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Hydrophobic Content and Lipid Interactions of Wild-Type and Mutant OmpA Signal Peptides Correlate with Their in Vivo Function†

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ABSTRACT: Peptides corresponding to the wild-type signal sequence of the *Escherichia coli* outer membrane protein OmpA and several mutants have been synthesized and characterized biophysically. The mutations were designed collaboratively with Inouye and co-workers to test the understanding of the critical characteristics of signal sequences required for their functions. The in vivo results for these mutants have been reported [Lehnhardt, S., Pollitt, S., & Inouye, M. (1987) *J. Biol. Chem. 262*, 1716-1719; Goldstein, J., Lehnhardt, S., & Inouye, M. (1990) *J. Bacteriol. 172*, 1225-1231; Goldstein, J., Lehnhardt, S., & Inouye, M. (1991) *J. Biol. Chem. 266*, 14413-14417], and the present paper compares the conformational and membrane-interactive properties of six of the OmpA signal peptides. Peptides corresponding to functional OmpA signal sequences in vivo are predominantly α-helical in membrane-mimetic environments and insert readily into phospholipid bilayers. Nonfunctional OmpA signal peptides may have high helical content but do not penetrate deeply into the acyl chain region of bilayers. The ability of the signal peptides to insert into membranes and their in vivo function correlate with the residue-average hydrophobicity of their hydrophobic cores. The results obtained on OmpA signal peptides parallel closely our previous observations on peptides corresponding to the LamB signal sequence and mutants, arguing that the critical biophysical properties of signal sequences are general despite their lack of primary sequence identity.

Despite considerable effort directed at elucidating the roles of signal sequences in protein export [for reviews see Gierasch (1989), Randall et al. (1987), Benson et al. (1985), Gennity et al. (1990), and Jones et al. (1990)], many questions remain. One of the major puzzles is how signal sequences, which lack primary sequence identity, can share several mechanistic steps in export. Recent genetic (Bieker & Silhavy, 1990) and biochemical (Hartl et al., 1990) evidence suggests that a multistage pathway is followed by a nascent exported protein: First, cytoplasmic chaperones such as SecB may bind the chain and help to keep it in a translocation-competent conformation (Collier et al., 1988; Kumamoto & Gannon, 1988). The signal sequence may be involved in this binding, but its presence is

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not essential at this step (Liu et al., 1988; Altman et al., 1990a,b). The nascent chain (perhaps with SecB or other chaperone) binds to SecA and is directed to the cytoplasmic membrane by virtue of the interaction of SecA with membrane proteins SecE and SecY. The signal sequence appears to interact with all three of these proteins. Clearly, the nascent chain binds SecA in the cytoplasm, an aqueous environment, and then must interact with the highly hydrophobic SecE and SecY species in the membrane. It may partition directly into lipid in order to facilitate these interactions. Direct lipid interaction of signal sequences has been suggested frequently (von Heijne & Blomberg, 1979; Engelman & Steitz, 1981; Briggs et al., 1985, 1986).

We have chosen to study isolated signal peptides to explore the required characteristics of functional signal sequences. Our goal has been to define the behavior of these sequences such that their roles in export may be better understood, including, in the long term, an understanding of what features of the signal sequence are required for each of the steps in the export pathway. We focused initially on the signal sequence of the *Escherichia coli* outer membrane protein, LamB. Peptides corresponding to functional LamB signal sequences showed a high tendency to adopt α-helical conformations in interfacial environments such as micelles or lipid vesicles (Briggs & Gierasch, 1984; McKnight et al., 1989). They also inserted spontaneously into lipid monolayers (Briggs et al., 1985; McKnight et al., 1989). From incorporation of the intrinsic fluorophore, tryptophan, into the LamB signal sequence at various positions, we find that the signal sequence is well inserted into the hydrophobic acyl chain region of the bilayer (McKnight et al., 1991).

While many of the same conclusions have been reached by researchers studying other examples of synthetic signal peptides [reviewed in Gierasch (1989)], in no other case has as extensive an analysis been carried out on a number of phenotypically characterized variants of a native signal sequence. The present study thus represents an extension of our work on synthetic signal peptides to a new family corresponding to the wild type and variants of the signal sequence of another *E. coli* outer membrane protein, OmpA.[1] In collaboration with Inouye and co-workers, we have designed mutants of the OmpA signal sequence that pose specific questions about structure/function relationships. Inouye and co-workers constructed the corresponding mutant strains to evaluate the in vivo effects of the designed mutations (Lehnhardt et al., 1987; Goldstein et al., 1990, 1991), while we have synthesized and studied the isolated signal peptides. The approach of design and directed mutagenesis offers the advantage of producing variants with a large range of phenotypes, from better than wild type to fully defective. By contrast, the LamB mutants we have studied to date all arose from selection schemes that were biased toward strongly defective phenotypes (Bieker & Silhavy, 1990).

We find that high helical propensity in interfacial environments and high tendency to insert into bilayers correlate with good in vivo function in the OmpA family of signal peptides, as they did in the LamB family. These properties appear to be general descriptors of what it takes to be a "good" signal sequence. Although we cannot fully separate the contributions of length, conformation, and hydrophobicity, the present results emphasize the importance of a minimum threshold hydrophobicity for effective function of a signal sequence and provide an estimate of what that threshold is. In one of the mutants examined in this study, an asparagine residue was incorporated in the hydrophobic core. This seemingly minor change led to a total loss of function, attributable, we believe, to a defect at the level of membrane interaction. A more detailed study of this particular mutant is presented elsewhere (Hoyt & Gierasch, 1991).

