Effect of Charged Residue Substitutions on the Membrane-Interactive Properties of Signal Sequences of the *Escherichia coli* LamB Protein

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ABSTRACT Although the central role of the signal sequence in protein export is well established, the molecular details underlying signal sequence in vivo function remain unclear. As part of our continuing effort to relate signal sequence phenotypes to specific biophysical properties, we have carried out an extensive characterization of the secondary structure and lipid interactions for a family of peptides corresponding to the wild-type *E. coli* LamB signal sequence, and mutants that harbor charged residue point mutations in the hydrophobic core region. We used membrane-resident fluorescence quenching according to the parallax method to determine the relative depth of insertion of tryptophan-labeled analogs of these peptides into the acyl chain region of bilayer vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol. Also, restriction of acyl chain motion upon peptide binding was evaluated using steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. Each of these peptides showed evidence of insertion into the acyl chain region, although most likely not in a transmembrane orientation. The mutant peptides were shown to have a reduced insertion potential relative to the wild-type peptide. Furthermore, tryptophan spectral properties indicated that insertion of the wild-type and mutant peptides enhances bilayer hydration. This effect was particularly pronounced with peptides harboring negatively charged aspartate point substitutions. The results are discussed in relation to the potential roles of signal sequences in mediating protein translocation.

INTRODUCTION

The leader (or signal) sequence constitutes the most universal requirement for protein export from the cytosol in bacteria and eukaryotes. Biochemical and genetic studies have demonstrated that both the "hydrophobic core" region and net N-terminal basicity are required for export activity (for recent reviews see Gierasch, 1989; Jones et al., 1990; Gennity et al., 1990). Although the precise mechanism by which signal sequences mediate protein export remains unknown, they are postulated to interact with soluble and membrane-bound proteinaceous components of an energy-utilizing translocation apparatus. In *Escherichia coli*, several studies support direct interaction of the signal sequence with the peripheral membrane ATPase SecA (Bankaitis and Bassford, 1985; Puziss et al., 1989; Akita et al., 1990) as well as with the integral SecY/E protein complex (Emr et al., 1981; Ito, 1984). These proteins are postulated to act in unison as components of the "translocon" (Brundage et al., 1990).

The presence of the hydrophobic core region, along with net positive charge, suggests interaction of signal sequences with membrane phospholipid. Several models for export envision signal peptide insertion mediating export directly through the lipid phase (von Heijne and Blomberg, 1979; Wickner, 1980). M13 procoat (Wolfe et al., 1985) and honeybee prepromelittin (Colet et al., 1989) have indeed been shown to translocate in the absence of protein factors. However, for the vast majority of cases, there is an absolute dependence on proteinaceous components for proper export. Nonetheless, signal sequence insertion into the lipid portion of the membrane may be necessary for the signal sequence to gain access to a binding site on an integral membrane protein. Also, by its high affinity for the cytoplasmic membrane, the signal sequence may restrict a secretory protein complex to two-dimensional diffusion at the membrane before interaction with the putative translocon. Regardless of the specific mechanism of translocation, the signal sequence is likely to interact with membrane lipid during the transport process and, thus, it is necessary to understand signal sequence-lipid interactions to describe adequately the energetics of the export process.

Previous studies from this laboratory showed that peptides derived from functional signal sequences of the *E. coli* LamB (Briggs et al., 1985; McKnight et al., 1989, 1991; Wang et al., 1993) and OmpA (Hoyt and Gierasch, 1991a, b; Rizo et al., 1993) proteins share the ability to adopt high proportions of α-helix in membrane-mimetic environments and to insert into the acyl chain region of model membranes. Similar results have been obtained by de Kruijff and co-workers for the *E. coli* PhoE signal peptide (Batenburg et al., 1988a, b; Killian et al., 1990). In the present study, we have examined the structure and lipid interactions of a family of signal

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Abbreviations used: t-Boc, tert-butyloxycarbonyl; CD, circular dichroism; DPH, 1,6-diphenyl-1,3,5-hexatriene; 5- or 12-doxylPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; Fmoc, 9-fluorenylmethoxycarbonyl; LUV, large unilamellar vesicle; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TLC, thin layer chromatography.

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peptides corresponding to mutated mutant LamB signal sequences that harbor arginine (Arg) and aspartate (Asp) residues in the hydrophobic core region. The in vivo export activities of these sequences have been determined by Stader et al. (1986). They found that export activity depends significantly on both the nature and position of the charged residue (see Results). We have used fluorescence spectroscopy to carry out a detailed analysis of the interaction of this signal peptide family with bilayer vesicles. These studies address the following questions: 1) What is the nature of the association of these point mutant peptides with model membranes? Do they share the ability of the wild-type sequence to insert into the lipid acyl chain region, or are they restricted to the lipid interfacial region? 2) Where is the region of the peptide bearing the charged residue located upon membrane interaction? Are the charged mutant peptides able to insert significantly into the bilayer? 4) How does binding/insertion of these peptides affect lipid properties such as bilayer hydration and lipid dynamics? Answers to these questions are essential to develop a molecular model describing the potential membrane interaction of these signal sequences during protein export.

Fluorescence quenching measurements using membrane-resident quenchers were carried out on this peptide family as well as on model surface-bound and transbilayer peptides. Also, steady-state anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was evaluated for the LamB WT and transbilayer peptides. The results indicate a relatively deep insertion of the WT signal peptide into the lipid acyl chain region. However, comparison of the fluorescence properties of the LamB signal peptide to those of the model peptides suggests that the signal peptide most likely does not span the bilayer in a stable fashion. The mutant peptides all have reduced insertion potential relative to the WT peptide. The mode of lipid binding of these peptides is described in terms of a model in which the signal peptides insert partially into the bilayer acyl chain region, with differing average insertion depths. We postulate that a reduced ability to insert into the lipid hydrocarbon region is in part responsible for the decreased in vivo export activity of the charged mutant peptides. In an accompanying manuscript, we describe the thermodynamics of interaction of this signal peptide family with bilayer vesicles. We show that the net hydrophobic component of binding is significantly less than that expected from theoretical estimates, which is consistent with a substantial energy cost associated both with burying polar residues and with possible lipid perturbation effects. These results collectively yield a quantitative description of signal peptide-lipid interactions and thus provide insight into potential in vivo lipid interactions. Furthermore, a molecular description of the action of integral protein factors, which may function to modify the energetics of the signal sequence/secretory protein-lipid interaction, is developed based on our results.

MATERIALS AND METHODS

Materials

All phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Lipid purity was routinely checked by thin layer chromatography (TLC) in $CHCl_3$/MeOH/H_2O (65:35:5) and visualized with primulin spray.

