Tryptophan Fluorescence Study on the Interaction of the Signal Peptide of the *Escherichia coli* Outer Membrane Protein PhoE with Model Membranes

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ABSTRACT: The interaction of the signal peptide of the *Escherichia coli* outer membrane protein PhoE with different phospholipid vesicles was investigated by fluorescence techniques, using a synthetic mutant signal peptide in which valine at position −8 in the hydrophobic sequence was replaced by tryptophan. First it was established that this mutation in the signal sequence of prePhoE does not affect in vivo and in vitro translocation efficiency and that the biophysical properties of the synthetic mutant signal peptide are similar to those of the wild-type signal peptide. Next, fluorescence experiments were performed which showed an increase in quantum yield and a blue shift of the emission wavelength maximum upon interaction of the signal peptide with lipid vesicles, indicating that the tryptophan moiety enters a more hydrophobic environment. These changes in intrinsic fluorescence were found to be more pronounced in the presence of phosphatidylglycerol (PG) or cardiolipin (CL) than with phosphatidylcholine (PC). In addition, quenching experiments demonstrated a shielding of the tryptophan fluorescence from quenching by the aqueous quenchers iodide and acrylamide upon interaction of the signal peptide with lipid vesicles, a shielding in the case of acrylamide that was more pronounced in the presence of negatively charged lipids. Finally it was found that acyl chain brominated lipids incorporated into phospholipid bilayers were able to quench the tryptophan fluorescence of the signal peptide, with the quenching efficiency in CL vesicles being much higher than in PC vesicles. The results clearly demonstrate that the PhoE signal peptide interacts strongly with different lipid vesicles. From the intrinsic fluorescence measurements as well as from the various quenching experiments, it is evident that the interaction of the signal peptide with negatively charged lipids is stronger than with the zwitterionic PC. The results are consistent with a deeper penetration of the tryptophan-containing part of the signal peptide into the hydrophobic interior of the bilayer when negatively charged lipids are used. It is proposed that the presence of negatively charged lipids in *Escherichia coli* membranes is essential for a functional interaction of the PhoE signal sequence with these membranes.

Signal sequences play an essential but as yet poorly understood role in the export of newly synthesized proteins out of the cytoplasm of bacterial cells [see Gierasch (1989) for a recent review]. These 20–25 amino acids long highly hydrophobic sequences are synthesized as temporary N-terminal extensions, which may be involved in various steps of the secretion process, such as recognition by proteinaceous cytosolic or membrane-bound factors (Oliver & Beckwith, 1982; Ryan & Bassford, 1985; Fikes & Bassford, 1989; Lill et al., 1988), prevention of folding of the precursor protein into a translocation-incompetent conformation (Park et al., 1987), or interaction with the lipids in the target membrane [reviewed by De Vrije et al. (1990)]. A variety of biophysical studies on the properties of isolated signal peptides have shown that these peptides have a strong interaction with lipids in model membrane systems (Batenburg et al., 1988a,b; Bruch et al., 1989; Cornell et al., 1989; Nagaraj et al., 1987; Reddy & Nagaraj, 1989; Briggs et al., 1986; Killian et al., 1990). They perturb lipid structure (Batenburg et al., 1988b; Nagaraj et al., 1987) and affect the macroscopic organization of membrane lipids (Batenburg et al., 1988b; Killian et al., 1990). In model membranes composed of the total lipid extract of *Escherichia coli* membranes, signal peptides were found to promote the formation of type II nonbilayer lipid structures (Killian et al., 1990) in accordance with suggestions (De Vrije et al., 1990) that signal sequences can act by inducing a local change in lipid structure that facilitates the movement of the mature region of the protein across the membrane. Although up till now a direct interaction of the signal sequence with membrane lipids during protein translocation has not been demonstrated, there is ample experimental evidence that membrane lipids are involved in the translocation process (De Vrije et al., 1990). Especially the observation that the negatively charged lipid phosphatidylglycerol (PG)[1] is involved in translocation of the precursor of the *E. coli* outer membrane protein PhoE across the inner membrane (De Vrije et al., 1988) is intriguing in view of recent monolayer experiments which demonstrated that a synthetic signal peptide, corresponding to the signal sequence of prePhoE, exhibits a specific interaction with negatively charged lipids (Batenburg et al., 1988b). An additional indication for lipid specificity of the signal peptide/lipid interaction was derived from circular dichroism (CD) studies in a micellar environment, which demonstrated different conformations of the PhoE signal peptide in the presence of either the negatively charged sodium dodecyl sulfate (SDS) or the

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[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; CL, cardiolipin; PIPES, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; CD, circular dichroism; TFE, trifluoroethanol; SDS, sodium dodecyl sulfate.

