Interaction of the *Saccharomyces cerevisiae* α-Factor with Phospholipid Vesicles As Revealed by Proton and Phosphorus NMR†

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ABSTRACT: Proton and phosphorus-31 nuclear magnetic resonance (^{1}H and ^{31}P NMR) studies of the interaction between a tridecapeptide pheromone, the α-factor of *Saccharomyces cerevisiae*, and sonicated lipid vesicles are reported. ^{31}P NMR studies demonstrate that there is interaction of the peptide with the phosphorus headgroups, and quasielastic light scattering (QLS) studies indicate that lipid vesicles increase in size upon addition of peptide. Previous solution (aqueous and DMSO) studies from this laboratory indicate that α-factor is highly flexible with only one long-lived identifiable structural feature, a type II β-turn spanning the central portion of the peptide. Two-dimensional (2D) ^{1}H nuclear Overhauser effect spectroscopy (NOESY) studies demonstrate a marked ordering of the peptide upon interaction with lipid, suggesting a compact N-terminus, in addition to a stabilized β-turn. In contrast to our results in both solution and lipid environment, Wakamatsu et al. [Wakamatsu, K., Okada, A., Suzuki, M., Higashijima, T., Masui, Y., Sakakibara, S., & Miyazawa, T. (1986) *Eur. J. Biochem. 154*, 607–615] proposed a lipid environment conformation, on the basis of one-dimensional transferred NOE studies in D_2O, which does not include the β-turn.

The α-factor is a 13 amino acid peptide (NH_2-Trp^1-His^2-Trp^3-Leu^4-Gln^5-Leu^6-Lys^7-Pro^8-Gly^9-Gln^{10}-Pro^{11}-Met^{12}-Tyr^{13}-COOH) secreted by α-mating type cells of the yeast *Saccharomyces cerevisiae*. It interacts with a receptor on α-mating type cells and elicits hormone-like responses necessary for the mating of the two haploid cell types (*a* and α) (Thorner, 1980).

A variety of physicochemical studies have been performed to determine α-factor conformation in the solution and lipid-bound states and to relate these structures to biological activity (Higashijima et al., 1983, 1984; Jelicks et al., 1988; Masui et al., 1977; Wakamatsu et al., 1986, 1987). Previously reported NMR[1] studies from this laboratory have shown that the solution structure of active peptides contains a type II β-turn spanning residues 7–10 (Jelicks et al., 1988). This structural feature does not appear in any inactive peptide studied. Other than this β-turn, we found no evidence for any long-lived ordered conformation in either aqueous solution or organic solvent (dimethyl sulfoxide). Our conclusions differ from those of Higashijima et al. (1984), who reported that the peptide assumes a defined structure in solution, including β-turns and a helical N-terminus.

One goal of our studies is to elucidate the structure of the pheromone during interaction with the membrane-bound receptor. As a step in this direction, we began an investigation into the influence of phospholipids on the conformation of α-factor. Circular dichroism and fluorescence measurements have indicated conformational changes of the pheromone upon interaction with lipids, relative to aqueous solution, and these changes appear to be more significant for active peptides than for those that are inactive (Higashijima et al., 1983).

Phosphorus-31 (^{31}P) NMR is sensitive to membrane structure and dynamics (Cullis & de Kruijff, 1979; Cullis & Hope, 1978; Smith & Ekiel, 1984). In addition, quasielastic light scattering (QLS) can be useful in the physical characterization (size and polydispersity) of phospholipid aggregates (Prendergast et al., 1982). Two-dimensional (2D) ^{1}H nuclear magnetic resonance spectroscopy, especially nuclear Overhauser effect spectroscopy (NOESY), has proven to be a very powerful technique for deducing peptide/protein conformation (Leach et al., 1977; Wüthrich, 1984; Zuiderweg et al., 1983). The nuclear Overhauser effect (NOE) connectivities expected for α-helices, 3_{10} helices, β-turns, and β-sheets have been studied in detail, and they are frequently used to identify these structures (Wüthrich et al., 1984). In this paper we report on the use of such techniques to provide information on the structure of α-factor bound to phospholipid vesicles and to study the interaction of the peptide with the headgroup of the lipids.

