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Lipid and peptide specificities in signal peptide–lipid interactions in model membranes

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The present data show the critical importance of the anionic lipid content in monomolecular layers for the interaction with PhoE signal peptide. At 37°C and 100 mM NaCl the interaction is maximal at 30–40 mol% anionic lipid. The results correlate with the reduced translocation competence of *Escherichia coli* strain HD3122, which has a much lower anionic lipid content as compared to the wild- type strain SD12 (De Vrije et al. (1988) Nature 334, 173–175). PhoE signal peptide analogs as *N*-formyl PhoE signal peptide, PhoE signal peptide +(1–7) and PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$ show the same lipid preference as PhoE signal peptide. On the other hand the affinity for an anionic lipid interface is strongly reduced for PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$, which correlates with the less efficient translocation of PhoE protein carrying this signal sequence. At limiting anionic lipid concentrations there is a temperature and salt effect on the observed interaction, which is related to a conformational change of the peptide. Signal sequences show clearly conformational flexibility in responds to environmental conditions. Under the conditions used in this study FTIR spectra of PhoE signal peptide-DOPG monolayers show a high content of β-structure and β-turn.

Introduction

Escherichia coli proteins which have to be transported over the inner membrane are synthesized with a N-terminal signal sequence. This sequence is required for translocation over the membrane [1,2]. Although there is little primary sequence homology [3] there are several common structural elements in signal sequences. One or more positively charged amino acids are present at the N-terminal part, followed by a central hydrophobic segment comprising 8–15 residues and a region with higher polarity at the C-terminal end [4]. It is believed that the conformational state of signal sequences are functionally recognized in the translocation process. The products of overlapping internal deletions in both the PhoE gene [5] and OmpA gene [6,7] were all exported out of the cytoplasm leading to the conclusion that the signal sequences contain all the information required for export of these proteins [8]. In the prokaryotic system apart from the leader peptidase [9] no protein components directly interacting with the signal peptide have yet been identified. In several models for translocation it is proposed that the signal sequence interacts directly and functionally with lipids in the membrane [10,11]. A negatively charged lipid-specific insertion of signal peptides in model membranes has been demonstrated for synthetic signal peptides of M13 coat [13,14], LamB [15] and PhoE protein [13]. Consistent with these models is the observation that reduction of the level of PG the major anionic membrane phospholipid, using *E. coli* mutants in the phospholipid biosynthesis, led to a less effective pre-PhoE translocation [16]. To study the levels of anionic lipids required for the interaction of PhoE signal peptide with lipid membranes, monomolecular layers were used with varying anionic lipid content. By this method also the involvement of the peptide cationic groups were further analyzed using the N-terminal formylated peptide and signal peptide analogs in which the lysine residues are replaced by aspartic acid which in vivo and in vitro results in a less efficient translocation [17]. In addition, a polypeptide consisting of the wild-type PhoE signal peptide and seven amino acids of the mature sequence

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was studied to understand the importance of the N-terminal part of the mature sequence on the signal peptide–lipid interaction. Fluorescence spectroscopy is commonly used to characterize tryptophan-containing peptides. To test the effect of introducing a tryptophan residue in the signal peptide on signal peptide–lipid interactions, such a peptide was included in this study. The pressure changes of the lipid monolayers at the air/water interface demonstrate penetration into the hydrophobic core, whereas temperature effects indicate that conformational changes of the peptide are involved in the membrane interaction.

Materials and Methods

Materials

The synthesis of PhoE signal peptide (MKKSTLALVVMGIVASASVQA), PhoE signal peptide +(1–7) (containing the additional C-terminal sequence: AEIYNKD); PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$; PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$ and N-terminal formylated PhoE signal peptide was performed by Dr. D. Olshefski (University of California, San Diego, CA, U.S.A.). The peptides were pure as determined by HPLC.

Radiolabeling of lysine residues by reductive methylation was essentially as described before [13]. After being dried from a solution of trifluoroacetic acid (TFA) the peptides were dissolved in dimethyl sulfoxide or trifluoroethanol (TFE) at concentrations of 1 mg/ml. PhoE signal peptide +(1–7) was also dissolved in aqueous HCl (10 mM), pH 2, after pretreatment with TFA (a procedure developed for OmpA signal peptide by Hoyt, D.W. and Gierasch, L.M., personal communication). Peptide concentrations were determined by quantitative amino acid analysis.

Dioleoylphosphatidylcholine (DOPC); dioleoylphosphatidylethanolamine (DOPE); dioleoylphosphatidylserine (DOPS); dielaidoylphosphatidylethanolamine (DEPE); dioleoylphosphatidylglycerol (DOPG); dielaidoylphosphatidylglycerol (DEPG); dioleoylphosphatidic acid (DOPA) were synthesized according to established methods [18,19]. Beef heart cardiolipin (CL) [20] and *E. coli* lipids [13] were isolated and purified essentially as described.

