level.

Two approaches to the isolation of signal peptide mutations have been taken: genetic selection and site-directed mutagenesis. The major advantage of classic genetic selection is that no preconceived hypothesis concerning signal peptide structure or function need be postulated. With the appropriate selection, the isolation of mutants should be rapid and technically simple. LacZ fusions have proven to be a very useful tool in signal peptide mutant isolation (Beckwith and Silhavy, 1984). Fusion of the cytoplasmic and translocation-incompetent protein β-galactosidase to the mature portion of a signal peptide-containing exported protein causes accumulation of hybrid protein in the bacterial inner membrane. This hybrid protein accumulation inhibits cell growth. Lesions within the signal peptide of the fused export protein prevent entry into the export pathway and consequent cell toxicity. This provides a positive selection for mutations which result in export-defective signal peptides. The isolation of intragenic pseudorevertants which suppress a lethal signal peptide defect is also possible.

Site-directed mutagenesis can be employed to produce mutations anywhere within the signal peptide. This permits the direct evaluation of the structural or functional importance of specific signal peptide residues. However, it is first necessary to establish a working hypothesis concerning signal peptide function which can then be tested by mutagenesis. Consequently, an unbiased approach to mutant isolation is not possible. In comparison to genetic selection, unlimited numbers of mutations including amino acid replacements, deletions, insertions, and their combinations can be isolated using site-directed mutagenesis. Further, suppresion mutations can be also isolated using appropriate expression systems by genetic selection (see Bieker *et al.*, this volume).

Basic Region Signal Peptide Mutations

The importance of the basic region of the signal peptide has been investigated by systematically varying the net positive charge at the N-terminus of several signal peptides.

Table I. Lipoprotein Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	↓	21	22	23	24
	Met	*Lys*	*Ala*	*Thr*	*Lys*	*Leu*	*Val*	*Leu*	*Gly*	*Ala*	*Val*	*Ile*	*Leu*	*Gly*	*Ser*	*Thr*	*Leu*	*Leu*	*Ala*	*Gly*		*Cys*	*Ser*	*Ser*	*Asn*
Basic Region:																									
D3 (+1) [1]			Asp																						
d2(+1) [1]		[ ]																							
d2D3(0) [1]		[ ]	Asp																						
E2D3(−1) [1]		Glu	Asp																						
N5(+1) [2]					Asn																				
d2N5(0) [2]		[ ]			Asn																				
E2D3N5(−2) [2, 3, 4]		Glu	Asp		Asn																				
E2D3N5R9 [4]		Glu	Asp		Asn				Arg																
E2D3N5D9 [4]		Glu	Asp		Asn				Asp																
E2D3N5R14 [4]		Glu	Asp		Asn									Arg											
E2D2N5D14 [4]		Glu	Asp		Asn									Asp											
Hydrophobic Region:																									
d9 [5]									[ ]																
d14 [5]														[ ]											
d9d14 [5, 6]									[ ]					[ ]											
d7d9d14 [3, 6]							[ ]		[ ]					[ ]											
d9d13d14 [3, 6, 7]									[ ]				[ ]	[ ]											
d7d9d13d14 [3, 6]							[ ]		[ ]				[ ]	[ ]											
D9 [4]									Asp																
R9 [4]									Arg																
D14 [4, 8, 9]														Asp											
R14 [4]														Arg											
V9 [5]									Val																
V14 [5]														Val											
V9V14 [5]									Val					Val											
V9d14 [5]									Val					[ ]											
d9V14 [5]									[ ]					Val											
A15 [10]															Ala										
A16 [10]																Ala									
A15A16 [3, 10]															Ala	Ala									

Cleavage Region:																									
S20 [11]																			Ser						
T20 [11]																			Thr						
V20 [11]																			Val						
L20 [11]																			Leu						
A20 [12]																			Ala						
d20 [12, 16]																			[ ]						
G21 [13, 16]																				Gly					
d18 [14]																	[ ]								
17L18 [14]																	+ [Leu]								
20G21 [14]																				+ [Gly]					
I23I24 [15]																							Ile	Ile	
A20I23I24 [15]																			Ala				Ile	Ile	
I23K24 [15]																							Ile	Lys	
A20I23K24 [15]																			Ala				Ile	Lys	
	Met	*Lys*	*Ala*	*Thr*	*Lys*	*Leu*	*Val*	*Leu*	*Gly*	*Ala*	*Val*	*Ile*	*Leu*	*Gly*	*Ser*	*Thr*	*Leu*	*Leu*	*Ala*	*Gly*	*Cys*	*Ser*	*Ser*	*Asn*	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20 ↓	21	22	23	24	

[a]The arrows indicate the signal peptide cleavage site. Empty brackets or a lower case d denote amino acid deletions. Bracketed amino acids indicate insertions. Numbers in parentheses denote the net charge of the signal peptide N-terminus. Numbers in brackets denote references: [1] Inouye *et al.* (1982); [2] Vlasuk *et al.* (1983); [3] Lunn and Inouye (1987); [4] C. Sung, S. Pollitt, G. Vlasuk, and M. Inouye, unpublished results; [5] Inouye *et al.* (1984); [6] Pollitt *et al.* (1985); [7] Pollitt and Inouye, (1988); [8] Lin *et al.* (1978); [9] Lee *et al.* (1983); [10] Vlasuk *et al.* (1984); [11] Pollitt *et al.* (1986); [12] Inouye *et al.* (1983b); [13] Inouye *et al.* (1983a); [14] S. Inouye, S. Pollitt, and M. Inouye, unpublished results; [15] Inouye *et al.* (1986); [16] Ghrayeb *et al.* (1985).

Lipoprotein

The signal peptide of the major outer membrane lipoprotein of *E. coli* was the first prokaryotic signal peptide of which the amino acid sequence was determined (Inouye *et al.*, 1977). Then it was pointed out that its signal peptide has unique features including a net positive charge of +2 due to lysines at positions 2 and 5 (Table I). Site-specific mutagenesis has been utilized to gradually reduce the net charge to −2 (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983). Essentially normal processing is observed in mutations which are neutral or have a net positive charge at the N-terminus (Δ3, Δ2N5, Δ3D3, Δ2N5).

In contrast, when the overall charge of this region is less than 0, severe effects on precursor modification and processing are observed. The E2D3 mutation (−1 charge), which retains one positively charged residue (K5) while adding two negative charges, had only a small amount of lipoprotein assembled in the membrane while a large amount of glycerol-unmodified precursor accumulated in the cytoplasm. This soluble prolipoprotein was gradually post-translationally translocated. The result is the same when all positive charges are removed, as in the E2D2N5 mutant (−2 charge), except the rate of post-translational translocation is even slower. A negative effect on processing for the E2D3N5 mutant was also observed for a lipo-β-lactamase hybrid, where the lipoprotein signal sequence plus nine residues of the mature lipoprotein is fused to the mature β-lactamase protein (Lunn and Inouye, 1987).

The defective E2D3N5 mutant can be supressed by substituting the glycine at position 9 with an arginine (E2D3N5-R9; C. Sung, S. Pollott, G. Vlasuk, and M. Inouye, unpublished results). The rate of processing in this mutant became much improved, most likely because the positively charged arginine is close enough to the negatively charged amino-terminal end of the signal sequence to restore function. The E2D3N5 translocation defect can also be suppressed by introduction of arginine at position 14 of the hydrophobic core (R14). The presence of an arginine residue in the hydrophobic region leads to blocked processing, but translocation is possible. When this lesion is combined with the E2D3N5 mutation, translocation of the mutant protein and outer membrane localization is observed, although processing remains blocked. Therefore, the R14 mutation is able to restore translocation competence to a translocation-defective −2 charged N-terminal mutant lipoprotein signal peptide. Substituting an aspartate at either position 9 or 14 (E2D3N5-D9 and E2D3N5-D14) resulted in a complete translocation defect (C. Sung, S. Pollitt, G. Vlasuk, and M. Inouye, unpublished results).

OmpA

Most of the OmpA signal peptide mutants discussed in this review are constructs of hybrid proteins. The OmpA signal sequence is fused with a small

linker to either the *Staphyloccocus* nuclease A (OmpA-nuclease) or TEM β-lactamase (OmpA-β-lactamase) mature proteins. These hybrid proteins are efficiently processed and localized to the periplasmic space. The OmpA-nuclease precursor has a half-life of 45 sec, and the OmpA-β-lactamase precursor about 90 sec (Lehnhardt *et al.*, 1987).

The overall net charge of the OmpA signal sequence is +2 due to two lysines at positions 2 and 3 (Table II). Changing the threonine at position 4 to serine (S4) is a conservative substitution which had no effect on the β-lactamase hybrid, yet slowed processing for the nuclease hybrid. When one of the lysines were removed to yield a net charge of +1 (Δ3S4), the efficiency of precursor processing was as good as wild type for both hybrid proteins. A net charge of 0 (Δ2-3S4) and −1 (Δ2E3S4) in the signal peptide was defective for both hybrid proteins which yielded a proteolytic product localized in either the cytoplasm or membrane (Lehnhardt *et al.*, 1988).

MBP

A series of maltose-binding protein (MBP) N-terminal mutants has been constructed by oligonucleotide-directed site-specific mutagenesis (Puziss, *et al.*, 1989). In this series the N-terminal net charge varies from +3 to −3 in a stepwise fashion (Table III). Strains harboring the mutations are phenotypically Mal^+. This indicates that all of the mutants export functional protein to the periplasm. However, the kinetics of MBP processing is considerably affected. Markedly slower protein processing was observed in mutants which had a neutral or net negative N-terminus. Mutants retaining a net positive charge, irrespective of the magnitude of that charge, exhibit essentially wild-type processing kinetics. Introduction of a hydrophobic core deletion mutant (Δ12–18, Table III) in *cis* to these basic region mutations intensified the processing defect. This was taken to be an indication that there is some overlap in the function of basic and hydrophobic regions in signal peptides. A processing-proficient hydrophic region could compensate to some extent for the acidic N-terminal mutations, while a truncated hydrophobic region could not.