Materials and Methods

α-Factor was synthesized as previously described (Tallon et al., 1987). L-α-Dipalmitoylphosphatidylcholine (DPPC),

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[1] Abbreviations: DMSO, dimethyl sulfoxide; DOPC, L-α-dioleoylphosphatidylcholine; DOPE, L-α-dioleoylphosphatidylethanolamine; DPPA, L-α-dipalmitoylphosphatidic acid; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PI, soybean phosphatidylinositol; PS, bovine brain phosphatidylserine; QLS, quasielastic light scattering; ROESY, rotating frame nuclear Overhauser effect spectroscopy; T_c, lipid phase transition temperature; 2D, two-dimensional.

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L-α-dioleoylphosphatidylcholine (DOPC), L-α-distearoylphosphatidylcholine (DSPC), L-α-dioleoylphosphatidylethanolamine (DOPE), and L-α-dipalmitoylphosphatidic acid (DPPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Soybean phosphatidylinositol (PI) and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL).

Phospholipids were dissolved in chloroform and stored as stock solutions below 0 °C. Phospholipids were prepared for sonication by evaporating chloroform under N_2 gas from the aliquots of stock solution and desiccating under vacuum overnight. The lipid films thus formed were rehydrated with 5 mM sodium acetate buffer (warmed to 10–15 °C above T_c, the lipid phase transition temperature). Vortexing with glass beads for approximately 1 min aided in dispersal of the lipids. The samples were transferred to polycarbonate vials, and vesicles were formed by low-power sonication with a Heat Systems W-380 sonicator equipped with a cup horn, following the procedure described by Barrow and Lentz (1980). Bath temperatures were maintained at 10–15 °C above the transition temperatures, and typically 15 min–1 h of sonication was required for optical clarity to be reached. Other researchers have used similar sonication procedures and have found no evidence of significant degradation of the lipid (Berden et al., 1975; Stockton et al., 1976). Furthermore, we observed no evidence of degradation products in the ^1H NMR spectra of the pure lipid vesicles. Sonication was performed in the absence of peptide, thus eliminating chemical effects due to peptide–lipid interaction. In the one case when vesicles (DSPC) were sonicated in the presence and absence of peptide no difference in spectral parameters was noted. After sonication, samples were centrifuged at 13 000 rpm and were stored at room temperature for no more than 1 week. Vesicle diameters and size dispersion were determined by QLS measurements obtained with a Lexel Model 65 ion laser and Langley Ford Model 1096 correlator. NMR samples were diluted 1/100 for the QLS studies.

All NMR experiments were performed on the JEOL GX-400 NMR spectrometer of the CUNY NMR Facility (located at Hunter College), which operates at a ^1H resonance frequency of 400 MHz. ^{31}P spectra were obtained at 161.8 MHz with broadband Waltz decoupling of protons. ^{31}P chemical shifts are reported relative to 85% H_3PO_4 at 0.0 ppm. ^1H shifts are reported relative to TSP [3-(trimethylsilyl)propionate, Na^+ salt] at 0.0 ppm.

NOESY spectra were acquired with the $(90°-t_1-90°-\tau_m-90°-t_2-\text{acquire})_n$ pulse sequence using a 400-ms mixing time, τ_m. Pure absorption spectra were obtained by the method of States et al. (1982) and were processed by using the FTNMR software package (Hare Research). 80° shifted sine squared bell multiplication was applied in both dimensions of the 2D data sets.

Samples were 16 mM lipid:1 mM peptide for the ^{31}P spectra. Various lipid–peptide ratios were used in the ^1H experiments as indicated in the text. Peptide ^1H resonances had been assigned previously (Jelicks et al., 1988); assignments in the presence of lipids were confirmed via COSY (correlated spectroscopy) spectra. All ^{31}P spectra were obtained on samples at pH 7; NOESY spectra were acquired for samples at both pH 4.6 and pH 7 and gave identical results (aqueous solution studies previously reported were performed on pH 4.6 samples). Peptide–lipid samples were prepared by adding peptide to the sonicated lipid solution (sonicated lipid to peptide for binding studies) and allowing the mixture to equilibrate 10–15 °C above T_c for at least 15 min. Samples were equilibrated in the NMR spectrometer for at least 15 min at a given temperature before acquisition of spectra; specific temperatures are given in the figure legends.

