PhoE Signal Peptide Inserts into Micelles as a Dynamic Helix−Break−Helix Structure, Which Is Modulated by the Environment. A Two-Dimensional ^{1}H NMR Study†

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Received March 22, 1995; Revised Manuscript Received June 6, 1995⊗

ABSTRACT: Proteins that are destined for export out of the cytoplasm of *Escherichia coli* cells are synthesized as precursor proteins with N-terminal extensions or signal sequences, which are essential for translocation of the protein across the inner membrane. Signal sequences contain very little primary sequence homology, and therefore recognition of these sequences is thought to involve specific folding. To assess the conformational flexibility of signal sequences, we have studied the signal peptide of PhoE (MKKSTLALVVMGIVASASVQA) by two-dimensional nuclear magnetic resonance and circular dichroism in different membrane mimetic environments. The secondary structure of the PhoE signal peptide was analyzed via interresidue nuclear Overhauser enhancement measurements, chemical shifts of backbone protons, and by measuring amide proton exchange. The membrane mimetic environments studied were trifluoroethanol (TFE) and micelles of sodium dodecyl sulfate (SDS) or dodecylphosphocholine (DPC). In all systems α-helix formation was observed. In TFE, the α-helix stretches from the positively charged N-terminus to Ser_{18}. In SDS and DPC micelles, the N- and C-terminal α-helical half are separated from each other by a kink at the Gly_{12} position, with the helical content being higher at the N-terminus and lower at the C-terminus. In zwitterionic DPC micelles, the C-terminal region has a less regular or more flexible structure compared to SDS. The insertion of the PhoE signal peptide into the hydrophobic environment of the micelles was demonstrated by the effect of spin-labeled 12-doxylstearate on the line widths of the peptide proton resonances. It is proposed that conformational rearrangements at Gly_{12} induced by the lipid environment play a role in the export process of the precursor protein.

In *Escherichia coli*, proteins which are destined for the periplasm or outer membrane are synthesized as precursor proteins carrying amino-terminal extensions or signal sequences (Oliver & Beckwith, 1982; Gierasch, 1989). These signal sequences are the most general requirement for the export of proteins from both eukaryotic and prokaryotic cells. Several lines of evidence argue that signal sequences from various organisms work in much the same way (Gierasch, 1989). Although hardly any sequence homology exists among signal sequences from different proteins, there is a common structural motif consisting of a positively charged N-terminus, followed by a stretch of 8−15 hydrophobic amino acids and a more polar C-terminal region, containing the cleavage site (Von Heijne, 1985). This suggests that overall characteristics such as hydrophobicity and conformation are more important factors for their function than a specific residue sequence.

Signal sequences are known to interact with several proteinaceous components of the export machinery, such as SecA (Lill et al., 1990; Akita et al., 1990) and SecY (Hartl et al., 1990). The presence of positively charged residues at the N-terminus and a hydrophobic core region makes signal sequences prone to interaction with the membrane lipids. Indeed, experimental evidence has been reported that during the translocation process the signal sequence interacts with the membrane lipids (Phoenix et al., 1993a,b; Kusters et al., 1994). This interaction is believed to be essential for the initiation of translocation (De Kruijff, 1994). It can be expected that signal sequences will have to adopt specific folding patterns to allow them to undergo specific recognition with the different components of the export machinery.

CD[1] studies showed that signal peptides from different precursors can adopt an α-helical conformation upon interaction with phospholipid vesicles (Keller et al., 1992; Wang et al., 1993) and micelles (Biggs & Gierasch, 1984; Batenburg et al., 1988a; Yamamoto et al., 1990; Hoyt & Gierasch, 1991; Rizo et al., 1993) and in bulk solvent mixtures that also mimic membrane environments (Rizo et al., 1993; Yamamoto et al., 1990). More detailed information was

†This study was carried out under the auspices of the Foundation for Biophysics with financial aid from the Netherlands Organization for Scientific Research.

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1995.

[1] Abbreviations: CD, circular dichroism; 1D, one dimensional; 2D, two dimensional; DPC, dodecylphosphocholine; DPC-d_{38}, [$^2H_{38}$]-dodecylphosphocholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, 2D nuclear Overhauser enhancement spectroscopy; SDS, sodium dodecyl sulfate; SDS-d_{25}, [$^2H_{25}$]dodecylsulfate; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFE-d_3, [$^2H_3$]trifluoroethanol; TOCSY, 2D total correlation spectroscopy; TPPI, time-proportional phase increment.

0006-2960/95/0434-11617$09.00/0

obtained for the bacterial OmpA and LamB signal peptides (Rizo et al., 1993; Bruch et al., 1989; Bruch & Gierasch, 1990; Wang et al., 1993) and for simplified artificial signal sequences (Yamamoto et al., 1990) by using high-resolution 2D NMR. These studies showed that, both in TFE/water and in SDS micelles, the helix begins after the positively charged N-terminal residues and is most stable in the hydrophobic core.

For the synthetic signal peptide analogue of the *E. coli* outer membrane protein PhoE, it was shown by CD that the signal peptide adopts an α-helical structure in negatively charged phospholipids and detergents, but β-structure in neutral phospholipids and detergents (Batenburg et al., 1988a; Keller et al., 1992). On the basis of monolayer experiments, the "unlooping" model was proposed to explain how the signal sequence could be mechanistically involved in the initiation of the translocation process (De Vrije et al., 1990). In this model, the signal sequence inserts initially into the membrane in a conformation which is looped around a break in the α-helix in the hydrophobic core region. In the presence of negatively charged lipids unlooping of the signal sequence occurs, resulting in a transmembrane conformation.