Methods

Interfacial measurements were performed in a thermostatically controlled box (±0.1°C) [21]. As a subphase, a buffer consisting of 10 mM Pipes (pH 7.0), 1 mM EDTA was used and a NaCl concentration as indicated. The monomolecular lipid layers were spread from a $CHCl_3/CH_3OH$ (8:2, v/v) solution to give an initial surface pressure of 20 mN/m. The surface pressure was measured by the Wilhelmy method. The teflon dish had a volume of 19 ml and a surface area of 29.6 cm^2. Peptide was added to the subphase through a hole at an extended corner of the dish. The subphase was continuously stirred with a magnetic bar. The pressure changes were followed for 90 min. Attenuated total reflectance-Fourier transform infrared spectra (ATR-FTIR) were obtained on a Perkin-Elmer 1720X spectrophotometer equipped with a liquid nitrogen cooled MCT detector at a resolution of 2 cm^{-2} 512 scans were averaged. Every four scans of the sample, reference spectra of a clean germanium plate were automatically recorded and the ratio against the recently run sample spectra was calculated using an automatic sample shuttle accessory. The spectrophotometer was continuously purged with dry air. For polarization experiments, a Perkin-Elmer silver bromide polarizer was placed before the sample and before the reference plate. The germanium plate was sealed in an universal sample holder (Perkin-Elmer 186–0354) and partial H/D exchange was obtained by flushing the chamber of the holder with D_2O saturated nitrogen for 2 h at room temperature. As dicussed earlier [27] a limited H/D exchange is sufficient to perform a secondary structure analysis of the peptide. The secondary structure was obtained after spectral resolution enhancement by Fourier self-deconvolution followed by a least-square curve fitting analysis of amide I^1 as described [27]. A teflon trough (15 × 5 × 0.5 cm) with a dipping well was used for film transfer to germanium ATR crystals (50 × 10 × 2 mm, 45°; Harrick Scientific Co., Ossining, NY) for infrared spectroscopy. The germanium plates were made hydrophilic by cleaning in a plasma clearner prior to use. When the monolayer was deposited the surface pressure was kept constant with a computer-controlled, stepped motor. The germanium crystal was raised at a rate of 3 mm · min^{-1}.

Results

The effect of membrane charge

In a previous study using mutants of *E. coli* it has been shown that a reduction of the anionic lipid content, drastically diminished translocation and processing of pre-PhoE [16]. In *E. coli* strain SD12 the PG and CL content of the inner membrane is 20.8 and 4.8 mol%, respectively. On the other hand in the mutant strain HD3122 the PG and CL content in the inner membranes dropped to 2.3 and 0.8 mol%. In the latter membrane also 2.8 mol% PA is present [16]. To study the influence of the negatively charged lipid content on the interaction with PhoE signal peptide, monomolecular layers of DOPE with increasing amounts of DOPG were used. After injection of PhoE signal peptide underneath a monomolecular layer of pure DOPE, having a surface pressure of 20 mN/m, the pressure increased rapidly with 6 mN/m (Fig. 1). For a pure DOPG monolayer also a rapid initial pressure increase with

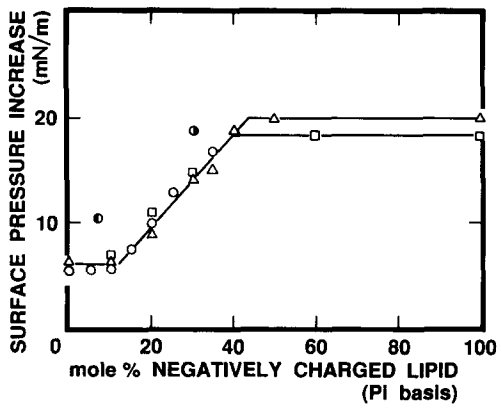

Fig. 1. Monolayer surface pressure increase as a function of anionic lipid content (based on phosphorus). Monolayers of DOPE-DOPG (Ⓓ); DOPE-CL (□); DOPC-DOPG (◑); lipid extracts of inner membrane of *E. coli* strain SD12 (○) and strain HD3122 (◐) were formed, of 20 mN/m initial pressure. 10 nmol of PhoE signal peptide was injected, the subphase contained 19 ml of 10 mM Pipes (pH 7.0) 100 mM NaCl, 1 mM EDTA. The temperature was maintained at 37°C.

