Mode of Insertion of the Signal Sequence of a Bacterial Precursor Protein into Phospholipid Bilayers As Revealed by Cysteine-Based Site-Directed Spectroscopy

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Received August 10, 1995; Revised Manuscript Received November 17, 1995[⊗]

ABSTRACT: The interactions between a bacterial precursor protein and phospholipids in bilayer-based model membrane systems is addressed in this study. The precursor−lipid interactions were assessed from the side of the lipid phase by fluorescence and electron spin resonance spectroscopy, using the precursor of the *Escherichia coli* outer membrane protein PhoE. The role of the signal sequence, as part of the precursor, in this interaction was investigated by using cysteine-based site-directed spectroscopy. For this purpose, purified cysteine-containing mutants of prePhoE, which were made by site-directed mutagenesis of the signal sequence part and of the mature part, and defined lipids were used. The location of the fluorescently labeled cysteine residues was established by resonance energy transfer and quenching experiments and those of the corresponding spin-labeled cysteine residues by paramagnetic relaxation enhancement. It was demonstrated that precursor−phospholipid interactions exist in model membrane systems and also that these interactions were dependent on the presence of anionic phospholipids and resulted in a deep insertion of (parts of) the precursor into the lipid bilayer. Furthermore, the results with the cysteine mutations in the signal sequence of the precursor indicate that both termini of the signal sequence are located near or at the membrane surface, with only the fluorescence of the labeled cysteines in the signal sequence part being protected against aqueous quenchers. The results demonstrate that, when part of the intact precursor, the signal sequence experiences similar lipid−protein interactions as do isolated signal peptides. They also indicate that the signal sequence inserts entirely as a looped structure into the membrane. In addition, the data also indicate that the mature part of the precursor has an affinity for the membrane.

Protein secretion across the bacterial cytoplasmic membrane has been characterized both *in vivo* by genetic approaches and *in vitro* by biochemical studies [see Arkowitz and Bassilana (1994) for a review]. Proteins destined to be transported out of the cytosol are synthesized as precursors with an N-terminal extension, the signal sequence (Milstein et al., 1972; Inouye & Beckwith, 1977). The protein components involved in prokaryotic secretion (the Sec proteins) are well defined in *Escherichia coli* [see Tokuda (1994) for a review]. Furthermore, it is well established that anionic phospholipids play an essential role in efficient protein translocation (de Vrije et al., 1988; Lill et al., 1990; Cabelli et al., 1991; Hendrick & Wickner, 1991; Kusters et al., 1991, 1994). One of the intriguing questions is how to explain the involvement of anionic phospholipids at a molecular level. At present, two explanations for the anionic phospholipid requirement of the translocation process are likely.

One possibility is that SecA−lipid interactions are involved. SecA is a peripheral membrane ATPase (Oliver & Beckwith, 1982; Cunningham & Wickner, 1989) that is active in a dimeric form (Akita et al., 1991; Driessen, 1993). The existence of SecA−lipid interactions that are dependent on anionic phospholipids has been demonstrated by several approaches (Lill et al., 1990; Breukink et al., 1992, 1993; Ulbrandt et al., 1992), leading to the proposal of a functional SecA insertion−deinsertion cycle (Breukink et al., 1992). In support of this model, SecA was found to be able to traverse (partly) the cytoplasmic membrane during protein translocation (Kim et al., 1994; Economou & Wickner, 1994). This seems to be an intrinsic property of the SecA protein, since it was demonstrated using model membrane experiments that part of SecA inserts deeply into a phospholipid bilayer (Keller et al., 1995a) and was partly accessible to trypsin, which was enclosed in phospholipid vesicles (Ahn & Kim, 1994). Despite this substantial amount of experimental evidence, it is unlikely that SecA−phospholipid interactions are the only explanation for the observed anionic phospholipid dependence of protein translocation. For example, recently it was demonstrated that a protein, whose translocation is Sec-independent, was still dependent on anionic phospholipids for its translocation (Kusters et al., 1994).

The other likely explanation for the anionic phospholipid involvement in protein translocation is a signal sequence−lipid interaction. The signal sequence generally has several positive charges in the N-terminal region, which is followed by a hydrophobic stretch of 8−15 amino acids and a more polar C-terminal region containing the cleavage site (Von

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

0006-2960/96/0435-3063$12.00/0

Heijne, 1985). Using synthetic signal peptides, it was demonstrated that signal peptide−lipid insertion was dependent on anionic phospholipids (Demel et al., 1990; Killian et al., 1990a,b; McKnight et al., 1991; Breukink et al., 1993). It was also demonstrated that the adoption of an α-helical conformation by the signal peptide of the precursor protein prePhoE upon interaction with lipids depended on the presence of anionic phospholipids (Keller et al., 1992). The conformational properties of signal peptides in a membrane-like environment have been characterized in great detail (Bruch et al., 1989; Rizo et al., 1993; Wang et al., 1993). A high propensity both for α-helix formation and for insertion into phospholipid membranes is characteristic of a functional signal sequence (Hoyt & Gierasch, 1991). Additionally, it was found that signal sequence mutants of a precursor that follows the Sec route, which were characterized by enlarged hydrophobic sequences, could translocate even with very low levels of anionic phospholipids (Phoenix et al., 1993a,b), also indicating a direct signal sequence−phospholipid interaction.

Despite the large amount of biophysical data on phospholipid interactions with isolated signal peptides, virtually nothing is known about the interactions between an intact precursor protein and phospholipids. Yet such knowledge might contribute materially to an understanding of the role of signal sequence−phospholipid interactions in the translocation process, especially in view of a recent paper which demonstrated a direct contact of the signal sequence with the lipids during membrane translocation (Martoglio et al., 1995). Therefore, in the present study, the lipid−protein interactions experienced by the intact precursor of the *E.* coli outer membrane protein PhoE were investigated in bilayer-based model systems. First, precursor−lipid interactions were studied by fluorescence and electron spin resonance spectroscopy using wild-type prePhoE. Secondly, the role of the signal sequence in this interaction was investigated. The strategy used to address this latter issue was to introduce unique cysteines at different positions in the precursor protein, which allowed specific labeling of the precursor with fluorescent or spin labels. Purified cysteine-containing mutant forms of prePhoE, made by site-directed mutagenesis, and defined lipids were used.