Table I: QLS Data for the Pure Lipid and Lipid plus α-Factor at 23 °C[a]

lipid sample	diameter (nm)	polydispersity index (V) (%)
DPPC	108	27
+α-factor	510	42
DSPC	108	12
+α-factor	1360	47
DPPA	110	76
+α-factor	350	114
brain PS	113	60
DOPC	47	51
+α-factor	51	39
DOPE	61	30
+α-factor	305	24

[a] The values reported represent an average for at least two measurements on each sample.

Results

The interactions of α-factor with a variety of neutral (DPPC, DOPE, DSPC) and anionic (PI, PS, DPPA) lipids were investigated. These lipids are all present, to varying extent, in the *S. cerevisiae* plasma membrane (Henry, 1982). In the studies reported herein, α-factor was added to preformed vesicles of a given lipid at temperatures above the reported lipid phase transition temperature, T_c. It should be noted that "phase" refers to the fluidity of the hydrocarbon chains, i.e., the more rigid gel phase or the more mobile liquid-crystalline phase, and not to the lipid packing arrangements (e.g., bilayer, hexagonal_{II}). When interaction with the gel phase was measured, the sample was cooled to the appropriate temperature. Sonicated phospholipid vesicles were chosen as the model membrane system since their small size and, therefore, shorter tumbling times enable the observation of well-resolved ^1H spectra. Because there is a high degree of curvature in the vesicle bilayer, some investigators have questioned whether the packing of the phospholipid molecules in a vesicle is similar to that found in the biological membrane (Sheetz & Chan, 1972; Chan et al., 1973). De Kruijff et al. (1975) measured T_c for dispersions and sonicated vesicles. T_c is highly sensitive and should change appreciably when the packing of the phospholipid molecule is changed. De Kruijff et al. observed no significant change in T_c upon sonication and have suggested that the packing in sonicated vesicles is no more disordered than in large lamellar structures. We have measured T_c for a DPPC dispersion and sonicated DPPC vesicles by DSC (Naider et al., 1989) and have confirmed that the T_c of both samples was 41 °C, as expected (Papahadjopoulos et al., 1975). The appearances of the ^1H and ^{31}P resonances are all consistent with the lipid phase expected at the given experimental temperature. Thus, we conclude that sonicated phospholipid vesicles are a reasonable model for biological membranes.

Effects of α-Factor on Lipid. QLS measurements were performed on the vesicle preparations both in the absence and in the presence of α-factor and are reported in Table I. Most vesicle preparations consisted of vesicles ~100 nm in diameter, although the unsaturated lipids, DOPC, and DOPE, formed smaller vesicles (~50 nm). Upon addition of α-factor all vesicles increased in size, although DOPC vesicle size increased only by a very small amount. The polydispersity, which is a measure of the uniformity of the vesicle size distribution, increased upon addition of α-factor for the saturated lipids and decreased for the unsaturated lipids.

Table II: ^{31}P NMR Chemical Shifts and Line Widths[a]

sample	temp[b] (°C)	δ	line width (Hz) without α-factor added	δ	line width (Hz) with α-factor added	Δδ	% increase in line width
DPPC	25	−0.31	175	−0.39	225	−0.08	29
	41	−0.24	85	−0.29	150	−0.04	88
	50	−0.17	65	−0.19	130	−0.02	100
DSPC	25	−0.39	175	−0.58	220	−0.19	26
	58	−0.09	65	−0.19	150	−0.10	131
	65	−0.07	50	−0.15	130	−0.08	160
DPPA	25	1.08	55	1.30	115	+0.22	109
	67	1.24	20	1.39	36	+0.15	80
	75	1.30	18	1.45	25	+0.15	19
DOPC	24	−0.87	120	−0.95	190	−0.07	58
PI	24	−0.44	20	−0.48	23	−0.04	15
DOPE	24	−0.13	85	c	c	c	c

[a] Reported line widths are accurate to ±5–10 Hz; chemical shifts are accurate to ±0.03–0.06 ppm. [b] Where three temperatures are reported, the first is lipid in the gel state, the second is near T_c, and the third is in the liquid-crystalline state. When only one temperature is reported, it is the liquid-cyrstalline state. [c] The DOPE spectrum was broadened to base line upon addition of α-factor; therefore, these parameters could not be determined.