approx. 10 mN/m was observed followed by an additional increase such that the final increase was 20 mN/m (the kinetics are discussed in the forthcoming Fig. 4 and Ref. 13). This corresponds to a final surface pressure of 40 mN/m. The added amount of PhoE signal peptide gave the maximal pressure increase. For mixed monolayers of DOPE-DOPG the pressure increase induced by PhoE signal peptide was critically dependent on the PG content. A maximal pressure increase of 20 mN/m was only found for mixtures containing more than 40 mol% DOPG. Mixtures containing up to 10 mol% DOPG showed a low pressure increase comparable to that found for pure DOPE. Mixed monolayers containing between 10 and 40 mol% DOPG showed a linear increase in surface pressure (Fig. 1). Also for mixtures of DOPE and CL a similar charge dependency between 10 and 40% anionic lipid was observed. Also mixtures of DOPE-DOPS and DEPE-DEPG behaved comparatively (not shown). No difference in the interaction was observed between the zwitterionic lipids DOPE and DOPC in mixtures with DOPG (Fig. 1).

Monomolecular layers formed of the lipid extracts from the inner membrane of *E. coli* strain SD12 showed after interaction with PhoE signal peptide a pressure increase of 19 mN/m (Fig. 1), which is very close to the maximal pressure increase. Taking into account the total content of negatively charged lipids (based on phosphorus), the pressure increase with the SD12 lipid extract is 4 mN/m higher than for the DOPE-DOPG mixture containing 30.4 mol% DOPG. However, upon injection of PhoE signal peptide underneath a monolayer of DOPE/DOPG/CL (molar ratio 70:25:5) results in an identical pressure increase as observed for *E. coli* strain SD12 membrane lipids (not shown). In a similar experiment using the lipid extract of the mutant strain HD3122, the pressure increase was only 10.3 mN/m (Fig. 1) consistent with the lower content of negatively charged lipids. The 4 mN/m difference in pressure increase between the lipid extract of mutant strain HD3122 inner membranes and a DOPE-DOPG mixture containing 6.7 mol% DOPG (corresponding with the amount of negatively charged lipids in HD3122 inner membrane) can be compensated by using a ternary lipid mixture of DOPE/DOPG/DOPA (molar ratio 92.4:4.2:2.5). A practical implication of this observation is that a PA impurity in PE will lead to an apparent discrepancy between PE and PC in interaction with the signal peptide, which will vanish at pH 5.9 when PA dissociation is suppressed.

The effect of temperature

The interaction between PhoE signal peptide and mixed monolayers of DOPE and DOPG proved to be very temperature sensitive, particularly at PG concentrations in the physiological range. Lowering the temperature from 37°C to 22°C reduced the pressure increase of a pure DOPE monolayer after addition of PhoE signal peptide by < 1 mN/m (Fig. 2). The reduced interaction is most evident between 10 and 60 mol% DOPG. At 22°C higher PG concentrations are required to obtain a maximal pressure increase of 20 mN/m than at 37°C. At PG concentrations > 60 mol% there is no significant effect of lowering the temperature to 22°C.

The effect cannot be ascribed to temperature effects on the molecular packing of DOPE or DOPG. The changes in molecular area of DOPE and DOPG at 20 mN/m by lowering the temperature from 37°C to 22°C are 2.4 and 2.3 Å^2/molecule, respectively, whereas no

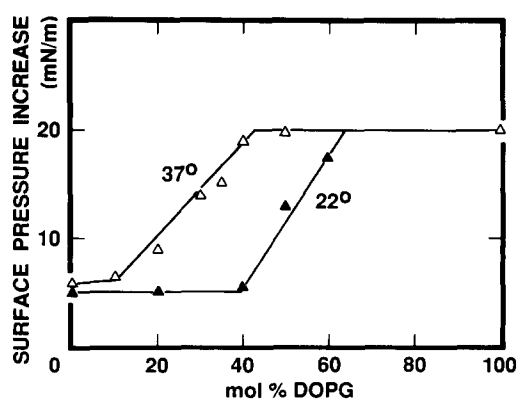

Fig. 2. Effect of temperature and anionic lipid content on the surface pressure increase after addition of PhoE signal peptide. Monolayers of DOPE-DOPG, initial pressure 20 mN/m, were formed at 37°C (△) and 22°C (▲). Experimental conditions as described in Fig. 1.

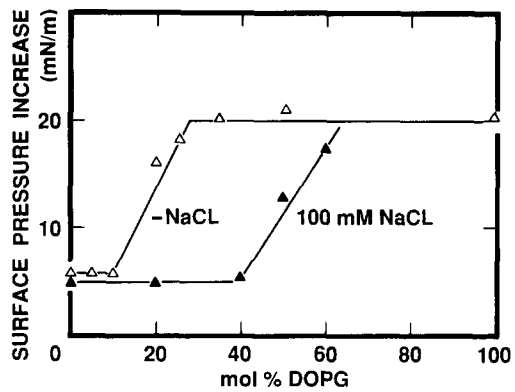

Fig. 3. Effect of ionic strength and anionic lipid content on the surface pressure increase after addition of 10 nmol PhoE signal peptide. Monolayers of DOPE-DOPG, initial pressure 20 mN/m, were formed at a subphase of 10 mM Pipes (pH 7.0), 1 mM EDTA (△) and 10 mM Pipes (pH 7.0), 1 mM EDTA, 100 mM NaCl (▲). The temperature was maintained at 22°C.

significant differences in pressure increase induced by the PhoE signal peptide are observed for the pure lipids.