Synthesis and purification of peptides

Peptides were synthesized via standard methodology using either N-terminal t-Boc protected amino acids (Erickson and Merrifield, 1976; Barany and Merrifield, 1979) or N-terminal Fmoc-protected amino acids (Dryland and Sheppard, 1986). Peptides were deprotected and cleaved from the resin using either anhydrous HF (t-Boc) or TFA (Fmoc). Peptides were purified on Vydac C_4 and/or phenyl columns eluted with acetonitrile/water gradients containing 0.1% TFA. Amino acid content and sequence were verified by quantitative amino acid analysis and peptide sequencing.

Preparation of vesicles

All experiments were carried out with large unilamellar vesicles (LUVs) prepared in the following manner. Appropriate amounts of the desired lipids were initially co-solubilized in $CHCl_3$, and the solvent was removed by a slow nitrogen purge followed by drying under vacuum for at least 4 h. The resulting lipid film was hydrated with a buffered solution. LUVs were prepared by the freeze-thaw extrusion method as described by Mayer et al. (1986) using 0.1-μM filters. This procedure yields vesicles of approximately 90 nm in diameter. Lipid phosphate concentrations were determined as described by Bartlett (1959). Unless otherwise noted, vesicle composition was 65 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 35 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPE/POPG 65:35 mol%).

Fluorescence measurements

Fluorescence experiments were performed on a steady-state, photon-counting spectrofluorimeter (Model Greg PC from ISS Inc., Champaign, IL) operating in the ratio mode. Uncorrected tryptophan (Trp) emission spectra (excitation 280 nm) were recorded in a 1 cm quartz cuvette, with continuous stirring of the solution. Correction was made for excitation light scattering by subtracting background spectra. Where necessary, corrections for emission scattering effects were made by analysis of pure Trp spectra. All measurements were carried out at 25 ± 0.2°C.

Nitroxide quenching experiments

To perform studies with a membrane-resident quencher, vesicles containing 20 mol% of either 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine (5- or 12-doxylPC) were prepared. Control vesicles contained 20 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). I_o/I values were determined by comparing the intensity at the emission maximum in the quencher-containing and the control vesicles. Quenching data were analyzed to yield peptide penetration depths as described in Results.

Steady-state fluorescence anisotropy

Anisotropy was measured using the L-format with excitation and emission wavelengths of 360 and 430 nm, respectively. A vesicle solution containing 0.5 mM lipid was doped with 1,6-diphenyl-1,3,5-hexatriene (DPH; dissolved in tetrahydrofuran) to give a DPH/lipid ratio of approximately 1:400. The sample was equilibrated for 30 min before initial measurement. Subsequent measurements were recorded after addition of peptides as indicated in Results. Parallel and perpendicular intensities were used to calculate anisotropy according to the standard relationship:

$$r = (I_\parallel - I_\perp)\{I_\parallel + 2I_\perp\}^{-1}.$$

Light scattering was subtracted by using reference samples with no DPH.

RESULTS

Peptides studied

The in vivo export activity of the family of LamB signal sequences containing charged residues has been characterized in detail by Stader et al. (1986). Substitution of an Asp

residue in the hydrophobic core at position 13 (A13D) caused a severe export defect, whereas this residue substituted for glycine at position 17 (G17D) yielded near wild-type export activity. An Arg residue at position 17 (G17R) resulted in a kinetic defect in export, which is less severe than that for A13D. These results suggest that substitution of a charged residue in the central region of the core inhibits export more strongly than placement of a charge at the core periphery. Also, introduction of a positively charged residue in the core region reduces export efficiency to a greater degree than a negatively charged residue, based on results at position 17.

The signal peptides selected for study are shown in Table 1 with their relative export activities. The A13R and M24D variants have not been characterized in vivo. A13R was designed to complement studies with G17R, and the M24D variant was designed as a control to distinguish the effects of placing a negatively charged residue in the core versus changing the bulk electrostatic properties of the peptide. All signal peptides were prepared with Trp substituted for valine at position 18 for fluorescence studies. The Trp18 variant has wild-type export activity in vivo (D. Jackson and T. Silhavy, personal communication). The WT and WT-AM peptides were also studied with Trp substituted at position 24 instead of 18 to monitor the behavior of the C-terminal region (this construct is lethal in vivo, most likely because of problems with signal peptidase cleavage; see McKnight et al., 1991). The TM peptide was designed to serve as an idealized transmembrane peptide. The five N-terminal lysine residues were added to enhance solubility of this highly hydrophobic sequence. This peptide was prepared with Trp substitutions at the 6, 15, and 25 positions to model fluorescence properties of both inserted and interfacial Trp residues. The KWK-AM peptide was designed as a control for analysis of the fluorescence properties of a surface-bound peptide, which has net charge equivalent to that of the N-terminal region of the signal peptides, but is not expected to insert deeply into the bilayer.

It is evident from the composition of this signal peptide family that substantial complexity is to be expected regarding their interaction with phospholipid vesicles. The charged residue substitutions result in changes in both the electrostatic and hydrophobic components of lipid binding. This peptide family, therefore, offers a rich system for addressing the relative contribution of various forces to the interaction of these peptides with lipid vesicles.

Properties of peptide-vesicle complexes

The wild-type (WT) and charged mutant peptides do not show significant differences in their ability to adopt high proportions of α-helix in membrane-mimetic environments (see Discussion and McKnight et al., 1989). Therefore, because the different in vivo activities of the WT and point mutant signal sequences probably do not arise from altered conformational tendencies, we examined whether insertion into a phospholipid membrane is affected when a charged residue is introduced in the core region. We used fluorescence spectroscopy employing the Trp-labeled versions of the peptides to characterize the relative insertion potential of these sequences into POPE/POPG (65:35 mol%) LUVs. All experiments reported in this study were carried out under conditions where peptides are quantitatively bound to vesicles (at least 90% binding based on binding affinities reported in the companion paper).

The Trp emission spectrum is sensitive to hydration, with the emission maximum moving to a lower wavelength (blue-shift) upon displacement from aqueous to hydrophobic environments (Lakowicz, 1983). Thus, peptide binding to lipid vesicles can be monitored by the resulting blue shift (Surewicz and Epand, 1984). This phenomenon is illustrated by the emission spectra of WT-AM18W in aqueous buffer and in vesicles shown in Fig. 1. The Trp emission maximum shifts from the solution value of 356 to 322 nm in vesicles under low ionic strength conditions (see below for discussion of ionic strength effects on peptide-vesicle complexes). This relatively large blue-shift indicates that the Trp-labeled region of the peptide penetrates into the lipid acyl chain region. In addition, Trp displacement from the aqueous phase to the lipid interior results in a significant intensity increase, which can arise from changes in both absorptivity and quantum yield (Markello et al., 1985).