MATERIALS AND METHODS

Lipids. 1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG),[1] 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were obtained from Avanti Polar Lipids (Birmingham, AL). 1-Hexadecanoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3,4a-diaza-s-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine (β-BODIPY FL C12-HPC), 1-hexadecanoyl-2-(4,4-difluoro-5,7-diphenyl-4-bora-3,4a-diaza-s-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine (β-BODIPY 530/550 C12-HPC), and 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (coumarin maleimide) were purchased from Molecular Probes, Inc. (Eugene, OR). 3-Maleimido-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (5-MSL) was from Aldrich (Milwaukee, WI). [[*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]methyl]thio (CPS) and (2-stearoyl-CPS)-oleoylphosphatidylcholine [(12-CPS)-18-PC] were synthesized as described (Silvius et al., 1987). 1-Acyl-2-stearoylphosphatidylglycerol spin-labeled at either the 5-, 12-, or 14-position in the *sn*-2 chain (5-, 12-, and 14-PGSL, respectively) were synthesized by B. Angerstein as described in Marsh and Watts (1982). 4-Phosphatidyl-2,2,6,6-tetramethylpiperidine-1-oxyl (T-PASL) was prepared by H. Eibl and A. Watts at the Max Planck Institute, according to the method of Eibl (1978). All other chemicals were at least of analytical grade.

Proteins. Wild-type prePhoE was isolated and purified as described previously (Kusters et al., 1989). The wild-type signal sequence does not contain any cysteines and has the following sequence: MKKSTLALVVMGIVASASVQA. The mature domain of the protein does not contain any cysteines. The signal sequence mutant prePhoE forms, A-5C and ∇C-19, with a cysteine near the C- and the N-terminus of the signal sequence, respectively, were created by site-directed mutagenesis (Nouwen et al., 1994). The mutant proteins were overexpressed in *secA* mutant strain MM52, carrying the plasmids pNN101 and pNN103, respectively, which contain the mutant genes under control of the *tac* promoter. The mutant protein ∇C159, with an insertion of four residues, including a cysteine, in the fourth exposed loop of the PhoE mature region, was overexpressed by growth of the strain MM52 carrying plasmid pMR08 (Agterberg et al., 1987) under phosphate limitation. All mutant proteins were isolated and purified as described for the wild-type protein (Kusters et al., 1989), except that DTT (15 mM) was added immediately during isolation to prevent disulfide cross-linking of the cysteines upon storage. The cysteines of the mutant prePhoE proteins were labeled with either fluorescent- or spin-label derivatives. Before the labeling could be performed, the excess of DTT present in the protein stock was removed by TCA-precipitation (30% w/v final concentration, 30 min, 0 °C), and the protein was collected by centrifugation (5 min, 10 000 rpm, Eppendorf 5415C). The prePhoE pellet was resuspended in the presence of 200 μM DTT (protein concentration 5−7 mg/mL in 10 mM Tris-HCl, 8 M urea, pH 8.0). The labeling (2 h at 0 °C) was started by the addition of label reagent from a 100 mM stock solution of either coumarin or rhodamin maleimide in dimethylformamide or, in the case of 5-MSL, from an ethanol solution (final label concentration 5 mM). The reaction was terminated by the addition of 10 mM DTT, and, after subsequent TCA precipitation, the nonreacted label was removed by two washing steps using dimethylformamide or acetone. After a final washing step in water, the proteins were stored in a 10 mM Tris-HCl, 8 M urea, pH 8.0 solution at −20 °C. The fluorescent label content was quantified by measuring the absorbance, while the spin label was quantified by double integration of the ESR signals. The functionality of the mutant proteins before and after labeling with fluorescent or spin probes was tested in a translocation assay (Kusters et al., 1989) and a processing assay. In the

[1] Abbreviations: β-BODIPY FL C12-HPC, 1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3,4a-diaza-s-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine; β-BODIPY 530/550 C12-HPC, 1-hexadecanoyl-2-(4,4-difluoro-5,7-diphenyl-4-bora-3,4a-diaza-s-indacene-3-dodecanoyl)-*sn*-glycero-3-phosphocholine; CW, continuous wave; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DTT, dithiothreitol; ESR, electron spin resonance; L/P, molar lipid to protein ratio; LUVs, large unilamellar vesicles; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-pyrrolidine-*N*-oxyl; PGSL,1-acyl-2-[*n*-(4,4-dimethyl-oxazolidine-*N*-oxyl)]-stearoyl-*sn*-glycero-3-phosphoglycerol; pmf, proton motive force; RET, resonance energy transfer; T-PASL, 4-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

processing assay, purified leader peptidase (40 μg), dissolved in 0.1% β-octylglucoside, was used, and the extent of processing was determined after the addition of prePhoE (60 μg) out of 8 M urea. Incubation times of 0, 10, 30, and 60 min were used.

Fluorescence Measurements. All fluorescent measurements were carried out on a SLM Aminco SPF-500C fluorometer. Fluorescence spectra were collected using excitation and emission slit widths of 5 nm. In all cases, control samples were prepared without fluorescent probes to correct for contributions of light scattering to the observed fluorescence signal (usually less than 1%). Vesicles were prepared by mixing DOPG with 2 mol % BODIPY-lipid in chloroform. After evaporation under a stream of nitrogen and overnight storage under vacuum, the lipid film was dispersed (concentration 5 mM) in a buffer containing 100 mM NaCl and 10 mM Tris-HCl, pH 7.6. This lipid dispersion was either bath sonicated for 5 min (Silvius et al., 1987) or passed through a 400 nm filter in an extruder (Hope et al., 1985) in the case of the protein−lipid experiments. For the resonance energy transfer (RET) experiments the coumarin-labeled protein (stock concentration of 3 mg/mL) was added from 8 M urea (Kusters et al., 1991) to DOPG vesicles (100 μM phospholipid), containing 2 mol % BODIPY-lipid resulting in a protein/lipid molar ratio of 1/100. The energy transfer efficiency E was defined as

$$E = 1 - F_D/F_D^o \quad (1)$$

where F_D and F_D^o are fluorescence intensities of the donor (coumarin) in the presence and absence of acceptor (BODIPY), respectively. Measurements were performed at 23 °C.