The effect of ionic strength

The interaction between PhoE signal peptide and negatively charged membranes is also influenced by salt concentrations particularly at lower temperatures. At 22°C the interaction is strongly enhanced when NaCl is absent in the concentration range of 10 to 40 mol% DOPG (Fig. 3). In the absence of NaCl a maximal pressure increase is found at about 25 mol% DOPG, whereas in the presence of 100 mM NaCl 50 mol% DOPG is required. At 37°C there is little effect of 100 mM NaCl on the interaction (data not shown). Only between 0 and 10 mol% DOPG there is a higher pressure increase (data not shown). It is most likely that both temperature and salt concentration mainly affect the peptide structure or conformational changes involved in the interaction with the negatively charged membrane interface.

Kinetics of interaction

Previously we reported two phase kinetics for the insertion of the PhoE signal peptide in CL monolayers [13]. To test the possible functional significance of this observation, we analyzed the kinetics of the insertion, using ^{14}C-labeled PhoE signal peptide. At the physiological temperature of 37°C the lipid extract obtained for inner membranes of strain SD12, and not for strain HD3122, displayed two phase kinetics of insertion (data not shown). The data obtained for strain SD12 at 37°C resembled the tracing shown in Fig. 4 for the total lipid extract of *E. coli* strain SD12 at 47°C. The temperature was raised to compensate for the decreased anionic lipid content compared to the inner membranes [12]. There is a first rapid pressure increase from 20.4 mN/m to 31.7 mN/m and a simultaneous increase in surface radioactivity. After 40 min the surface pressure started to increase to a final surface pressure of 40.8 mN/m. At the same time the surface radioactivity increased four times. The conditions were chosen such that, in separate experiments, the monolayer could be collected at the lower and higher pressure increase. The molecular area of PhoE signal peptide that can be calculated for the two stages of penetration was 371 and 153 Å^2/molecule, respectively. This is in agreement with the values found before for CL monolayers [13]. In a similar experiment, performed at 37°C a limiting molecular area of 168 Å^2/molecule was found for PhoE signal peptide +(1–7) after penetration of a DOPG monolayer to give a final surface pressure of 41 mN/m (Table I).

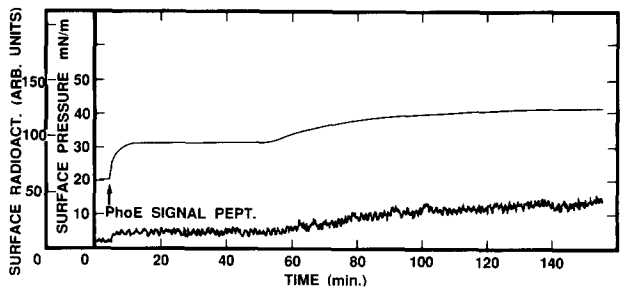

Fig. 4. Kinetics of PhoE signal peptide interaction with a monomolecular layer of *E. coli* strain SD12 total lipid extract. The initial surface pressure was 20 mN/m and subphase conditions as described in Fig. 1. 20 nmol [^{14}C]PhoE signal peptide was injected as indicated. The temperature was maintained at 47°C.

A molecular area of approx. 160 Å^2/molecule is not found when mixed monolayers of anionic lipid and PhoE signal peptide are compressed after spreading at the interphase. At low surface pressures, approx. 5 mN/m, the peptide molecular area was 350 Å^2/molecule, but at high surface pressures (42 mN/m) the molecular area was still 250 Å^2/molecule even at low compression rates. This suggests that the conforma-

TABLE I

Biophysical parameters of PhoE signal peptides

Signal peptide	Molecular area		K_d (nM) [c]
	at 31 mN/m [a]	at 40 mN/m [b]	
PhoE signal peptide	371	153	79
PhoE signal peptide +(1–7)		168	174
Formyl PhoE signal peptide			190
PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$			190
PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$			398

[a] Molecular area (Å^2/molecule) after insertion in an anionic lipid containing monolayer increasing the pressure from 20 to 31 mN/m.
[b] The pressure increase was from 20 to 40 mN/m.
[c] For insertion in a DOPG monolayer at 37°C, the aqueous phase contained Pipes buffer (pH 7.0), 100 mM NaCl.