TABLE 1 Peptide sequences studied

		Activity
Export characterized signal peptides		
WT	M M I T L R K L P L A V A V A A G V M S A Q A M A	+++++
WT-AM	M M I T L R K L P L A V A V A A G V M S A Q A M Aa	++++
G17R	M M I T L R K L P L A V A V A A **R** V M S A Q A M A	++
A13D	M M I T L R K L P L A V **D** V A A G V M S A Q A M A	+
G17D	M M I T L R K L P L A V A V A A **D** M S A Q A M A	++++
Control peptides		
KWK-AM	K W Ka	
A13R	M M I T L R K L P L A V **R** V A A G V M S A Q A M A	
M24D	M M I T L R K L P L A V A V A A G V M S A Q A **D** A	
TM-AM	K K K K K A L A L A L A L A L A L A L A L A L A La	

The signal peptides all harbor a tryptophan for valine substitution at position 18 for fluorescence studies. WT and WT-AM were also prepared with tryptophan replacing methionine at position 24. The TM peptide was synthesized with tryptophan at positions 6, 15, and 25 for fluorescence studies. Relative export activities are estimated based on data from Stader et al. (1986).

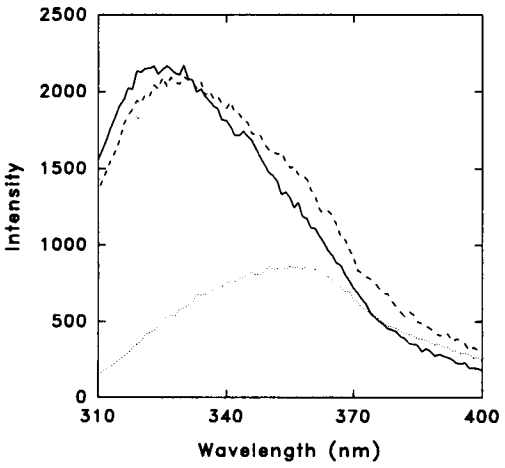

FIGURE 1 Uncorrected emission spectra of WT-AM18W under the following conditions: 5 mM Tris, pH 7.3 (·····); 5 mM Tris, pH 7.3; 0.5 mM POPE/POPG 65:35 mol% LUV (——); 5 mM Tris, pH 7.3, 0.1 M NaCl; 2.0 mM POPE/POPG 65:35 mol% LUV (- - - -). Peptide concentration is 5 μM in each case.

In principle, blue-shifts can be used to compare the relative insertion depths of a family of peptides, because a deeply buried Trp is expected to yield a larger blue-shift than an interfacial Trp residue. However, as discussed more fully below, changes in bilayer hydration upon peptide insertion may also influence the magnitude of the blue-shift and, therefore, caution must be applied in inferring the relative position of Trp residues in the bilayer.

The relatively large blue-shifts observed for WT-AM18W, WT18W, A13R18W, and G17R18W (Table 2) suggest that these peptides insert relatively deeply in the bilayer. However, the result for KWK-AM (blue-shift of 22 nm) indicates that surface binding can cause a significant blue-shift. In contrast to these results, the core Asp mutants show substantially reduced blue-shifts, which indicates a more hydrated environment for the Trps in these peptides. This result is consistent with either reduced insertion or increased bilayer hydration concomitant with binding and/or insertion of these peptides. The relatively large blue-shift obtained for the M24D18W peptide demonstrates that placement of the negatively charged residue in the hydrophobic core is responsible for the reduced blue-shift at the Trp 18 position, as opposed to a general electrostatic repulsion arising from the presence of an additional negatively charged residue in the peptide.

TABLE 2 Tryptophan emission maxima and blue-shifts for vesicle-bound peptides under low ionic strength conditions

Peptide	Vesicle λ_{max} (nm)	Vesicle blue-shift* (nm)
WT-AM18W	323	33
WT18W	326	30
A13R18W	332	24
G17R18W	332	24
A13D18W	350	6
G17D18W	347	9
M24D18W	328	28
WT24W	350	6
WT-AM24W	337	19
KWK-AM	334	22
TM-AM6W	323	33‡
TM-AM15W	311	45‡
TM-AM25W	325	21‡

Conditions are as follows: vesicle composition 65%/35% POPE/POPG prepared in 5 mM Tris, pH 7.3. Uncertainty levels are approximately ±2 nm.
* Blue-shifts for the Trp residues on the signal peptides and KWK-AM are given from the solution emission maximum (356 nm).
‡ The Trp residues on the transmembrane peptide analogs show blue-shifts from the observed maximum of 356 obtained for the other peptides (and free Trp) in aqueous solution. The λ_{max}s in solution are as follows: TM-AM15W; TM-AM5W, 337; and TM-AM25W, 353. The blue-shift values given above are referenced to 356 nm for the purpose of quantitative comparison to the signal peptides.

Reduced blue-shifts for the core Asp mutants could result from enhanced bilayer hydration, which is likely necessary to achieve an intrabilayer proton activity high enough to protonate the Asp carboxyl group upon interaction with acidic vesicles. Conversely, ion pairing between negatively charged PG head groups and protonated amines can satisfy charge-charge interactions for the Arg mutants and, thus, a smaller effect on bilayer hydration is expected. This hypothesis was supported in experiments in which the pH was lowered. At pH 5.0 (5 mM sodium acetate), G17D18W exhibited a 30 nm blue-shift and A13D18W showed 23 nm. At pH 4.5, the blue-shift of A13D18W was further increased to 28 nm. Thus, the driving force for increased bilayer hydration is minimized when the bulk proton concentration is increased. Detailed analysis of the pH dependence is complicated by the potential for protonation of the free carboxy terminus. Future studies on the same peptide with an amidated C-terminus are necessary to assess quantitatively the pH dependence of binding and bilayer insertion properties. Nonetheless, this pH dependence, in conjunction with the result obtained for M24D18W, strongly suggests that increased bilayer hydration upon insertion of the core Asp mutants results in reduced blue-shifts. Our results do rule out low blue-shifts arising from specific Asp-Trp interactions, or from oligomerization of Asp-containing peptides in the membrane. However, the bilayer hydration hypothesis is also supported by comparison of blue-shift and quenching results obtained for the WT and transmembrane peptides (see below).

The WT versions of the peptide were also Trp-labeled at position 24 to monitor insertion of the C-terminus (McKnight et al., 1991). Again, the low blue-shift (6 nm) for WT24W could arise from either an interfacial environment for this residue or increased bilayer hydration caused by the proximity of the free carboxyl group. The value of 19 nm for WT-AM24W indicates that a substantial blue-shift is restored upon amidation of the C-terminus, although this value remains lower than those observed for WT and WT-AM labeled with Trp at position 18.

The large blue-shift for the model transmembrane peptide TM-AM15W (45 nm) indicates that this peptide samples an average environment that is much more hydrophobic than that of the signal peptides (blue-shifts referenced to the theoretical solution value, Table 2). Thus, TM-AM15W occupies

a more deeply buried position in the bilayer, or insertion of this peptide does not increase bilayer hydration to the same degree as the signal peptides. The blue-shift of 33 nm for the TM-AM6W peptide is identical to that observed previously for the LamB5W peptide (McKnight et al., 1991), which suggests that the N-terminal regions of these peptides occupy similar environments in the bilayer. Placement of the Trp residue adjacent to lysine residues may result in an enhanced blue-shift, because of a local effect of the hydrophobic region of the lysine side chains. Also, it is reasonable to assume, in view of the results with the core Asp mutants, that decreased hydration occurring along with electrostatic complex formation may also enhance blue-shifts. This hypothesis is supported by the observation of a 22-nm blue-shift for KWK-AM. The blue-shift of 21 nm for the TM-AM25W is identical within experimental error to that obtained for WT-AM24W and suggests that Trps adjacent to free amide groups may sample similar environments.