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neutral detergent Lubrol (Batenburg et al., 1988a).

The aim of the present study was to get more insight into the interaction of the PhoE signal peptide with lipid bilayers as well as into the lipid dependency of this interaction. A useful approach to study such peptide/lipid interactions is by measuring changes in tryptophan fluorescence properties of the peptide upon interaction with model membranes (Surewicz & Epand, 1984; Jain et al., 1985). However, the amino acid composition of the wild-type PhoE signal sequence ($MK^{-20}KSTLALVVMGIVASASVQA^{-1}$) does not include any tryptophan residues, and therefore we used a synthetic analogue of this peptide in which valine at position −8 was substituted by tryptophan. The position of the tryptophan was chosen such that it is located well away from the signal peptidase cleavage site near position −1, and sufficiently into the hydrophobic region of the peptide to expect that penetration of the peptide into the hydrophobic core of the lipid bilayer will lead to significant changes of the tryptophan fluorescence. Using this [Trp^{-8}]-PhoE signal peptide, we studied the interaction of the peptide with sonicated vesicles of the zwitterionic lipid phosphatidylcholine (PC) and of the negatively charged lipids PG and cardiolipin (CL). First we show that the $Val^{-8} \rightarrow Trp^{-8}$ substitution does not affect the in vivo and in vitro translocation efficiency of prePhoE and that the biophysical properties of the synthetic Trp^{-8} analogue are similar to those of the wild-type PhoE signal peptide. From changes in the intrinsic fluorescence properties of the [Trp^{-8}]-PhoE signal peptide as well as from quenching experiments using both aqueous and lipidic quenchers, we next demonstrate that the PhoE signal peptide interacts strongly with the various types of model membranes but that the interaction with negatively charged lipids is much more pronounced, most likely reflecting a deeper penetration into the hydrophobic core of the bilayer and indicating a lipid specificity of the mode of insertion of the signal peptide into the membrane.

It is proposed that the requirement of PG for efficient in vivo and in vitro translocation of prePhoE (De Vrije et al., 1988) is related to a functional interaction of the PhoE signal sequence with the negatively charged lipids in the *E. coli* inner membrane.

Experimental Procedures

Plasmids and DNA Manipulations. Plasmid pJP300 (Tommassen et al., 1987) (Figure 1) is a derivative of pJP29 (Bosch et al., 1986) in which the *Eco*RV site upstream of *phoE* is changed into a *Bam*HI site. Plasmid DNA was prepared by using the mini prep kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Large-scale purifications were performed as described by Birnboim and Doly (1979) followed by CsCl–ethidium bromide isopycnic centrifugation. Recombinant DNA techniques were performed essentially as described by Maniatis et al. (1982). Restriction endonuclease reactions, treatment of DNA with Klenow fragment of DNA polymerase, and bacteriophage T4 DNA ligase treatments were performed as described by the manufacturers of the enzymes. DNA fragments were analyzed on 0.6% (w/v) agarose gels.

The mutagenic oligonucleotide was synthesized on a Biosearch 8600 DNA synthesizer. Site-directed mutagenesis was carried out via the gapped duplex approach (Kramer et al., 1984). The duplex consisted of phage M13 DNA which carried in the (+) strand the *phoE* insert and amber mutations to allow selection against the nonmutagenized strand (Carter et al., 1985). Single-stranded M13 DNA was prepared as described before (Messing & Vieira, 1982). DNA sequencing was performed according to Sanger et al. (1977).

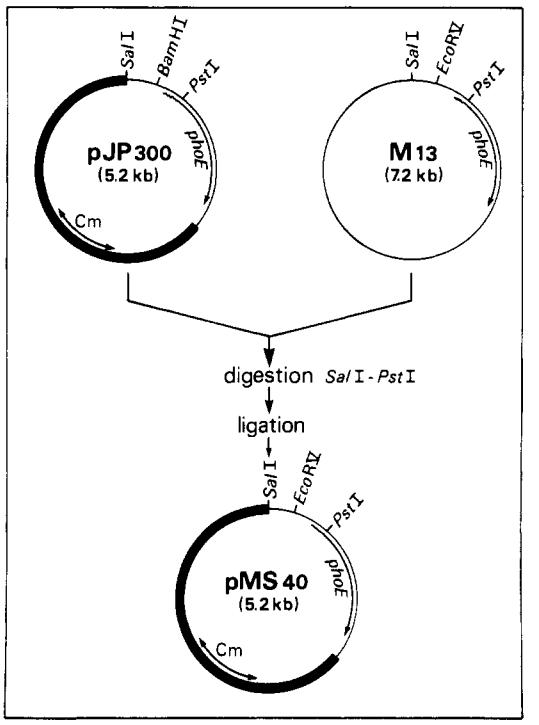

FIGURE 1: Construction of plasmid pMS40. The *SalI–PstI* fragment of pJP300 was replaced by the corresponding segment of double-stranded M13 DNA containing the desired signal sequence mutation. The indicated *Eco*RV site was used to select the desired recombinant plasmid. The position of the chloramphenicol resistance marker (Cm) is also indicated.