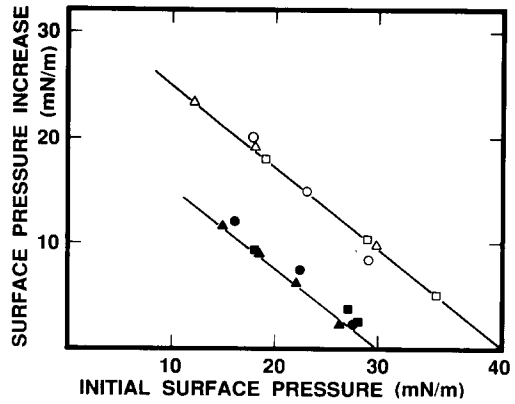

Fig. 5. Signal peptide-induced surface pressure increase as a function of the initial pressure. Open symbols, DOPG; closed symbols, DOPC. The subphase was 10 mM Pipes (pH 7.0), 100 mM NaCl, 1 mM EDTA, 37°C, 30 nmol peptide was injected. PhoE signal peptide +(1–7), (△, ▲); formyl PhoE signal peptide (□, ■); PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$ (○,●).

tional change is dependent on the proper interfacial insertion of the signal peptide.

Although it is clear that the presence of negatively charged lipid is a prerequisite for the interactions resulting in a high pressure increase by PhoE signal peptide little is known of the charge requirements of the peptides for interaction with lipids. In PhoE signal peptide the polar residues are all located at the N-terminal part, whereas the overall properties are those of a very hydrophobic peptide. A charge distribution at both the N- and C-terminal side of the peptide is found in PhoE signal peptide +(1–7). The additional seven amino acids of the mature sequence of the protein increased the polarity such that this peptide could now also be dissolved in water at pH 2.

PhoE signal peptide analogs

Injection of the extended signal sequence underneath monomolecular layers of DOPG or DOPC at different initial pressures gave pressure increases comparable to those found previously for PhoE signal peptide (compare Fig. 5 with Ref. 13). In all cases there is a clear preference for the anionic lipid interface. The pressure increase observed with PhoE signal peptide +(1–7) was essentially the same whether it was injected underneath the lipid monolayer from TFE, DMSO or from an aqueous pH 2 solution.

The limiting pressure for penetration of a DOPG monolayer is 44 mN/m and of a DOPC monolayer 31 mN/m. The signal sequence with the physiological N-terminus, i.e. formyl PhoE signal peptide, has a reduced positive charge content which renders the peptide even more hydrophobic. However, the interaction with DOPG and DOPC monolayers is not affected as the same pressure increases and anionic lipid specificity is found as for PhoE signal peptide (Fig. 5). The rate of pressure increase is slower for PhoE signal peptide +(1–7) and formyl PhoE signal peptide than for PhoE signal peptide.

The single amino acid replacement PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$ also did not affected the lipid specificity of the interaction (Fig. 5). Only the rate of pressure increase was enhanced as compared to PhoE signal peptide (not shown). The importance of the charge interaction of PhoE signal peptide with anionic lipids is underlined in the experiment shown in Fig. 6, where the charge of the signal peptide is reversed. Addition of PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$ to monolayers of DOPG showed a strong reduction in surface pressure change compared to the parent signal peptide. The pressure increment induced by the PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$ is lower for the anionic lipid than for the zwitterionic DOPC monolayer (Fig. 6).

The K_d values determined from the maximal surface pressure change of DOPG monolayers at increasing peptide concentrations showed the lowest value of 79 nM for PhoE signal peptide. PhoE signal peptide +(1–7), formyl PhoE signal peptide and PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$ showed K_d values of 174, 190 and 190 nM, respectively, whereas a much higher value of 398 nM was found for PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$ (Table I).

Conformational properties of PhoE signal peptide

The secondary structure of PhoE signal peptide samples prepared from different solutions was determined by FTIR. The amide I^1 absorption band occurring in the 1620–1660 cm^{-1} spectral region being a sensitive indicator of the peptide's secondary structure. The results were in qualitative agreement with previous CD measurements [22]. The FTIR spectrum of PhoE signal peptide preparation from H_2O shows a high absorption at 1621 cm^{-1} typical for β-structure [23], whereas also a high percentage unordered structure is found (Fig. 7; curve 1). The addition of SDS after the injection of the

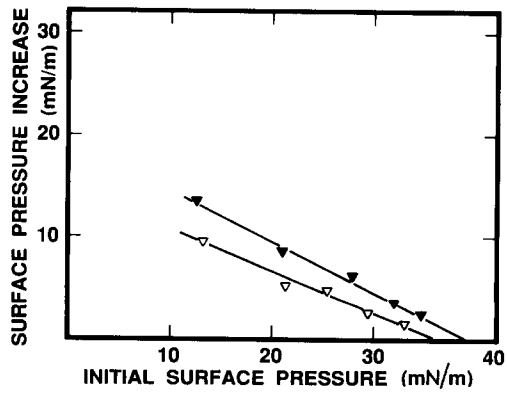

Fig. 6. PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$ induced surface pressure increase as a function of the initial pressure. DOPG (▽); DOPC (▼). Experimental conditions as described in Fig. 4.