The aim of this study was to characterize the structure of the PhoE signal peptide on an atomic level and to investigate in detail how this structure can be modulated by the lipid environment. For this purpose the signal peptide was incorporated in different membrane mimetic environments and studied by CD and 2D NMR. TFE was used as a solvent mimicking the homogeneous hydrophobic part of the membrane. Micelles formed from the detergents SDS and DPC were used as membrane models providing a heterogeneous amphiphilic environment with a negatively charged or electrically neutral lipid−aqueous interface. Because of their small size, micelles undergo isotropic motion, yielding a high-resolution spectrum for any peptide that is incorporated in their interior. The results indicate that the secondary structure of the PhoE signal peptide is modulated by membrane-like environments. In TFE, the signal peptide behaves as a rod. In SDS and DPC micelles, the N- and C-terminal half are separated from each other by a kink at the Gly_{12} position. In zwitterionic DPC micelles, the C-terminal region has a less regular or more flexible structure than in SDS.

MATERIALS AND METHODS

Materials. Perdeuterated SDS-d_{25} and DPC-d_{38} were obtained from MSD Isotope (Montreal, PQ). SDS-d_{25} was treated with chelating resin Chelex 100 (Bio-Rad), filtrated, and lyophilized. TFE-d_3, methanol-d_4, and ^2H_2O were from Isotec Inc. (Miamisburg, OH). TFE and TFA were from Aldrich (Steinheim, Germany). 12-Doxylstearic acid was obtained from Sigma (St. Louis, MO). The PhoE signal peptide, corresponding to the signal sequence of the *E. coli* outer membrane protein PhoE, was synthesized by Dr. D. Olshevski (San Diego, CA). The amino acid composition and sequence of the signal peptide were confirmed by mass spectroscopy.

Sample Preparation. Samples were prepared as described previously (Killian et al., 1994). The signal peptide was first dissolved in TFA (100 mg/mL) and dried under a stream of nitrogen. Residual TFA was removed by dissolving the signal peptide in TFE (50 mg/mL) and evaporating off the solvent under a stream of nitrogen. Next a 5 mM solution of the signal peptide in TFE was prepared and was added to an equal volume of an aqueous 550 mM solution of SDS-d_{25} or DPC-d_{38}. Water was then added to yield a 10:1 ratio of water to TFE by volume. The sample was mixed by vortexing and lyophilized by rapid freezing followed by drying overnight under high vacuum. The dry samples were rehydrated in H_2O, containing 10% D_2O, or in D_2O to form a solution with 5 mM signal peptide and 550 mM detergent. The pH was adjusted as will be indicated to 4.5, 3.0, or 1.6 at 25 °C (direct uncorrected pH-meter reading) by addition of 0.1 N HCl or 0.1 N NaOH.

NMR Measurements. NMR spectra were recorded on Bruker AMX spectrometers operating at 500 or 600 MHz proton frequencies (the latter at the SON hf-NMR facility, Nijmegen University, The Netherlands) at 25 °C. Chemical shifts were measured relative to the water resonance at 4.75 ppm and to the residual resonance from protonated TFE at 3.88 ppm. TOCSY spectra were recorded with a clean MLEV-17 mixing sequence (Bax & Davis, 1985; Griesinger et al., 1988) with total mixing times of 30−50 ms which include the delays of the clean-TOCSY pulse scheme. NOESY spectra (Jeener et al., 1979; Kumar et al., 1981) were recorded with a mixing time of 250 ms for the TFE-d_3 spectrum and 125 ms for the spectra of the micelle systems. All 2D spectra were recorded in a phase-sensitive absorption mode using TPPI in t_1 (Marion et al., 1983). The cross-peaks around 4.6 ppm, which are normally irradiated together with the water line were made observable using the SCUBA method (Brown et al., 1988). Two-dimensional spectra were collected as a 400−512 (t_1) real and 1024 (t_2) complex point time domain matrix with a spectral width of 5000 Hz (^1H = 500 MHz) or 6000 Hz (^1H = 600 MHz) in both dimensions and 80−160 scans per t_1 increment. Data were transformed after zero-filling in the F_1 dimension, into 1024 and 1024 real points in the F_1 and F_2 dimension frequency-domain spectra. Sixth order polynomial baseline corrections in each domain were applied after the double Fourier transformation was completed (Boelens et al., 1985). The data were processed with the Triton software library (Bijvoet Center for Biomolecular Research, Utrecht University, the Netherlands).

Amide deuterium exchange rates were monitored by recording TOCSY spectra after 2 h and NOESY spectra after 28 h of exchange after the signal peptide was dissolved in TFE-d_3 or after the lyophilized samples were dissolved in D_2O (final pD 1.6).