A DNA fragment containing the desired mutation was transferred from the M13 DNA to an expression plasmid as depicted in Figure 1. The resulting plasmid is designated as pMS40.

Translocation Assays. E. coli K-12 strain CE1224 (Tommassen et al., 1983) was used to study the processing of PhoE precursors in vivo. This strain is deleted for its chromosomal *phoE* gene and does not express the related OmpF and OmpC proteins as a result of an *ompR* mutation. This strain was transformed with plasmids pJP29 and pMS40. Cells were grown under aeration at 37 °C in a synthetic medium in which the phosphate concentration can be varied (Tommassen & Lugtenberg, 1980) and which was supplemented with chloramphenicol (25 μg/mL). Cells, which were induced for the synthesis of PhoE protein by growth under phosphate starvation, were labeled for 30 s at 37 °C with [^{35}S]methionine and subsequently chased with an excess of nonradioactive methionine as described (Bosch et al., 1986).

In vitro transcription, translation, and translocation were carried out essentially as described (De Vrije et al., 1987). In brief, 1 μg of plasmid DNA was transcribed by RNA polymerase for 15 min at 37 °C. The mRNA was used to direct the translation of prePhoE protein. The translation was carried out for 25 min at 37 °C using an S-135 extract of *E. coli* strain MRE600 (Cammack & Wade, 1965). Cotranslational translocation was investigated by adding inverted vesicles of the cytoplasmic membrane of strain MRE600, 4 min after initiation of protein synthesis. Import of the proteins into the vesicles was demonstrated by their protection against externally added protease K.

Protein patterns were analyzed by autoradiography after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Lugtenberg et al., 1975). For quantitation of protein bands, the gels were scanned with a densitometer (LKB Ultrascan XL).

Synthetic Signal Peptides. The PhoE signal peptide (MKKSTLALVVMGI*V*ASASVQA) and the [Trp^{-8}]-PhoE signal peptide (MKKSTLALVVMGI*W*ASASVQA) were prepared by solid phase synthesis by D. Olshevski (University of California, San Diego). The amino acid composition of both peptides was checked by amino acid analysis on a Kontron Liquimat III amino acid analyzer. The presence of tryptophan in the [Trp^{-8}]-PhoE signal peptide was demonstrated by its characteristic absorbance spectrum. In ethanol and water at 280 nm, a molar extinction coefficient (ϵ_{280}) was found of 5500 L mol^{-1} cm^{-1}, which is very close to the value measured for free tryptophan and thus indicates that one tryptophan is present per signal peptide molecule. HPLC profiles of the signal peptides, obtained as described previously (Batenburg et al., 1988a), demonstrated that both peptides were more than 95% pure.

Phospholipids. Cardiolipin was isolated from bovine heart and purified according to Smaal et al. (1985). Egg phosphatidylcholine was purified according to standard methods (Van Duijn et al., 1984). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized as described before (Van Deenen & De Haas, 1964) and converted to the corresponding phosphatidylglycerol (DOPG) by the phospholipase D catalyzed base-exchange method (Comfurius & Zwaal, 1979). DOPG and DOPC were purified according to Geurts van Kessel et al. (1981). 1-Palmitoyl-2-(2-bromostearoyl)-*sn*-glycero-3-phosphocholine (2-Br-PC) and 1-palmitoyl-2-(*n*,*n*+1-dibromostearoyl)-*sn*-glycero-3-phosphocholines (6,7-Br_2-PC, 9,10-Br_2-PC, and 11,12-Br_2-PC) were synthesized and purified as described elsewhere (De Kroon et al., 1990). *E. coli* phospholipids were isolated and purified as described before (Batenburg et al., 1988b) from strain SD12 (wild type) and from the mutant strain HD3122, which has a reduced content of negatively charged lipids (De Vrije et al., 1988).

Sample Preparation for Fluorescence Measurements. 1.5 mM stock solutions of the wild-type and [Trp^{-8}]-PhoE signal peptide in dimethyl sulfoxide (DMSO) were freshly prepared after first dissolving the peptides in trifluoroacetic acid (20 mg/mL) and evaporating off this solvent under a stream of N_2. For fluorescence measurements, the [Trp^{-8}]-PhoE signal peptide was mixed with the wild-type signal peptide in a 1/5 molar ratio for reasons that will be outlined under Results.