The transmembrane peptide has proven extremely useful in the present study as a membrane-active model peptide. The peptide readily binds vesicles and demonstrates specific properties that reveal the environment at the position of the Trp label. The Trp-labeled versions of this peptide exhibited blue-shifts in solution that varied depending on the position of the Trp label. Solution blue-shifts were previously observed for single Trp-containing lipophilic alamethicin analog peptides (Voges et al., 1987). Also, this peptide adopts substantial α-helical content in aqueous buffer (not shown). This highly hydrophobic sequence may form micellar aggregates in solution, or it is possible that the relatively long, continuous hydrophobic segment induces α-helical structure and, thus, solution blue-shifts arise from intramolecular effects. Further studies are necessary to determine the solution behavior of this model peptide. However, ESR spectroscopy results indicate that the peptide adopts a monomeric transmembrane orientation when vesicle-bound (Sankaram and Jones, 1994). This conclusion is supported by fluorescence quenching results in the current study (see below). Thus, if solution aggregates form, the peptide must be in rapid equilibrium between monomers and micelle-bound forms and, therefore, peptide monomers associate with added lipid vesicles.

Depth-dependent quenching studies

Analysis of blue-shifts suggests that the spectral properties of Trps in vesicle-bound peptides are a complex function of peptide insertion and peptide-induced bilayer hydration. These data, therefore, allow little to be concluded with regard to specifics of peptide topology. Thus, membrane-resident quenching using nitroxide-labeled lipids was carried out to determine the position of the Trp residue in the bilayer. Procedures have been developed (Chattopadhyay and London, 1987; Chattopadhyay, 1990; Abrams and London, 1992) to determine the depth of insertion of a fluorescent probe from the difference in quenching by two quenchers located at different vertical positions in the bilayer, i.e., using the degree of parallax of membrane-resident quenching. This method has been used previously to locate Trp residues in several membrane-associated peptides and proteins (Voges et al., 1987; Meers, 1990; Clague et al., 1991; Jiang et al., 1991; Chattopadhyay and McNamee, 1991; Chung et al., 1992). Data were analyzed according to the procedure described by Chattopadhyay and London (1987) to obtain the average position of a fluorescent label. A more complex data treatment, which considers probe and quencher movements, is given by Abrams and London (1992). However, in the absence of a suitable description of peptide dynamics, comparison of different peptides by these procedures would be highly speculative. Furthermore, Abrams and London (1992) concluded that these dynamic effects typically had little effect on average insertion depths of the systems studied. Average values are thus determined for the present system. The equation relating observed quenching values to probe depth (Z_{1F}), under the assumption of *cis* quenching, i.e., a probe is quenched only by quenchers in the same bilayer leaflet, is

$$Z_{1F} = \frac{(1/-\pi C)\ln(F_1/F_2) - L_{21}^2}{2L_{21}},$$

where C is the mole fraction of quencher divided by lipid area, F_1 and F_2 are fluorescence intensities in the presence of the shallower and deeper quencher, respectively, and L_{21} is the difference in depth of the two quenchers. The distance from the center of the bilayer (z_{cF}) is given by $z_{cF} = z_{1F} + L_{cl}$, where L_{cl} represents the distance from the bilayer center to the shallow quencher. Probe insertion depth (Trp in our case) is then given by (bilayer thickness/2) $- z_{cF}$.

The penetration depths given in Table 3 indicate that the Trp residues of WT18W and WT-AM18W peptides insert

TABLE 3 Nitroxide quenching of peptides under low ionic strength conditions

Peptide	I_0/I 5	I_0/I 12	I_0/I 12/5	Depth (Å)*
WT-AM18W	2.13	3.27	1.54 ± 0.19	10.0
WT18W	1.92	2.68	1.40 ± 0.13	9.0
A13R18W	2.41	2.28	0.95 ± 0.11	5.5
G17R18W	2.50	2.20	0.88 ± 0.13	5.0
A13D18W	1.19	1.30	1.09 ± 0.07	7.0
G17D18W	1.34	1.48	1.10 ± 0.04	7.0
M24D18W	2.74	2.39	0.87 ± 0.02	5.0
KWK-AM	3.16	2.02	0.64 ± 0.07	2.0
TM-AM6W	2.14	1.61	0.75 ± 0.01	3.5
TM-AM15W	1.88	2.40	1.28 ± 0.07	8.0
TM-AM25W	2.40	1.88	0.78 ± 0.10	4.0
WT-AM24W	2.56	3.15	1.23 ± 0.03	8.0
WT24W	1.74	2.18	1.25 ± 0.02	8.0

Peptide and vesicle lipid concentrations were 5 μM and 1 mM, respectively. Errors are given as SDs from at least duplicate experiments and range from less than 5 to approximately 15%. These errors in 12:5 ratios correspond to average depth errors of approximately 0.5 Å.

* Values are given as depths of penetration into the bilayer acyl-chain region. These distances are calculated according to the treatment described in the results section, assuming the following parameters: diameter of the bilayer hydrocarbon region, 30 Å; lipid area, 70 Å^2; L_{21}, 6.30 Å; L_{cl}, 12.15 Å.

most deeply into vesicles (9 and 10 Å, respectively), with KWK-AM showing the expected interfacial binding (2 Å). The Trp labels on the Arg and Asp mutants all show significant penetration into the bilayer interior. This result supports the assertion that the low blue-shift observed for the core Asp mutants is caused by increased bilayer hydration upon insertion, rather than by an interfacial location of these peptides. Results for the different TM peptide analogs with the Trp residue near the termini and in the core region strongly support a transmembrane orientation for this peptide, because the Trps on the TM-AM6W and TM-AM25W peptides are near the interface and that of TM-AM15W is well inserted. One would ideally expect 15W to lie closer to the center of the bilayer (depth near 15 Å) for a transmembrane peptide. However, because of uncertainties inherent in calculation of Trp insertion depth, and the difficulty in concluding peptide backbone position from this measurement, interpretation of Trp insertion data beyond "interfacial" and "well buried" is speculative (see below and Discussion). As given above, these results are calculated using the *cis* (same leaflet) quenching model. If opposite leaflet quenching is considered by using the *trans* quenching model (Chattopadhyay and London, 1987) with a critical separation distance (R_c) of 11 Å, results for WT18W and WT-AM18W were within experimental error of the values in Table 3. Thus, it is not necessary to consider *trans* quenching for any of the peptides studied.