To determine the insertion of the signal peptide into micelles, the effect of a hydrophobic spin-labeled 12-doxylstearic acid on the ^1H signal peptide resonances was investigated. After recording of the TOCSY spectrum in the absence of the spin-labeled acid, the NMR sample (500 μL) was mixed with 3 μL of the 12-doxylstearic acid in methanol-d_4 to yield a 80:1 molar ratio of detergent to 12-doxylstearic acid, and the TOCSY spectrum was recorded. The effect of the 12-doxylstearic acid was measured by comparing the intensities of the TOCSY cross-peaks in the presence and in the absence of the spin-labeled acid. All NMR experiments were performed in an N_2 atmosphere to avoid possible oxidation of the spin label.

Circular Dichroism. CD measurements were carried out on a JASCO 600 spectropolarimeter, using a 0.1 mm path

Table 1: Proton Chemical Shifts for PhoE Signal Peptide in TFE, SDS, and DPC Micelles

	SDS					DPC	TFE
	NH	αH	βH	γH	others	αH	αH
Met_1		4.31	2.26, 2.17	2.66, 2.56	CH_3-2.08	4.18	4.30
Lys_2	8.46	4.57	2.15, 1.94	1.69, 1.60	δCH_2-1.79, ϵCH_2-3.09, ζNH_2-7.42	4.52	4.45
Lys_3	8.71	4.02	2.07, 1.85	1.74, 1.52	δCH_2-1.82, δ′CH_2-1.75, ϵCH_2-3.11, ζNH_2-7.54	4.09	4.14
Ser_4	8.27	4.35	4.07, 4.02			4.25	4.27
Thr_5	7.73	3.97	4.32	1.29		4.06	4.12
Leu_6	7.74	4.02	2.07, 1.81	1.74	δCH_3-0.98, δ′CH_3-0.95	4.04	4.14
Ala_7	8.17	3.93	1.51			3.93	4.04
Leu_8	7.79	4.10	2.05, 1.89	1.67	δCH_3-0.98, δ′CH_3-0.96	4.12	4.19
Val_9	8.15	3.70	2.36	1.11, 0.96		3.75	3.75
Val_{10}	8.31	3.55	2.22	1.07, 0.93		3.57	3.65
Met_{11}	8.60	4.12	2.26, 2.14	2.76, 2.55	CH_3-2.05	4.19	4.15
Gly_{12}	8.38	3.91, 3.82				4.04, 3.91	3.95, 3.92
Ile_{13}	8.26	3.77	2.10	CH_2-1.97, 1.11; CH_3-0.92	δCH_3-0.86	3.82	3.74
Val_{14}	8.45	3.60	2.23	1.09, 0.98		3.60	3.62
Ala_{15}	8.61	4.18	1.56			4.32	4.08
Ser_{16}	7.93	4.32	4.07, 4.00			4.35	4.22
Ala_{17}	8.12	4.37	1.52			4.24	4.20
Ser_{18}	8.06	4.48	3.96			4.31	4.36
Val_{19}	7.72	4.19	2.21	1.01		4.16	4.14
Gln_{20}	8.14	4.43	2.19, 2.03	2.43	δNH-7.49, δ′NH-6.80	4.40	4.31
Ala_{21}	8.06	4.32	1.44			4.19	4.40

length cell, with a 1 nm bandwidth, 0.1 nm resolution, 1 s response time, and a scan speed of 20 nm/min. Typically, four scans were added and averaged, followed by subtraction of the CD signal of empty micelles, recorded under the same conditions. Spectra shown are from NMR samples diluted to a final concentration of the signal peptide of 100 μM. Identical results were obtained when samples were prepared freshly in the desired concentration at pH 1.6. The helix content was estimated by the method of Greenfield and Fasman (1969).

RESULTS

The PhoE signal peptide has a high tendency to aggregate in aqueous solution even in the presence of detergents, which makes it difficult to obtain a stable solution in the mmolar concentration range suitable for NMR measurements. Homogenous incorporation of the PhoE signal peptide into micelles was achieved by using a new method (Killian et al., 1994). The signal peptide was mixed with detergent in a water−TFE solution, diluted with water to form micelles, lyophilized, and rehydrated with appropriate amounts of D_2O or H_2O/D_2O (9:1). The detergent/peptide ratio used was more than 100 to 1 to provide the average occurrence of less than one peptide molecule per micelle. Rehydrated samples could be again lyophilized and rehydrated, yielding identical 2D NMR spectra. Therefore, the same sample could be used for amide exchange studies. For SDS we were able to obtain clear and stable solutions in the pH range of 1.5−4.5. 2D NMR measurements showed that the secondary structure of the signal peptide in SDS micelles does not depend on the pH in the range of 1.5−4.5 (data not shown). For DPC a low pH (less than 2) was necessary to avoid significant aggregation of the signal peptide. Despite the occurrence of some aggregation, time-independent NMR spectra were obtained for the signal peptide in DPC micelles at pH 1.6. DPC is still zwitterionic at this pH, as concluded from ^{31}P NMR titration measurements (data not shown), which showed that the pK_a of DPC is below 1.