Sonicated lipid vesicles were prepared as stock solutions (10 mM lipid) by hydrating dry lipid films in the appropriate amount of a 100 mM NaCl, 10 mM PIPES, and 2 mM EDTA buffer at pH 7.4 and allowing them to swell for 1 h at room temperature. Next the lipid dispersion was sonicated in ice under a N_2 atmosphere for 5–10 min until clear, employing a Branson B12 tip sonicator with an input power of 50 W and a 50% dutycycle. Titanium particles were removed by centrifugation for 15 min at 30000*g* at 4 °C. Lipid concentrations in the supernatant were measured by the method of Rouser et al. (1975). Cardiolipin concentrations are expressed on the basis of their phosphorus content.

Sonicated vesicles of the *E. coli* total lipid extracts were prepared at pH 8.5, resulting in the formation of a stable vesicle solution.

Fluorescence Measurements. Fluorescence spectra were recorded on an SLM Aminco SPF-500 C fluorometer at an excitation wavelength of 280 nm and using a bandwidth of 5 nm. The tryptophan fluorescence of the signal peptide was measured 10 min after addition of 20 μL of a 1.5 mM stock solution of the wild-type/[Trp^{-8}]-PhoE (5/1 molar) signal peptide mixture in DMSO to 2 mL of a vesicle suspension, which had been diluted to the desired lipid concentration. Vesicle preparations of *E. coli* lipids at pH 8.5 were diluted with buffer at pH 7.4 just before addition of the signal peptide, resulting in a maximum increase of pH of 0.1 unit. The final concentration of [Trp^{-8}]-PhoE signal peptide was 2.5 μM in all experiments. Corrections for scattering were made by subtracting spectra obtained under identical conditions using the pure wild-type PhoE signal peptide. Corrections for the inner filter effect were applied according to $F = F_m 10^{(A_{ex}+A_{em})/2}$ (Parker, 1968) in which F is the corrected fluorescence intensity, F_m is the measured fluorescence intensity after correction for scattering and for the added volume, and A_{ex} and A_{em} are the absorbances measured at the excitation wavelength and at the emission maximum, respectively. The absorbance of the samples was measured by using a Hitachi U-3200 spectrophotometer. All measurements were carried out at 25 °C.

Quenching Experiments. All quenching experiments were carried out under the conditions described above and at a peptide/lipid molar ratio of 1/30, unless otherwise stated. Quenching by acrylamide and iodide was measured as follows: 10 min after addition of the signal peptide to the vesicle suspension (450 μM lipid), a titration was started by adding increasing amounts of the quenchers from a 4 M solution in water. The KI solution contained in addition 1 mM $Na_2S_2O_3$; in the case of acrylamide quenching, an excitation wavelength of 295 nm was used, and an inner filter correction for the absorbance by acrylamide was made. Fluorescence intensities were read 1 min after each addition of quencher. Similar results were obtained when separate samples were used for each data point.

To monitor the accessibility of the tryptophan indole ring to water, 30 μL of the signal peptide stock solution was added to the vesicle suspension (900 μM lipid) in 1.5 mL of buffer. Five minutes after addition of the signal peptide, 1.5 mL of H_2O or D_2O was added, and after 30 s, the emission spectra were recorded.

Quenching by brominated lipids was measured by incorporating different amounts of brominated PC in egg PC (10, 25, and 40 mol %) and in CL (10, 20, and 30 mol % brominated PC). Fluorescence spectra were recorded 2 min after addition of the peptide; all other conditions were as described for the fluorescence measurements. The relative quenching efficiency was defined as $1 - [(F - F_{sc})/(F_0 - F_{0-sc})]$, in which $F - F_{sc}$ is the measured fluorescence intensity and $F_0 - F_{0-sc}$ is the fluorescence intensity in the absence of brominated PC, both corrected for scattering. Corrections for scattering were performed as described above, using a final concentration of 15 μM wild-type PhoE signal peptide. The quenching efficiencies at 30 mol % brominated PC incorporation were calculated by using the linear Stern–Volmer relationship ($F_0/F = 1 + K_{SV}[Q]$) (Eftink & Ghiron, 1981) which was obtained by plotting F_0/F (F_0 and F both corrected for scattering) versus brominated PC content in egg PC or CL for each brominated PC. In the experiments with CL, the value of F_0 was derived from control measurements in which respectively 10, 20, and 30 mol % DOPC was incorporated in cardiolipin. Experiments with egg PC samples were carried out at a molar ratio of peptide to lipid of 1/30, while for the cardiolipin samples a 1/20 molar ratio was used.