Results for the point mutants are consistent with their occupying a partially inserted configuration, with the charged group of the Asp and Arg residues near the interface (see Discussion and Fig. 3). Insertion depths of the Trps on the WT18W (and WT-AM18W) peptide(s) are consistent, however, with either partial insertion or an average penetration of the Trp residue that is deeper than those in the charged mutants, or with a transmembrane configuration. Data at the 24 position, however, suggest a relatively deep penetration of the C-terminal region for both WT-AM24W and WT24W. This result is not consistent with a linear, stable transmembrane orientation of the peptide, with the C-terminus near the *trans* interface. These data do not rule out, however, partial peptide insertion with the C-teminus extending into the *trans* leaflet (see Discussion).

The errors given in Table 3 reflect estimations of random error. Systematic errors arising from inaccuracies in estimating quencher position and/or local concentration of doxyl-labeled lipids in the quaternary system (POPE, POPG, doxyl-lipid, peptide) may also contribute to the results. The bulk concentration of doxyl lipids was verified by analyzing penetration depth of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) in POPC vesicles; results agreed with those previously reported (Chattopadhyay and London, 1987) within ±10–15%. Perturbation of quencher position by inserted peptides is of course very possible. Likewise, the local concentration of the doxyl-labeled PC lipid in the vicinity of the peptide may not precisely reflect its bulk concentration in the vesicles. These potential problems, in addition to the uncertainty imposed by the length of the Trp group, demand that caution be applied in drawing absolute peptide penetration depths from these data. However, the fact that this method clearly identifies KWK-AM as interfacial, and yields generally expected penetration depths for the different regions of the TM peptide, demonstrates the reliability of these results for determination of approximate insertion potential of the various signal peptides.

It should be noted that the absolute magnitudes of the quenching at a given position vary substantially. In particular, quenching values for the core Asp mutants are much lower than for the other peptides. Potentially reduced lifetimes for the Trp residues in these peptides should not affect quenching, because dynamic quenching is negligible for membrane-resident nitroxides (London and Feigenson, 1981; London, 1982). It is possible that the quenching of Trps on the core Asp mutants, by virtue of local hydration effects, is characterized by a lower critical separation between probe and quencher (reduced R_c value; see Chattopadhyay and London, 1987) than those typically observed, or that quenching does not follow an "all or none" mechanism. However, the parallax treatment has been shown to apply for static quenching with realistic distance dependencies (Chattopadhyay and London, 1987). Thus, although analysis of Trp lifetimes would be necessary to rule out dynamic quenching definitely, we are confident that this treatment is valid for this system.

Peptide effects on lipid order

To provide additional information on the topology of vesicle-localized peptides, the effect of peptide addition on lipid acyl chain motion was examined by measuring steady-state DPH anisotropy. Although time-resolved measurements are required to distinguish changes in rate of motion (dynamics) from changes in lipid order (amplitude of motion) upon addition of a perturbant, steady-state measurements most frequently reflect the order term (for review, see Lentz, 1989). Thus, we will use the term "lipid order" to discuss changes in acyl chain motion upon peptide addition. The integral membrane protein cytochrome *c* oxidase was shown to induce an increase in DPH anisotropy upon insertion into lipid (Rigell et al., 1985). Also, results from this laboratory showed that insertion of the OmpA WT signal peptide (Hoyt and Gierasch, 1991b) induced a marked increase in DPH anisotropy in bilayers, where two nonfunctional mutant peptides showed no effect on anisotropy. Trp fluorescence results (Hoyt and Gierasch, 1991a) and ESR spectral analysis (Sankaram and Jones, 1994) support a transmembrane orientation for the OmpA WT peptide, and restricted insertion for the mutants. These results suggest that insertion of transmembrane polypeptides restricts acyl chain motion in the bilayer interior and, thus, lipid order measurements represent a potential method to determine the mode of peptide-lipid interactions.

Results shown in Fig. 2 indicate that the model TM-AM peptide (TM-AM6W) induces a significant increase in DPH anisotropy at P/L ratios of approximately 1:100. This result

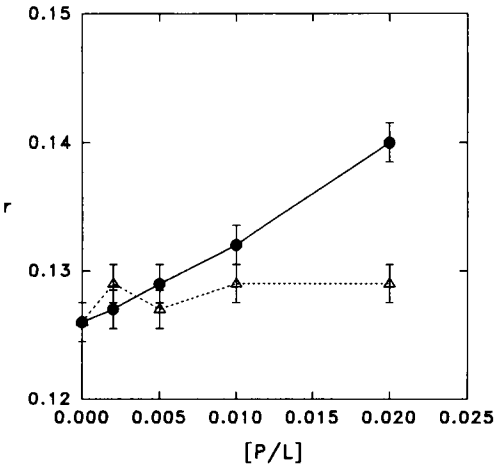

FIGURE 2 DPH anisotropy as a function of peptide/lipid ratio for: WT-AM18W (△- - -); TM6W (●——). Vesicle lipid concentration is 0.5 mM.

is consistent with the expected transmembrane orientation of this peptide. In contrast, no significant change in anisotropy is observed for the WT-AM18W peptide up to P/L ratios of 1:50. Similar results were obtained for the other LamB signal peptides (data not shown). Hence, this signal peptide family does not enhance lipid order upon binding, as evidenced by the DPH anisotropy method. Lack of acyl chain ordering by these peptides argues against deep insertion into the bilayer center. Thus, although quenching results suggest insertion of these peptides into the membrane apolar region, the DPH anisotropy results do not support a transmembrane arrangement for this signal peptide family.

Ionic strength effects on peptide-vesicle complexes

We have demonstrated that increasing ionic strength markedly reduces the affinity of most of the peptides examined in this study for these vesicles (see companion paper). However, increasing salt concentration was not observed to have a substantial effect on the mode of peptide-lipid interactions. The emission spectrum of WT-AM18W in vesicles at 0.1 M NaCl (Fig. 1) is similar to that obtained under low ionic strength conditions. The magnitude of the blue-shift is slightly reduced, and there is slight broadening of the spectrum near the emission maximum. This result was also obtained for A13R18W, G17R18W, and M24D18W, with blue-shifts approximately 3–4 nm less than those observed at low ionic strength. This decrease in blue-shift of vesicle-associated peptides at high ionic strength may reflect an alteration in bilayer hydration with changes in salt concentration, rather than a significant effect on the potential of the peptide to insert into the lipid acyl chain region (see below).

The depth of bilayer insertion for the Trp residue in WT18W and the Arg mutants at high ionic strength was monitored by carrying out membrane-resident quenching using the doxyl-labeled lipids as described above (Table 4).

TABLE 4 Nitroxide quenching of peptides under high salt conditions

Peptide	I_o/I 5	I_o/I 12	I_o/I 12/5	Depth (Å)
WT18W	1.79	2.08	1.16	7.5
A13R18W	2.14	2.18	1.02	6.0
G17R18W	2.30	2.34	1.02	6.0

Peptide and vesicle lipid concentrations were 10 μM and 2 mM, respectively. Experiments were carried out at 25°C in 5 mM Tris, pH 7.3; 0.1 M NaCl. Uncertainty levels are similar to those given in Table 3.
* Penetration depths are calculated as described in Results section assuming the parameters given under Table 3.