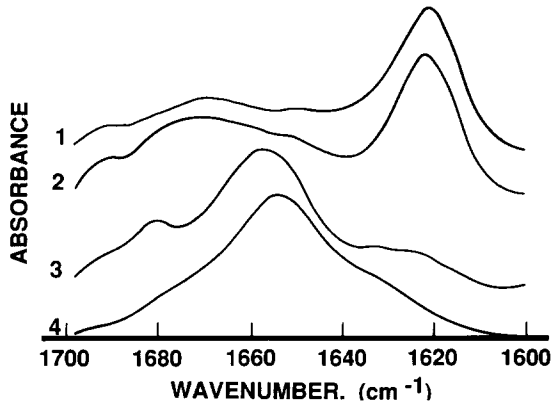

Fig. 7. FTIR spectra of PhoE signal peptide deposited from a solution on germanium ATR crystals. Two μl of 10 mg/ml TFE solution of the peptide were added to 50 μl H_2O (curve 1), to 50 μl H_2O, SDS was added thereafter at a final concentration of SDS of 0.8% (curve 2), to 50 μl H_2O containing 0.8% SDS (curve 3). The sample was then dried under a N_2 stream on the germanium crystal. For curve 4, the TFE solution was directly evaporated on the germanium crystal. H/D exchange was performed on the film as described in Methods.

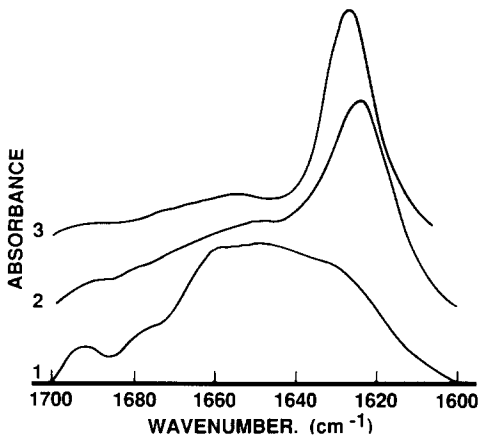

Fig. 8. FTIR spectra of PhoE signal peptide containing monolayers transferred to germanium ATR crystals. The initial pressure of the monolayer before peptide injection was 20 mN/m. Monolayer of DOPC transferred at 26 mN/m, curve 1; monolayer of DOPG transferred at 27 mN/m, curve 2; monolayer of DOPG transferred at 38 mN/m, curve 3.

peptide into the aqueous phase has little effect (Fig. 7; curve 2). On the other hand, if the peptide is directly dissolved in SDS solution the maximum absorption is shifted to the range typical for α-helix structure (Fig. 7; curve 3). An even higher percentage of α-helix (83%) is found in samples prepared from a TFE solution (Fig. 7; curve 4). From this experiment it can be concluded that the ATP germanium plate induces no effect on the peptide structure. A mainly α-helix or mainly β-sheet conformation could be found on the ATR plate depending only on the conformation of the peptide in the solution state.

The FTIR spectra of lipid-PhoE signal peptide monolayers transferred to germanium crystals are shown in Fig. 8. Before transfer the subphase was refreshed to eliminate unbound peptide. By double label experiments it was established that the lipid-peptide ratios in the monolayer and on the germanium plate were equal. The monolayer of DOPC after interaction with PhoE signal peptide was collected at a surface pressure of 26 mN/m. The spectrum shows a broad maximum from 1620 to 1670 cm^{-1}, which is indicative for a combination of α-helix, β-turn and β-structure [23] (Fig. 8; curve 1). The actual spectrum estimated as described in [27] contains 15% α-helix, 35% β-sheet and 50% turns and unordered structure. The monolayers of DOPG-PhoE signal peptide interaction products were transferred at a surface pressure of 27 and 38 mN/m. The spectrum of the monolayer collected at the lower pressure showed the maximum absorption at 1625 cm^{-1}, but there was still a considerable absorption in the region 1630–1670 cm^{-1} (Fig. 8; curve 2). Analysis of this spectrum indicates that the secondary structure is considerably enriched in β-sheet, which amounts now to 53%, the α-helix representing 22% and the turns and unordered structure 25%. The β-sheet content increases even further when the monolayer is collected at 38 mN/m (Fig. 8; curve 3). The secondary structure arrived at is then 63% β-structure, 12% α-helix and 25% turns and unordered structures.