Staphlokinase

Similar results have been obtained in an investigation employing site-specific mutagenesis of staphlokinase to generate basic region mutations (Iino *et al.*, 1987). As long as the N-terminus has a net positive charge, essentially normal processing and export of prostaphlokinase occurs (Table IV). Signal peptide processing becomes increasingly defective as the net charge decreases from neutral to −2. Significant quantities of prostaphlokinase were found to

Table II. OmpA Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	↓	22	23	24	25	26
	Met	*Lys*	*Lys*	*Thr*	*Ala*	*Ile*	*Ala*	*Ile*	*Ala*	*Val*	*Ala*	*Leu*	*Ala*	*Gly*	*Phe*	*Ala*	*Thr*	*Val*	*Ala*	*Gln*	*Ala*		*Gly*	*Ile*	*Pro*	*Ser*	*Leu*
Basic Region:																											
S4(+2) [1]				Ser																							
d3S4(+1) [1]			[ ]	Ser																							
d2-3S4(0) [1]		[ ]	[ ]	Ser																							
d2E3S4(−1) [1]		[ ]	Glu	Ser																							
Hydrophobic Region:																											
G5 [7]					Gly																						
P5 [7]					Pro																						
P5, 5G6 [7]					Pro+[Gly]																						
A8 [2]								Ala																			
M8 [2]								Met																			
V9 [2]									Val																		
L9 [2]									Leu																		
L6L8 [2]						Leu		Leu																			
I7V9 [2]							Ile		Val																		
V7V9 [2]							Val		Val																		
V7d8 [2]							Val	[ ]																			
d8V9 [2]								[ ]	Val																		
d8 [2]								[ ]																			
d9 [3]									[ ]																		
d8-9 [3]								[ ]	[ ]																		
d7-9 [3]							[ ]	[ ]	[ ]																		
d6-9 [3]						[ ]	[ ]	[ ]	[ ]																		
N6 [7]						Asn																					
N8 [7]								Asn																			
Q8 [7]								Gln																			
N9 [7]									Asn																		
N10 [7]										Asn																	
N12 [7]												Asn															
P13 [7]													Pro														
A14 [7]														Ala													
P14 [7]														Pro													

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	↓	22	23	24	25	26
	Met	*Lys*	*Lys*	*Thr*	*Ala*	*Ile*	*Ala*	*Ile*	*Ala*	*Val*	*Ala*	*Leu*	*Ala*	*Gly*	*Phe*	*Ala*	*Thr*	*Val*	*Ala*	*Gln*	*Ala*		*Gly*	*Ile*	*Pro*	*Ser*	*Leu*
R14 [7]														Arg													
d14 [7]														[ ]													
S1 [4]							+[AlaArg]																				
S3 [4]							+[AlaArgAlaArgAlaArg]																				
P1 [4]							[ ]+[ValAlaIleAlaThr]																				
P2 [4]							[ ]+[ValAlaIleAlaIleAlaIleAlaThr]																				
Cleavage Region:																											
d24 [5]																									[ ]		
d24-25 [5]																									[ ][ ]		
K22d24-25 [5]																							Lys		[ ][ ]		
K22 [5]																							Lys				
IV-97 [6]																					Val						
II-3e [6]				Ile	Val				Val					Val									Val	Leu			

[a]The arrows indicate the signal peptide cleavage site. Empty brackets or a lower case d denote amino acid deletions. Bracketed amino acids indicate insertions. Numbers in parentheses denote the net charge of the signal peptide N-terminus. Numbers in brackets denote references: [1] Lehnhardt *et al* (1988); [2] Goldstein *et al.* (1990); [3] Lehnhardt *et al.* (1987); [4] Freudl *et al.* (1988); [5] Duffaud and Inouye (1988); [6] Freudl *et al.* (1985); [7] J. Goldstein, S. Lehnhardt, and M. Inouye, unpublished results.

Table III. Maltose-Binding Protein Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	↓	27
	Met	*Lys*	*Ile*	*Lys*	*Thr*	*Gly*	*Ala*	*Arg*	*Ile*	*Leu*	*Ala*	*Leu*	*Ser*	*Ala*	*Leu*	*Thr*	*Thr*	*Met*	*Met*	*Phe*	*Ser*	*Ala*	*Ser*	*Ala*	*Leu*	*Ala*		*Lys*
Basic Region:																												
TL1(+2) [1]				Ile																								
TL2(+1) [1]		Thr		Ile																								
TL3(0) [1]		Thr		Ile				Leu																				
TL5(−1) [1]		Glu		Ile				Leu																				
TL6(−2) [1]		Glu	Glu	Ile				Leu																				
TL8(−3) [1]		Glu	Glu	Glu				Leu																				
Hydrophobic Region:																												
mal10-1 [2]										Pro																		
mal10-2 [3]										Arg																		
mal11-1 [3]											Pro																	
mal11-2 [3]											Ser																	
mal11-3 [3]											Glu																	
mal14-1 [2]														Glu														
mal14-2 [3]														Pro														
mal16-1 [2]																Lys												
mal18-1 [2]																		Arg										
mal19-1 [2]																			Arg									
d12-18 [3]												[	][	][	][	][	][	][	]									
Cleavage Region:																												
mal24-1 [4]																								Asp				
mal24-1, 19-1 [4]																			Arg					Asp				
	Met	*Lys*	*Ile*	*Lys*	*Thr*	*Gly*	*Ala*	*Arg*	*Ile*	*Leu*	*Ala*	*Leu*	*Ser*	*Ala*	*Leu*	*Thr*	*Thr*	*Met*	*Met*	*Phe*	*Ser*	*Ala*	*Ser*	*Ala*	*Leu*	*Ala*		*Lys*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	↓	27

[a]The arrow indicates the signal peptide cleavage site. Empty brackets or a lower case d denote positions of amino acid deletions. Numbers in parentheses denote the net charge of the signal peptide N-terminus. Numbers in brackets denote references: [1] Puziss *et al.* (1989); [2] Bedouelle *et al.* (1980); [3] Emr and Bassford (1982); [4] Fikes and Bassford (1987).

Table IV. Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27 ↓
STAPHLOKINASE	*Met*	*Leu*	*Lys*	*Arg*	*Ser*	*Leu*	*Leu*	*Phe*	*Leu*	*Thr*	*Val*	*Leu*	*Leu*	*Leu*	*Leu*	*Phe*	*Ser*	*Phe*	*Ser*	*Ser*	*Ile*	*Thr*	*Asn*	*Glu*	*Val*	*Ser*	*Ala*
Basic Region:																											
C1(0) [1]			Asn	Gly																							
C2(−1) [1]			Glu	Gly																							
C3(+1) [1]			Gln																								
C4(0) [1]			Glu																								
C6(0) [1]				Glu																							
C7(−1) [1]				Glu	Asp																						
C5(−1) [1]			Glu	Arg	Asp																						
C8(−1) [1]			Glu	Lys	Asp																						
C9(−2) [1]			Glu	Gln	Asp																						
Hydrophobic Region:																											
H-1 [1]															Glu												

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25 ↓
RIBOSE BINDING PROTEIN	Met	Asn	Met	Lys	Lys	Leu	Ala	Thr	Leu	Val	Ser	Ala	Val	Ala	Leu	Ser	Ala	Thr	Val	Ser	Ala	Asn	Ala	Met	Ala
Hydrophobic Region:																									
rbsB103 [2]									Pro																
Suppressor of rbsB103:									*Pro*																
pAI27s [2]											Phe														

[a]The arrow indicates the signal peptide cleavage site. Numbers in parentheses denote the net charge of the signal peptide N-terminus. Numbers in brackets denote references: [1] Iino *et al.* (1987); [2] Iida *et al.* (1985).

accumulate in the cytoplasm and membranes of cells harboring the plasmid C2 which encodes a mutant protein with an N-terminal net −1 charge.

Staphlokinase altered through a hydrophobic core lesion (H-1, Table IV) inhibits the rate of processing of wild-type proOmpA *in vivo* (Iino, *et al.*, 1987). This blockage of wild-type protein export is believed to be caused by prolonged mutant protein occupancy of the export machinery. When the C2 mutant with N-terminal −1 net charge is combined *in cis* to the H-1 hydrophobic region lesion, suppresion of the inhibition of proOmpA maturation is observed. These results indicate that mutations which eliminate the net positive charge at the signal peptide N-terminus do not enter the export pathway efficiently.

β-Lactamase

In vitro mutagenesis of the TEM β-lactamase has yielded two basic region mutations (Table V) (Kadonaga *et al.*, 1985). Both of these mutations (M61, M8) decrease the positive charge of the signal peptide only minimally and have only minor effects upon the export of active β-lactamase to the periplasm. In order to introduce a restriction site into the signal peptide, the second amino acid residue serine was replaced by the positive residue arginine. This introduces an extra positive charge into the N-terminal region of the protein. This additional basic residue itself does not alter processing. However, by increasing the net positive charge at the N-terminus, it could serve to obscure effects of the M8 and M61 mutations on processing.

Effects on Protein Synthesis

Alterations in the rate of protein synthesis have also been observed in a number of basic region mutants. The S6 mutation of the outer membrane LamB protein exhibits reduced protein synthetic rates (Table VI). This reduction is not due to an alteration in the secondary conformation of the LamB mRNA, since the introduction of a point mutation in the 5′ noncoding region of S6 restores wild-type mRNA structure, but not synthetic rates (Hall *et al.*, 1983). This reduced synthesis is proposed to reflect an interaction of the signal peptide with a putative component of the protein export pathway which purportedly functions in the translation arrest of exported proteins. Thus, a direct coupling of protein synthesis and translocation as observed in eukaryotic endoplasmic reticulum secretion is envisioned.