Results and Discussion

Effect of the Val → Trp Substitution on the Functional Activity of the PhoE Signal Sequence and on the Biophysical Properties of the Synthetic Signal Peptide. First, the effect of the Val^{-8} → Trp^{-8} substitution of the functionality of the signal sequence of prePhoE was assessed by in vivo and in vitro

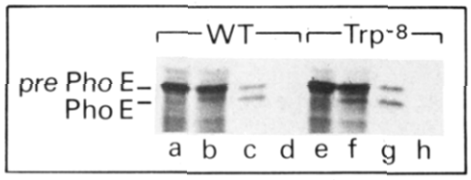

FIGURE 2: Gel electrophoresis patterns showing the in vitro co-translational translocation of wild-type prePhoE (lanes a–d) and of [Trp^{-8}]prePhoE protein (lanes e–h). Translation products of pJP29 and pMS40 (lanes a and e) were incubated with inverted *E. coli* inner membrane vesicles (lanes b and f) and subsequently treated with proteinase K (lanes c and g) or proteinase K and Triton X-100 (lanes d and h). The positions of the wild-type PhoE precursor and of mature PhoE are indicated in the figure.

translocation experiments. In vivo, the signal sequence is cleaved off from precursor proteins by leader peptidase at the periplasmic side of the inner membrane. Therefore, processing of the precursors can in general be considered as an indication for translocation. The processing kinetics of wild-type and mutant prePhoE were determined in in vivo pulse–chase experiments. In agreement with previous experiments (Bosch et al., 1986), both the wild-type mature PhoE and its precursor could readily be detected on the autoradiograms after pulse labeling for 30 s of CE1224 cells containing pJP29 (not shown). The precursor was rapidly processed and could no longer be detected after a chase period of 1 min. Importantly, a very similar behavior was observed for [Trp^{-8}]-prePhoE encoded by plasmid pMS40 (not shown). Since it is possible that the organism compensates for a possible translocation defect by overproduction of proteinaceous components of the export machinery (Oliver & Beckwith, 1982), we next compared the translocation efficiency of both precursor proteins in an in vitro system, which is much more sensitive to small differences in translocation activity (Bosch et al., 1989). Figure 2 (lanes a and e) shows the translation products in the absence of vesicles for the wild-type prePhoE and the pMS40-encoded precursor, respectively. A small difference can be observed between the positions of the two precursor proteins, indicating that the $Val^{-8} \rightarrow Trp^{-8}$ substitution slightly lowers the electrophoretic mobility of the protein. The same effect was observed in the in vivo pulse-labeling experiments (not shown). Upon addition of inverted vesicles of the cytoplasmic membrane in both systems, a band occurs at the position of mature PhoE (lanes b and f), demonstrating that the leader peptidase is able to cleave off the signal sequence of wild-type prePhoE as well as that of the pMS40-encoded precursor. Import of the precursors into the membrane vesicles can be demonstrated by their protection against externally added protease (lanes c and g). The translocation activity, defined as the percentage of total PhoE synthesized that is resistant to protease digestion, was found to be 21% for both precursor proteins. Furthermore, by comparing the intensities of the bands of precursor protein and mature PhoE in lanes c and g, it can be calculated that under our experimental conditions 52 and 44% of the translocated wild-type prePhoE and [Trp^{-8}]-prePhoE are processed, respectively. Upon lysis of the vesicles by treatment with Triton X-100, complete degradation occurs of all proteins in both translation systems (lanes d and h). Thus, it can be concluded from the in vivo as well as from the in vitro translocation experiments that both the wild type and the [Trp^{-8}]-prePhoE behave similarly and that translocation activity is not affected by the substitution.

Next it was tested whether the substitution had an effect on the behavior of the synthetic signal peptide. Competition experiments, carried out as described elsewhere (De Vrije et al., 1989), showed that the wild-type signal peptide and the [Trp^{-8}]-PhoE signal peptide were able to inhibit translocation of prePhoE across inner membrane vesicles to the same extent (not shown), indicating that both peptides behave similarly on a functional level. Furthermore, monolayer experiments demonstrated a similar increase in surface pressure upon penetration of the [Trp^{-8}]-PhoE signal peptide in PG and PC monolayers as upon insertion of the wild-type PhoE signal peptide (Demel et al., 1990). Finally, CD experiments were performed as described previously (Batenburg et al., 1988a) to monitor the conformational behavior of both signal peptides in trifluoroethanol (TFE) and in a micellar environment using the neutral detergent Lubrol and the negatively charged SDS. Again, very similar behavior of the [Trp^{-8}]-PhoE signal peptide was observed (R. C. A. Keller, unpublished observations), as found previously for the wild-type PhoE signal peptide, showing the presence of an α-helix in TFE and SDS and dominantly β-structure in Lubrol.