^{31}P measurements were made on several of the phospholipid vesicle preparations in the presence and absence of α-factor (Table II). As would be expected with increased vesicle size and hence decreased overall rotational diffusion constant, the lipid ^{31}P spectra were broadened upon addition of peptide at all temperatures studied. This line-broadening effect was most pronounced in the DOPE spectrum, which was broadened beyond detectability when α-factor was added (increasing the spectral width did not aid in the observation of this resonance); the greatest observable percent increase in line width was observed for DSPC near T_c and in the liquid-crystalline state. In the neutral lipids DPPC (Figure 1 and Table II) and DSPC, there was greater broadening in the liquid-crystalline phase relative to the gel phase, while for the negatively charged DPPA broadening was much greater in the gel phase than in the liquid-crystalline phase (Table II). The effect on the spectrum of soybean PI (also negatively charged) was quite small (3 Hz) and within experimental error. In the spectra of DPPC (Figure 1), of DSPC at 25 °C, and of DPPA at 75 °C (data not shown), a second ^{31}P peak appeared upon addition of α-factor. In DPPC it was a very small, downfield shifted (~1 ppm) peak; in DSPC the new peak was more intense than in DPPC and was shifted downfield by ~2.5 ppm. At 75 °C the spectrum of negatively charged DPPA with peptide had a small, distinct, broad peak shifted upfield of the primary resonance by ~0.3 ppm.

α-Factor Binding to Phospholipids. Addition of lipid to α-factor led to a broadening and upfield shifting of most peptide ^{1}H resonances. The effects of lipid on peptide resonances are illustrated with the DPPC–peptide spectra in Figure 2. The onset of resonance broadening and disappearance of multiplet structure was observed at DPPC–peptide ratios of 1:1. Note that the lipid resonances are not observable at 25 °C until a very high concentration has been reached. This phenomenon has also been observed by Sheetz and Chan (1972). They found that ^{1}H resonances of unilamellar vesicles (~90 nm) were unobservable for gel-phase lipids. Broad ^{1}H resonances for the DPPC $N(CH_3)_3^+$ and $(CH_2)_n$ did appear as the temperature was increased to T_c. The ^{1}H resonances of smaller vesicles (30 nm) were observed in the gel phase. We observed only one peak for each peptide resonance, indicating fast exchange between free and bound peptide, and for each given lipid–peptide ratio, increase in temperature resulted in the sharpening of the peptide resonances (Figure 3). Similar behavior was observed with addition of the other lipids to α-factor. In contrast, Wakamatsu et al. (1987) reported that proton resonances of α-factor were broadened near

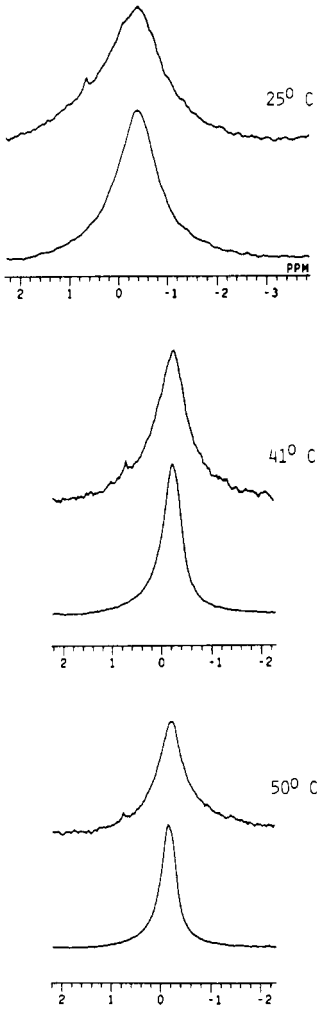

FIGURE 1: ^{31}P NMR spectra of DPPC vesicles with (top spectrum) and without (bottom spectrum) α-factor added. At 25 °C pure DPPC is gel phase, at 41 °C it undergoes the transition to the liquid-crystalline phase, and at 50 °C it is liquid crystalline.

T_c (DPPC) relative to resonances in either gel or liquid-crystalline environments.