This interpretation is supported by the isolation of suppressor mutations in the S6 signal sequence which will restore the wild-type arginine residue (MH8028) without restoring the original codon (Table VI) (Benson *et al.*, 1987). This argues that the protein synthesis inhibition caused by the serine replacement is due to a perturbation in protein and not mRNA structure. A

Table V. β-Lactamase Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	↓	24	25	26
	Met	*Ser*	*Ile*	*Gln*	*His*	*Phe*	*Arg*	*Val*	*Ala*	*Leu*	*Ile*	*Pro*	*Phe*	*Phe*	*Ala*	*Ala*	*Phe*	*Cys*	*Leu*	*Pro*	*Val*	*Phe*	*Ala*		*His*	*Pro*	*Glu*
Basic Region:																											
M61 [1]		Arg			Tyr																		Gly				
M8 [1]		Arg					His																Gly				
Hydrophobic Region:																											
M7 [1]		Arg						Ile															Gly				
M3 [1]		Arg							Thr														Gly				
M65 [1]		Arg								Phe													Gly				
M9 [1]		Arg													Thr								Gly				
M16 [1]		Arg														Thr							Gly				
M63 [1]		Arg					His											Tyr					Gly				
M18 [1]		Arg										Ser							Phe				Gly				
M13 [1]		Arg							Val	Phe		Leu			Val								Gly				
Cleavage Region:																											
M17 [1]		Arg																		Ser			Gly				
M14 [1]		Arg																			Ile		Gly				
M20 [1]		Arg					His														Ile		Gly				
M52 [1]		Arg						Ile													Ile		Gly				
M15 [1]		Arg										Ser								Phe			Gly				
Mature Protein:																											
K082 [2]																									(+2)LysArg		
	Met	*Ser*	*Ile*	*Gln*	*His*	*Phe*	*Arg*	*Val*	*Ala*	*Leu*	*Ile*	*Pro*	*Phe*	*Phe*	*Ala*	*Ala*	*Phe*	*Cys*	*Leu*	*Pro*	*Val*	*Phe*	*Ala*	↓	*His*	*Pro*	*Glu*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		24	25	26

[a]The arrow indicates the signal peptide cleavage site. Numbers in parentheses denote the net charge of the mature protein N-terminus. Numbers in brackets denote references: [1] Kadonaga *et al.* (1985); [2] Yamane and Mizushima (1988).

Table VI. LamB Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	1	15	16	17	18	19	20	21	22	23	24	25
	Met	*Met*	*Ile*	*Thr*	*Leu*	*Arg*	*Lys*	*Leu*	*Pro*	*Leu*	*Ala*	*Val*	*Ala*	*Val*	*Ala*	*Ala*	*Gly*	*Val*	*Met*	*Ser*	*Ala*	*Gln*	*Ala*	*Met*	*Ala*
Basic Region:																									
S6 [1]						Ser																			
Hydrophobic Region:																									
12D [2]												Asp													
13D [2]													Asp												
14D [3]														Asp											
15E [3]															Glu										
16E [3]																Glu									
17R [4]																	Arg								
17D [3]																	Asp								
19R [4]																			Arg						
19K [2]																			Lys						
S78 [3]									[	][	][	][	][	]											
S60 [3]									[	][	][	][	][	][	][	][	][	][	][	][	][	][	][	][	]
Suppressors of S78:									[	][	][	][	][	]											
S78, 9L [5]									Leu																
S78, 17C [5]																	Cys								
Suppressors of S6:						*Ser*																			
MH8028 [6]						Arg																			
MH8021 [6]															Glu										
MH8023 [6]									[	][	][	][	][	][	][	][	][	][	][	][	][	][	][	][	]
	Met	*Met*	*Ile*	*Thr*	*Leu*	*Arg*	*Lys*	*Leu*	*Pro*	*Leu*	*Ala*	*Val*	*Ala*	*Val*	*Ala*	*Ala*	*Gly*	*Val*	*Met*	*Ser*	*Ala*	*Gln*	*Ala*	*Met*	*Ala*
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	15	16	17	18	19	20	21	22	23	24	25

[a] The arrow indicates the signal peptide cleavage site. Empty brackets denote amino acid deletions. Numbers in brackets denote references: [1] Hall *et al.* (1983); [2] Stader *et al.* (1986); [3] Emr and Silhavy (1980); [4] Emr *et al.* (1978); [5] Emr and Silhavy (1983); [6] Benson *et al.* (1987).

second suppressor isolated in the same study has a large portion of the hydrophobic region deleted (MH8023). This deletion mutant is export defective, yet exhibits wild-type levels of expression. The MH8023 mutation may affect an export step prior to the S6 translation block. This is further evidence in support of a coupling of protein translation to secretion.

All of the mutations which resulted in a lowered net charge from the +2 charge of the wild-type lipoprotein yielded a decreased rate of lipoprotein synthesis (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983; Table I). Furthermore, a trend is apparent where the lower the net charge, the slower the rate of synthesis. the synthesis level of the E2D3N5 mutant can be restored to a greater rate than wild type by the addition of the -R14, -D9, or -D14 substitutions (C. Sung, S. Pollott, G. Vlasuk, and M. Inouye, unpublished results).

Similar to the lipoprotein signal sequence charge mutations, the OmpA mutants for both hybrid proteins also exhibited a decreasing rate of synthesis as the amino-terminal region became more negative (Lehnhardt *et al.*, 1988; Table II).

A marked decrease in the rates of protein synthesis was also noted in MBP mutants with an acidic N-terminus (Puziss *et al.*, 1989). However, it is not clear whether the mutations induce changes in mRNA structure and stability.

Two Staphlokinase basic region mutations were found to reduce protein synthetic rates (Iino *et al.*, 1987). This was attributed to increased stability of the mRNA for the mutant with a net +1 charge (C3). The C8 lesion (net −1 charge) is not expected to alter mRNA secondary structure, yet markedly lower protein synthesis is exhibited by this mutation.

Conclusions

Basic region signal peptide mutations have clearly demonstrated that a net positive charge at the N-terminus is required for optimal processing and translocation. This is consistent with the loop model of signal peptide function which envisions a direct interaction between the positive signal peptide basic region and negative phospholipid bilayer (Inouye *et al.*, 1977; Inouye and Halegoua, 1980). However, it is also apparent that neutral and even negative signal peptides can be processed, albeit at reduced rates. A positively charged N-terminus is therefore not an absolute requirement for signal peptide function. The observation that mutant proteins with a net charge of +3 function as effectively as those with a +1 charge, suggests that a redundancy may exist in the basic region of the signal peptide (Puziss, *et al.*, 1989). This redundancy presumably mitigates against possible loss of function

due to a mutation which eliminates a sole positive residue. This rationale is confirmed by the dearth of genetically selected basic region mutants. The only example of such a genetically selected mutation is the LamBS6 lesion.

Mutations which reduce the net positive charge of the signal peptide basic region have been found to affect protein translation rates in a variety of proteins. For lipoprotein and OmpA, there is a close correlation between the severity of the processing defect and extent of protein synthesis inhibition. The effect of these mutations on mRNA structure and stability is largely unknown. It is therefore not possible to conclusively attribute protein synthetic defects to changes in mutant signal peptide structure. However, the observation of signal peptide mutants with lowered protein synthetic rates is an enticing indication of a possible coupling between translation and translocation of secretory proteins, which is deserving of further investigation.

Hydrophobic Region Signal Peptide Mutations

Both genetic selection and site-localized mutagenesis have been employed in the isolation of mutations in the hydrophobic region of the signal peptide. A wealth of signal peptide mutants have been obtained by genetic selection utilizing LacZ fusions to two proteins of the maltose operon (Bankaitis *et al.*, 1987). LacZ fusion to the periplasmic MBP and outer membrane porin LamB result in hybrid proteins the expression of which is induced by maltose. Maltose induction leads to cellular growth inhibition (maltose sensitivity), apparently due to the entrance of a nonexportable hybrid protein into the protein export pathway. This offers a simple positive selection for maltose-resistant mutants, a large proportion of which contain lesions in the hydrophobic region of the signal peptide.

Lipoprotein

The hydrophobic region of all known natural signal sequences is devoid of charged residues. An aspartate was found to substitute for the glycine at position 14 (D14) in a lipoprotein signal sequence which was isolated as a globomycin-resistant mutant (Lin *et al.*, 1978; Table I). Although this mutant was translocated to and assembled in the outer membrane in an *lpp*$^-$ strain, there was no glyceride modification or processing. In an *lpp*$^+$ strain, the mutant precursor remained predominantly in the cytoplasm (Lee *et al.*, 1983). This indicates that the mutant unprocessed lipoprotein can be translocated. However, signal peptide affinity for components of the cellular export machinery is markedly reduced by substitution of a charged residue in the hydrophobic region. Translocation of the D14 mutant protein is in

marked contrast to results obtained with similar MBP and LamB mutant proteins (discussed below) which are totally nonexportable. Replacing the glycine at position 14 with an arginine (R14) similarly resulted in a processing-defective, but translocation-competent, mutant protein (C. Sung, S. Pollitt, G. Vlasuk, and M. Inouye, unpublished results). Substituting the glycine at position 9 with either aspartate (D9) or arginine (R9), in contrast, had little effect on precursor processing (C. Sung, S. Pollott, G. Vlasuk, and M. Inouye, unpublished results). Such a tolerance for a charged residue at this position may be due to the location occurring early enough in the hydrophobic region so as not to significantly disrupt hydrophobicity or structure.

The glycine residues at positions 9 and 14 have also been systematically deleted or substituted with valine (V9, Δ9, V14, Δ14, V9V14, V9Δ14, Δ9V14, and Δ9Δ14; see Inouye *et al.*, 1984). Most of these mutations had no effect on signal peptide function, demonstrating that glycine is not necessary in this region. The exceptions were the Δ14 and V9Δ14 mutants which exhibited much slower glyceride modification and signal peptide processing at 42°C. Interestingly, the Δ9 mutation could suppress the Δ14 defect. It was subsequently determined that deleting three (Δ7Δ9Δ14) and even four residues (Δ7Δ9Δ13Δ14) from the hydrophobic core resulted in an effective signal peptide, indicating a surprising length flexibility in this region (Pollitt *et al.*, 1985). It should be noted, however, that the Δ7Δ9Δ13Δ14 mutation in lipo-β-lactamase resulted in a slower rate of processing than for this hybrid protein with a wild type signal peptide (Lunn and Inouye, 1987).