Steady-State Fluorescence Anisotropy. The determination of the fluorescence anisotropy was essentially performed as described (Breukink et al, 1993). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was added to 25 μM DOPG LUVs in a 1:250 molar ratio. DPH fluorescence was measured on a SPF 500 C spectrophotometer (SLM instruments Inc., Urbana, IL), with an excitation wavelength of 360 nm and an emission wavelength of 430 nm. Emission and excitation slit widths were 5 nm. Increasing amounts of prePhoE were added to a DPH-containing lipid solution and the anisotropy ratio (A) of each sample was calculated according to

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

where $I_{||}$ and $I_{\perp}$ are the fluorescence emission intensities with polarizations parallel and perpendicular, respectively, to that of the exciting beam. Samples were continuously stirred and measured at room temperature. Each measured ratio is the average of at least four different experiments.

ESR Sample Preparation. Preparation of samples was performed essentially as described previously (Görrissen et al., 1986; Keller et al., 1995a). A dried lipid film (0.4 mg) with 1 mol % spin-labeled lipid was hydrated in 6.0 mL of 50 mM Tris and 100 mM NaCl, pH 7.6. prePhoE was added (3 mg/mL), and after incubation for 60 min at 25 °C the lipid−protein complexes were isolated by ultracentrifugation at 45 000 rpm (SS34 rotor) for 45 min at 4 °C. After centrifugation, the pellets were transferred to ESR capillaries and concentrated further by centrifugation (Labofuge II, Heraeus, 15000*g*, 3 min). The complexes of unlabeled lipids with spin-labeled prePhoE were prepared essentially in the same way. After the ESR measurements, the protein and phospholipid concentrations were determined as described previously (Keller et al., 1995a).

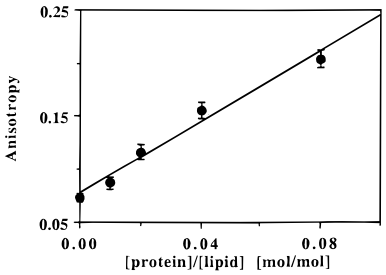

FIGURE 1: Steady-state fluorescence anisotropy of DPH in DOPG vesicles as a function of the mount of wild-type prePhoE added (3 mg/mL stock). The lipid concentration was 25 μM.

ESR Spectroscopy. The ESR measurements were performed on a Varian E-12 Century Line 9 GHz ESR spectrometer. The temperature was regulated with a pure nitrogen gas flow system, and the sample capillaries were centered in the microwave cavity in a standard 4 mm quartz tube which contained light silicone oil for thermal stability. Conventional, in-phase, absorption spectra were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.25 G peak to peak. The total scan width was 100 G. In the case of the 5-PGSL, the ESR spectra were quantitated in terms of the hyperfine splitting between the outer extrema ($2A_{max}$) and for 14-PGSL by spectral subtraction (Marsh, 1982). CW power saturation ESR experiments using either spin-labeled proteins or spin-labeled lipids were performed as described previously (Snel & Marsh, 1993). Samples were saturated with either pure oxygen or pure argon, in order to determine the relaxation enhancement by paramagnetic molecular oxygen. The accessibility parameter to oxygen is then defined by (Snel & Marsh, 1993)

$$\mathrm{AP} = [(1/T_1T_2)_{O2} - (1/T_1T_2)_0]/\delta \qquad [\times 10^{13}\ \mathrm{s}^{-2}\ \mathrm{G}^{-1}] \quad (3)$$

where $(1/T_1T_2)_{O2}$ and $(1/T_1T_2)_0$ are the reciprocal values of the T_1T_2 relaxation time product obtained from fitting the power saturation curves for samples in the presence and absence of oxygen, respectively, and δ is the peak-to-peak line width of the central spin-label line. This quantity is directly proportional to the local oxygen concentration at the site of the spin label and is generally higher in a hydrophobic than in an aqueous environment.

RESULTS

Characterization of Precursor−Phospholipid Interactions. In order to determine the existence of precursor−phospholipid interactions, DPH fluorescence anisotropy measurements were performed. For this purpose, WT-prePhoE was added to DOPG vesicles doped with DPH. Phosphatidylglycerol was selected as test lipid since it represents the most abundant anionic phospholipid in *E. coli* and since it has been found to interact strongly with isolated signal peptides (Killian et al., 1990a,b; Hoyt & Gierasch, 1991; Keller et al., 1992). From Figure 1, it is clear that prePhoE causes a progressive increase in the DPH fluorescence anisotropy, indicating an interaction between the precursor and the lipid bilayer. Furthermore, these results can be interpreted [cf. Hoyt and Gierasch (1991)] as insertion of prePhoE into the

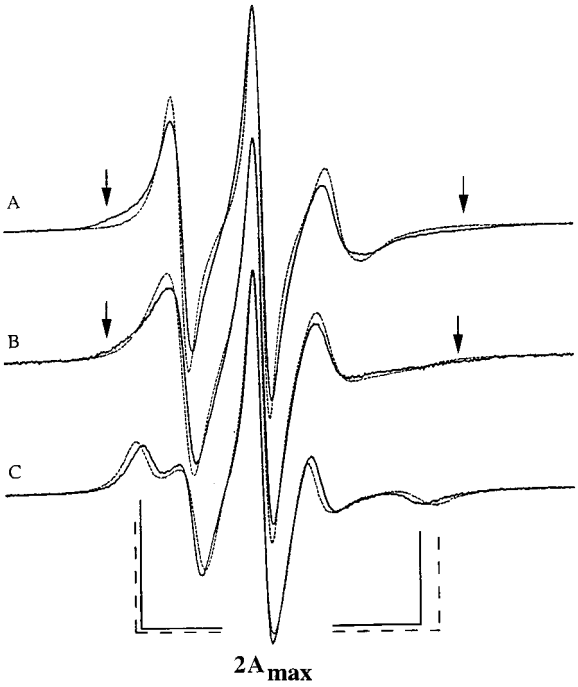

FIGURE 2: Effect of prePhoE binding on the ESR spectra of DOPG dispersions containing spin-labeled phosphatidyl glycerol. ESR spectra of spin-labeled phospholipids 14-PGSL (A), 12-PGSL (B), and 5-PGSL (C) in complexes of DOPG with wild-type prePhoE. Spectra of the spin labels in DOPG lipid dispersions alone are given as dashed lines (except for C, where the absence of protein is indicated in solid line). prePhoE was bound to DOPG bilayers at a lipid to protein ratio of 60 mol/mol. The spectra are recorded at 20 °C. Total scan width = 100 G. The motionally restricted components are indicated by arrows: also the outer splitting ($2A_{max}$) is indicated.