The conformational behavior of the PhoE signal peptide upon interaction with membrane mimetic environments was

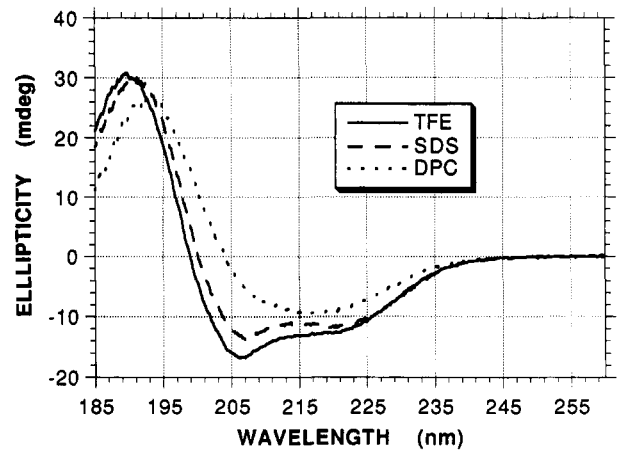

FIGURE 1: CD spectra of the PhoE signal peptide upon interaction with TFE, SDS, and DPC micelles.

investigated using CD and 2D NMR. The CD results are shown in Figure 1. For TFE, a spectrum with two minima at 208 and 222 nm and a cross-over point at about 200 nm is observed which is characteristic of a highly α-helical conformation. For SDS micelles, the CD spectrum is rather similar to that in TFE. In contrast, upon interaction with the zwitterionic detergent DPC, the CD spectrum exhibits only one minimum at 218 nm and a cross-point at about 208 nm, indicating a substantial decrease of the amount of α-helical structure and the formation of β-structure. The helix content in TFE, SDS, and DPC micelles was estimated to be 55, 60, and 30%, respectively.

For 2D NMR data analysis, the standard method proposed by Wüthrich (1986) was used to carry out the sequential assignment of all the proton resonances of the signal peptide in different environments. TOCSY spectra were used to identify spin systems, and NOESY spectra were used to obtain interresidue connectivities and to distinguish equivalent spin systems. Unambiguous assignments were obtained with the help of sequential NH(i)−NH(i+1), Hα(i)−NH(i+1), and other NOE connectivities characteristic of a helical conformation. The assignment for all the protons of the peptide in SDS micelles is shown in Table 1. In this table

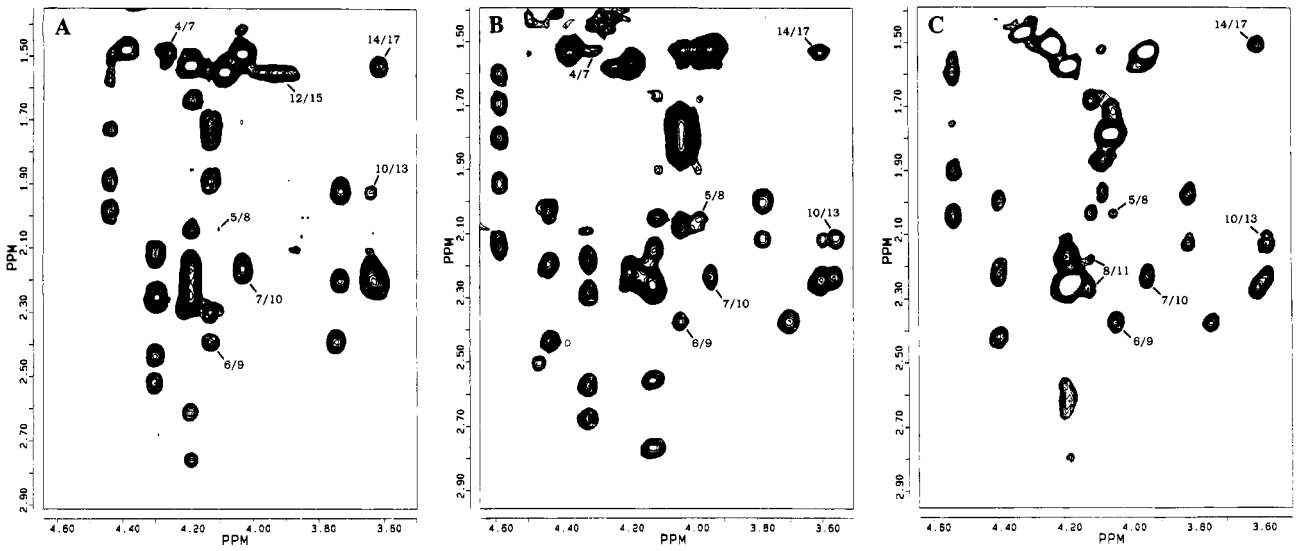

FIGURE 2: Hα−Hβ region of the NOESY spectra of the PhoE signal peptide upon interaction with TFE (A), SDS (B), and DPC (C) micelles (pH 1.6). Interresidual medium-range Hα(*i*)−Hβ(*i*+3) NOEs are indicated.

also chemical shift values of the Hα protons of the peptide are compared in TFE, SDS, and DPC micelles.

Determination of the secondary structure of the PhoE signal peptide in different membrane-like environments involved identifying NOE cross-peaks between protons on nonneighboring residues, chemical shifts of backbone protons, and measuring amide proton exchange. The characteristic NOE connectivity pattern for an α-helix is the presence of medium Hα(*i*)−Hβ(*i*+3) and weak Hα(*i*)−NH(*i*+3) medium-range contacts. On the basis of the resonance assignments, a large number of sequential and medium-range NOE cross-peaks could be identified in the NOE spectra. Figure 2 shows the Hα(*i*)−Hβ(*i*+3) connectivities for the PhoE signal peptide in different environments. The medium-range NOE connectivities are summarized in Figure 3 for the PhoE signal peptide in TFE, SDS, and DPC micelles. These data suggest that in TFE the α-helix starts at Lys_3 or Ser_4 and extends till Ala_{17} or Ser_{18}. In SDS micelles some differences are observed: the α-helix now begins at Lys_2, and the connectitivity between Gly_{12} and Ala_{15} is missing, indicating a destabilization of the helix around the Gly_{12} position. In DPC micelles fewer NOE connectivities are observed; the helix starts after Lys_3, and again a break occurs near Gly_{12}. In both micelle environments the number of observed NOE connectivities and their intensity is higher for the N-terminal half of the helix than for the C-terminal half, indicating a higher helical content near the N-terminus.