Another three residue deletion mutant, Δ9Δ13Δ14, was interestingly found to cause depolarization of the electrochemical potential across the cytoplasmic membrane, which is known to be required for efficient protein export (Pollitt and Inouye, 1988). The result was the cessation of cell growth 10 min after mutant protein induction and rapid cell death caused by severe effects on cell physiology. The mutant precursor was capable of secretion at a rate slower than the wild type, and there was also slower processing observed for several other exported proteins. The reason for the membrane depolarization is not known, but it is interesting that also deleting the valine at position 7 (Δ7Δ9Δ13Δ14) suppresses this effect (Lunn and Inouye, 1987).

The region consisting of residues 14–17 of the lipoprotein signal sequence has a higher probability for β-turn structure than for α-helical structure, which may be important for signal peptide function in this region (Vlasuk *et al.*, 1984). When the threonine residue at position 16 is substituted with alanine (A16), there are similar structural probabilities and no effect on secretion. On the other hand, when the serine at position 15 is replaced by an alanine (A15), there is an initial accumulation of membrane-bound unmodified precursor, which eventually matures to lipoprotein. The double alanine

mutant (A15A16) exhibits even greater precursor accumulation. Interestingly, the A15A16 mutant has a higher α-helical than β-turn probability in this region. It should be noted, however, that the A15A16 mutation in lipo-β-lactamase had no effect on secretion for this hybrid protein (Lunn and Inouye, 1987).

OmpA

Just as a charged residue is never found naturally in the hydrophobic region of signal sequences, a noncharged, polar residue such as asparagine or glutamine is very rare. To study what effect such a residue would have on signal peptide function, various substitution mutations have been made in the hydrophobic region of the OmpA signal peptide: N6, N8, Q8, N9, N10, and N12 (Table II). Almost all of these mutants when fused to nuclease were totally defective in secretion (J. Goldstein, S. Lehnhardt, and M. Inouye, unpublished results). The N6 mutant did exhibit very slow pocessing of this hybrid protein, probably because the lesion is early enough in the hydrophobic region so as not to cause such a serious defect. The same defective result for N8 was found for the OmpA-β-lactamase hybrid.

The hydrophobic region of the OmpA signal peptide was also systematically shortened to yield the Δ9, Δ8–9, Δ7–9, and Δ6–9 mutations (Lehnhardt *et al.*, 1987). Processing of the mutant hybrid proteins was dependent upon both the length of the hydrophobic region and the protein to which the mutant signal peptide was fused. For both the nuclease and β-lactamase fusions, the three- and four- residue deletions resulted in no processing. For the nuclease hybrid, the Δ9 mutant had no effect while the Δ8–9 mutant resulted in significantly slower processing. On the other hand, deleting the alanine at position 9 resulted in significantly slower processing for the β-lactamase hybrid., while also deleting the isoleucine at position 8 supressed the defect. Furthermore, it was subsequently found that deleting the isoleucine at position 8 by itself (Δ8) resulted in slow processing for the nuclease hybrid but had no effect on the β-lactamase hybrid. The defect for nuclease harboring the Δ8-OmpA mutation could be suppressed slightly by also substituting the alanine at either position 7 or 9 with valine (V7Δ8 and Δ8V9; see Goldstein *et al.*, 1990).

As listed in Table II, various substituition mutations have been made in the OmpA signal peptide hydrophobic region which are not severe to the point of being totally defective, but do decrease or even increase the efficiency of processing (Goldstein *et al.*, 1990). The V9, I7V9, V7V9, and L6L8 mutants all exhibited faster processing than the wild type for the nuclease fusion. The fastest was the V9 mutant, which had a precursor half-life of 25 sec, almost twice as fast as the wild type which had a half-life of 45 sec.

The A8 and M8 mutants had slower processing (the precursor half-life of A8, for example, was 144 sec). These results indicate that relatively small changes in hydrophobicity and/or secondary structure (in general, there is an increase in hydrophobicity and an increase in the probability of β-sheet structure over α-helix for the faster-processing mutants) in the hydrophobic region may affect precursor processing effeciency. In contrast, the V9, A8, and M8 OmpA signal peptide mutants had no effect on processing when fused to β-lactamase.

Certain regions of the signal peptide have been proposed to need a degree of flexibility for efficient functioning. One such location is just after the amino-terminal charged region. When the alanine at position 5 was changed to glycine (G5), there was no effect on secretion for both the nuclease and β-lactamase hybrid proteins. The more rigid proline at this position (P5), however, resulted in much slower processing. To restore flexibility to the P5 mutant, a glycine was inserted after the proline (P5, 5G6). Although this mutant was not processed as rapidly as the wild type, it did exhibit faster processing than did the P5 mutant alone for both hybrids (S. Lehnhardt, J. Goldstein, and M Inouye, unpublished results).

Another region which is thought to require flexibility is at the end of the hydrophobic region just prior to the cleavage region. Replacing the glycine with proline at position 14 (P14) of the OmpA signal sequence resulted in normal processing for both nuclease and β-lactamase hybrids. Changing the alanine at position 13 to proline (P13), however, resulted in only slight processing. This may be due to the creation of a more rigid β-turn and the loss of structural flexibility. The A14 mutation had no effect on secretion, while Δ14 exhibited much slower processing. Arginine at postition 14 (R14) was found to be defective for both hybrids, perhaps because the charge is still located within the hydrophobic region (S. Lehnhardt, J. Goldstein, and M. Inouye, unpublished results).

Using oligonucleotide insertions, Freudl *et al.* (1988) were able to reduce or extend the hydrophobic core of the OmpA signal peptide (residues 5–16). The OmpA precursor (OmpA signal sequence and mature peptide) has been shown to be processed partially co-translationally and post-translationally. When the hydrophobic core was reduced from 12 to 10 residues in length (SI) by an oligonucleotide insertion which added a positive charge at the amino terminus, the processing rate became significantly slower, exhibiting only post-translational processing. On the other hand, extending the hydrophobic core to 16 (P1) or 20 (P2) residues resulted in much faster cotranslational processing. These mutants demonstrate the length flexibility of the signal sequence since a 16- or 20-residue hydrophobic core yielded such efficient processing and export.

MBP

Mutants in the hydrophobic region of the MBP signal peptide have been grouped into two classes (Table III). Class I mutations are Mal^- on indicator plates, show a 50–95% block in MBP export which results in defective protein localization, and exhibit a slow post-translational form of processing. Such mutants suffer amino acid replacements which introduce charged residues at positions 14, 16, 18, and 19 of the signal peptide or contain a deletion covering this apparently critical region. Class II mutations block hybrid MBP-LacZ export, but are silent when recombined into wild-type MBP and accumulate lower levels of precursor MBP than Class I mutants. Mutants which introduce charged, proline or serine residues at positions 10 and 11 and proline at position 14 of preMBP fall into this category.

All of the class I mutations introduce hydrophobic residues, or would be predicted to disrupt the secondary structure of the core. The severity of these mutations can be explained in two ways. The hydrophobicity and secondary structure of the hydrophobic region may be important in conferring to the signal peptide the physical properties required for melting into the phospholipid bilayer of the cytoplasmic membrane. Alternatively, the area of the hydrophobic region defined by the critical residues at positions 14, 16, 18, and 19 forms a recognition site for entrance into the protein export pathway.

Analysis of numerous intragenic mutations which suppress the export defects of hydrophobic region lesions tends to support the contention that hydrophobicity and a putative α-helical structure at the core are essential for signal peptide function (Ryan *et al.*, 1986). Isolated mutations suppress the export defect by one of five mechanisms (Table VII): (1) substitution of a hydrophobic for neutral amino acid (mal19–1, R5), (2) insertion of hydrophobic residues to increase the length of the hydrophobic core (mal19–1, R2), (3) extension of the hydrophobic core toward the N-terminus by removal of arginine at position 8 (mal19–1, R7), (4) extension of the putative core α-helix into the mature N-terminus (mal19–1, R6), and (5) substitution of charged residues by uncharged residues (mal19–1, R9). These suppressors emphasize the importance of overall hydrophobicity in the central region of the signal peptide.

The careful kinetic analysis of mutant MBP processing has led to the conclusion that a proportion of the preMBP is never exported from the cytoplasm (Ryan and Bassford, 1985). This proportion of export-defective preMBP increases with the severity of the signal peptide lesion. This is believed to represent the folding of preMBP into an export-incompetent form.

The SecB protein is thought to promote MBP export by interacting directly with preMBP to prevent folding to an export-incompetent form. The observation that the processing defect of the mal16–1 lesion is exacerbated in

the *secB*$^-$ background supports this contention (Collier and Bassford, 1989). Many suppressors of the mal16–1 lesion selected in a *secB*$^-$ background were found to map to the MBP signal peptide. Again these suppressors function by increasing the hydrophobicity of the signal peptide core (Table VII). However, the deletion mutants MBP172 and MBP173 were found to exhibit improved SecB-independent export. This indicates that certain signal peptide mutations can partialy supplant SecB function. Such suppressor mutations could increase the rate at which preMBP enters the secretion pathway or may serve directly to hinder folding of preMBP into a form incompatible with translocation.

LamB

Genetically selected mutations in the signal peptide core of LamB resemble closely MBP lesions (Bankaitis *et al.*, 1987). A class of severe processing defects could be observed (Table VI). These mutants were the result of charged residue introduction at positions 14, 15, 16, or 19 or deletions in the hydrophobic core. Less severe class II defects were conferred when charged residues were introduced at the adjacent positions 12, 13, and 17. Intragenic suppressor mutants of the S78 deletion could be explained by postulating that they restored a putative α-helical structure to the hydrophobic region. This interpretation was strengthened by physical studies of synthetic signal peptides which indicated that the wild-type and suppressor signal peptide exhibited α-helical structure *in vitro* while the deletion mutant did not (Briggs *et al.*, 1985). In addition, one mutation in the hydrophobic region (16E) resulted in decreased LamB synthesis levels as well as slowed processing. It remains unclear whether this is due to alterations in the structure of the mRNA or the protein it encodes.