Interaction of the Synthetic PhoE Signal Peptide with Lipid Vesicles. Thus, having established that the $Val^{-8} \rightarrow Trp^{-8}$ substitution does not significantly affect the functional properties of the PhoE signal sequence, fluorescence measurements were performed to further investigate the interaction of the signal peptide with various lipids. These measurements were carried out by using a mixture of the wild-type signal peptide and the [Trp^{-8}]-PhoE signal peptide in a 5/1 molar ratio. When this procedure was used, the fluorescence emission intensity of the peptide in buffer remained constant in time up to 30 min after addition of the signal peptide from the stock solution in DMSO, and the fluorescence intensity was linear with the peptide concentration in the range of 0–30 μM (i.e., 0–5 μM for the [Trp^{-8}]-PhoE signal peptide). When the pure [Trp^{-8}]-PhoE signal peptide was used, a time-dependent decrease in fluorescence intensity was observed to about 40% of the initial intensity 10 min after addition of this peptide to the buffer solution. A possible explanation is that aggregation of the peptide occurs, involving intermolecular tryptophan–tryptophan interactions, which lead to self-quenching of the tryptophan fluorescence. Consistent with this explanation is the observation that upon mixing the [Trp^{-8}]-PhoE signal peptide with the wild-type signal peptide the loss of signal intensity decreased with increasing relative amounts of wild-type PhoE signal peptide. Since at a 5-fold molar excess of the wild-type signal peptide the fluorescence intensity was stable in time, we used this 1/5 molar ratio of [Trp^{-8}]-PhoE signal peptide to wild-type signal peptide for all fluorescence measurements.

First the interaction of the signal peptide with various lipids was investigated by measuring the fluorescence quantum yield and the wavelength of the emission maximum in the absence and presence of different concentrations of lipid vesicles. The lipid systems tested were PG and CL, the dominant negatively charged lipids in *E. coli* membranes, and PC. PC was used as a model for the zwitterionic *E. coli* lipid phosphatidylethanolamine (PE). Figure 3 shows that interaction of the signal peptide with the various lipid vesicles in all cases results in an increase in fluorescence quantum yield and a blue shift of the emission maximum. Such changes in fluorescence properties typically occur when tryptophan enters a more hydrophobic environment, reflecting penetration of the signal peptide into the hydrophobic part of the bilayer. For PC, a maximum increase in quantum yield of about 1.8 and a maximum blue shift of 8 nm are observed at a molar ratio of peptide to lipid of 1/20 and lower (Figure 3A). For both negatively charged lipids, the effects on quantum yield and blue shift are more pronounced, and less lipid is required to

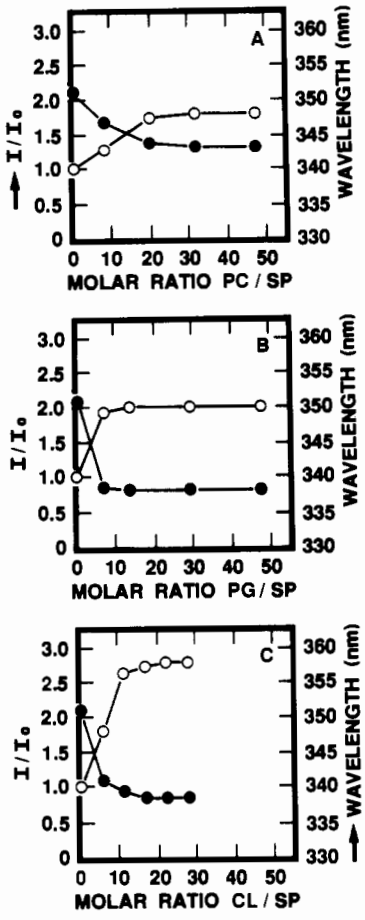

FIGURE 3: Fluorescence quantum yield (○) and wavelength emission maximum (●) of [Trp^{-8}]-PhoE signal peptide in the presence of vesicles of PC (A), PG (B), and CL (C) as a function of the total lipid/peptide ratio. See text for details.

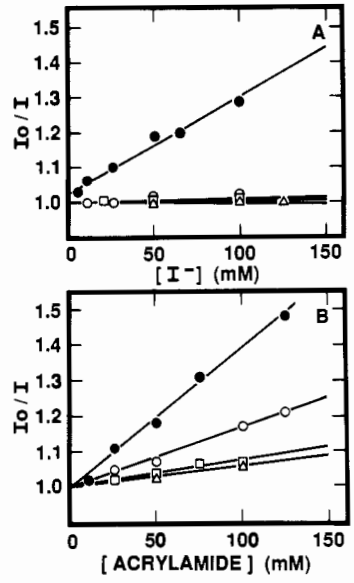

FIGURE 4: Stern–Volmer plots, showing the tryptophan fluorescence quenching of [Trp^{-8}]-PhoE signal peptide in buffer (●) and in the presence of vesicles of PC (○), PG (□), and CL (△) by the aqueous quenchers I^- (A) and acrylamide (B). See text for details.