acyl chain region of the bilayer. Next, we investigated the extent of insertion of prePhoE and its effects on the chain packing and mobility in more detail. For this purpose, ESR measurements were performed with spin-labeled lipids. The ESR spectra of negatively charged phosphatidylglycerols spin-labeled at the 5th, 12th, or 14th C-atom position in the s*n*-2 chain (5-, 12-, and 14-PGSL) incorporated in DOPG, with and without WT-prePhoE, are given in Figure 2. In the absence of prePhoE, the spectra evidence the chain mobility gradient within the bilayer, with the most anisotropic (motionally restricted) spectrum arising from the spin label at the C5 position and the most nearly isotropic (least motionally restricted) spectrum from the C14 position. The binding of WT-prePhoE causes several effects. A clear increase in anisotropy of the hyperfine splittings was observed in the spectrum for the 5-PGSL spin-label, as became apparent by the increase of the $2A_{max}$. A similar effect was observed also for one of the two components (the more mobile) in the spectra from the 12-PGSL and 14-PGSL spin-labeled phosphatidylglycerols (as indicated in Figure 2). In addition, a second, more motionally restricted component was observed in the spectra from these latter two spin-labeled lipids. This second component, which is visible as partially resolved peaks or shoulders in the outer wings of the spectra, is attributed to lipid chains that directly contact membrane-penetrating parts of the protein (Marsh & Watts, 1982; Görrissen et al., 1986).

These results indicate that (parts of) the intact prePhoE precursor can insert into a DOPG bilayer and that this insertion is sensed up to the 14th position of the lipid acyl

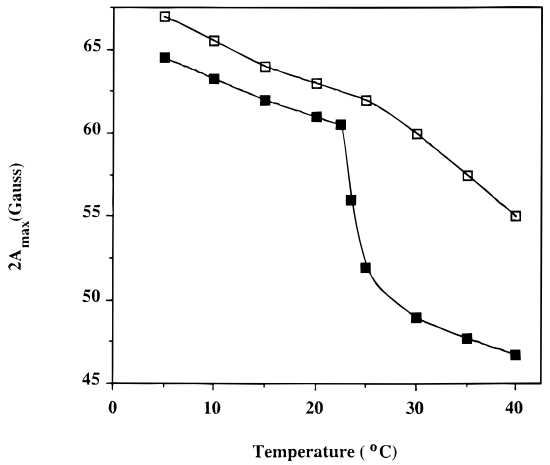

FIGURE 3: Phase behavior of DMPG dispersions doped with 1 mol % 5-PGSL, in the absence and presence of wild-type prePhoE. The temperature dependence of the outer hyperfine splitting ($2A_{max}$) in the ESR spectrum of 5-PGSL is shown for DMPG dispersions in the absence of prePhoE (filled squares) and in the presence of prePhoE (open squares). The lipid to protein ratio of the samples containing prePhoE was 55 mol/mol.

chains. The gel-to-liquid crystalline bilayer phase transition of DMPG offers another tool to study the membrane interaction of prePhoE. In the absence of prePhoE, the outer hyperfine splitting ($2A_{max}$) of 5-PGSL in DMPG bilayers changes abruptly at around 23 °C, which is the phase transition temperature of that lipid (Figure 3). Binding of the prePhoE protein led to an almost complete elimination of this chain-melting phase transition and resulted instead in a gradual decrease of the outer hyperfine splitting with increasing temperature. This result is consistent with the penetration of (part of) the prePhoE protein into the hydrophobic region of the bilayer, hence disrupting the cooperative packing of the lipid chains in the gel phase. Even at temperatures corresponding to the gel phase of the lipid bilayers alone, the outer hyperfine splitting is greater in the presence of WT-prePhoE than in its absence.

Recently, accessibility studies of spin-labeled phospholipids and spin-labeled proteins toward paramagnetic relaxation agents (Snel & Marsh, 1993; Snel et al., 1994) were used to determine the extent of penetration of proteins into the bilayer. In a continuous wave power saturation experiment, ESR spectra are recorded at increasing incident microwave powers. At low powers, the spectral intensity increases linearly with the square root of the microwave powers, whereas at higher powers the intensity levels off due to saturation. In the latter case, the rate of microwave absorption is so great that the spin−lattice relaxation processes are unable to maintain the Boltzmann equilibrium spin population difference, and the microwave absorption by the sample saturates. Alleviation of saturation can be caused by interactions between the nitroxide spin labels and fast-relaxing paramagnetic agents. The extent to which this occurs depends on the accessibility of the paramagnetic agent to the spin label. We explored this method to study the prePhoE−phospholipid interaction by measuring the accessibility of paramagnetic molecular oxygen to spin labels attached at various positions in the lipid chain. As depicted in the upper part of Table 1, there is a decrease in the oxygen accessibility parameters of the spin-labeled lipids upon addition of WT-prePhoE to DOPG vesicles, as determined by continuous wave (CW) power saturation ESR spectros-

Table 1: Accessibility Parameters to Oxygen, AP, of Spin Labels Attached to Phospholipids or to prePhoE in DOPG Bilayers[a]

spin label	protein	AP (in units of 10^{13} s^{-2} G^{-1})
spin-labeled lipids		
T-PASL		6.2
T-PASL	prePhoE (WT)	2.2
5-PGSL		8.0
5-PGSL	prePhoE (WT)	3.1
14-PGSL		10.5
14-PGSL	prePhoE (WT)	5.4
spin-labeled proteins		
5-MSL	A-5C	1.0
5-MSL	∇C-19	2.9
5-MSL	∇C159	5.2

[a] Accessibility parameters are as defined in Snel and Marsh (1993) and are proportional to the difference in spin label relaxation rates for samples saturated with oxygen and in the absence of oxygen.