Apparent affinity constants, K_a, were determined as described by Deber and Benham (1985) by using

$$K_a = M_f/[(1 - M_f)(L - M_f P_0)]$$

$$M_f = (\delta_{obs} - \delta_{free})/(\delta_{bound} - \delta_{free})$$

where M_f is the mole fraction of bound peptide, L is the molar

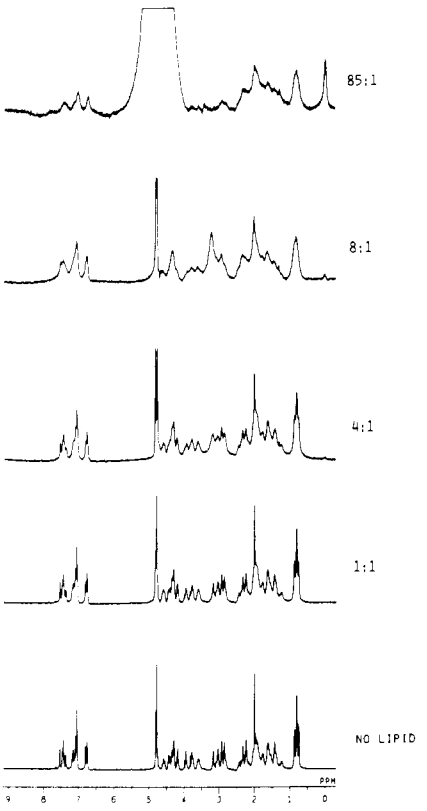

FIGURE 2: ^{1}H NMR spectra of α-factor with varying amounts of DPPC vesicles added (25 °C, D_2O, pH 7). Initial α-factor concentration was 4 mM; lipid was added in small, concentrated aliquots.

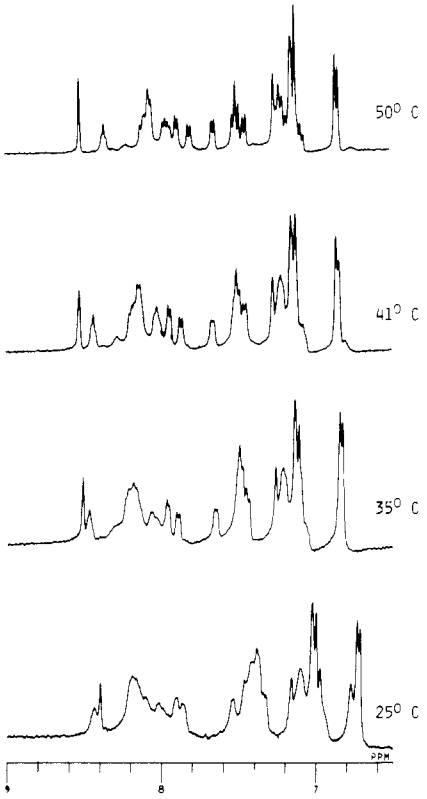

FIGURE 3: Expansions of the ^{1}H NMR spectra of α-factor in DPPC vesicles at specified temperatures.

concentration of lipid, P_0 is the initial molar concentration of peptide, δ_{obs} is the observed chemical shift, δ_{free} is the chemical shift of free peptide, and δ_{bound} is the chemical shift of bound peptide. The peptide was assumed to be fully bound when the resonance position did not change upon addition of more

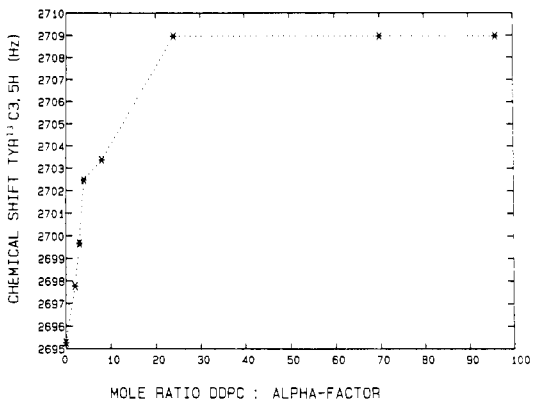

FIGURE 4: Tyr^{13} C3,5H resonance position (hertz) versus DOPC–α-factor ratio; initial peptide concentration was 4 mM.