The value obtained for WT18W (7.5 Å) indicates insertion into the acyl chain region. Given the limitations of the parallax method described above, it is difficult to distinguish this value from the insertion depth obtained at low salt (10.0 Å). Also, the derived penetration depths for the G17R18W and A13R18W were very similar to those obtained at low salt, which indicates that the presence of 0.1 M NaCl does not significantly reduce the insertion depth for the Trps on these peptides. These results indicate that increased ionic strength does not substantially inhibit peptide penetration and, thus, does not alter the mode of interaction of the peptides with vesicles.

DISCUSSION

The primary structural motif of signal sequences, a basic N-terminal region followed by a relatively short (10–12 residue) hydrophobic core region, is distinct from membrane-interactive transbilayer and amphipathic peptides. The hydrophobic region of signal peptides is too short to span the bilayer in an α-helical conformation (about 20 residues of α-helix are necessary to span the hydrocarbon region of the bilayer (30 Å)). However, the highly hydrophobic nature of signal peptides represents a significant driving force for insertion into the bilayer acyl chain region. Our analysis of the membrane-interactive properties of the WT LamB signal peptide, along with the charged mutant peptides, is thus aimed at defining features of peptide-lipid interactions characteristic of these unique sequences as well as yielding insight into phenotypic differences exhibited among this signal sequence family in protein export.

Our results indicate that the WT peptide inserts into the bilayer acyl chain region. However, comparison of LamB WT and the model surface-bound and transbilayer peptides in terms of membrane-resident quenching and effects on lipid order does not support a stable transmembrane arrangement for WT. Rather, the peptide probably inserts only partially into the bilayer hydrocarbon region. The charged mutant peptides also show insertion into the bilayer interior, although their average penetration depth is somewhat reduced relative to WT. Furthermore, no significant differences in membrane interactive properties were observed between

peptides with a given charged residue at either the 13 or 17 positions. We will discuss this model in terms of peptide-lipid interactions and in vivo function of signal sequences.

Peptide orientation in the bilayer

Model peptide topography

Our model transmembrane peptide is designed along the lines of previously studied transmembrane peptides, which have a contiguous hydrophobic stretch of approximately 20 residues, with basic residues at the termini (Davis et al., 1982; Vogel et al., 1988; Bolen and Holloway, 1990; Zhang et al., 1991). Our TM peptide was synthesized, however, with the basic residues only at the N-terminus to most appropriately mimic insertion of the signal peptides, when added exogenously to pre-formed vesicles. The uneven charge distributions on both the TM-AM and signal peptides should kinetically bias insertion toward more rapid insertion of the relatively nonpolar C-terminus than the polybasic N-terminus. At equilibrium, the peptide termini will be symmetrically distributed with respect to the vesicle inner and outer surfaces, regardless of specific orientation adopted. However, we most likely observe a kinetically trapped state in terms of transleaflet equilibration, with the N-terminus located on the *cis* side of the vesicle. Model transmembrane peptides with basic residues only at the N-terminus have been shown previously to incorporate into phosphatidylcholine liposomes as stable transbilayer α-helices (Katakai et al., 1990).

Results with the membrane-resident quenchers permit approximate determination of the penetration depth of the monitored Trp residue. The depth is given as the distance from the top of the bilayer hydrocarbon region (hydrocarbon distance of leaflet 15 Å) to the center of the fluorescent indole group on Trp. The depth of 2.0 Å for the KWK-AM peptide is consistent with surface binding caused by electrostatic interaction. As discussed by Jacobs and White (1989), Trp is a relatively large group (about 8 Å) that can penetrate significantly without the peptide backbone entering the acyl chain region. Nonetheless, this result indicates that the parallax method accurately reflects the expected interfacial localization of this model peptide. Conversely, penetration depths for the model transbilayer peptide indicate that the central portion is well buried in the acyl chain region of the bilayer (depth of 8 Å for TM-AM15W), with the termini located near the bilayer interface (depths of 3.5 and 4.0 Å for TM-AM6W and TM-AM25W, respectively). These results are consistent with the presumed transbilayer orientation of this peptide. A model in which the peptide adopts a loop structure with both termini on the same face of the bilayer cannot be ruled out from these data. However, this orientation is highly unlikely on theoretical grounds in view of the large unfavorable energy associated with breaking backbone hydrogen bonds concomitant with reverse turn formation in the hydrophobic region of the bilayer (Jacobs and White, 1989). The observation of a high proportion of α-helix (about 75%) in membrane-mimetic environments argues against a turn in the bilayer interior and, thus, strongly supports a linear, transmembrane orientation for this peptide. This conclusion is also supported by the enhancement of lipid acyl chain order reflected by deeply buried probes (DPH- and doxyl-labeled lipids (Sankaram and Jones, 1994)) upon insertion of the TM-AM peptide.

A schematic illustration of our model for the interaction of TM-AM with POPE-POPG vesicles is given in Fig. 3. The peptide is shown spanning the bilayer as a transbilayer α-helix, with the basic N-terminal region interacting with the lipid headgroups. The specific geometry adopted by the peptide will enable maximum incorporation of hydrophobic side chains into the bilayer interior. The tilt angle illustrated reflects the possibility that an arrangement that is not normal to the plane of the bilayer may be most compatible with this condition. Indeed, transmembrane domains of integral membrane proteins are often observed to be tilted with respect to the bilayer normal (Henderson et al., 1990; Deisenhofer and Michel, 1991). Also, it is quite possible that the C-terminus confers some potential for "bobbing" in the bilayer, because of its relatively nonpolar nature. A tilted orientation may in part (along with specific Trp effects, discussed below) account for the fact that the Trp on TM-AM15W is not found in the bilayer center. The pentalysine N-terminal region is, of course, expected to remain anchored near the bilayer in-