The difference between the Hα proton chemical shifts in a protein structure and the chemical shifts in a random coil also correlates with protein secondary structure. An α-helix structure is indicated by an upfield chemical shift of more than 0.1 ppm with respect to random coil chemical shift values (Wishart et al., 1992). Figure 4 shows a graph of the Hα proton chemical shifts of the signal peptide in TFE, SDS, and DPC micelles. In all three environments the helix of the PhoE signal peptide appears to extend from Lys_3 to Ser_{18}, in close agreement with the observed NOE connectivities. In addition, the profile of the chemical shifts suggests that some weakening of the helix occurs around Gly_{12}, with a higher helical content at the N-terminus as compared to the C-terminus.

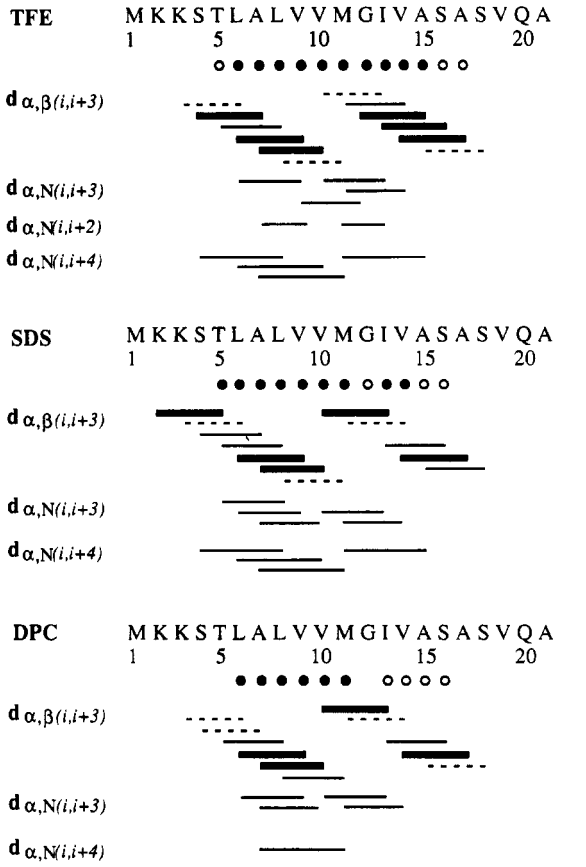

FIGURE 3: Medium-range NOE contacts observed in the NOESY spectra of the PhoE signal peptide in TFE, SDS, and DPC micelles. The thickness of the horizontal bars is a qualitative indication of the relative intensity of the NOEs as observed in NOESY spectra. Solid bars indicate NOEs that have been identified, while dashed lines indicate NOEs whose presence or absence cannot be assessed due to overlap with intraresidual and sequential connectivities. The slow exchange (>28 h) of amide resonances is indicated with filled circles, whereas open circles represent intermediate exchange (>2 h).

These conclusions were supported also by the amide proton exchange behavior of the signal peptide. The rate of exchange is dependent on the state of folding of the peptide. In an α-helix, the secondary fold is stabilized by the

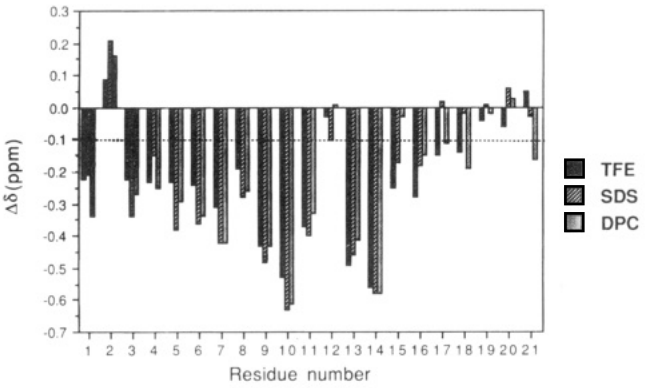

FIGURE 4: Difference between the Hα chemical shifts observed and the random coil chemical shifts described in Wüthrich (1986) is represented as a function of residue position for the PhoE signal peptide in TFE, SDS, and DPC micelles.