Other Proteins

Alkaline phosphatase (PhoA) signal peptide mutations in the hydrophobic core have been selected genetically using PhoA-LacZ fusions (Table VIII) (Michaelis *et al.*, 1986). Again most of the defects are due to the introduction of charged residues or deletions at the core. However, two of the mutants contained the polar, but uncharged residue glutamine in place of leucine. These are the only examples of mutants containing a polar residue in the hydrophobic region which have been isolated by genetic selection.

The importance of α-helical structure has been demonstrated by the construction *in vitro* of a PhoA signal peptide containing an idealized hydrophobic core (Table VIII) (Kendall *et al.*, 1986). This mutant (13A) hydrophobic region consists essentially of polyleucine which has a high probability of forming an α-helix. This mutation improves export. However, further

Table VII. Suppressors of Maltose-Binding Protein Signal Peptide Mutants[a]

	1 *Met*	2 *Lys*	3 *Ile*	4 *Lys*	5 *Thr*	6 *Gly*	7 *Ala*	8 *Arg*	9 *Ile*	10 *Leu*	11 *Ala*	12 *Leu*	13 *Ser*	14 *Ala*	15 *Leu*	16 *Thr*	17 *Thr*	18 *Met*	19 *Met*	20 *Phe*	21 *Ser*	22 *Ala*	23 *Ser*	24 *Ala*	25 *Leu*	26 *Ala*	↓ 27 *Lys*	45 *Gly*	309 *Tyr*
Suppressors of d12-18:											[	][	][	][	][	][	][	][	]										
R2 [1]								Leu																					
R3 [1]								Cys																					
R4 [1]											+[Leu]																		
R5 [1]											Val																		
R6 [1]																												Val	
R1 [1]																				+[LeuAlaMet]									
Suppressors of mal18–1:																		*Arg*											
R1 [2]														+[SerAlaLeu]															
R2 [2]															Met														
R3 [2]															+[PheAla]														
R4 [2]																					Phe								
R5 [2]								Cys																					
R6 [2]																		Ser											
R7 [2]																		Gly											
R8 [2]																		Met											
Suppressors of mal19–1:																			*Arg*										
R1 [2]																												Cys	
R2 [2]												+[CysIleLeuAla]																	
R3 [2]																Met													
R4 [2]																+[SerAlaLeu]													
R5 [2]													Phe																
R6 [2]																											Thr		
R7 [2]								Cys																					
R8 [2]																											Asn		
R9 [2]																			Ser										
R10 [2]																			Gly										
R11 [2]																			Met										
Suppressors of mal14–1:														*Glu*															
R1 [2]								Leu																					
R2 [2]														Ala															
mal2261 [3]																													Asp

	Met	*Lys*	*Ile*	*Lys*	*Thr*	*Gly*	*Ala*	*Arg*	*Ile*	*Leu*	*Ala*	*Leu*	*Ser*	*Ala*	*Leu*	*Thr*	*Thr*	*Met*	*Met*	*Phe*	*Ser*	*Ala*	*Ser*	*Ala*	*Leu*	*Ala*	↓	*Lys-Gly-Tyr*	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		27	45	309
Suppressors of mal10–1:										*Pro*																				
R1 [2]																+ [SerAlaLeu]														
R2 [2]										Leu																				
Suppressors of mal16–1:																*Lys*														
MBP170 [4]								Leu																						
MBP171 [4]								Ala																						
MBP172 [4]																[ ][ ]														
MBP173 [4]																[ ][ ][ ]														
MBP174 [4]													+ [LeuAlaLeu]																	
MBP177 [4]																+ [PheIleLeuAlaLeuSerAla]														
MBP201 [4]																Met														
MBP204 [4]													Phe																	
Revertants of d12–18, R2:								*Leu*				[ ][ ][ ][ ][ ][ ][ ]																			
CC13 [5]																					Pro									
CC15 [5]																										Asp				
CC17 [5]																											Arg			
CC18 [5]										Glu																				
CC19 [5]								Pro																						
CC21 [5]										Arg																				
CC23 [5]																			Arg											

"The arrow indicates the signal peptide cleavage site. Empty brackets or a lower case d denote amino acid deletions. Bracketed amino acids indicate insertions. Numbers in brackets denote references: [1] Bankaitis *et al.* (1984); [2] Ryan *et al.* (1986); [3] Cover *et al.* (1987); [4] Collier and Bassford (1989); [5] Fikes *et al.* (1987).

Table VIII. Alkaline Phosphatase Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	↓	22	23	24	25	26	27	28	29	30	31	32
	Met	*Lys*	*Gln*	*Ser*	*Thr*	*Ile*	*Ala*	*Leu*	*Ala*	*Leu*	*Leu*	*Pro*	*Leu*	*Leu*	*Phe*	*Thr*	*Pro*	*Val*	*Thr*	*Lys*	*Ala*		*Arg*	*Thr*	*Pro*	*Glu*	*Met*	*Pro*	*Val*	*Leu*	*Glu*	*Asn*	*Arg*
Hydrophobic Region:																																	
phoA68 [1]								Gln																									
phoA21 [2]									Glu																								
phoA93 [2]										His																							
phoA61 [1]														Arg																			
phoA73 [2]														Glu																			
phoA82 [2]								[	][	]																							
13A [3]							Leu		Leu			Leu			Leu																		
63A [3]							Leu		Leu			Leu			Leu																		
														+ [SerLeuLeuLeuLeuLeu]																				
Mature Protein:																																	
2AB [4]																							(+2)IleArgPro	[	][	][	][	][	][	][	]	
X2 [4]																							(0)IleGluPro	[	][	][	][	][	][	][	]	
X3 [4]																							(+1)IleThrPro	[	][	][	][	][	][	][	]	
X4 [4]																							(+2)IleLysPro	[	][	][	][	][	][	][	]	
X6 [4]																							(+1)GluArgPro	[	][	][	][	][	][	][	]	
	Met	*Lys*	*Gln*	*Ser*	*Thr*	*Ile*	*Ala*	*Leu*	*Ala*	*Leu*	*Leu*	*Pro*	*Leu*	*Leu*	*Phe*	*Thr*	*Pro*	*Val*	*Thr*	*Lys*	*Ala*		*Arg*	*Thr*	*Pro*	*Glu*	*Met*	*Pro*	*Val*	*Leu*	*Glu*	*Asn*	*Arg*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	↓	22	23	24	25	26	27	28	29	30	31	32

[a]The arrow indicates the signal peptide cleavage site. Empty brackets denote amino acid deletions. Bracketed amino acids indicate insertions. Numbers in brackets denote references: [1] Michaelis *et al.* (1983); [2] Michaelis *et al.* (1986); [3] Kendall *et al.* (1986); [4] Li *et al.* (1988).

extension of the leucine core by insertion of six amino acids (63A) inhibits processing by perhaps preventing proper alignment of cleavage site and signal peptidase.

A number of lesions in the hydrophobic region of β-lactamase have been generated through *in vitro* mutagenesis using methoxylamine (Table V) (Kadonaga *et al.*, 1985). Most of the hydrophobic region mutations are conservative replacements and exhibit only minor processing defects. However, the replacement of cysteine at position 18 with tyrosine in the double mutant M63 did block export almost completely. The histidine replacement at position 7 of this double mutant is considered to be unimportant since it had only a minimal inhibitory effect when singly present in the signal peptide (M8). Substitution of a threonine residue at position 15 (M9) caused a 70% increase in periplasmic β-lactamase. It is not known whether this reflects improved export or increased protein synthesis.

The ribose binding protein signal peptide core lesion isolated from a collection of mutants defective in ribose transport does not fit completely into the mold established by other hydrophobic region defects (Iida *et al.*, 1985). The proline replacement (*rbs103*) causes a complete processing block, perhaps by altering the secondary structure at the core (Table II). However, similar MBP and LamB mutations are of the milder class II type. The phenylalanine for serine substitution mutant isolated as a suppressor of the *rbs103* allele increases the hydrophobicity of the core, but is not expected to restore the wild-type secondary conformation.

Several mutations which introduce charged residues into the hydrophobic core of the putative signal peptide of MalM were selected using LacZ fusions (Table IX) (Rousset *et al.*, 1986). However, the processing defects associated with these lesions were not evaluated.

Effects on Protein Synthesis

A few of the lipoprotein hydrophobic region mutants exhibited a change in the rate of synthesis of the protein (Table I). The four-residue deletion, Δ7Δ9Δ13Δ14, was shown to have a much lower rate of synthesis. On the other hand, the Δ9Δ14, Δ7Δ9Δ14, Δ9Δ13Δ14, D14, and R14 mutants had a higher level of sythesis compared to the wild type (Pollitt, *et al.*, 1985; C. Sung, S. Pollott, G. Vlasuk, and M Inouye, unpublished results). It should be noted, however, that in lipo-β-lactamase, the Δ9Δ14, Δ7Δ9Δ14, and Δ9Δ13Δ14 mutants had no such increases in the rate of synthesis compared to the wild-type hybrid protein (Lunn and Inouye, 1987).