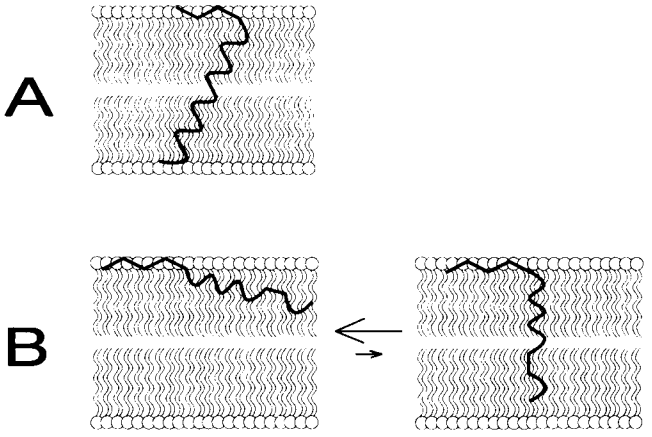

FIGURE 3 Schematic model of the topography for peptides bound to POPE/POPG vesicles. (*A*) Transmembrane (TM) peptide: this peptide is shown with the hydrophobic $(AL)_{10}$ segment adopting a transbilayer α-helical arrangement. We speculate that the peptide is most likely tilted with respect to the bilayer normal (see text). The basic N-terminal region is presumably tightly anchored to the bilayer headgroups via electrostatic interaction with POPG molecules. (*B*) LamB signal peptides: the predominant mode of bilayer interaction for these peptides is depicted as partial insertion into the *cis* leaflet (see text for specific penetration depths for the WT and point mutant LamB peptides). The LamB peptides are shown as mostly α-helical, with the helix stretching from about L(10) to the C-terminus. There is a break in the helix at about V(18)–S(20) that may impart a kink to the peptide and presumably results in enhanced chain flexibility toward the C-terminus. We speculate that LamB WT may have some tendency to adopt a transmembrane configuration, although not in a stable fashion. The N-terminal region of the LamB peptides is also shown as interacting strongly with the lipid headgroups.

terface, via charge-charge interaction between the lysine side chains and POPG headgroups. The nonpolar region of the lysine side chains may extend into the bilayer interior, as postulated below for the Arg mutant side chains.

Signal peptide topography

The fluorescence quenching results with the model peptides demonstrate the usefulness of the parallax method for determination of average Trp insertion. The results strongly support the reliability of application of this method to the problem of signal peptide insertion, despite potential caveats discussed in Results. We will develop our model for mode of membrane interaction of this signal peptide family by initially considering the charged mutant peptides, followed by discussion of the WT peptide.

The fact that the penetration depths of the Trp residue in the Arg-containing mutant peptides are significantly greater than that of KWK-AM suggests insertion of the peptide backbone into the hydrocarbon interior. As discussed above, potential errors arising from uncertainty regarding the orientation of the large Trp residue relative to the peptide backbone demand caution in drawing conclusions about peptide insertion. However, the intrinsic preference for Trp is very likely near the hydrated bilayer interface, because this residue has significant polar character in addition to being highly hydrophobic (Jacobs and White, 1989). This property of Trp is also reflected by its significantly greater tendency to be found on the surface of globular proteins than that of other highly hydrophobic residues (Janin et al., 1988). Thus, insertion of Trp labels on the signal peptides relative to the control KWK-AM peptide indicates insertion of the peptide backbone. The results are consistent with an orientation in which the peptide backbone is anchored to the bilayer interface via ion pairing between the basic Arg side chains and negatively charged PG headgroups, with the backbone penetrating into the bilayer acyl chain region. The feasibility of this model underscores the fact that significant peptide insertion is possible along with specific interactions between peptide side chains and lipid headgroups. This situation may arise because the length of amino acid side chains (typically 3–5 Å), as well as that of the large Trp residue, is quite significant with respect to bilayer leaflet thickness.

The Trps on the core Asp mutants show marginally deeper insertion than those for the anchored Arg mutants. These peptides are probably also partially inserted into the bilayer interior, with the presumably protonated side chain near the interfacial region. Somewhat surprisingly, the M24D18W mutant seems to be less well inserted than the core mutants. This result may reflect the fact that insertion of the C-terminal region of this peptide is strongly inhibited by the presence of the Asp residue at position 24 which, along with the free C-terminus, results in two free carboxyl groups in this region. Thus, if the peptide is restricted to a predominantly linear, helical conformation (see below), the helical axis must adopt a more interfacial position. Also, greater chain flexibility near the C-terminus allows a more interfacial location for the Trp residue in this peptide (Vogel et al. 1988). In any case, the quenching data indicate insertion of these charged mutant peptides into the bilayer acyl chain region. Previous surface tensiometry results on G17R and A13D likewise supported insertion into POPE-POPG monolayers (McKnight et al., 1989). Similar results were observed for G17D and A13R (K. Ng and L. M. Gierasch, unpublished data).

Among the signal peptides, the Trp residues of WT18W and WT-AM18W insert most deeply into the lipid acyl chain region. This result is consistent with a partially inserted configuration with, on average, deeper penetration than peptides with charged residues in the core region. Alternatively, these data are also consistent with a transbilayer arrangement with the 18 position in the *trans* leaflet of the bilayer. However, other lines of evidence argue against a transmembrane arrangement for this peptide. The lack of effect on DPH anisotropy indicates that insertion of the peptide does not perturb the central region of the bilayer. Preliminary ESR results likewise indicated that the LamB WT and WT-AM peptides did not order deeply labeled phospholipid probes when added exogenously to vesicles (M. B. Sankaram, J. D. Jones, L. M. Gierasch, unpublished data). These results suggest that the WT (and WT-AM) peptide does not penetrate deeply enough to order the central core region of the bilayer and, thus, argue against a transmembrane orientation for this peptide.

The penetration depth for the 24-position Trp suggests that the C-terminus of the WT peptide (and WT-AM) is relatively well buried in the bilayer acyl chain region. This observation is somewhat surprising, because burying the relatively polar C-terminus is not expected to be energetically favorable. However, as discussed above, the energetics associated with forming a loop structure with both termini on the same face of the vesicle are highly unfavorable. Also, because these peptides have a relatively short contiguous hydrophobic region, a transmembrane arrangement requires burying of polar residues in the bilayer hydrocarbon interior. Thus, partial insertion of the peptide in a tilted parallel orientation, which allows insertion of hydrophobic side chains while maintaining polar groups near the interface, may be the most favored topology (Jacobs and White, 1989; and see companion paper for a detailed discussion of the thermodynamics of the interaction of this peptide family with vesicles). This model is supported by our recent results in which we calculated backbone amide penetration depths by application of the parallax method to analysis of doxyl-labeled, lipid-induced line broadening of 2D-NMR intensities (Wang et al., 1993). We found that the hydrophobic core and C-terminal residues of a LamB analog with enhanced N-terminal basicity (KRR-LamB) insert well into the bilayer acyl chain region in PC/PG 99/1% vesicles. For example, Val^{14} and Ala^{16} showed estimated depths of 11 and 12 Å, respectively; however, depths of 9.4 and 11 Å were obtained for Gln^{22} and Met^{24}. By contrast, residues near the basic N-terminal region were shown to localize near the bilayer-water interface (depth of −2.4 Å for Thr^{4}).

The quenching results with WT-AM24W and WT24W (and line broadening with KRR-LamB) do not rule out an orientation perpendicular to the bilayer plane with the C-terminus extending partially into the *trans* leaflet (Fig. 3). However, this orientation places Ser^{20} and Gln^{22} well into the hydrocarbon interior and, thus, is not presumed to be energetically favorable. Also, as discussed above, the lack of lipid ordering induced by the WT versions of the peptide argues against an arrangement in which the peptide penetrates into the central region of the bilayer. Furthermore, preliminary results from a study in which we used the membrane-impermeant fluorescent quencher Tempo-choline to determine the orientation of the C-terminal region in NBD-labeled LamB (M24C-NBD LamB) and TM (A24C-NBD TM) peptide analogs indicate that the LamB C-terminus remains on the side of the vesicle from which it inserts. However, the TM C-terminus was found to be located in the vesicle interior, as expected for a membrane-traversing peptide with a basic N-terminus (J. D. Jones and L. M. Gierasch, unpublished data).