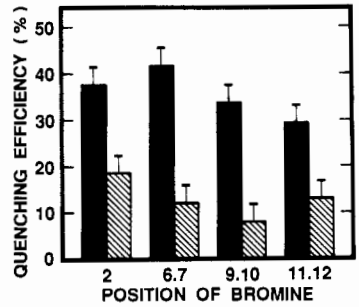

FIGURE 5: Quenching efficiency of the fluorescence of [Trp^{-8}]-PhoE signal peptide by brominated PC, incorporated at 30 mol % in CL (black bars) and egg PC (hatched bars) as a function of the position of bromine(s) in the fatty acyl chain. The error bars represent the estimated maximal error in the measurements.

reach the maximum effects, indicating a higher affinity of the signal peptide for these negatively charged lipids. Panels B and C of Figure 3 show that already at a molar ratio of peptide to lipid of about 1/10 a maximum blue shift is observed of about 13 nm for both lipid systems and a maximum quantum yield increase of 2.1 and 2.8 for PG and CL, respectively. These results suggest that in all three lipid systems investigated the signal peptide penetrates into the hydrophobic part of the bilayer but that there is a stronger interaction of the peptide with negatively charged lipids.

To further investigate the extent of penetration and the lipid specificity of the signal peptide/lipid interaction, quenching experiments were performed. Figure 4A,B shows Stern–Volmer plots of the effect of the aqueous quenchers I^- and acrylamide, respectively, on fluorescence intensity in the absence and presence of various lipids. Although I^- quenches the fluorescence of the signal peptide in buffer, no quenching is observed when lipids are present (Figure 4A), demonstrating that upon interaction with negatively charged as well as with zwitterionic lipid vesicles the tryptophan moiety of the signal peptide becomes inaccessible to I^-. A similar result was found upon investigating the effect of D_2O on the fluorescence quantum yield (not shown). Upon addition of D_2O to a solution of signal peptide in buffer, an increase in quantum yield of about 1.45 was observed. In the presence of either PG or PC lipid vesicles, however, no changes in quantum yield occurred, indicating that the tryptophan becomes inaccessible to water. In contrast, the interaction of the signal peptide with vesicles does not completely shield the tryptophan residues from quenching by acrylamide, and the extent of shielding is found to depend upon the type of lipid used. While in the presence of PC vesicles a marked decrease of the extent of quenching is found (Figure 4B), this shielding from quenching is much more pronounced in the presence of vesicles of the negatively charged PG or CL. Although the reason for the differences in apparent penetrating capacity for acrylamide on one hand and I^- and water on the other are not clear, it can be concluded from these quenching experiments that the tryptophan indole moiety of the signal peptide is shielded from the aqueous environment upon interaction with vesicles and that for acrylamide this shielding is more effective in the presence of negatively charged lipids. It should be noted that the quenching efficiency of I^- and acrylamide as found for the signal peptide in the absence of lipid is relatively low as compared to that found for other peptides (Eftink & Ghiron, 1977; Lehrer, 1971), indicating that the tryptophan indole rings in the signal peptide are not fully accessible to these quenchers. This could very well be a result of aggregation of this very hydrophobic polypeptide in an aqueous environment. The signal peptide/lipid interaction was further characterized by quenching experiments using PC's that were brominated at different positions along the acyl chain. These lipids act as collisional quenchers of tryptophan fluorescence and can in principle be used to obtain information about the extent and relative depth of penetration of the signal peptide into the hydrophobic core of the bilayer in the different lipid systems.