formation of hydrogen bonds between the carbonyl oxygen of residue *i* and the amide proton of residue *i*+4. This results in a slow amide proton exchange rate. Figure 5 shows the slowly exchanged amide proton resonances, which are observed in the NMR spectra after 28 h of exchange at 25 °C. In TFE, amide proton resonances of amino residues from 6 to 15 are present (Figure 5A). In SDS micelles, there are two regions, separated by Gly_{12}, that show a slow exchange (Figure 5B). The N-terminal part is represented by seven intense resonances (Thr_5, Leu_6, Ala_7, Leu_8, Val_9, Val_{10}, and Met_{11}), whereas the intensities and the number of amide proton resonances of the C-terminus (Ile_{13}, Val_{14}, and Ala_{15}) are relatively low. This supports the conclusion from the NOE and chemical shift data that the N-terminal half adopts a more stable α-helical conformation than the C-terminal half. In DPC micelles the rate of amide proton exchange at the C-terminus (Figures 5C) is even higher than in SDS, suggesting a less regular structure of this part of the signal peptide in DPC micelles. Figure 3 indicates the intermediate and slow exchange of the amide protons with open and filled circles, respectively. It should be noted here that a direct comparison of the amide proton exchange data is not trivial, because the kinetics could be affected by other factors, such as a difference in intrinsic exchange rates for the signal peptide in TFE, SDS, or DPC. Nevertheless, these data provide a quantifiable measure of dynamics throughout the entire backbone of the signal peptide.

In principle, it is possible to obtain additional conformational information from backbone coupling constants (Wüthrich, 1986). Unfortunately, in the micelle environments due to extensive line broading these constants could not be measured.

To determine the insertion of the PhoE signal peptide into micelles, the spin-labeled 12-doxylstearic acid was used to produce selective broadening of the residues buried into the hydrocarbon region (Brown et al., 1982; Papavoine et al., 1994). The spin label in 12-doxylstearic acid is covalently attached to the stearic acid at position 12, providing a location of the free radical in the center of the micelle. The 12-doxylstearic acid/detergent molar ratio used was 1:80 to provide an average occurrence of approximately one molecule of the relaxation reagent per micelle. The effect of the 12-doxylstearic acid was measured by comparing the intensities of the TOCSY cross-peaks in the absence (Figure 6A) and presence (Figure 6B) of the 12-doxylstearic acid. It is shown that the presence of the 12-doxylstearic acid drastically reduces the intensities of the signal peptide TOCSY cross-peaks of the amino residues located around the hydrophobic core of the signal peptide, while the intensities of the residues located at the N- and C-terminus are not affected. The difference of the spectra obtained in the absence and in the presence of the 12-doxylstearic acid shows almost exclusively residues that are located in the hydrocarbon core of micelles (Figure 6C). These data are consistent with a buried location for the hydrophobic core of the signal peptide within the micelles interior, while the N- and C-terminus are located at the water/detergent interface.

DISCUSSION

The structure of the PhoE signal peptide has not previously been examined using 2D NMR. The purpose of this work was to study the conformational behavior of the PhoE signal

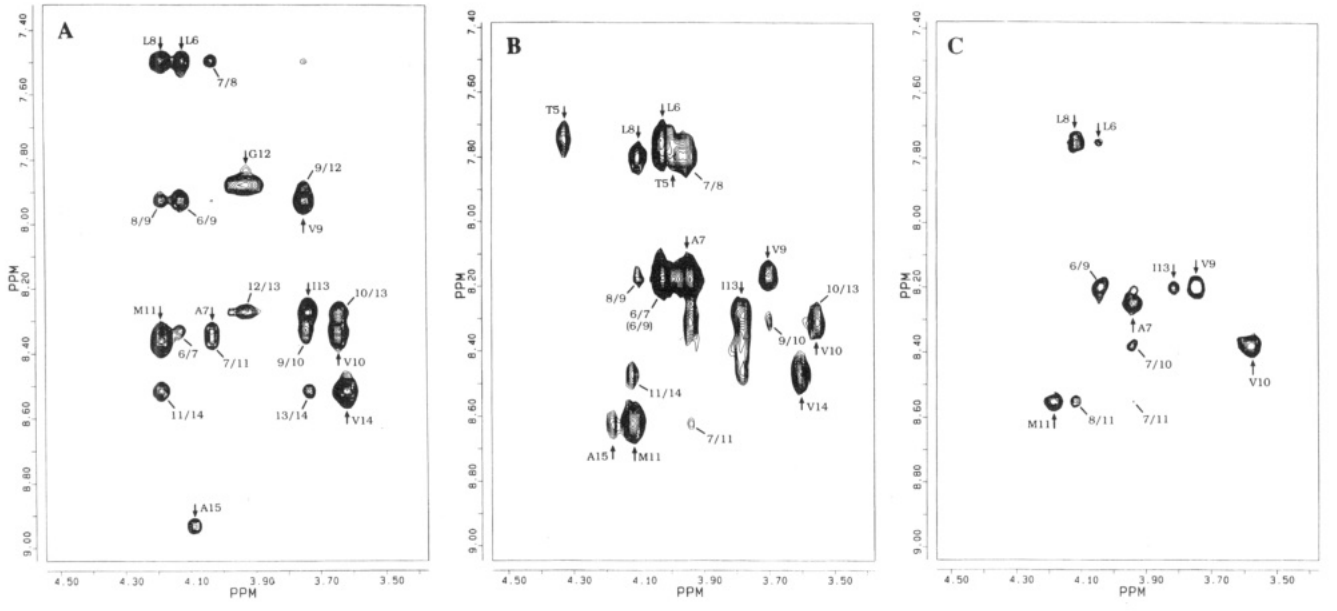

FIGURE 5: Hα−NH region of the NOESY spectra of the PhoE signal peptide after 28 h incubation in TFE-d_3 (A) and in SDS (B) and DPC (C) micelles in D_2O (pD 1.6). Intra- and interresidual sequential and medium-range NOEs are indicated.