A model for the mode of interaction of the LamB peptides with POPE-POPG vesicles is illustrated in Fig. 3. These peptides all insert to some degree into the bilayer acyl region. However, the results are most consistent with a mainly parallel orientation to the bilayer plane, with the peptide backbone tilted into the acyl chain region. We have previously demonstrated a high helical propensity (about 75%) for the WT, A13D, and G17R peptides in membrane-mimetic environments (McKnight et al., 1989; similar results were obtained for the other peptides in this family (K. Ng, J. Jones, L. Gierasch, unpublished data). 2D-NMR results with WT in membrane-mimetic environments (Bruch et al., 1989), and TrNOE data with KRR-LamB in lipid vesicles (Wang et al., 1993) both suggest that the helix extends from about Leu^{10} to near the C-terminus, with a break in the region encompassing G^{17}–M^{19}. The helix, however, is less stable near the C-terminus, which suggests greater chain flexibility in this region (Bruch et al., 1989). It is possible that the LamB peptides adopt a kink in this region. The WT peptide has a deeper average penetration depth than the charged mutants. It is quite likely that this peptide (particularly with the amidated C-terminus) samples a considerable fraction of the bilayer volume and may well exist in rapid equilibrium between transmembrane and partially inserted orientations (Fig. 3). The data are most consistent with a predominance of the partially inserted orientation. However, additional experiments are required to define rigorously the orientation and dynamics of the WT peptide. By contrast, the charged mutants are probably confined to one leaflet, in the partially inserted configuration.

The lipid perturbation results for the LamB peptides stand in contrast to those obtained previously in this laboratory for the *E. coli* OmpA signal peptide. As discussed in Results, DPH anisotropy and ESR spectral changes strongly support a stable transmembrane arrangement for the OmpA WT peptide. In view of the similarity of structure of the LamB and OmpA signal peptides, these studies collectively suggest that these signal peptides exist near an equilibrium energy point differentiating transmembrane and partially inserted structures. Present studies are aimed at distinguishing these structures by use of fluorescence quenching in asymmetric vesicles in conjunction with leaflet-specific labeling and proteolysis techniques. The OmpA peptides were synthesized with the first four residues of the mature protein (APKD), which may affect membrane insertion because of the additional charges at the C-terminus. We will examine the effect of addition of a few residues to the LamB sequence and subtraction of the APKD group from OmpA.

Peptide effects on bilayer hydration

The blue-shift for WT18W-AM is significantly lower than that for TM15W, although quenching suggests similar penetration depths for these peptides. This result suggests that binding/insertion of the signal peptides is associated with an increase in bilayer water content. As discussed above, the hydrophobic region of the TM peptide is long enough to insert stably into/across the bilayer. In contrast, because the hydrophobic region of the signal peptides is relatively short, insertion of these sequences is likely accompanied by partial insertion of polar groups, such as Ser^{20} and Gln^{22}. These hydrogen bonding groups on the signal peptides may act as an effective "force field" that increases bilayer hydration. The low blue-shifts found for Trps near free carboxyl groups may be viewed as representing the extreme case of this hydration phenomenon. Because of the potential for specific solvent effects at the Trp residue (Lakowicz, 1983), blue-shifts are very likely nonlinear with bilayer hydration and, thus, quantitative estimates of increased bilayer-associated water upon peptide binding are not feasible. However, increased bilayer hydration upon signal peptide insertion could have profound effects on protein-lipid and intrabilayer protein-protein interactions during the protein export process, because these may be modulated strongly by hydrogen bonds within the membrane.

Relation of results to translocation

The in vivo export defect induced by charged residue point mutations in the LamB signal sequence may arise from effects at various stages of the export pathway. For instance, interaction of the preprotein with cytoplasmic export factors may be adversely affected by charged residues in the core region. Also, these mutations may result in changes in the folded conformation of the preprotein in the cytoplasm and, thus, decrease the potential for subsequent interactions necessary for efficient export. However, until more information becomes available on the effects of point charge mutations on signal sequence/preprotein interaction with soluble factors and/or preprotein folding, these postulates must remain speculative.

It is also possible that these mutations act at the level of membrane interactions. These include changes in the affinity and mode of interaction with bilayer lipids as well as altered

interactions with peripheral (SecA) or integral (SecY/E) proteinaceous secretory components. Membrane insertion of the signal sequence may be necessary to ensure proper topology for efficient preprotein interaction with these proteins. These interactions could be specific for either the signal or mature regions of the preprotein. In either case, altered signal sequence insertion is a likely explanation for in vivo export defects caused by these mutations.

All of the point mutants examined in this study exhibited the ability to insert into the acyl chain region of PE-PG bilayers, although their insertion potential is reduced relative to WT. This inhibition of bilayer insertion may in part account for the reduced in vivo activities of these mutant peptides. No significant difference was observed between peptides with a given charged residue at either the 13 or 17 positions. By contrast, export efficiency for the Asp mutants depends strongly on the position of the Asp residue, with A13D showing significantly lower activity than G17D. Our results do not suggest a specific insertion defect for A13D. However, it must be considered that our studies only permit determination of the time-averaged state of the peptide in the bilayer, which may not be the functionally relevant state of the inserted peptide. Interaction of the signal peptide and/or the mature region with integral membrane components of the translocon may occur via a nonequilibrium process. Thus, a more relevant description of peptide insertion may arise from a complete distribution analysis of the inserted peptides. For example, although the average penetration depths of the WT and Arg mutant peptides are not greatly different, the WT peptide is very likely more dynamic in the bilayer, because it does not contain a charged (or polar) residue in the hydrophobic core region. Hence, the WT signal sequence may have a significantly higher potential to transiently insert deeply, with the concomitant insertion of the initial region of the preprotein and, thus, to facilitate interaction with an integral protein. Likewise, peptides with charged residues in the central part of the core region may have a substantially lower "dynamic insertion potential" than those with charged residues placed at the core periphery, although their average depths of insertion do not differ significantly. Future studies using rapid-kinetic and dynamic fluorescent techniques are necessary to address these points.

It is also possible that hindered interactions of these mutant peptides with membrane proteins involved in secretion occur independent of lipid interactions. In addition, the physiological membrane potential will almost certainly affect membrane interaction of these sequences. It is clear that future work, which will address interaction of preproteins harboring these signal sequences with membrane protein components of the secretory apparatus, as well as effects of membrane potential on insertion, is necessary to determine the physiological basis of export defects induced by these mutations. However, the information from the present study on the steady-state insertion potential of these peptides is an essential prerequisite for future studies on the behavior of these signal sequences and/or preproteins in more complex systems.

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