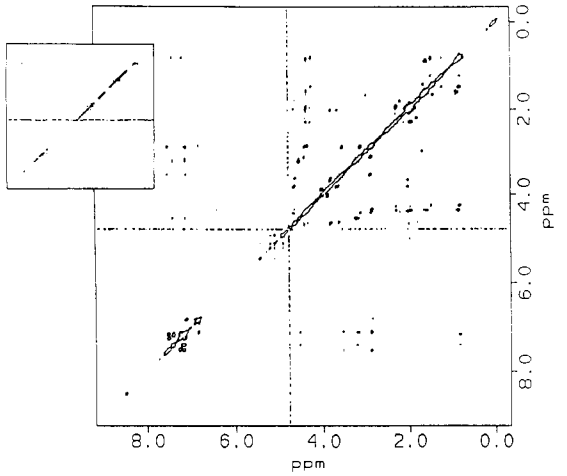

FIGURE 5: 400-ms NOESY spectrum of 4:1 DPPC–α-factor mixture (25 °C, D_2O, pH 4.6); insert is analogous spectrum of α-factor in D_2O (25 °C, pH 4.6) (Jelicks et al., 1988).

Table III: Affinity Constants Determined from Proton NMR Binding Studies at 25 °C[a]

sample	resonance	K_a (×10 M^{-1})
PS[b]	Tyr^{13}C3,5H	23.0
	Trp^{1,3}C2H/C5H[d]	25.0
DPPC[c]	Tyr^{13}C3,5H	4.8
	Trp^{1,3}C2H/C5H[d]	4.6
	Leu$^{4,6}\delta$ CH_3[d]	4.8
DOPC[b]	Tyr^{13}C3,5H	5.3
	Trp^{1,3}C2H/C5H[d]	2.5
DSPC[c]	Tyr^{13}C3,5H	5.1
	Trp^{1,3}C2H/C5H[d]	4.4
	Leu$^{4,6}\delta$ CH_3[d]	13.1

[a] All affinity constants were determined as described in the text. [b] Liquid-crystalline phase. [c] Gel phase. [d] Overlapping resonances.

α-factor (illustrated with DOPC vesicles in Figure 4). The affinity constants thus determined are reported in Table III. Only those resonances that could be followed in more than one type of lipid are reported therein.

Conformation of α-Factor Bound to Lipid Vesicles. α-Factor conformation in the presence of lipid was investigated via NOESY spectra. Figure 5 shows the 400-ms D_2O NOESY spectrum of α-factor in the presence of gel-phase DPPC vesicles. The comparable NOESY spectrum of α-factor in aqueous solution was virtually void of crosspeaks (Figure 5, insert) due to a nulling of the NOE because of the correlation time dependence (NOE approaches zero at $\omega_0\tau_c \sim 1$) and rapid motion (Jelicks et al., 1988). The lipid to peptide ratio in Figure 5 was 4:1. Under these conditions the peptide was exchanging between bound and free states. This sample

Table IV: Interresidue NOEs (i to j) for α-Factor Bound to DPPC Vesicles[a]

residue i	proton i	residue j	proton j	helix type and distance[b] (nm)
1	NH	4	δ	
1	NH	5	γ	
1	NH	3	NH	
1	NH	3	C4	
1	NH	4	NH	
1	β	2	NH	α, 0.25; 3_{10}, 0.29
1	C4	3	α	
1	C4	4	δ	
2	α	3	NH	α, 0.35; 3_{10}, 0.34
2	β	4	δ	
2	C2	5	γ	
3	NH	4	δ	
3	NH	5	γ	
3	β	4	δ	
3	β	5	NH	>0.45 ordered str
3	β	6	δ	>0.45 ordered str
3	α	7	NH	α, 0.42
3	β	7	NH	α, 0.50
4	NH	5	α	>4.5 ordered str
4	α	5	NH	α, 0.35; 3_{10}, 0.34
4	δ	5	α	
4	δ	5	γ	
4	NH	7	NH	
4	α	7	NH	α, 0.34; 3_{10}, 0.33
4	δ	lipid		
5	β	6	NH	α, 0.25; 3_{10}, 0.29
5	γ	6	NH	
5	γ	6	δ	
5	γ	7	NH	
6	NH	7	β	α, 0.45; 3_{10}, 0.43
6	δ	lipid		
7	NH	8	β	α, 0.45; 3_{10}, 0.43
7	CH	8	δ	
8	α	10	NH	β-turn 0.33
8	α	9	NH	β-turn 0.22
8	β	9	NH	β-turn 0.36–0.44
9	NH	10	NH	β-turn 0.24
9	α	10	NH	β-turn 0.32
10	NH	11	β	α, 0.45; 3_{10}, 0.43
10	α	11	δ	

[a] Conditions as in Figure 6. [b] Wüthrich et al. (1984).