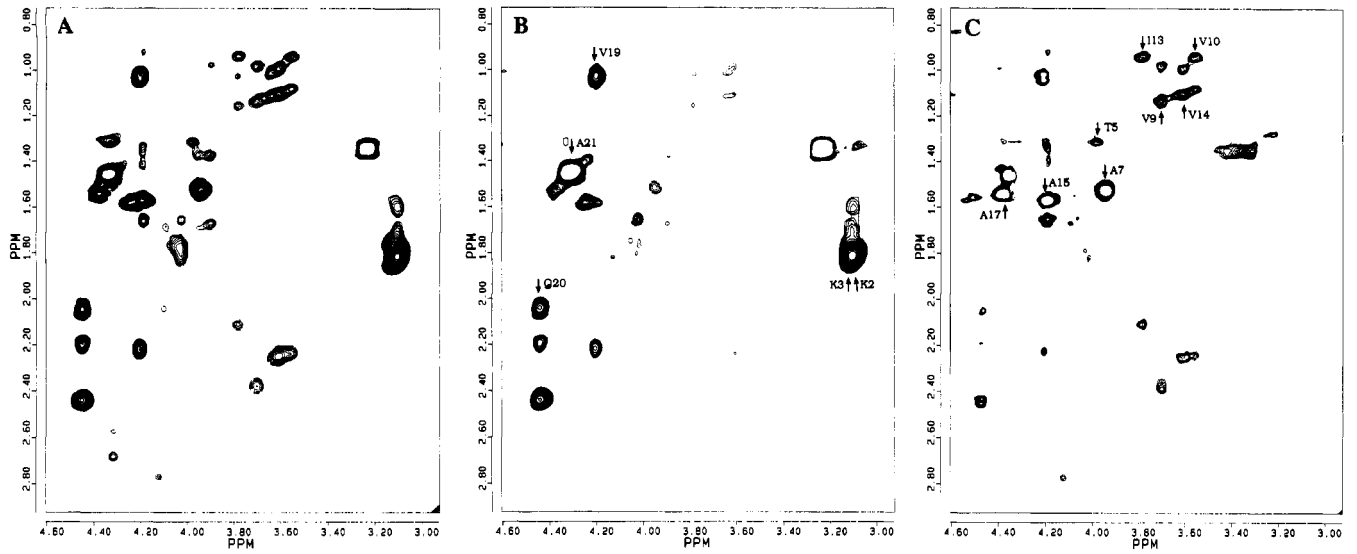

FIGURE 6: Comparison of expanded region of TOCSY spectra of the PhoE signal peptide in SDS micelles in the absence (A) and presence (B) of 12-doxylstearic acid (pH 4.5). Resonances which were not affected by the presence of 12-doxylstearate are indicated. The difference between A and B is represented on the right panel (C). Resonances which were affected by the presence of 12-doxylstearate are indicated.

peptide in different membrane mimetic environments. TFE provides a homogeneous membrane mimetic environment, while micelles provide a heterogeneous amphiphilic environment with a lipid−aqueous interface.

The presence of the medium-range NOEs, the chemical shifts of Hα protons, and the kinetics of amide proton exchange provide evidence for the presence of a dynamic helix−break−helix structure for the PhoE signal peptide in TFE, SDS, and DPC micelles. Although the secondary structure appears to be determined primarily by the intrinsic helical propensity of the signal peptide, our results show that it can be subtly modified by the specific membrane environment. The similarities and differences among the signal peptide structures in different environments are listed below:

(1) *TFE (Homogeneous Environment).* The helix stretches from the positively charged N-terminus near Lys_3 to Ser_{18}. The chemical shift data suggest that the helix is weaker at the glycine residue at position 12. However, the presence of a medium-range Hα(i)−Hβ(i+3) NOE between Gly_{12} and Ala_{15} and the slow exchange of the amide proton of Gly_{12} demonstrate that this residue is still involved in α-helix formation.

(2) *SDS Micelles (Anionic Lipid).* The helix stretches from Lys_2 to Ser_{18} and is clearly destabilized around Gly_{12}. Gly_{12} does not appear to be involved in any medium-range NOE connectivities, and the rate of exchange is relatively fast. Another difference as compared to TFE is that the helix starts more near the beginning of the N-terminus, as concluded from the presence of a medium-range Hα(i)−Hβ(i+3) NOE between Lys_2 and Thr_5 and from the slow exchange of the amide proton of Thr_5, suggesting that even Met_1 is to some extent involved in the formation of the α-helix. Furthermore, the helical content is now higher near the N-terminus as compared to the C-terminus, as suggested by the increased intensity of the NOEs, the exchange data, and the chemical shift profile.

(3) *DPC Micelles (Zwitterionic Lipid).* Like in SDS micelles an α-helix is formed, in which the N-terminal half and the C-terminal half are separated by a kink at the Gly_{12} position. However, the helix extends less far to the N-terminus, and the C-terminus has a less regular or more flexible structure compared to SDS micelles, as concluded from the weak medium-range NOEs and the relatively fast amide proton exchange of the C-terminus.