Table 2: Characteristics of Labeled Cysteine Mutants of prePhoE

protein	label	labeling efficiency[a]	translocation[b] (%)
A-5C	coumarin	1.0 ± 0.05	105
A-5C	rhodamine	1.15 ± 0.10	85
∇C-19	coumarin	1.05 ± 0.05	100
∇C-19	rhodamine	1.2 ± 0.05	85
∇C159	coumarin	1.0 ± 0.05	100
∇C159	rhodamine	1.1 ± 0.05	90

[a] Labeling efficiency is in mol of label/mol of protein. [b] For translocation (and processing), cysteine-containing mutant prePhoE proteins grown in the presence of [^{35}S]-methionine were used, which were labeled after isolation and purification. Translocation was quantified by cutting the protected bands out of the gel and counting the radioactivity with a scintilation counter. Results are based on at least two experiments (SD ± 5%). The results with wild-type prePhoE was taken as 100%.

copy (cf. later). This reduction in accessibility parameter is again an indication that upon interaction with DOPG lipids, the precursor decreased the acyl chain mobility up to the 14th position in the bilayer, hence reducing the local oxygen concentration [cf. Snel et al. (1994)]. Finally, experiments with the 14-spin-label in zwitterionic DOPC bilayers did not give rise to any spectral changes on adding WT-prePhoE (data not shown), indicating that anionic phospholipids are essential for deep penetration of (part of) the precursor protein into phospholipid bilayers. To determine the lipid specificity of the interaction in more detail and to detect whether the signal sequence of the precursor directly interacts with the membrane lipids, we used cysteine engineering of the protein in combination with fluorescence- and spin-labeling techniques.

Determination of the Membrane Interaction and Topology of the Signal Sequence of prePhoE. The mutants A-5C and ∇C-19, which introduce cysteines into the signal sequence, were shown both *in vivo* and *in vitro* to have no deleterious effect on the translocation of the precursor (Nouwen et al., 1994). This was also reported for the ∇C159 mutant protein, which contains a unique cysteine in the mature region and which is used here as a control system (Agterberg et al., 1987). Labeling with either fluorescent- or spin-labeled covalent reagents was performed and was found to be nearly stoichiometric in all cases (Table 2). In order to see whether the labeling procedure or the attached label affects the functionality of the precursor protein, translocation and processing experiments were performed (see Materials and Methods). For all mutant proteins, it was found that the processing was not disturbed compared to wild-type prePhoE (data not shown). The translocation of radioactive mutant precursors, labeled with fluorescent label, was comparable with wild-type prePhoE (Table 2).

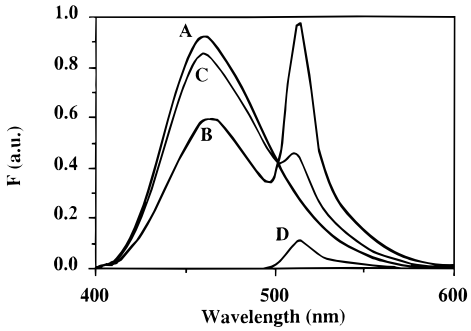

FIGURE 4: Fluorescence emission spectra of coumarin-labeled A-5C prePhoE without vesicles (curve A) or added to DOPG LUV (curve B) or DOPC LUV (curve C) doped with 2.5% β-BODIPY FL C12-HPC. The contribution of vesicles alone is also included (curve D). All spectra are measured at an excitation wavelength of 390 nm and recorded after 30 min of incubation.

We first studied the precursor−phospholipid interaction by using the novel energy transfer couple BODIPY−coumarin, the spectral properties of which were characterized recently (Keller et al., 1995b). This approach involves coumarin labeling of precursor cysteine-containing mutant proteins and BODIPY−lipids labeled in the acyl chain. As depicted in Figure 4, a strong energy transfer was observed upon addition of the coumarin-labeled mutant protein A-5C to DOPG vesicles doped with BODIPY-labeled lipids. For RET a typical decrease in the coumarin emission (473 nm) and a simultaneous increase in the BODIPY emission (513 nm) was found. To quantify the extent of energy transfer, this latter increase in BODIPY emission (F) was obtained by subtracting the coumarin emission contribution to the total emission scan. The kinetics of the interaction of the coumarin labeled mutant prePhoE A-5C with the DOPG vesicles are fast, with a half-time of less than a minute (Figure 5A). Figure 5B shows that saturation of interaction of the A-5C mutant protein with the vesicle bilayer occurs at around 100 μM DOPG which corresponds to a molar protein to lipid ratio of 1/65. Therefore, in subsequent experiments, a vesicle concentration corresponding to 100 μM lipid was used. From Figure 4 it is also clear that, in contrast to the results with DOPG, energy transfer is almost absent when similarly labeled DOPC vesicles are used. This suggests that in the intact precursor, which is overall negatively charged, it is the positively charged signal sequence that preferentially binds to anionic phospholipids. This result was also observed for the other signal sequence mutant ∇C-19 (Keller et al., 1995b). Figure 6 depicts the relation between the anionic phospholipid content and the BODIPY−coumarin energy transfer efficiencies for the A-5C prePhoE mutant with mixed DOPC/DOPG vesicles. From this figure, it is clear that anionic phospholipids are necessary for efficient energy transfer from the coumarin-labeled precursor to the BODIPY-labeled lipids.