Figure 5 shows the relative quenching efficiency as a function of the position of the label calculated for incorporation of 30 mol % brominated PC into egg PC and into CL vesicles. The most conspicuous feature of this figure is the much higher quenching efficiency when the brominated lipid is incorporated into the negatively charged CL than in a PC bilayer, indicating an average higher exposure of the signal peptides' indole moiety to the bromines. These differences are most likely due to either a higher extent of association or a deeper penetration of the signal peptide into CL bilayers. Unfortunately, the brominated PC quenching experiments do not allow a clear-cut assessment of the signal peptides' membrane topology in these lipid systems. However, taking into account that the quenching efficiency by 2-Br-PC is underestimated because in this lipid on average less than one bromine per PC is present, whereas the other brominated PC's contain two bromines per molecule (De Kroon et al., 1990), these experiments do indicate a preferential localization of the tryptophan near the lipid/water interface in both lipid systems, as has been found for other tryptophan-containing (model) peptides (De Kroon et al., 1990). Interestingly, the shape of the quenching profile obtained upon interaction of the signal peptide with PC is quite similar to that observed for the positively charged tripeptide KWK in membrane systems in which a surface localization of the peptide is expected (De Kroon et al., 1990). A careful comparison of the shapes of the quenching profiles in Figure 5 suggests that in the case of interaction of the signal peptide with CL the tryptophan penetrates more deeply into the hydrophobic part of the bilayer, in accordance with the much higher extent of quenching of the signal peptide by brominated lipids incorporated into CL vesicles than into PC vesicles. Control experiments showed that the fluorescence intensity of the free amino acid tryptophan is not at all affected by the incorporation of bromines into PC or CL bilayers (data not shown).

From the intrinsic fluorescence measurements as well as the quenching experiments, it is clear that the PhoE signal peptide has a different interaction with the negatively charged PG and CL than with the zwitterionic PC. To get insight into the question whether this apparent specificity for negatively charged lipids could be relevant to the interaction of the signal peptide with *E. coli* membranes, we next performed fluorescence experiments using sonicated vesicles of the total lipid extracts of *E. coli* strains SD12 and HD3122. In this latter strain, the content of anionic lipids is very low (about 6 mol % as opposed to about 26 mol % in the wild-type strain), and translocation activity is drastically reduced (De Vrije et al., 1988). Upon interaction of the signal peptide with vesicles prepared of lipids of the wild-type strain SD12 in a 1/70 molar ratio of peptide to lipid, a blue shift of the emission maximum of the tryptophan fluorescence is observed of about 13 nm, similar to that found for PG and CL vesicles under the same conditions. In contrast, upon interaction with vesicles composed of the total lipid extract of *E. coli* strain HD3122 containing about 94% of PE, the observed blue shift of 9 nm is similar to that found upon interaction of the signal peptide with vesicles of the zwitterionic PC. These results thus suggest that a change in lipid composition of *E. coli* membranes can result in a different interaction with the PhoE signal sequence.

Conclusions

The aim of this study was to get more insight into the possible role of signal peptide/lipid interactions and the importance of negatively charged lipids in protein translocation. The strategy we used was to investigate the mode of interaction and the lipid specificity of the signal peptide of PhoE with various model membranes employing a synthetic tryptophan-containing analogue of this signal peptide. Although it is known that minor changes in amino acid composition of the signal sequence can affect translocation efficiency as well as alter the biophysical properties of the isolated signal peptide (Gierasch, 1989; Nagaraj et al., 1987; Reddy & Nagaraj, 1989; Green et al., 1989; Silhavy et al., 1983), we showed that our approach of substituting Val^{-8} by Trp^{-8} did not affect the in vivo or the in vitro translocation efficiency of prePhoE nor did it significantly affect the biophysical properties of the isolated signal peptide. From changes in the fluorescence properties of this [Trp^{-8}]-PhoE signal peptide in the presence of lipid vesicles of PC, PG, and CL, it was demonstrated that the peptide exhibits a strong interaction with all these lipid systems. This could be concluded from (1) an increase in fluorescence intensity, (2) a blue shift of the emission wavelength maximum, (3) complete inaccessibility to the aqueous quencher I^- and a reduced accessibility to acrylamide, and (4) by quenching of the tryptophan fluorescence by brominated lipids upon interaction of the signal peptide with lipid vesicles. These same measurements demonstrated in addition that the interaction of the PhoE signal peptide is much stronger with negatively charged lipids, since all the effects were more pronounced when either cardiolipin or PG was used instead of the zwitterionic PC. The relatively high quenching efficiency of brominated lipids incorporated into cardiolipin vesicles, together with the different shapes of the quenching profiles obtained for cardiolipin and PC vesicles (Figure 5), suggests a deeper penetration of the tryptophan moiety of the signal peptide into the hydrophobic part of the bilayer when the lipids have a negative charge.

Interestingly, we showed that the PhoE signal peptide likewise has a different interaction with sonicated vesicles prepared of the total lipid extract from a mutant strain with a low content of anionic lipids than with vesicles of wild-type *E. coli* lipids. Our results therefore suggest that if upon translocation across the membrane a direct interaction occurs between the signal sequence of the precursor protein and the membrane lipids, the initial mode of interaction could depend on the presence of negatively charged lipids. Therefore, the observation that PG is involved in translocation of prePhoE across the *E. coli* inner membrane (De Vrije et al., 1988) could very well be due to a different mode of insertion of the signal sequence in membranes that contain low amounts of negatively charged lipids.

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