Table I: OmpA Signal Peptides Studied

	OmpA signal peptide[a]	export activity[b]
	1 5 10 15 20 1 4	
WT	M K K T A I A I A V A L A G F A T V A Q A / A P K D	++++ (46 s)
L6L8	L L	++++ (39 s)
Δ9	-	++++ (43 s)
Δ8	-	++ (246 s)
Δ6-9	-------	0
I8N	N	0

[a] A slash indicates the signal peptidase cleavage site; residues following correspond to the mature OmpA protein. Peptides were synthesized including four residues of the mature sequence to improve their solubility. [b] In vivo data are from Lehnhardt et al. (1987) and Goldstein et al. (1990, 1991). Results reported are for fusions of the OmpA signal sequence to staphylococcal nuclease. Times given are precursor half-lives.

Experimental Procedures

Reagents. Synthetic 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids. Sodium dodecyl sulfate (SDS) was electrophoretic grade from Bio-Rad. Crude carboxyfluorescein was obtained from Eastman Kodak and was purified before use by ethanol–water recrystallization. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes Inc. and was dissolved in spectral-grade tetrahydrofuran (J. T. Baker) prior to use. Sephadex G-10 was obtained from Pharmacia. Triton X-100 was purchased from Sigma.

Peptide Synthesis and Preparation. (A) Peptide Synthesis. The peptides studied (Table I) were synthesized by solid-phase methods using standard protocols (Stewart & Young, 1984). Using *tert*-butyloxycarbonyl (*t*-Boc) amino acid precursors, the OmpA WT and Δ6–9 peptides were synthesized on a Peptides International Synthor 2000-AT peptide synthesizer, and Δ8 and I8N peptides were synthesized on an automated Applied Biosystems Inc. (ABI) 430A peptide synthesizer. The extent of coupling was checked using the qualitative ninhydrin test (Kaiser et al., 1970). The peptides were cleaved from the resin by treatment with anhydrous HF containing 10% anisole at 0 °C for 45 min. The cleaved crude peptide was washed with anhydrous ether to remove the anisole scavenger and then extracted with acetonitrile and water before lyophilizing. The L6L8 and Δ9 peptides were synthesized on an automated Milligan 9050 continuous-flow peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) amino acid precursors and methods described by Dryland et al. (1986). Deprotection and cleavage of the peptides from the resin was accomplished using a 95:5 (v/v) trifluoroacetic acid (TFA)/phenol solution at room temperature for 2 h. Anhydrous ether was used to remove the phenol scavenger and to precipitate the peptide. Peptide precipitate was then centrifuged, separated, and dried under vacuum prior to purification and analysis.

[1] Abbreviations: ABI, Applied Biosystems Inc.; ACN, acetonitrile; *t*-Boc, *tert*-butyloxycarbonyl; CBXF, carboxyfluorescein; CD, circular dichroism; DPH, 1,6-diphenyl-1,3,5-hexatriene; Fmoc, 9-fluorenylmethoxycarbonyl; OmpA, outer membrane protein A; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

(*B*) *Peptide Purification.* All peptides were purified by high-pressure liquid chromatography (HPLC) on either a Vydac phenyl or a Vydac C-4 reversed-phase column (2.2 cm × 25 cm) at room temperature using water/acetonitrile (ACN) with 0.1% TFA (v/v) or water/methanol (MeOH) gradients with 0.1% TFA (v/v). The identity of each peptide was verified by peptide sequencing on an ABI 477A automated sequencer and by amino acid analysis on a Beckman 6300 analyzer. Peptide purity was confirmed by analytical HPLC.

(*C*) *Stock Preparation.* Peptide samples were dissolved in approximately 3.2 mM HCl (pH 2.5 H_2O) at approximately 1 mg/mL concentrations (250–400 μM), and then solutions were filtered through a 0.2-μm filter (Gelman) to remove particulate matter. All samples were prepared fresh, never frozen, and kept at room temperature. Estimates of stock solution concentration were made by UV measurements. An aliquot of sample was submitted for quantitative amino acid analysis to determine peptide stock concentration.

Lipid Vesicle Preparation. Stock solutions of lipid, dissolved in chloroform, were dispensed in amounts to form a 65/35 (mol %) mixture of POPE/POPG. Chloroform was evaporated under a slow nitrogen purge and then under vacuum. A multilamellar dispersion was prepared by hydration of the dried lipids with 5 mM Tris, pH 7.3, at room temperature to a final lipid concentration of either 10 or 20 mM, sparged for 15 min with nitrogen, and vortexed. Small, unilamellar vesicles (SUVs) were prepared according to the method of Huang (1969). The multilayer suspension was sonicated in a bath sonicator (Laboratories Supply Co., Inc.) until clear. Large, unilamellar vesicles (LUVs) were prepared according to the freeze–thaw/extrusion method of Mayer et al. (1986). The suspension was freeze–thawed (five times) using a liquid nitrogen bath and then extruded through two stacked 100-nm pore polycarbonate filters under nitrogen pressure (ten times) using an extrusion device (Lipex Biomembranes, Inc.). LUVs containing carboxyfluorescein (CBXF) were made by including 0.1 M CBXF in the hydration buffer and subsequently passing the extruded vesicles over a G-10 Sephadex column to remove extravesicular CBXF. Isoosmotic conditions were preserved by including 0.1 M NaCl in the 5 mM Tris, pH 7.3, elution buffer. Eluted fractions containing LUVs were determined by the addition of a drop of 10% (v/v) Triton X-100. Those fractions (excluding the first and last) which gave increased fluorescence intensity with Triton were pooled. Phospholipid concentrations of all vesicle samples were determined using an ashing/phosphate assay (Ames & Dubin, 1960).