We further used this RET method for the various cysteine-containing mutant proteins, together with fluorescence quenching and CW saturation ESR experiments, to determine the topology of the signal sequence in the membrane. First, we describe the results from the RET experiments. With BODIPY−lipid containing DOPG vesicles, the coumarin-

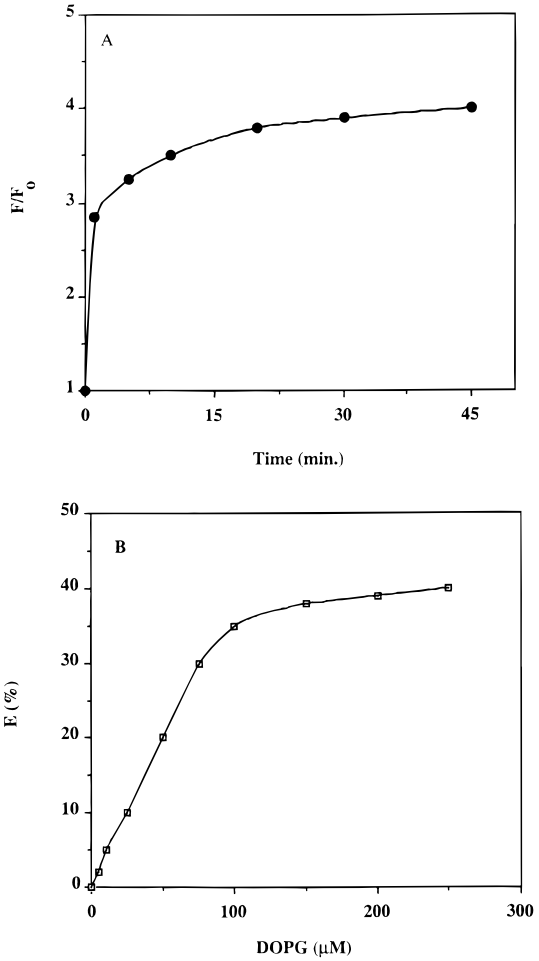

FIGURE 5: (A) Kinetics of the prePhoE-phospholipid interaction of A-5C cysteine mutant prePhoE, as quantified by the increase in BODIPY emission intensity (520 nm) after adding coumarin-labeled prePhoE. (B) Saturation curve of the precursor−phospholipid interaction using the A-5C cysteine mutant prePhoE with different concentrations with DOPG vesicles and quantified in terms of energy transfer efficiency, *E* (%).

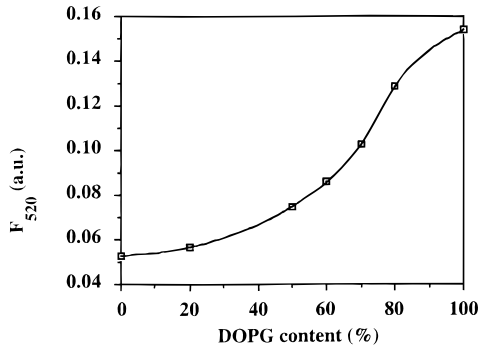

FIGURE 6: Anionic phospholipid dependence of the prePhoE−phospholipid interaction, on varying the amount of DOPG in DOPC vesicles. Depicted is the energy transfer from coumarin-labeled cysteine prePhoE mutant A-5C, as quantified by the BODIPY fluorescence emission increase at 520 nm of the labeled lipids.

labeled mutant proteins A-5C and ∇C-19 with the cysteines present in the signal sequence gave efficient energy transfer, i.e., 4.05 ± 0.18 and 3.81 ± 0.17, respectively, indicating that both termini of the signal sequence are located within the membrane. The coumarin-labeled mutant protein ∇C159 with the cysteine present in the mature part of the precursor also gave rise to energy transfer (3.72 ± 0.15). Similar results were obtained using the BODIPY analog β-BODIPY

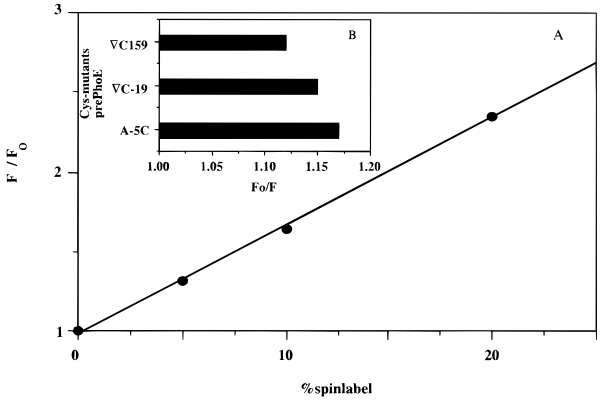

FIGURE 7: (A) Quenching of the fluorescence of the coumarin-labeled lipid (12-CPS)-18-PC (at 487 nm), incorporated at 1 mol % into bath-sonicated DOPG vesicles, by increasing molar percentages of spin-labeled lipid, 5-PGSL. (B) Quenching of the fluorescence intensity, *F*, of different coumarin-labeled cysteine mutants of prePhoE upon interaction with DOPG vesicles doped with 10% spin-labeled lipid.

530/550 C12-HPC, which together with coumarin was found to have a working distance R_o of approximately 40 instead of 50 Å for the coumarin-β-BODIPY FL C12-HPC couple (Keller et al., 1995b) (data not shown).

It is indicated (Lakowitz, 1983) that for collisional quenching contact between the fluorophore and quencher is necessary, with a sphere of action radius of 5−10 Å. Fluorescence quenching experiments were performed using spin-labeled lipids. First of all it was tested whether coumarin could be quenched efficiently by nitroxide spin labels. It is clear that the fluorescence of coumarin-labeled lipids incorporated in DOPG vesicles could be quenched efficiently by increasing amounts of spin-labeled lipids (see Figure 7A). Figure 7B gives the results obtained with the different mutant prePhoEs after addition of coumarin-labeled protein to DOPG vesicles containing 10% spin-labeled lipids. The fluorescence of the coumarin attached to the cysteines could be quenched by spin labels attached at the 5-position of the phospholipid chain. The fluorescence of mutant proteins labeled in the signal sequence could be quenched more efficiently than that of the ∇C159 mutant protein.