Many of the OmpA signal peptide hydrophobic region mutants exhibited a decreased rate of synthesis, while some resulted in increased rates (Table II). Each of the proline/glycine mutants at position 5 had decreased rates for both

Table IX. Signal Peptide Mutants[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	↓	23	24	25	26	27	
OMPF	*Met*	*Met*	*Lys*	*Arg*	*Asn*	*Ile*	*Leu*	*Ala*	*Val*	*Ile*	*Val*	*Pro*	*Ala*	*Leu*	*Leu*	*Val*	*Ala*	*Gly*	*Thr*	*Ala*	*Asn*	*Ala*		*Ala*	*Glu*	*Ser*	*Ser*	*Asn*	
Mature Protein:																													
pK107(+2) [1]																						Phe		Pro	Gly	(0)			
pK108(+2) [1]																						Phe		Arg	Gly	(+1)			
pK109(+2) [1]																						Phe		Lys	Gly	(+1)			
pK110(+2) [1]																						Phe		Arg	Lys	(+2)			
pK112(−1) [1]			Asn	Leu	Glu																	Phe		Pro	Gly	(0)			
pK113(−1) [1]			Asn	Leu	Glu																	Phe		Arg	Gly	(+1)			
pK114(−1) [1]			Asn	Leu	Glu																	Phe		Lys	Gly	(+1)			
pK115(−1) [1]			Asn	Leu	Glu																	Phe		Arg	Lys	(+2)			
021 [1]																									Lys			(+1)	
026 [1]																									Gly	Arg		Lys	(+2)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
MALM	*Met*	*Lys*	*Met*	*Asn*	*Lys*	*Ser*	*Leu*	*Ile*	*Val*	*Leu*	*Cys*	*Leu*	*Ser*	*Ala*	*Gly*	*Leu*	*Leu*	*Ala*	*Ser*	*Ala*	*Pro*	*Gly*	*Ile*	*Ser*	*Leu*	*Ala*	*Asp*
Hydrophobic Region:																											
M10 [2]														Glu													
M11 [2]												Ser															
M12 [2]									Asp																		
Cleavage Region:																											
M1c [2]																						Arg					

[a]The arrow indicates the signal peptide cleavage site. Numbers in parentheses denote the net charge of the signal peptide and mature protein N-terminus. Numbers in brackets denote references: [1] Yamane and Mizushima (1988); [2] Rousset *et al.* (1986).

nuclease and β-lactamase hybrid proteins. The A14 mutant also had decreased synthesis. On the other hand, the P13 and P14 mutants exhibited increased synthesis for both hybrids (S. Lehnhardt, J. Goldstein, and M. Inouye, unpublished results). For some of the mutants, however, the rate of synthesis depended on the mature protein to which the signal sequence was fused. The V9 and N8 mutants increased for nuclease only, whereas the R14 mutant increased for β-lactamase only. The A8 and M8 mutants decreased for nuclease only. The I7V9 mutant which increased sythesis and the V7V9 and L9 mutants which decreased synthesis have only been studied with the nuclease fusion (Goldstein *et al.*, 1990).

Conclusions

In summary, the overall hydrophobicity of the signal peptide core rather than any specific amino acid sequence appears to be essential for export function. The length of the core can vary considerably, within limits.

A specific secondary structure in the hydrophobic region is also needed for efficient export. Ample evidence suggests that an α-helical core may be required. However, OmpA mutations which increase the probability of predicted β-sheet structure show improvement in the processing rate. It will be important to correlate the *in vivo* export defects of hydrophobic region signal peptide mutations with the actual alterations they induce in signal peptide secondary structure. Isolated OmpA mutations have also illustrated the need for maintenance of a flexible secondary structure at the signal peptide juncture points between basic and hydrophobic regions and hydrophobic and cleavage regions.

The isolation of hydrophobic region mutations which lead to altered protein synthesis rates suggests a possible coupling of translation and export. Although potentially quite important, considerable additional work will be required to understand this phenomenon. A sometimes pronounced contrast in export defect can be associated with the same OmpA signal peptide mutation when fused to differing mature proteins. The mature protein clearly influences signal peptide function. Whether this is through a direct interaction is not known.

The function of the hydrophobic region remains unresolved. It may serve as a recognition site to direct preproteins into the export pathway, directly interact with the lipid bilayer to initiate translocation, or serve to maintain the preprotein in an export-competent folded state. In any case it is important to note that the signal peptide is likely to dynamically change its secondary and tertiary structures during the secretion process.

Cleavage Region Signal Peptide Mutations

Signal peptides are cleaved from the mature portion of the protein by one of two signal peptidases present in the cytoplasmic membrane of the cell. Lipoproteins must first be modified at their mature N-terminal cysteine residue before signal sequence cleavage by Signal Peptidase II (Wu, 1987). There is strong conservation among bacterial lipoproteins of the sequence LeuAlaGlyCys with cleavage occurring between the Gly-Cys residues. This strongly conserved cleavage site is referred to as the lipoprotein box and may also serve as recognition site for lipid modification enzymes. Nonlipid modified proteins are processed by Signal Peptidase I (Wu, 1987). A generalized cleavage site recognized by this enzyme is A-X-B/ (the slash indicates the cleavage site) (von Heijne, 1983). Residue B is usually alanine or glycine. There is a strong tendency to find alanine as residue A, but a variety of other aliphatic amino acids can occupy this position. There is low specificity for residue X, which can be almost any amino acid. The presence of a β-turn may be required for recognition of the cleavage site (Perlman and Halvorson, 1983).

Lipoprotein

Most signal peptides contain either a glycine or alanine, and sometimes a serine, at the cleavage site, suggesting that only residues with small side chains are tolerated at this position (Inouye and Halegoua, 1980; von Heijne, 1986). The glycine at position 20 of the lipoprotein signal sequence has been altered to determine how rigid this requirement is (Pollitt *et al.*, 1986; Inouye *et al.*, 1983b; Table I). Alanine (A20) and serine (S20) at this site were comparable to wild type in phenotype. A valine (V20) or leucine (L20), on the other hand, resulted in the accumulation of unmodified precursor. A threonine at the cleavage site (T20) exhibited slow lipid modification and no detectable processing of lipoprotein, indicating that the size of the threonine side chain is boarderline as a substrate for signal peptidase II. There appears to be a structural requirement as well since deleting the glycine (Δ20), which placed a normally acceptable alanine at the cleavage site, resulted in the abolition of processing.

Although inserting an additional glycine (20G21) yielded a functional signal peptide (S. Inouye, S. Pollitt, and M. Inouye, unpublished results), changing the cysteine at position 21 (the first residue of the mature lipoprotein) to a glycine (G21) resulted in a mutant prolipoprotein which was lethal to the cell upon induction (Inouye *et al.*, 1983a). Large amounts of the precursor accumulated in the outer membrane, indicating that signal peptidase II requires a glycerol-modified cysteine at the cleavage site.

Interestingly, the Δ20 and G21 mutants which exhibited no processing for lipoprotein did have some processing occur for the lipo-β-lactamase hybrid protein, although less effeciently than for the unaltered hybrid (Ghrayeb *et al.*, 1985). It was found that these mutant signal peptides were cleaved five amino acid residues downstream of the normal cleavage site. This new cleavage site was resistant to globomycin, indicating that signal peptidase I and not II became the processing enzyme. Thus, when the normal cleavage site is blocked, such as occurs here where there is no lipid modified cysteine for recognition by signal peptidase II, an alternate pathway of processing may take place.

By the rules of Chou and Fasman (1978), a β-turn structure is predicted at the cleavage site in the mature region of the protein (Inouye *et al.*, 1986). When the serine and asparagine residues at positions 23 and 24 were changed to isoleucine (I23I24), β-turn probability decreases in this region. Processing in this mutant is slow at 30°C and severley inhibited at 42°C. Also, changing the glycine at position 20 of the signal sequence to alanine (A20I23I24), further disrupting β-turn structure, resulted in a complete block in lipid modification and processing at all temperatures. The mutant I23K24 replaces the hydrophobic isoleucine of the mutant I23I24 with the hydrophilic lysine. This mutant, which also decreases β-turn structure, yields a more effective signal peptide than the I23I24 mutant, although processing is still slower than wild type. The A20I23K24 mutant is as defective as the I23I24 mutant.

As mentioned above, deleting the glycine at position 20 (Δ20) resulted in precursor accumulation. The same result was obtained for the leucine deletion at position 18 (Δ18). Inserting a glycine at the cleavage site (20G21) or a leucine within the box (17L18) resulted in mutants which were functional (S. Inouye, S. Pollitt, and M. Inouye, unpublished results). The lipoprotein box may be a recognition sequence for the glyceryl transferase enzyme involved with lipid modification. The deletion mutations may thus disrupt this recognition sequence while the insertions leave it intact.

OmpA

In order to decrease the probability of β-turn structure at the cleavage site of the mature region of the OmpA-nuclease hybrid protein, one (Δ24) and two (Δ24Δ25) residues were deleted from the linker portion separating the two proteins (Duffaud and Inouye, 1988; Table II). Deleting the proline (Δ24) resulted in slower processing than wild type, and also removing the serine (Δ24Δ25) resulted in an even slower rate of processing. Changing the glycine at position 22 to lysine (K22Δ24Δ25) disrupts β-turn structure still further, and resulted in even slower processing. The K22 mutant by itself was as efficient as wild type. Such results indicate that proper functioning of signal

peptidase I requires a β-turn structure at the cleavage site of precursor proteins that are to be exported.

Freudl *et al.* (1985), using the native OmpA mature protein and signal peptide, constructed the IV-97 and II-3e mutants by chemical mutagenesis. These lesions were isolated by selecting mutants which caused cell lysis upon induction. The IV-97 mutant had a valine at the cleavage site of the signal peptide, position 21, and resulted in very slow processing. The II-3e mutant had four substitutions in the signal sequence and two at the cleavage site of the mature polypeptide. Small amounts of precursor were observed associated with the plasma membrane, while a reduced rate of processing yielded some mature mutant OmpA in the outer membrane.

Other Proteins

Several cleavage site mutants of the M13 procoat signal peptide have been isolated by localized mutagenesis with hydroxylamine (Table X) (Kuhn and Wickner, 1985). These mutant proteins were synthesized and inserted into the cytoplasmic membrane normally, but not processed. The mutations isolated support the proposal that the residues at position -1 and -3 are critical for cleavage. The substitution of threonine at the -1 or phenylalanine at the -3 position introduces large or aromatic residues at sites which are important for putative β-turn structure. In addition, a serine replacement for proline at position -6 was found to prevent cleavage. It has been proposed that a proline or glycine residue at the -4 to -6 position of the signal sequence is important for breaking the hydrophobic core α-helix (Perlman and Halvorson, 1983). The serine substitution may permit α-helix extension into the cleavage site. This may disrupt the conformation here and prevent cleavage.