Circular Dichroism. All CD spectra were obtained on an AVIV Model 60DS spectropolarimeter. The amplitude of the CD signal was calibrated using a 0.1% (w/v) solution of *d*-(+)-camphorsulfonic acid (Aldrich) (Cassim & Yang, 1969), and the wavelength of the CD signal was set using standard absorbance peaks of benzene vapor (Aldrich, spectral grade). Temperature was regulated by a Hewlett-Packard 89100A temperature controller, and measurements were performed at 25 ± 0.1 °C. Peptide concentrations were determined by quantitative amino acid analysis. CD spectra were obtained on peptide samples of 5 μM concentration in a 5-mm cell for bulk solvents and 2-mm cell for SUVs unless stated otherwise. All solutions were buffered with 5 mM Tris, pH 7.3, unless specifically stated otherwise. All CD spectra were base line corrected and smoothed using software provided by Aviv Associates. Helical content was estimated using CD signal intensity according to the method of Chen et al. (1974). This calculation compares the experimental ellipticity at 222 nm ($[\theta]_{222}$) to a theoretical $[\theta]_{222}$. The theoretical $[\theta]_{222}$ is empirically adjusted to account for differences in peptide length and is based on experimental CD data from a series of proteins with known crystal structures. Since both the curve shape and magnitude are important in analysis of a CD spectrum for secondary structure contributions, we also considered qualitatively the contributions to the spectral shapes from different secondary structures using reference curves for poly(L-lysine) (Greenfield & Fasman, 1969). This latter method was used in particular to follow tendencies to adopt β-structure.

Carboxyfluorescein (CBXF) Leakage Assay. Peptide-induced leakage of the vesicle-entrapped CBXF dye from LUVs (prepared as described above) was measured by monitoring the emission at 520 nm (excitation wavelength 480 nm) as a function of time. The fluorescent CBXF dye self-quenches at the high concentrations inside the vesicles. For each experiment maximal leakage was determined by adding a drop of 10% Triton X-100 to the peptide/vesicle solution (solubilizing the vesicles) and recording the final fluorescence intensity. The addition of Triton X-100 completely solubilized the vesicle membranes, and the self-quenching of the CBXF was relieved upon dilution with the outside medium. Fluorescence measurements were made on an ISS Inc. steady-state spectrofluorimeter (Model Greg PC) using 1-cm quartz fluorescence cuvettes (Helma). Samples were thermostated at 25 ± 0.2 °C and were continuously stirred. Emission and excitation slit widths were 0.5 mm.

Steady-State Fluorescence Anisotropy. LUVs (65/35 POPE/POPG prepared in either 5 mM Tris, pH 7.3, or 0.1 M NaCl and 5 mM Tris, pH 7.3) were diluted to a 1 mM lipid concentration (1200 μL final volume) in sample and reference quartz fluorescence cuvettes (1 cm) containing the appropriate Tris buffer. To the sample cell was added 2 μL of 2 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) (dissolved in tetrahydrofuran) in a ratio of 1:300 DPH:lipid. The sample was equilibrated at least 30 min before addition of peptide. DPH fluorescence was measured with an ISS steady-state spectrofluorimeter (Model Greg PC) equipped with excitation and emission polarizers using the L format. Excitation and emission wavelengths were 360 and 430 nm, respectively. After each addition of peptide to the sample, the resulting emission intensities were then used to calculate the anisotropy value according to the equation:

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

Light scattering was subtracted by adding peptide to a reference sample containing no DPH. Samples were continuously stirred and thermostated at 25 ± 0.2 °C unless stated otherwise. When DPH anisotropy was recorded as a function of temperature, samples were equilibrated at each point for 45 min.

Results

OmpA Signal Sequences Studied. The OmpA wild-type signal sequence (WT, Table I) has a typical pattern of residues (von Heijne, 1985; Goldstein et al., 1990). Its hydrophobic core [alanine 5 (A5) to alanine 13 (A13)] has an alternating X-A repeat with X being Ile, Val, or Leu. This X-A repeat may be conducive to β-sheet formation with large hydrophobic residues (mostly β-branched) on one side and alanines on the other. The hydrophobic core region of other signal sequences, including LamB, has been proposed to fold as an α-helix (Emr & Silhavy, 1983; von Heijne, 1985; Briggs et al., 1986). Chou–Fasman rules for secondary structure prediction (Chou & Fasman, 1974a,b) reveal that the hydrophobic core segment of OmpA (as well as that of other signal sequences) has a high

predicted probability for both α-helix and β-sheet. Thus, mutations predicted to alter the secondary structure were constructed and characterized in vivo. These changes were also considered in light of their effect on the overall hydrophobicity of the signal sequence. We have synthesized the corresponding peptides, including four residues of the mature sequence in order to improve solubility (Table I). The rationale for design and the phenotype of each of the modified OmpA signal sequences is summarized below.

Single-residue deletions in the hydrophobic core lead to quite distinct phenotypes depending on the residue removed. For example, a single-residue deletion of Ala in position 9 (Δ9) is predicted to exhibit a slight decrease in α-helix and a slight increase in β-sheet and to have a slightly lower hydrophobicity than the native sequence. In vivo this mutation is silent, showing wild-type processing efficiency (Table I; Lehnhardt et al., 1987). By contrast, a single-residue deletion of Ile in position 8 (Δ8) results in slow processing kinetics (processing half-life of 247 s) as compared to WT (46 s; Goldstein et al., 1990). The Δ8 mutant has a lower predicted β-sheet content and hydrophobicity while only a slight decrease in α-helix is expected. The two deletion mutants Δ9 and Δ8 are intriguing because although the length of each hydrophobic segment is identical, small differences in amino acid sequence yield different phenotypes.

A double substitution of leucines for isoleucines in positions 6 and 8 (L6L8) is predicted to confer a slightly higher probability for α-helical structure, a slightly lower probability for β-structure, and a slightly lower hydrophobicity than WT. The L6L8 mutant seems to be processed at about the same rate as the wild-type sequence (processing half-life of 39 s as compared to 46 s) (Goldstein et al., 1990). The substitution of isoleucines with leucines was also examined in the alkaline phosphatase (PhoA) signal sequence system by Chou and Kendall (1990). Again, no dramatic changes were observed in phenotype when isoleucine was replaced with leucine.