In addition aqueous quenchers were used. It was found that the fluorescence of the coumarin-label was insensitive to potent aqueous quenchers such as iodide, but that efficient quenching was observed for rhodamine-maleimide labeled prePhoE mutants (see Figure 8). The figure shows that F_o/F is linearly dependent upon the concentration of quencher in all cases, and these Stern−Volmer plots yield slopes equal to K_Q. From Figure 8 the Stern−Volmer constants were found to be 2.9 and 0.4 M^{-1} for the ∇C-19 mutant in the absence and presence of DOPG, respectively, and 2.7 and 2.6 M^{-1} for the ∇C159 mutant in the absence and presence of DOPG, respectively. It can therefore be concluded that the label on position −19 (N-terminus) of the signal sequence is shielded from iodide ions in the presence of DOPG vesicles, while the label on position +159 of the mature domain is fully accessible to the quencher when bound to the DOPG vesicles.

In order to obtain further information on the membrane topology of the signal sequence of the prePhoE protein, CW power saturation ESR spectroscopy of the spin-labeled cysteine-containing mutant proteins was performed. This technique enables the determination of the membrane loca-

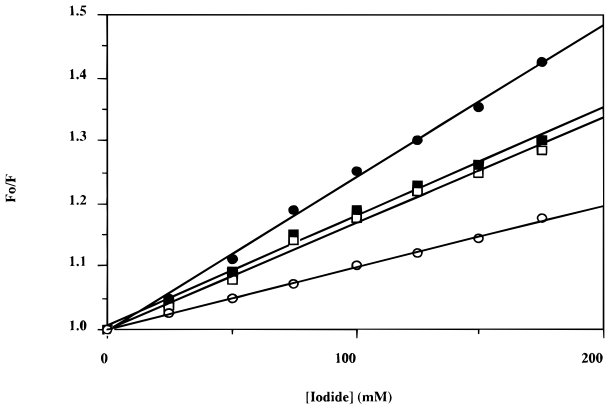

FIGURE 8: Quenching of the fluorescence intensity, *F*, of rhodamine-labeled cysteine mutants of prePhoE, ∇C-19 (circles), and ∇159 (squares) by increasing concentrations of iodide in the presence (open symbols) or absence (closed symbols) of DOPG vesicles.

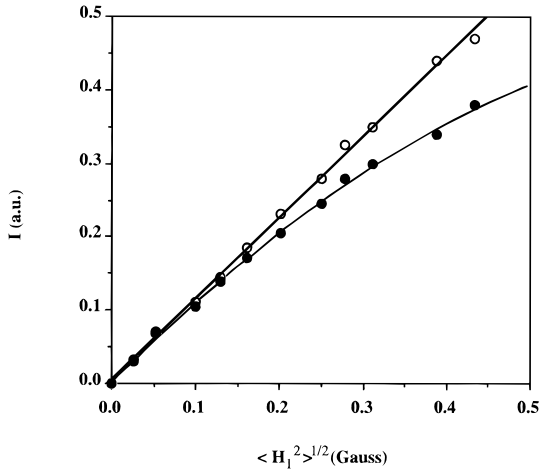

FIGURE 9: Dependence of the double-integrated intensity, *I*, of the ESR spectrum of the spin-labeled cysteine mutant ∇C-19 upon interaction with DOPG in the presence (open circles) and in the absence (filled circles) of oxygen on the root-mean-square microwave magnetic field, $\langle H_1^2 \rangle^{1/2}$. $T = 25$ °C. The signal intensities are normalized relative to the number of spin-labels in each sample (arbitrary units). The solid lines show the results of the nonlinear least-squares fits of the saturation curves according to Páli et al. (1993).

tion of a spin label attached to a protein, as has been demonstrated recently, for example, for apocytochrome *c* (Snel et al., 1994). In the present study, the position of the spin label, attached to the cysteines in the signal sequence and mature domain of the mutant precursors, was determined from the exposure of the nitroxide spin label to the paramagnetic agent oxygen. Figure 9 shows typical microwave power saturation curves obtained in a CW ESR experiment. The increase in spin-label relaxation rate by interaction with paramagnetic oxygen is clearly evident in this figure. The intensity of the ESR spectrum saturates far less readily with increasing microwave power in the presence of oxygen than in its absence. The saturation curves are fitted to yield the effective T_1T_2 relaxation time products (Páli et al., 1993), and from these the increase in relaxation rate and hence a parameter for the accessibility to oxygen can be deduced (Snel & Marsh, 1993). The oxygen accessibility parameters for the different spin-labeled lipids in the presence and absence of prePhoE bound to DOPG and for the various spin-labeled cysteine mutant PhoE precursors in DOPG bilayers are summarized in Table 1. The oxygen accessibility for 14-PGSL is greater than that for 5-PGSL, which is in turn greater than that for a spin-label in the lipid polar head group (T-PASL) because of the preferential localization of oxygen in the fluid hydrocarbon interior of the lipid bilayer. This forms a basis for determining the membrane location of the spin labels on the mutant prePhoEs. For this, the measurements on the spin-labeled lipids in the presence of wild type prePhoE constitute the appropriate calibration. Comparison of the accessibility of the spin label attached to the protein with that of the spin label attached at various positions in phospholipid molecules in the presence of unlabeled prePhoE (WT) (upper part of Table 1) indicates that the label in the signal sequence mutant ∇C-19 is located near the 5-position of the lipid chains and that of the signal sequence mutant A-5C is most likely located near the surface of the membrane. According to the high accessibility parameter, the label of the mutant protein ∇C159 is located near the 14-position of the acyl chain. Alternatively, the spin label might be located in a hydrophobic pocket of the mature part itself. This would be a situation analogous to that found for spin-labeled Cys-102 of yeast cytochrome *c*, where the oxygen accessibility parameter in aqueous solution was found to be 4.8×10^{13} s^{-2} G^{-1} (Snel et al., 1994). This value is comparable with that found here for ∇C159-prePhoE and much larger than that of 2.5×10^{13} s^{-2} G^{-1} found for unstructured spin-labeled apocytochrome *c* in solution (Snel & Marsh, 1993).

DISCUSSION

The signal sequence and synthetic signal peptides have been subjected to extensive studies regarding their role in protein translocation [see Izard and Kendall (1994) for a review]. Increasing evidence points to the ability of signal sequences to interact functionally with anionic phospholipids [see de Kruijff (1994) and Martoglio et al. (1995) for reviews]. The present study is focused on the role of the signal sequence in the interactions of the intact precursor with phospholipids.