To get insight into the orientation of the secondary structural elements of the PhoE signal peptide polarization experiments were carried out as described in [27]. Both spectra in which the incident light is polarized at

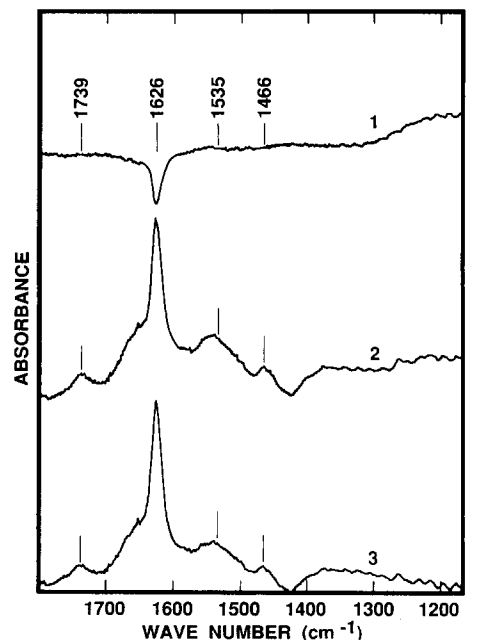

Fig. 9. IR spectra of the PhoE signal peptide (as described in Fig. 8 for curve 3) recorded with the incident light polarized at 90° (curve 2) and at 0° (curve 3). Curve 1 is the dichroic spectrum 90°–0°. Each curve is plotted on the same ordinate scale.

0° and 90° show the strong amide I^1 absorption band at 1626 cm^{-1} typical for β-structure (Fig. 9). In the difference spectrum the dichroic nature of this band is clearly manifested. Whereas the bands at 1739, 1535 and 1466 cm^{-1} which are assigned to the phospholipid carbonyls and the peptides amide II and II^1 bands, respectively, are cancelled in the difference spectrum the negative sign of the amide I^1 band strongly indicates that the carbonyl group of the peptide bond in the β-sheet lays parallel to the plane of the membrane. The calculated [27] dichroic ratio, R, of 0.91 implies that this orientation is nearly absolute. It should be realized that such an orientation can be accomplished with the long axis of the β-sheet either parallel or perpendicular to the plane of the membrane. However, an orientation of the short axis of the sheet perpendicular to the plane of the membrane can be excluded.

Discussion

The anionic lipid content of model membranes proves to be of critical importance for the interaction with PhoE signal peptide. The 20.8 mol% PG and 4.8 mol% CL present in the inner membrane of *E. coli* wild-type strain SD12 are obligatory for a maximal pressure change of the monolayer after signal peptide addition at 37°C and 100 mM NaCl. The kinetics of the PhoE signal peptide interaction with lipid monolayers was also highly dependent on the content of negatively charged lipids. For lipid monolayers with an initial pressure of 20 mN/m and an anionic lipid content of more than 40 mol% there is a rapid pressure increase of 18–21 mN/m in 10–15 min after injection of PhoE signal peptide in the subphase. At 0–10 mol% anionic lipid the pressure increase was rapid but limited to about 6 mN/m. At intermediate concentrations of negatively charged lipids there was a rapid initial pressure increase of 8–11 mN/m followed by a second pressure increase. The lag time of the latter can be as much as 30–60 min. For different mixtures of anionic and zwitterionic lipids similar pressure increases were observed although a higher rate was observed for CL than for PG or PS. The observed anionic charge dependency is not caused by a particular fatty acid composition of the compounds. Also for mixtures of DEPE and DEPG a similar effect of the anionic lipid was noticed. It is therefore striking that the ultimate pressure increase was higher for the *E. coli* membrane lipids consisting of different anionic lipids than for the synthetic lipid mixtures containing a single anionic lipid specie (Fig. 1). On the other hand, when a synthetic mixture was formed of DOPE/DOPG/CL (molar ratio 75:20:5) the same pressure increase was observed as with the lipid extract of natural translocation competent membranes. The difference in pressure increase between the monolayers formed of the lipid extract of *E. coli* mutant strain HD3122 inner membranes and the corresponding mixture of DOPE-DOPG can be explained by the absence of PA in the latter mixture. These results could indicate that the complex anionic lipid composition is important for the optimal interaction with PhoE signal peptide. The different responses on PhoE signal peptide injection of monolayers formed of *E. coli* wild-type strain SD12 and mutant strain HD3122 inner membrane lipids correlate well with the translocation competence of the first strain and reduced translocation of the latter strain measured in in vivo and in vitro translocation experiments [12,16]. It is tempting to suggest that the conditions leading to a final surface pressure of 38–41 mN/m after PhoE signal addition are requirements for a functional insertion of the peptide in biological membranes.