The removal of four amino acids from the hydrophobic core (IAIA) generates a mutant (Δ6–9) with a substantially lower predicted α-helix and β-sheet content as well as a significantly lower hydrophobicity. This mutant was modeled after a similar four-residue deletion in the hydrophobic core of the LamB signal sequence which was known to be completely export-defective (Emr & Silhavy, 1983). Analogously to the LamB deletion mutant, the OmpA Δ6–9 mutant demonstrated no detectable processing in vivo even after 10 min (Lehnhardt et al., 1987).

While it is known that charged residues introduced in the hydrophobic core of signal sequences cause severe export defects (Bankaitis et al., 1984; Stader et al., 1986), the impact of a neutral polar residue has rarely been examined. Asn was substituted for Ile in the eighth position (I8N) to explore this type of change. Incorporation of an Asn is expected to decrease the propensity to take up secondary structure, whether α-helix or β-structure, as well as to decrease the hydrophobicity appreciably. The Ile 8 to Asn mutation leads to a complete export block in vivo (viz., no processing up to 10 min). Biophysical studies of the I8N mutant are covered in more detail elsewhere (Hoyt & Gierasch, 1991).

Impact of Mutations on Conformational Properties. Signal peptides were examined in bulk aqueous solutions as well as in solvents that offer an aqueous/hydrophobic interface. These environments were used to compare peptide conformation under conditions that approximate the cytoplasm and inner membrane. In an aqueous buffer, at dilute peptide concentrations, all the OmpA signal peptides showed circular dichroism (CD) spectra characteristics of a lack of ordered secondary structure (Figure 1, solid lines).

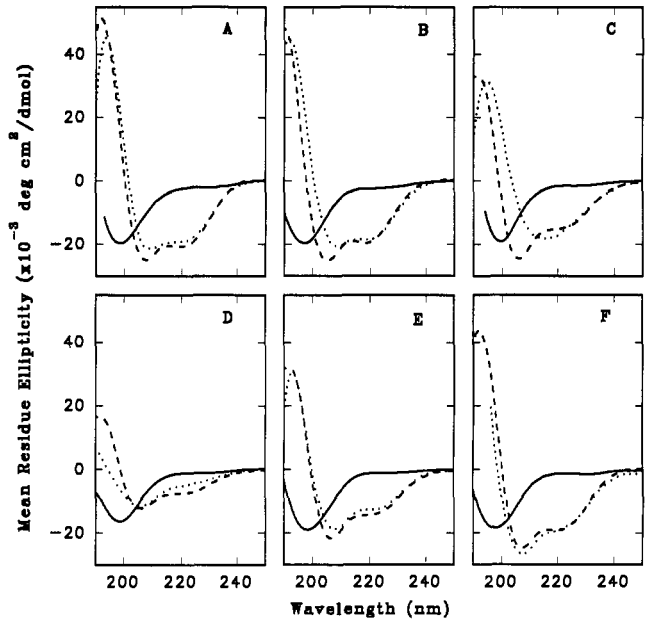

FIGURE 1: CD spectra of OmpA signal peptides in buffer, SDS, and phospholipid vesicles. All peptide concentrations were 5 μM. 5 mM Tris, pH 7.3 (solid lines); 40 mM SDS, pH 7.3 (dashed lines); 1 mM lipid, 65/35 (mol %) POPE/POPG SUVs, pH 7.3 (dotted lines). (A) WT; (B) L6L8; (C) Δ9; (D) Δ6–9; (E) I8N; (F) Δ8.

Table II: α-Helix Contents of OmpA Signal Peptides[a]

signal peptide	% α-helix		
	aqueous buffer[b]	SDS	vesicles
WT	7	64	60
L6L8	8	60	58
Δ9	5	50	55
Δ8	4	60	60
Δ6–9	4	24	17
I8N	4	44	40

[a] Helical contents were estimated using the method of Chen et al. (1974), as described in the text. All values are ±5%. [b] Aqueous buffer is 5 mM Tris, pH 7.3. SDS is 40 mM. Vesicles are SUVs prepared as described in the text.

The CD spectrum of the wild-type OmpA signal peptide indicated a substantial content of β-structure (single minimum in the ellipticity occurring between 212 and 222 nm; Greenfield & Fasman, 1969) in aqueous buffered environments at high peptide concentrations (≥50 μM) and neutral pH (Figure 2). However, under these conditions the peptide apparently exists in a multimeric form, as indicated by increased light scattering and formation of a visible precipitate over time. Peptide aggregate formation in aqueous solution appeared to be concomitant with the appearance of β-structure. As expected, the rate of the conformational transition from the unordered random coil to nearly 100% β is increased by higher peptide concentrations (Figure 2A,B), but slowed by reduced pH (Figure 2C). Furthermore, peptide aggregation was enhanced at temperatures below 15 °C and above 45 °C (data not shown). Similar conformational equilibria between random and β-structure have been previously reported for other signal peptides (Rosenblatt et al., 1980; Batenburg et al., 1988a), and we suspect on the basis of our studies that they may have arisen from peptide–peptide interactions.

SDS micelles, which provide a simple hydrophobic/hydrophilic interface, were used to mimic a membrane environment. The CD spectrum for each peptide in 40 mM SDS indicates adoption of at least some α-helical structure with its charac-