Two mutations at the cleavage site of the MBP signal peptide have been isolated as maltose-resistant revertants using an MBP-LacZ fusion which contains the Δ12–18, R2 signal peptide (Table VII) (Fikes *et al.*, 1987). Both mutations when *cis* to the Δ12–18, R2 signal peptide exhibit a strong export deficiency, but the aspartate replacement at the -3 position completely blocks cleavage whereas this residue at the -2 position permits normal cleavage. When the aspartate was introduced at the -3 position of the wild-type MBP signal peptide by site-directed mutagenesis (Table III) (Fikes and Bassford, 1987), preMBP was found to be normally exported to the periplasm, but unprocessed. The preprotein remained attached to the cytoplasmic membrane, presumably through the signal peptide acting as a membrane anchor. These results clearly indicate that a signal peptide cleavage site is not essential for translocation. In good agreement with what would be

Table X. Signal Peptide Mutants[a]

M13 PROCOAT	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	↓
	Met	*Lys*	*Lys*	*Ser*	*Leu*	*Val*	*Leu*	*Lys*	*Ala*	*Ser*	*Val*	*Ala*	*Val*	*Ala*	*Thr*	*Leu*	*Val*	*Pro*	*Met*	*Leu*	*Ser*	*Phe*	*Ala*	
Cleavage Region:																								
H9 [1]																		Ser						
H5 [1]																					Phe			
H10 [1]																							Thr	
COLICIN A LYSIS PROTEIN																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	↓	19				
	Met	*Lys*	*Lys*	*Ile*	*Ile*	*Ile*	*Cys*	*Val*	*Ile*	*Leu*	*Leu*	*Ala*	*Ile*	*Met*	*Leu*	*Leu*	*Ala*	*Ala*		*Cys*				
Cleavage Region:																								
pAK31 [2]																		Pro						
pAL16 [2]																				Thr				

[a]The arrow indicates the signal peptide cleavage site. Numbers in brackets denote references: [1] Kuhn and Wickner (1985); [2] Cavard *et al.* (1987).

predicted from the concensus cleavage site, the presence of a charged residue at the −3 position inhibits cleavage, but can be tolerated at the −2 position.

Mutations have been isolated by *in vitro* mutagenisis near the cleavage site of the β-lactamase signal peptide (Kadonaga *et al.*, 1985). Mutations which substitute serine or phenylalanine for proline at position −4 exhibit inhibited processing which could be due to cleavage inhibition (Table V). This region of the signal peptide may be important for breaking the hydrophobic core α-helix. However, the defect may affect translocation since these mutants exhibit very low periplasmic β-lactamase activities. The conservative replacement of valine by isoleucine at the −3 position as expected does not affect cleavage. However, this substitution confers a severe export defect.

An arginine for glycine substitution in MalM has been isolated which may be at the cleavage site of the peptide (Table IX) (Rousset *et al.*, 1986). However, this mutation is difficult to evaluate due to uncertainty concerning the precise location of signal sequence cleavage and the lack of any direct assessment of processing phenotype.

The colicin A lysis lipoprotein contains a typical lipoprotein box with the minor variation that alanine is present at the −1 position in place of glycine (Cavard *et al.*, 1987). Replacement of this alanine residue by proline blocks both lipid modification and cleavage (Table X). Lipid modification and subsequent processing is obviously prevented by the substitution of threonine for the mature N-terminal cysteine residue which is the site of lipid modification. The protein with the threonine replacement was observed to undergo processing at an alternate site, perhaps due to cleavage by signal peptidase I.

Conclusions

Taken together, these results define certain stuctural requirements for cleavage by signal peptidase I and II. Only small amino acids (alanine, glycine, or serine) can be accommodated at the −1 position, and small to moderately sized aliphatic amino acids at the −3 position. A wide range of amino acids can be in position −2 of the signal peptidase I cleavage site. Insertion in the lipoprotein box of the same residue to generate an amino acid duplication can be tolerated by signal peptidase II. However, deletions alter the lipoprotein box sufficiently to prevent cleavage.

The requirement for small residues at the cleavage site of both enzymes suggests that a β-turn may be present in this region. Mutations which are designed to alter this secondary structure support this conclusion. Virtually any residue may occupy the mature N-terminal position of a signal peptidase I substrate, as long as it does not disturb secondary structure. This suggests

that this residue is not required for recognition of the cleavage site by signal peptidase I. The requirement for an α-helix breaking residue(s) at the −4 to −6 position remains questionable.

Mature Protein Mutations

Mutations within the mature portion of the protein have recently been shown to dramatically affect signal peptide function *in vivo*. Mutant PhoA proteins constructed by site-directed mutagenesis to contain a net charge of +2 at the mature N-terminus were found to be severely export defective (Table VIII) (Li, *et al.*, 1988). The wild-type mature N-terminus is neutral. Mutant proteins with a mature N-terminal net charge of 0 or +1 were exported normally. A mutation which increased the net positive charge of the signal peptide N-terminus did not suppress defective processing due to the mature N-terminus lesion.

Quite similar results have been obtained employing a noncleavable OmpA-lipoprotein hybrid (net mature N-terminal charge of −1) (Yamane and Mizushima, 1988). Introduction of a +2 charge totally blocks export *in vitro*, and a +1 charge partially interferes with it (Table IX). OmpF-lipoprotein with a neutral mature N-terminus is exported effectively. These same results were obtained *in vivo* when the mutations were introduced into a cleavable OmpF-lipoprotein hybrid. Decreasing the signal peptide N-terminal charge from +2 (wild type) to −1 exacerbated the defective translocation.

Identical results were obtained with β-lactamase (Yamane and Mizushima, 1988). A mutant with mature N-terminal net charge of +2 was not processed *in vivo* (Table V).

These results clearly indicate that a significant net positive charge at the mature N-terminus severely inhibits translocation. This could be due to some requirement for a net neutral or negative charge at the mature N-terminus. Alternatively, the charge distribution across the signal peptide may be altered by the mutations. The correct signal peptide charge distribution has been postulated to be essential for proper membrane interaction (Von Heijne, 1986; Hartmann *et al*, 1989). It has been proposed that either of these possibilities could block translocation, by causing insertion of the mutant protein into the membrane in the reversed orientation (Li *et al.*, 1988) or by preventing the mature protein from following the signal peptide hydrophobic core into the membrane translocation site (Yamane and Mizushima, 1988). The observation that basic mature N-terminal residues can block export could help to explain the export incompetency of certain hybrid proteins (Li *et al.*, 1988).

Mutations deep within the mature MBP protein have been genetically isolated as suppressors of export-defective signal peptide lesions (Ryan and Bassford, 1985). Two such mutations substitute valine or cysteine for glycine residue 45 (+ 19 of the mature protein) (Table VII). The MBP2261 mutation replaces tyrosine 309 (+ 283 of the mature protein) with aspartate. This latter mutant improves export of MBP containing a defective signal peptide, but does not affect export of an otherwise wild-type MBP (Cover *et al.*, 1987). Large in-frame deletions of the LamB protein which remove portions of the first 28 mature N-terminal amino acid residues inhibit protein export (Rasmussen and Silhave, 1987).

Such mature region mutations may indicate that a direct interaction between the signal peptide and mature protein may be required for efficient export. The signal peptide may help to maintain the preprotein in an export-competent form. Alternatively, these mutations may directly affect protein export competency by altering folding.

Future Directions

The identification and purification in recent years of various proteins of the cellular export machinery has made possible the establishment of *in vitro* systems capable of precursor protein membrane translocation. These *in vitro* systems make possible the study of protein secretion at the molecular level. It should now be possible to study *in vitro* the interaction of defective precursor proteins with the various components of the export pathway. The correlation of *in vivo* defects and *in vitro* activities with specific signal peptide mutations should lead to a far better understanding of the precise function of each component region of the signal peptide.

When expressed, a number of signal peptide mutations generated *via* site-directed mutagenesis significantly inhibit cell growth. By employing a simple positive selection for growing clones, this observation can be exploited to isolate intra-and extragenic suppressors (Pollitt and Inouye, 1988). Such suppressors should help to elucidate the function of specific areas of the signal peptide and should be useful in identifying as yet unknown cellular components required for protein secretion.

The phenomenon of signal peptide–mature protein incompatibility must be investigated further. When fused to functional signal peptides, numerous proteins have been found to be export incompetent. A significant body of research already exists which suggests that charged areas within the mature protein can significantly inhibit the export of hybrid proteins (discussed above). In addition, a direct interaction between the signal peptide and mature protein may be critical for proprotein folding to an export-competent

state. Recent experiments employing fusions of the OmpA signal peptide and Staphlococcal nuclease as mature protein have clearly demonstrated that a signal peptide can drastically retard the kinetics of protein folding (Chatterjee and Inouye, unpublished results). Understanding the basis of signal peptide–mature protein compatibility is a major goal with significant practical consequences for biotechnology.

Synthetic signal peptides have been extensively employed to study the biophysical properties of signal sequences (Briggs and Gierasch, 1986). This approach can be extended to directly investigate signal peptide interaction with components of the export pathway. Synthetic peptides can be utilized quite successfully as probes of biological function, as inhibitors of *in vitro* translocation (Chen *et al.*, 1987; DeVrije *et al.*, 1989), or as artificial signal peptidase substrates (Caulfield *et al.*, 1989). It may be possible to purify protein components of the secretion machinery using a synthetic peptide as high-affinity ligand.

The signal peptide has been found to function as a stop-transfer signal when internalized in the mature protein structure (Coleman *et al.*, 1985). The nature of the N-terminal signal peptide and its distance from the internalized signal peptide determine the role (signal or stop-transfer sequence) that the internal sequence will assume. This observation can be extended to study structural determinants of membrane protein orientation.

In vitro translocation systems can be employed to test the tolerance or flexibility of the secretion machinery for proproteins carrying signal peptide mutations. The prokaryotic lipoprotein signal peptide (both wild-type and export-defective mutant) can be recognized by SRP and efficiently translocated *in vitro* by a eukaryotic translocation system (Garcia *et al.*, 1987). Further comparative studies using heterologous systems consisting of a mixture of prokaryotic and eukaryotic secretion components may reveal conserved structural features of the signal peptide.