The results from 2D NMR are consistent with the CD data, in which also the helical content is decreased in the order TFE ≅ SDS > DPC. An apparent discrepancy is that in DPC micelles the CD data in addition suggested the formation of β-structure, probably due to a partial aggregation of the peptide. A likely explanation is that the size of such aggregates would make them "invisible" for high-resolution NMR, while they still could contribute to the CD spectrum. β-Sheet formation might be facilitated by the high mobility and relatively irregular structure of the C-terminus in DPC micelles. This is supported by the observation that raising the temperature leads to an increase in turbidity, suggesting aggregation of the signal peptide. A high temperature will decrease the structural order in the peptide and may accelerate its aggregation due to peptide−peptide interactions.

Let us now compare our results with literature data on the structure of other signal peptides. 2D NMR studies have been reported on the signal sequences (and mutants) of LamB (Bruch et al., 1989; Bruch & Gierasch, 1990) and OmpA (Rizo et al., 1993) and on artificial signal sequences (Yamamoto et al., 1990) in TFE/water mixtures and in SDS micelles. In all cases a helix was observed with a break in the hydrophobic core around either a Gly or Pro residue, similar to what we found with the PhoE signal peptide. However, for these other signal peptides no comparison was made, nor was it evident from the data, of how the different environments affect the stability of the helix near the break. Another general feature of signal peptides appeared to be that the N-terminal half has a higher helical content than the C-terminal half, again similar to what we observed for the PhoE signal peptide. In SDS micelles this N-terminal half was found to be more stable than in TFE/water, probably due to a propagation of the helix toward the N-terminus as a consequence of the favorable interaction between the helix dipole and the negatively charged sulfate groups (Rizo et al., 1993). This is supported by our results, which show that also for the PhoE signal peptide such a propagation

occurred in SDS micelles, but not in the zwitterionic DPC micelles.

The present study adds two important conclusions concerning the conformational behavior of the PhoE signal peptide, which may be valid for signal sequences in general. (1) A lipid/water interface can destabilize the helix near the position of a helix-breaker such as glycine or proline. (2) Different lipid environments can modulate the structure of the signal peptide. The general validity of the first conclusion is supported by the recent observation that a lipid/water interface also destabilizes the helix near a helix-breaker in alamethicin, a channel forming peptide which is structurally related to signal sequences (Kelsh et al., 1992; Franklin et al., 1994). Also the second conclusion is most likely general, since CD (Batenburg et al., 1988a; Reddy & Nagaraj, 1989; Keller et al., 1992), infrared (Demel et al., 1990), fluorescence (Killian et al., 1990), and monolayer experiments (Batenburg et al., 1988b; Demel et al., 1990) on different types of signal peptides showed that the structure of signal peptides is sensitive to the lipid environment. The results obtained from these various types of model membrane systems thus correspond with those obtained from micelle systems in the present study, suggesting that micelles mimic well the membrane environment. This is further supported by the spin-label measurements, demonstrating that the peptide inserts into the micelles, and by transferred NOE experiments (Wang et al., 1993) of a signal peptide in the presence of vesicles, for which similar general structural properties were found as described above for signal peptides in micelles.

Let us now discuss the possible functional relevance of the conformational behavior as observed for the signal peptide of PhoE and its modulation by the lipid environment. Using *E. coli* cells with different lipid composition, it was shown that anionic lipids are essential for efficient translocation of the precursor of PhoE across the *E. coli* inner membrane (De Vrije et al., 1988; Kusters et al., 1991). Subsequent studies with different types of precursor proteins indicated that the signal sequence requires anionic lipids for its functional activity (Phoenix et al., 1993a; Kuster et al., 1994). On the basis of monolayer measurements, it was suggested that the signal peptide adopts a looped conformation in zwitterionic lipids, whereas anionic lipids induce a shift in the equilibrium to an unlooped or extended conformation, thereby providing the first step in translocation of the protein across the membrane (Batenburg et al., 1988b). Such a stabilization of the unlooped structure in the presence of anionic lipids may be a direct consequence of the decreased flexibility of the C-terminus in the presence of an anionic lipid/water interface, as observed in the present study. The importance of the conformational flexibility of the signal sequence for protein translocation was shown in *in vitro* experiments with a number of mutant precursors of the PhoE protein, in which the conformational flexibility was excluded or reduced. It was found that fixing the signal sequence into a looped structure by disulfide cross-linking arrests translocation at a very early stage (Nouwen et al., 1994), while precursor proteins with α-helix-stabilizing amino acids (Ala, Leu, Cys) at the position of Gly_{12} are less efficiently translocated than the wild-type precursor protein (Nouwen et al., 1995). In addition, such substitutions relieve the transmembrane potential dependency of translocation (Nouwen et al., 1995).

Taken together all these data indicate that conformational changes of the signal sequence induced by a specific lipid/water interface play an important role in the export process. Lipids are structurally flexible membrane constituents and their two-dimensional diffusion does not require a high activation energy. It can be expected that at the site of membrane insertion of the signal sequence local and temporal changes in lipid composition can occur as a result of interaction of both the signal sequence (Batenburg et al., 1988b) and the Sec A protein (Lill et al., 1990; Breukink et al., 1992) with the membrane. This could result in specific conformational changes in the signal sequence which in conjunction with the transmembrane potential could trigger the translocation machinery.

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BI950647+