First of all, it was established that interactions do occur between the whole precursor and phospholipids. This was done by using DPH-fluorescence anisotropy and ESR measurements. The ESR spectra of the spin-labeled phospholipids, together with the DPH-fluorescence anisotropy data, indicate a deep insertion of (part of) the precursor protein into a DOPG bilayer. This was further confirmed by using CW saturation ESR experiments. Furthermore, comparison of the ESR results obtained with DOPG and DOPC gave a first indication for an anionic phospholipid specificity of the precursor−phospholipid interaction. This is particularly interesting because previously a strong anionic phospholipid dependence was also found for interaction of the isolated signal peptide with phospholipids (Demel et al., 1990; Killian et al., 1990; Keller et al., 1992).

The specific introduction of cysteine residues in the signal sequence and mature domain of prePhoE by site-directed mutagenesis (Agterberg et al., 1987; Nouwen et al., 1994) results in unique labeling sites in the mutant precursor as wild-type prePhoE does not contain any cysteines. It was demonstrated that neither the mutations nor the subsequent labeling affected the functionality of the precursors. Additionally, we have shown that coumarin labeling of the signal sequence did not disturb functionality, as was also found previously for the mature part of proOmpA (Lecker et al., 1990).

The RET data revealed that the signal sequence of prePhoE was inserted in the membrane. Furthermore, as reported before for synthetic signal peptide−phospholipid interactions (Demel et al., 1990; Keller et al., 1992), this precursor−phospholipid interaction was found to depend on anionic phospholipids, although in the former case a true threshold value of negatively charged lipid content for insertion was observed. These results indicate that the signal sequence in the complete precursor exhibits the same features as the synthetic signal peptide analogs. The quenching experiments revealed that labeled cysteines at both termini of the signal sequence could be quenched by lipids spin-labeled in the acyl chain and not by aqueous quenchers in the presence of lipid vesicles. From the CW power saturation ESR experiments, we could estimate the approximate depth of insertion into the membrane, and it was concluded that the two cysteines placed in the signal sequence of prePhoE are located near the membrane surface or inserted into the hydrophobic part of the membrane (up to the 5-position). These results are consistent with a looped structure of the signal sequence, with both termini located at the water−membrane interface, as was postulated from studies using both synthetic signal peptides (Batenburg et al., 1988; Demel et al., 1990; Wang et al., 1993; Jones & Gierasch, 1994) and signal sequence mutants of prePhoE (Nouwen et al., 1994). Although our data do not exclude a transmembrane orientation with the residues at positions −5 and −19 on opposite sides of the bilayer, this is unlikely because it would require the polar N-terminal region of the mature part to partition into the hydrophobic core of the membrane, which is energetically unfavorable. In the "unlooping" model for signal sequence function (de Vrije et al., 1990), the signal sequence inserts initially into the membrane in a looped conformation, around a break in the α-helix of the hydrophobic core region, and finally unlooping of the signal sequence occurs, resulting in a transmembrane conformation. 2D ^{1}H NMR experiments performed recently demonstrated that the signal peptide of prePhoE inserts into micelles as a dynamic helix−break−helix structure, which is modulated by the environment (Chupin et al., 1995). *In vitro* studies suggested that the conformational flexibility of the signal sequence is required for efficient pmf-dependent translocation (Nouwen et al., 1996).

A mutant protein with a cysteine inserted in the mature part of the precursor was used here for control purposes. Substantial fluorescence energy transfer was found, indicating an affinity of the mature part of the precursor for phospholipids. It can be argued that the working distance of 50 Å for the coumarin-β-BODIPY FL C12-HPC couple (Keller et al., 1995b) is too large to discriminate between small distance differences between the various mutant proteins studied. However, similar results were obtained with the BODIPY analog β-BODIPY 530/550 C12-HPC, which has a smaller working distance, i.e., approximately 40 Å (Keller et al., 1995b). In addition, the experiments with spin-label quenching of fluorescence, which is a short range process (5−10 Å), were found to be consistent with the RET data.

Finally, the results of the CW saturation ESR experiment and the quenching experiment with iodide gave rather contradictory results for the ∇C159 mutant protein. While no protection against iodide was found in the presence of phospholipids, the accessibility for oxygen seemed to indicate a hydrophobic environment for this residue. In order to determine the behavior and topology of this part in detail, more cysteine mutants need to be studied. Taking all results together, the data suggest that the mature part exhibits affinity for phospholipids. Consistent with this view, recently performed monolayer experiments unambiguously demonstrated extensive insertion of the mature protein into the lipid phase, which interestingly could not be prevented by SecB (Van Raalte et al., 1996). Such an affinity of the overall negatively charged, mature part of prePhoE for the membrane could have either a positive or negative effect on the protein translocation process. In the latter case, interactions of the mature part of prePhoE with the lipids in the inner membrane both at the cytoplasmic and the periplasmic side need to be avoided during translocation. Especially precursor−SecA/SecB complexes might play an important role in this respect, together with the SecY/E/G complex.

Recent NBD-fluorescence studies revealed a hydrophilic environment of the mature part of a eukaryotic precursor protein during its translocation across the ER membrane (Crowley et al., 1994). Furthermore, in a similar study, the environment of the positively charged N-terminus of the signal sequence was probed and found to be hydrophilic (Crowley et al., 1993). This result is consistent with our results on the localization of the N-terminus, which was found to be positioned near the head group region of the phospholipids. The importance of understanding signal sequence−phospholipid interactions has become even more apparent, because recently it was demonstrated that the hydrophobic core of the eukaryotic signal sequence is in direct contact with the lipids during translocation across the ER membrane (Martoglio et al., 1995). This is especially significant because there are indications that protein translocation across the ER membrane in eukaryotes and across the cytoplasmic membrane in bacteria are highly related [see Jungnickel et al. (1994) for a review], and the signal sequences in both systems show strong similarity (Von Heijne, 1984).

ACKNOWLEDGMENT

We are grateful to Dr. J. R. Silvius for his generous gift of the coumarin-labeled lipids. R.K. was supported by a short-term EMBO fellowship during the course of part of this work.

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BI951870+