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References

Bankaitis, V. A., Rasmussen, B. A., and Bassford, P. J. (1984). *Cell* **37**, 243–252.

Bankaitis, V. A., Altman, E., and Emr, S. D. (1987). In *Bacterial Outer Membranes as Model Systems* (Inouye, M., ed.), Wiley, New York, pp. 75–116.

Batenburg, A. M., Brasseur, R., Ruysschaert, J., van Scharrenburg, G. J. M., Slotboom, A. J., Demel, R. A., and de Kruijff, B. (1988). *J. Biol. Chem.* **263**, 4202–4207.

Bechwith, J., and Silhavy, T. (1984). In *Methods in Enzymology* (Fleischer, S., and Fleischer, B., eds.), Academic Press, New York, pp. 3–11.
Bedouelle, H., and Hofnay, M. (1981). In *Membrane Transport and Neuroreceptors* (Oxender, D., *et al.*, eds.), A. R. Liss, New York, pp. 399–403.
Bedouelle, H., Bassford, P. J., Fowler, A. V., Zabin, I., Beckwith, J., and Hofnung, M. (1980). *Nature (London)* **285**, 78–81.
Benson, S. A., Hall, M. N. and Rasmussen, B. A. (1987). *J. Bacteriol.* **169**, 4686–4691.
Blobel, G., and Dobberstein, B. (1975). *J. Cell. Biol.* **67**, 835–851.
Briggs, M. S., and Gierasch, L. M. (1986). *Adv. Protein Chem.* **38**, 109–180.
Briggs, M. S., Gierasch, L. M., Zlotnick, A., Lear, J. D., and DeGrado, W. F. (1985). *Science* **228**, 1096–1099.
Caulfield, M. R., Duong, L. T., Baker, R. K., Rosenblatt, M., and Lively, M. O. (1989). *J. Biol. Chem.* **264**, 15813–15817.
Cavard, D., Baty, D., Howard, S. P., Verheij, H. M., and Lazdunski, C. (1987). *J. Bacteriol.* **169**, 2187–2194.
Chen, L., Tai, P. C., Briggs, M. S., and Gierasch, L. M. (1987). *J. Biol. Chem.* **262**, 1427–1429.
Chou, P. Y., and Fasman, G. D. (1978). *Annu. Rev. Biochem.* **47**, 251–276.
Coleman, J., Inukai, M., and Inouye, M. (1985). *Cell.* **43**, 351–360.
Collier, D. N., and Bassford, P. J. (1989). *J. Bacteriol.* **171**, 4640–4647.
Cover, W. H., Ryan, J. P., Bassford, P. J., Walsh, K. A., Bollingen, J., and Randall, L. L. (1987). *J. Bacteriol.* **169**, 1794–1800.
DeVrije, T., Batenburg, A. M., Jordi, W., and DeKruijff, B. (1989). *Eur. J. Biochem.* **180**, 385–292.
Duffaud, G., and Inouye, M. (1988). *J. Biol. Chem.* **263**, 10224–10228.
Emr, S. D., and Bassford, P. J. (1982). *J. Biol. Chem.* **257**, 5852–5860.
Emr, S. D., and Silhavy, T. J. (1980). *J. Mol. Biol.* **141**, 63–90.
Emr, S. D., and Silhavy, T. J. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 4599–4603.
Emr, S. D., Schwartz, M., and Silhavy, T. J. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 5802–5806.
Fikes, J. D., and Bassford, P. J. (1987). *J. Bacteriol.* **169**, 2352–2359.
Fikes, J. D., Bankaitis, V. A., Ryan, J. P., and Bassford, P. J. (1987). *J. Bacteriol.* **169**, 2345–2359.
Freudl, R., Braun, G., Hindennach, I., and Henning, U. (1985). *Mol. Gen. Genet.* **201**, 76–81.
Freudl, R., MacIntyre, S., Degen, M., and Henning, U. (1988). *J. Biol. Chem.* **263**, 344–349.
Garcia, PO. D., Ghrayeb, J., Inouye, M., and Walter, P. (1987). *J. Biol. Chem.* **262**, 9463–9468.
Goldstein, J., Lehnhardt, S., and Inouye, M. (1990). *J. Bacteriol.* **172**, 1225–1231.
Ghrayeb, J., Lunn, C. A., Inouye, S., and Inouye, M. (1985). *J. Biol. Chem.* **260**, 10961–10965.
Hall, M. N., Gabay, J., and Schwartz, M. (1983). *EMBO J.* **2**, 15–19.
Hartmann, E., Rapaport, T. A., and Lodish, H. F. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 5786–5790.
Iida, A., Groarke, J. M., Park, S., Thom, J., Zabicky, J. H., Hazelbauer, G. L., and Randall, L. L. (1985). *EMBO J.* **4**, 1875–1880.
Iino, T., Takahashi, M., and Sako, T. (1987). *J. Biol. Chem.* **262**, 7412–7417.
Inouye, M., and Halegoua, S. (1980). *CRC Crit. Rev. Biochem.* **7**, 339–371.
Inouye, S., Wang, S., Sekizawa, J., Halegoua, S., and Inouye, M. (1977). *Proc. Natl. Acad. Sci. USA* **74**, 1004–1008.
Inouye, S., Soberon, X., Franceschini, T., Nakamura, K., Itakura, K., and Inouye, M. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 3438–3441.
Inouye, S., Franceschini, T., Sato, M., Itakura, K., and Inouye, M. (1983a). *EMBO J.* **2**, 87–91.
Inouye, S., Hsu, C-P. S., Itakura, K., and Inouye, M. (1983b). *Science* **221**, 59–61.
Inouye, S., Vlasuk, G. P., Hsiung, H., and Inouye, M. (1984). *J. Biol. Chem.* **259**, 3729–3733.
Inouye, S., Duffaud, G., and Inouye, M. (1986). *J. Biol. Chem.* **261**, 10970–10975.
Kadonaga, J. T., Plueckthun, A., and Knowles, J. R. (1985). *J. Biol. Chem.* **260**, 16192–16199.
Kendall, D. A., Bock, S. C., and Kaiser, E. T. (1986). *Nature (London)* **321**, 706–708.
Kuhn, A., and Wickner, W. (1985). *J. Biol. Chem.* **260**, 15914–15918.
Lee, N., Yamagata, H., and Inouye, M. (1983). *J. Bacteriol.* **155**, 407–411.

Lenhardt, S., Pollitt, S. and Inouye, M. (1987). *J. Biol. Chem.* **262**, 1716–1719.
Lehnhardt, S., Pollitt, N. S., Goldstein, J., and Inouye, M. (1988). *J. Biol. Chem.* **263**, 10300–10303.
Li, P., Beckwith, J., and Inouye, H. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 7685–7689.
Lin, J. J. C., Kanazuwa, H., Ozols, J., and Wu, H. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 4891–4895.
Lunn, C. A., and Inouye, M. (1987). *J. Biol. Chem.* **262**, 8318–8324.
Michaelis, S., Inouye, H., Oliver, D., and Beckwith, J. (1983). *J. Bacteriol.* **154**, 366–374.
Michaelis, S., Hunt, J. F., and Beckwith, J. (1986). *J. Bacteriol.* **167**, 160–167.
Nikaido, H., and Wu, H. C. (1984). *Proc. Natl. Acad. Sci. USA* **81**, 1048–1052.
Perlman, D., and Halvorson, H. O. (1983). *J. Mol. Biol.* **167**, 392–409.
Pollitt, N. S., and Inouye, M. (1988). *J. Bacteriol.* **170**, 2051–2055.
Pollitt, S., Inouye, S., and Inouye, M. (1985). *J. Biol. Chem.* **260**, 7965–7969.
Pollitt, S., Inouye, S., and Inouye, M. (1986). *J. Biol Chem.* **261**, 1835–1837.
Pugsley, A. P. (1988). In *Protein Transfer and Organelle Biogenesis* (Das, R. C., and Robbins, P. W., eds.), Academic Press, New York, pp. 607–652.
Puziss, J. W., Fikes, J. D., and Bassford, P. J. (1989). *J. Bacteriol.* **171**, 2303–2311.
Rasmussen, B. A., and Silhavy, T. J. (1987). *Genes Dev.* **1**, 185–196.
Rousset, J. P., Gilson, E., and Hofnung, M. (1986). *J. Mol. Biol.* **191**, 313–320.
Ryan, J. P., and Bassford, P. J. (1985). *J. Biol. Chem.* **260**, 14832–14837.
Ryan, J. P., Duncan, M. C., Bankaitis, V. A., and Bassford, P. J. (1986) *J. Biol. Chem.* **261**, 3389–3395.
Stader, J., Benson, S. A., and Silhavy, T. J. (1986). *J. Biol. Chem.* **261**, 15075–15080.
Vlasuk, G. P., Inouye, S., Ito, H., Itakura, K., and Inouye, M. (1983). *J. Biol. Chem.* **258**, 7141–7148.
Vlasuk, G. P., Inouye, S., and Inouye, M. (1984). *J. Biol. Chem.* **259**, 6195–6200.
von Heijne, G. (1983). *Eur. J. Biochem.* **133**, 17–21.
von Heijne, G. (1986a). *Nucleic Acids Res.* **14**, 4683–4690.
von Heijne, G. (1986b). *J. Mol. Biol.* **192**, 287–290.
Watson, M. E. E. (1984). *Nucleic Acids Res.* **12**, 5145–5164.
Wickner, W. (1979). *Annu. Rev. Biochem.* **48**, 23–45.
Wu, H. C. (1987). In *Bacterial Outer Membranes as Model Systems* (Inouye, M., ed.), Wiley, New York, pp. 37–71.
Yamaguchi, K., Yu, F., and Inouye, M. (1988). *Cell* **53**, 423–432.
Yamane, K., and Mizushima, S. (1988). *J. Biol. Chem.* **263**, 19690–19696.