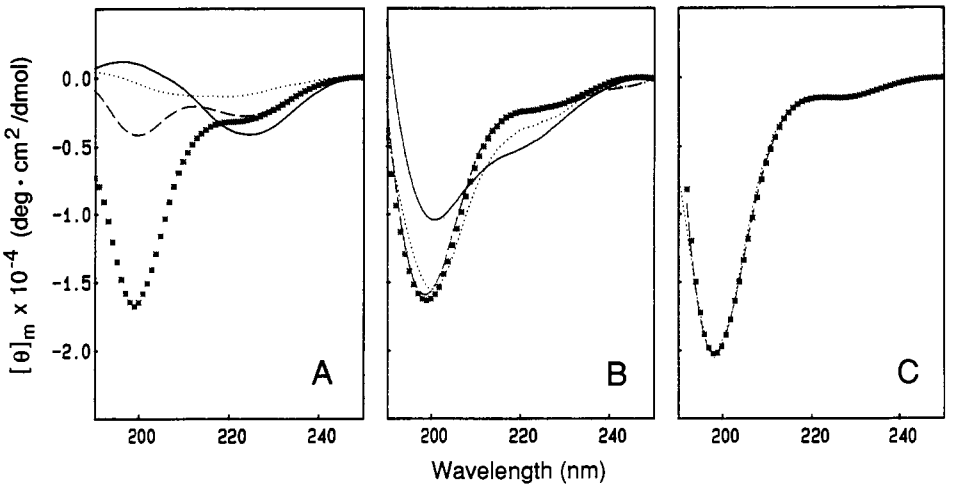

FIGURE 2: Time-dependent intermolecular association of OmpA signal peptides in buffer as a function of concentration and pH; 10 mM phosphate buffer used throughout. (A) 50 μM WT, pH 7.3: 0.5 h (***); 2 h (dashed line); 3.5 h (dotted line); 10 h (solid line). (B) 5 μM WT, pH 7.3: 1.5 h (***); 4.5 h (dashed line); 6 h (dotted line); 15 h (solid line). (C) 100 μM WT, pH 3.0: 0.5 h (***); 3.5 h (dashed line); 17.5 h (dotted line).

teristic minima at 208 and 222 nm (Woody, 1985) (Figure 1, dashed lines). The WT and other export-competent signal peptides (Δ9, L6L8, Δ8) yield spectra with generally higher α-helical content than export-incompetent signal peptides (Δ6–9, I8N) (Table II). The Δ6–9 signal peptide demonstrated the lowest helix content of about 25 ± 5%. The CD spectrum of the I8N peptide shows higher helical content (45 ± 5%) than does the Δ6–9 peptide, but slightly less than seen for export-competent signal peptides (50–65 ± 5%). Very little difference was found between the helix contents of the Δ8, Δ9, and L6L8 peptides; they were all essentially the same as wild type.

In order to examine signal peptides in a lipid bilayer environment, peptides were added to a suspension of small unilamellar vesicles. These vesicles mimic the plasma membrane lipid headgroup composition of *E. coli* (65% POPE/35% POPG) (Tanford, 1980). Again, the CD spectra demonstrate that each peptide adopts at least partial α-helical secondary structure (Figure 1, dotted lines). OmpA WT was found to be predominantly α-helical when added to vesicles at the low peptide/lipid (P/L) ratio of 1/200 (Figure 1A, dotted line). However, raising the concentration of WT resulted in increased β-sheet content in the CD spectrum (data not shown), in a manner analogous to the concentration-dependent conformational equilibrium seen in aqueous solution. The Δ9 peptide appears to be a mixture of β-sheet and α-helix even at the low P/L ratio of 1/200 (Figure 1C, dotted line). Interestingly, this peptide was predicted by Chou–Fasman rules to have a slightly higher propensity for β-structure than the WT. Δ8 and L6L8 exhibit as much helix as WT in vesicles. Although the CD spectrum of the Δ6–9 reflects a significantly lower α-helical content than the peptides corresponding to export-competent phenotypes, the other export-incompetent peptide I8N has a helical content close to that of the WT sequence in both vesicles and SDS (Table II). Thus, analogously to the LamB signal peptide family, the ability to adopt α-helical structure in membrane-mimetic environments seems necessary but not sufficient for in vivo function.

CBXF Leakage Assay. The CD results above indicate that all of the OmpA signal peptides undergo at least some conformational change upon the addition of small, unilamellar phospholipid vesicles. Whether this change is due to a structure formed upon binding to the vesicle surface or arises from a more substantial interaction with the lipid acyl chain region cannot be distinguished from these data. To investigate

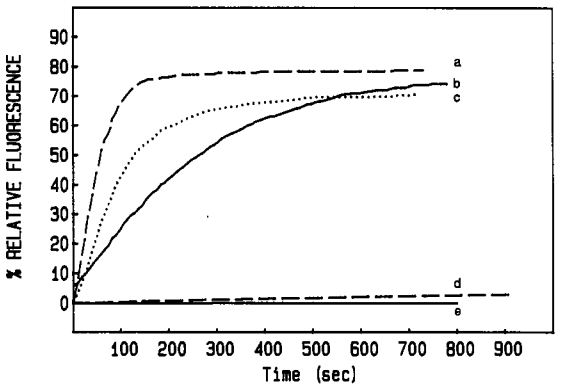

FIGURE 3: Carboxyfluorescein (CBXF) leakage assay of OmpA signal peptides. Peptide concentrations were 15 μM. Vesicles were 250 μM lipid and contained 65/35 (mol %) POPE/POPG. The entrapped vesicle volume contained 0.1 M CBXF and 5 mM Tris, pH 7.3; the extravesicular buffer was 0.1 M NaCl and 5 mM Tris, pH 7.3. Peptides were added at 0 s. At the end of the experiment, 100% maximal carboxyfluorescein fluorescence was determined by complete solubilization of the vesicles after addition of Triton X-100 (not plotted). (a) WT; (b) L6L8; (c) Δ9; (d) Δ8; (e) Δ6–9 and I8N.

the ability of each OmpA signal peptide to perturb phospholipid bilayers, an assay using large, unilamellar vesicles (LUVs) containing a self-quenching fluorescent dye, carboxyfluorescein, was employed (Weinstein et al., 1977). Perturbation of the vesicle bilayer is detected by the large increase in fluorescence which accompanies dequenching of the carboxyfluorescein fluorescence upon dilution of the dye in the bulk aqueous medium. The results are displayed as the percent increase in carboxyfluorescein fluorescence at 520 nm, relative to the maximal change determined by the addition of Triton X-100, vs time (Figure 3). The peptide to lipid ratio was 1 to 17. Addition of OmpA signal peptides corresponding to functional sequences in vivo (WT, L6L8, and Δ9) resulted in carboxyfluorescein leakage from LUVs (L6L8 not shown). The kinetically defective mutant, Δ8, caused only marginal leakage even at a high P/L ratio (1/10). The two peptides with completely export-defective in vivo phenotypes, Δ6–9 and I8N, did not cause any measurable leakage (even at P/L = 1/10). A poly-L-lysine control peptide (Sigma) did not cause any leakage. These results demonstrate that the ability of OmpA signal peptides to perturb model lipid bilayers correlates closely with the export phenotype of the corresponding signal sequences.