Temperature effects on the PhoE signal peptide-membrane interaction are apparent at limiting anionic lipid concentrations. The effect is not due to drastic changes in molecular packing since the very limited reductions in molecular area are similar for DOPE and DOPG. It is likely that these effects originate from conformational changes of the PhoE signal peptide which are inhibited at lower temperatures when anionic lipid content and interaction rate is low. This seems also the most obvious explanation for the salt effect observed at 22°C. At physiologically relevant anionic lipid concentrations a maximal pressure increase is observed in the absence of NaCl whereas the pressure increase is suppressed in the presence of 100 mM NaCl at 22°C (Fig. 3). At 37°C, however, there is hardly an effect of ionic strength (data not shown). Considering also the K_d value of PhoE signal peptide-DOPG interaction of 79 nM it is not likely that ionic interactions between lipid and peptide are strongly affected by 100 mM NaCl. K_d values of this order were also found for LamB signal sequence interaction with monolayers of 65% PE and 35% PG [24]. The fluid state of the membrane is essential since interaction is strongly reduced when the anionic lipid in the gel state [13] by affecting penetration by and conformational changes of the peptide.

Sequences related to the wild type as N-terminal formylated PhoE signal peptide where the charge is reduced and the PhoE signal peptide +(1–7) with charged amino acids at both the N- and C-terminal end showed affinities for DOPG identical to that of PhoE signal peptide [13], only the K_d values were increased to 170–190 nM. Otherwise the pressure increase profile was identical for all these peptides whether the peptides were added from TFE or DMSO or in case of PhoE signal peptide +(1–7) also from aqueous solution. These results are compatible with the similar inhibition of PhoE translocation in an in vitro translocation experiment by PhoE signal peptide and PhoE signal peptide +(1–7) [12]. The PhoE signal peptide $Val^{-8} \rightarrow Trp^{-8}$

showed a similar preference for DOPG as the other signal peptides mentioned above. Also the K_d value was in line. Only the kinetics of the pressure increase were more rapid.

Reversal of the charge of the signal peptide as in PhoE signal peptide $Lys^{-19,-20} \rightarrow Asp^{-19,-20}$ decreased strongly the induction of a pressure change in DOPG whereas the K_d increased to 398 nM, but in DOPE the pressure change was greater than measured for the positively charged signal peptides (Figs. 4 and 5). The diminished affinity of PhoE ($Asp^{-19,-20}$) for negatively charged interfaces correlates with the retardation of PhoE translocation when this peptide was in front of wild-type mature PhoE as was shown in in vivo pulse label and in vitro translocation experiments [17] and less effective in inhibition of PhoE translocation than wild-type signal peptide [12].

Signal sequences show clearly conformational flexibility in responds to environmental conditions. The secondary structure of PhoE signal peptide showed in aqueous medium a FTIR and CD spectrum [22] typical for β-structure. In trifluoroethanol and 0.8% SDS the spectrum is indicative for α-helix structure. However, CD spectra showed that in neutral detergent, Lubrol PX and lysophosphatidylcholine β-structure was apparent [22]. The molecular area determined for the PhoE signal peptide in soy-PC and CL was 345 and 165 $Å^2$/molecule, respectively [13]. On the assumption of α-helix formation in the lipid monolayer this would reconcile with a looped and unlooped α-helical structure, respectively [13].

A high percentage of α-helix but also β-conformation was observed for LamB signal peptide in the surface of lipid monolayers [25], which we could confirm with our experimental set up (unpublished observations). Thermodynamic considerations argue for an α-helical conformation for signal sequences in order to span the membrane [11]. A recent study on the signal sequences of chicken lysozyme; *E. coli* proteins alkaline phosphatase and lipoproteins indicates a marked preference for β-structure at increasing order of hydrophobicity. It was concluded that the ability of these signal sequences to adopt β-structure in highly hydrophobic environment is important for function [26].

For PhoE signal peptide +(1–7), after interaction with a DOPG monolayer, a molecular area of 168 $Å^2$/molecule was measured. This would be in accordance with β-structure, assuming both polar endings at the interface. A similar molecular area was found for PhoE signal peptide after interaction with an anionic lipid containing interface. A structure with a β-turn at the glycine moiety could also be suggested for PhoE signal peptide and would be in agreement with the high percentage of β-structure and β-turn measured by FTIR and consistent with the orientation determined by this method.

Although in monomolecular layers, under the conditions described, a high content of β-structure is found for PhoE signal peptide, as well as for other signal peptides in a highly hydrophobic environment, it still has to be proven that this is the functional conformation. The possibility that β-structure formation is the result of signal peptide aggregation should be seriously considered.

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