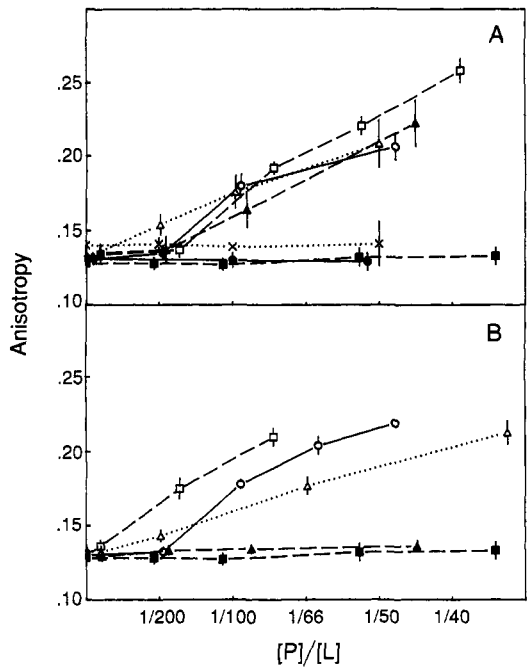

FIGURE 4: Steady-state fluorescence anisotropy of DPH as a function of OmpA signal peptide concentration for 65/35 (mol %) POPE/POPG LUVs. Lipid concentration for all experiments was 1 mM. (A) Peptides added to vesicles in 5 mM Tris, pH 7.3: WT (open squares); L6L8 (open circles); Δ9 (open triangles); Δ8 (filled triangles); Δ6–9 (filled circles); I8N (filled squares); poly(L-lysine) (×). (B) Peptides added to vesicles in 100 mM NaCl, 5 mM Tris, pH 7.3: WT (open squares); L6L8 (open circles); Δ9 (open triangles); Δ8 (filled triangles); I8N (filled squares).

DPH Anisotropy. To assess whether the OmpA wild-type and mutant signal peptides can insert into the fatty acyl chain region of the bilayer, steady-state fluorescence anisotropy was used. A well-characterized fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was incorporated into LUVs and was used to detect changes in the order of the fatty acyl chains of the phospholipid (Lakowicz, 1983; Lentz, 1989). An increase in anisotropy reflects restrained motion in the acyl chain region and indicates peptide insertion. DPH resides in the middle of the bilayer at or near the ends of the acyl chains (Lentz et al., 1976). This molecule is exquisitely sensitive to acyl chain motion and thus is a good probe for detecting peptide insertion (Rigell et al., 1985).

OmpA signal peptides were added to LUVs containing DPH at a probe/lipid ratio of 1/300. Anisotropy values increased upon addition of all fully export-competent OmpA signal peptides to the LUVs and did not increase upon addition of the export-defective peptides, whether the experiment was carried out at relatively low (5 mM Tris) or high (5 mM Tris plus 0.1 M NaCl) ionic strength (Figure 4). Interestingly, addition of the peptide corresponding to the kinetically defective mutant, Δ8, led to an anisotropy increase under the low ionic strength conditions (Figure 4A), but not the higher ionic strength (Figure 4B). Note by comparison that this peptide did not cause carboxyfluorescein release, but that this latter experiment was carried out at higher salt concentration. The peptides corresponding to the export-defective mutants I8N and Δ6–9 were added at or above ratios of peptide to lipid known from other results (Hoyt & Gierasch, 1991; Hoyt, Jones, and Gierasch, unpublished results) to be saturating for binding and still did not cause any change in anisotropy values. These results are compelling, because they demonstrate that an export-incompetent peptide, I8N, which still has substantial helical structure in the presence of vesicles (see CD results above) as compared to WT and Δ6–9, cannot penetrate deeply enough into the bilayer to change the environment of the fatty acyl chains of the vesicle phospholipids. Furthermore, with the result for the Δ8 mutant peptide, ability to perturb DPH anisotropy emerges as the most diagnostic property of export function in the OmpA signal peptides.

We checked whether the rise in anisotropy upon addition of peptide was a consequence of a shift of the lipid-phase transition to a higher temperature. The T_m of the LUVs (65% POPE/35% POPG) without peptide added was 15.5 °C, and with peptide [F15W, a fully export-competent analogue of OmpA WT, described in Hoyt and Gierasch (1991)] at P/L of 1/100, the T_m was slightly lowered to 14.6 °C. This result demonstrates that the increased anisotropy seen when the experiment was performed at 25 °C was well above the vesicle-phase transition of 14.6 °C. Therefore, the anisotropy rise is due to ordering of the liquid-crystalline phase by peptide as opposed to an increase in T_m.

Discussion

The six peptides examined here (WT, L6L8, Δ9, Δ8, Δ6–9, I8N) were designed to alter the potential to take up predicted secondary structure of the OmpA signal sequence to test the functional importance of conformation. Moreover, these changes also created a series of signal sequences that differ in hydrophobicity. The results of this study indicate that hydrophobicity and the resulting ability to insert into membranes correlate with in vivo competence for the series of OmpA mutants examined. The functional OmpA signal peptides (WT, L6L8, Δ9) are predominantly α-helical in membrane-mimetic environments, from CD analysis, and bind and insert into phospholipid vesicles, from carboxyfluorescein leakage and DPH anisotropy assays. Nonfunctional OmpA signal peptides may have high helical content and may bind to lipid surfaces, but do not penetrate the acyl chain region of bilayers significantly. A marginally functional signal peptide showed ionic strength dependent insertion ability, suggesting that it has a borderline potential for membrane insertion. In toto, these results are consistent with studies of LamB wild-type and mutant signal peptides (Briggs et al., 1985, 1986; Cornell et al., 1989; McKnight et al., 1989) as well as the wild-type signal peptide of PhoE (Batenburg et al., 1988a,b).

The availability of several variants of the OmpA signal sequence with quite subtle modifications provides greater insight into the specific hydrophobicity requirements for export competence than available from previous studies. All of the peptides used in this work have the same net charge and were examined under identical conditions. Therefore, even though both electrostatic and hydrophobic forces contribute to their membrane binding, differences in the abilities of these peptides to interact with lipid bilayers most likely arise from differences in their hydrophobic properties. In DPH anisotropy experiments using a physiologically relevant salt concentration (100 mM NaCl), the WT, Δ9, and L6L8 OmpA signal peptides can insert into vesicles but Δ6–9, Δ8, and I8N cannot. The insertion ability (and the export competence) of Δ8 is borderline, since it does insert at low ionic strength. Note that higher ionic strength conditions reduce electrostatic forces and therefore would be expected to increase the role of hydrophobic forces in the interaction of peptide with lipid.

We considered what the discrimination among the peptides in terms of their export competence and their membrane insertion ability may mean in terms of quantitative measures of hydrophobicity. The deletion of an Ile at position 8 (Δ8) decreases the sequence hydrophobicity (compared to WT) more than the loss of an Ala at position 9 (Δ9) or substitution

Table III: Hydrophobicity of OmpA and LamB Signal Sequence Mutants[a]

Signal Peptide	Hydrophobicity: Kyte-Doolittle[b] Core	Mean	Tot Mean		Goldman-Steitz-Engelman[c] Core	Mean	Tot	Mean
OmpA Peptides[d,e]								
WT MKKTAIAIAVALAGFATVAQA	26.0	2.9	27.2	1.3	-19.6	-2.2	-15.8	-0.8
L6L8 L L	24.6	2.7	25.8	1.2	-19.0	-2.1	-15.2	-0.7
Δ9 -	24.2	3.0	25.4	1.3	-18.0	-2.3	-14.2	-0.7
Δ8 -	21.5	2.7	22.7	1.1	-16.5	-2.1	-12.7	-0.6
I8N N	*18.0*	*2.0*	*19.2*	*0.9*	*-11.7*	*-1.3*	*-7.9*	*-0.4*
Δ6-9 ----	*12.3*	*1.8*	*14.6*	*0.9*	*-12.4*	*-1.8*	*-6.4*	*-0.3*
LamB Peptides[d,e]								
WT MMITLRKLPLAVAVAAGVSMAQAMA	21.6	2.4	33.3	1.3	-17.0	-1.9	-21.5	-0.9
Δ78 ----	*13.8*	*2.0*	*21.7*	*1.0*	*-12.0*	*-1.7*	*-12.9*	*-0.6*
Δ78r1 ---- C	17.1	2.4	23.6	1.1	-13.0	-1.9	-13.9	-0.7
Δ78r2 L----	19.2	2.7	26.9	1.3	-15.0	-2.1	-15.9	-0.8
G17R R	21.6	2.4	29.2	1.2	-17.0	-1.9	-8.2	-0.3
A13D D	*16.3*	*1.8*	*28.0*	*1.1*	*-6.2*	*-0.7*	*-10.7*	*-0.4*

[a] Entries written in italics correspond to export-defective mutants. [b] Kyte–Doolittle parameters are termed hydropathicities and are unitless. They are calculated using data from Kyte and Doolittle (1982). [c] Goldman–Engelman–Steitz parameters are estimates of free energies of transfer from water to a hydrocarbon and are in kcal/mol (Engelman et al., 1986). [d] The hydrophobic core used in the calculation is underlined except in the sequence with four-residue deletions. In these cases, seven residues were considered to form the minimum-length core. The seven residues used in the calculation were the most hydrophobic, leaving at least seven residues between the end of the core and the cleavage site and not including the basic residues. [e] The phenotypes of the OmpA signal sequences are given in Table I. The in vivo data for these LamB mutants are summarized in Emr and Silhavy (1983) and in Stader et al. (1987). Briefly, Δ78 and A13D are export-defective, Δ78r1 exports at 50% wild-type levels, Δ78r2 exports at 90% wild-type levels, and G17R exports at wild-type levels but is kinetically deficient.

of the isoleucines at positions 6 and 8 (L6L8). According to the Goldman–Engelman–Steitz hydrophobicity scale, the transfer free energies from water to a nonaqueous environment (dielectric constant = 2, pH = 7) for the amino acids Ile and Ala are −3.1 and −1.6 kcal/mol, respectively (Engelman et al., 1986). Therefore the expected $\Delta\Delta G_{transfer}$ between Δ9 and Δ8 is −1.5 kcal/mol, which yields approximately a 12.8-fold difference in the theoretical partition coefficient. A more useful measure of the threshold hydrophobicity required for a signal sequence to function would be the net hydrophobicity of the entire sequence, or alternatively that of the hydrophobic core. Table III gives a summary of these parameters for the OmpA signal sequences studied here, as well as the LamB mutants we have examined in past work. There are no major differences between the results calculated with two scales of hydrophobicity, that of Kyte and Doolittle (1982) and that of Engelman et al. (1986). The mean residue hydrophobicity of the entire signal sequence roughly parallels the export competence of the signal sequence variant, but the mean residue hydrophobicity in the core yields a clear threshold for function that is consistent throughout both the OmpA and the LamB mutants: The average hydrophobicity of the residues in the core must be ≥2.4 (Kyte–Doolittle) or ≤−1.9 (kcal/mol; Goldman–Engelman–Steitz). It is clearly desirable to relate the deduced hydrophobicity threshold to an energetic requirement for productive signal peptide/membrane interaction. However, to do this requires a more detailed model for the membrane-inserted form of the peptide than is currently available.

Chou and Kendall (1990) also noted from their polymeric replacement mutants of the alkaline phosphatase signal sequence that mean residue hydrophobicity was a critical determinant of function. Interestingly, their results suggest that a somewhat reduced hydrophobicity may be compensated by an increased core length. They found that an all-Ala hydrophobic core functioned best when 20 residues in length, whereas one with only Leu was optimal at 10–15 residues. Others have shown that the length of the hydrophobic core must meet certain requirements. Genetic studies (Yamamoto et al., 1987, 1989; Chou & Kendall, 1990) and statistical analysis (von Heijne, 1985) have defined limits in the length of signal sequences. In most cases, seven residues can be considered the lower length limit for the core. An upper limit of 20 residues, depending somewhat on composition, corresponds to a conversion of a cleaved signal sequence to a membrane anchor (Chou & Kendall, 1990). Results from the OmpA peptides show that removal of one or two residues from the hydrophobic core may be tolerated in this signal sequence without any loss in function (e.g., OmpA Δ9). In contrast, signal sequences of the same length but of different hydrophobicity, like Δ8 and Δ9 or WT and I8N, can have noticeably different phenotypes and distinguishable physical behavior in vitro. From the arguments presented above, we would suggest that defects in vivo and in vitro will be observed if the mean hydrophobicity drops below the threshold values noted above. Therefore, one can impose a window of permissible lengths of the hydrophobic core (greater than 7 and well less than 20) and a threshold mean hydrophobicity value within the core

for a signal sequence to function optimally.

It is also of interest to examine more closely the conformational characteristics of the OmpA signal peptides, the impact of the mutations studied, and the comparative conformational properties of the OmpA and the LamB signal sequences. The CD results presented argue that OmpA signal peptides may exist in different secondary structures depending on the solvent environment, concentration of signal peptide, and available membrane surface area. We conclude that the OmpA signal peptide may exist in equilibrium among four major states: unordered ("random coil") in solution, a multimeric β-structure in solution, either multimeric or monomeric β-structure at the lipid headgroup interface, or inserted into lipid as an α-helix. The tendencies of the different sequence variants to exist in each of these states are a consequence of their conformational propensities as well as their membrane insertion abilities. The analysis of the OmpA WT signal peptide revealed that it had a strong tendency to adopt β-structure, either in aqueous solution, as a multimer, or upon interaction with a membrane surface. From the results of the present study, we cannot determine whether the membrane-associated form is multimeric or not. The L6L8 signal peptide, while showing near-wild-type behavior in terms of membrane insertion and in vivo activity, did not adopt the β-structure seen for wild type. Hence, we conclude that the Ile to Leu replacements shift the conformational preference of the peptide away from β, as anticipated from Chou–Fasman predictions. The Δ8 mutant, while suffering from a lowered overall hydrophobicity (see above), shows no tendency to self-associate, eitner in aqueous solution or at the membrane surface, consistent with its expected reduced tendency to adopt β-structure. By contrast, the Δ9 peptide manifested an even higher predilection than wild type for taking up β-structure upon interaction with vesicles, indicating, as expected from deletion of an Ala, that its conformational equilibrium had shifted toward β-structure. The Asn mutant showed no association tendency and, by CD, only a slightly reduced helical content. This observation underlies the importance of the membrane insertion ability as a requirement for signal sequence function, since the I8N signal peptide appears to meet the conformational requirement for a high content of α-helix. In order to assess the importance of all of these conformational changes in more detail, we are carrying our NMR studies of the OmpA peptides as we have for the LamB series (Bruch et al., 1989; Bruch & Gierasch, 1990).

While the LamB signal peptides showed no tendency to associate into β-structure in aqueous solution, direct physical evidence for a lipid surface associated β-structure was obtained for the LamB WT signal peptide in lipid monolayers using CD and Fourier transform infrared spectroscopy (Cornell et al., 1989). It is difficult to assign the relative importance of the preferred conformational states of isolated signal peptides in vivo since it is not easy to mimic all the environments offered by the export pathway. Nonetheless, we earlier had suggested (Briggs et al., 1986) that surface-associated β-structure was the initial form of the signal peptide upon interaction with membrane lipids, followed by α-helix when inserted. The results with OmpA reinforce the general conclusion that functional signal peptides can adopt β-structure as well as α-helix depending on their environment and that the β-structure may be of functional importance.

The implications of biophysical characterization such as the present work on OmpA for the mechanism of protein export must be cautiously extracted. First, we have learned in this study that the conformational properties of all signal sequences studied to date are very similar and that they include both a high tendency to adopt α-helix in interfacial environments and a capacity to form β-structure depending on environment. The interactions of signal sequences with various proteinaceous components of the export pathway may exploit both of these conformational tendencies. The detailed understanding of the specific steps in which the known conformational properties of signal sequences are required must await dissection, either genetically or biochemically, of the signal sequence binding to SecA, SecE, SecY, and possibly other constituents. If, for example, allele-specific suppression of signal sequence defects from mutations in any of the export components can be described, then we may be able to deduce features of the binding interaction. Alternatively, physical methods can be sought to characterize binding of signal sequences to isolable components of the export pathway.

Despite the difficulties of pinning down steps at which particular properties of signal sequences are required in the export pathway, all of the work done on isolated signal peptides has consistently revealed that they *must be able to insert into membranes* in order to act as competent signal sequences in vivo. As discussed in the introduction, the transfer of the nascent chain from SecA to the membrane protein SecE and SecY moves the export complex into the lipid environment. We are persuaded that one of the critical roles of the signal sequence is to facilitate partitioning of the nascent chain into the two-dimensionally restricted domain of the membrane. Certainly, all of the characteristics of signal sequences that enable them to insert into lipid bilayers in model systems are conserved among different native signal sequences and are retained in functional mutants. The OmpA results add considerably to the available evidence for a tight correlation between membrane insertion and function and, in fact, have provided a quantitative measure of this characteristic.

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