condition was chosen because fully bound peptide resonances were too broad to resolve in the 2D spectrum. Likewise, the lipid–lipid crosspeaks at higher lipid concentration overwhelmed the peptide–peptide crosspeaks. Connectivities appearing in the presence of DPPC must be due to a transfer of NOE from bound to free peptide and must represent the conformation of the peptide in the lipid-bound state (Clore & Gronenborn, 1982). Thus, NOESY spectra of α-factor interacting with DPPC vesicles were acquired to obtain detailed structural information, via transferred NOE, of the lipid-bound peptide. Data were acquired in H_2O–D_2O (9:1) so that exchangeable proton resonances could be observed. Table IV lists the interresidue α-factor NOEs observed in the presence of DPPC; observed intraresidue NOEs that arise from close contacts in the primary sequence are not reported. Figure 6 is a one-dimensional slice through the Gly^9 amide proton resonance from the NOESY spectrum of α-factor and DPPC in H_2O–D_2O. NOEs between this proton and the Pro^8 α-proton, Pro^8 β-protons, and the Gln^{10} amide proton are expected for a type II β-turn. The significance of this structure and the NOEs listed in Table IV are discussed below.

NOESY spectra were also acquired for α-factor in the presence of several lipids in addition to DPPC (DOPC, DOPE, PS, and DSPC) at 25 °C. Since these spectra were acquired in D_2O, rather than H_2O, some potentially significant NOEs were transparent. The interresidue NOEs involving nonexchangeable protons are listed in Table V; intraresidue NOEs similar to those observed in the presence of DPPC were also seen. At 25 °C, PS, DOPC, and DOPE are in the liquid-crystalline state and DSPC is in the gel state. Many of the side chain–side chain crosspeaks observed upon interaction with DPPC were also observed upon interaction with PS and DSPC, although those in the presence of PS were much less intense. The lipid resonances in the DOPC sample were so intense that very few peptide resonances were observed, and in DOPE no crosspeaks were seen (in this sample the lipid resonances do not overwhelm the peptide resonances—the diagonal is clearly observed). A NOESY spectrum of α-factor in the presence of DPPC at 41 °C (near T_c of pure DPPC) was also acquired and like the PS sample contained crosspeaks similar, but with decreased intensity, to those observed with DPPC at 25 °C. Because many of the same conformationally significant crosspeaks appeared in the spectra of α-factor in the presence of lipids, regardless of whether the lipid was in the gel or liquid-crystalline phase or was near T_c, we believe the reduced crosspeak intensity and the absence of some crosspeaks in liquid-crystalline lipids are due to a decreased correlation time (more rapid motion) in this more fluid phase and not an indication of lack of structure. We have obtained similar results in aqueous solution as contrasted to DMSO solvent; more rapid

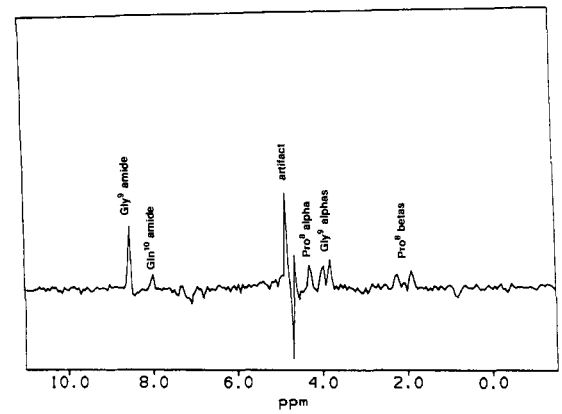

FIGURE 6: Slice through the Gly^9 NH diagonal peak of 4:1 DPPC–α-factor NOESY spectrum (25 °C, H_2O–D_2O 9:1, pH 4.6), showing NOE connectivities indicating type II β-turn. Artifact is due to the irradiation of water.

Table V: Interresidue NOEs (i to j) for α-Factor Bound to Various Phospholipids

lipid	residue i	proton i	residue j	proton j
DPPC, 41 °C	1	β	3	CH4
	1	α	3	C7H
	2	α	3	C7H
DSPC	1	α	3	C4H
	1	C4H	3	α
	1	C4H	4	δ
	2	C2H	5	γ
	2	β	4	δ
	3	β	6	δ
	4	δ	5	α
	4	δ	5	γ
	4	δ	lipid	
	5	γ	6	δ
	6	δ	7	ε
	6	δ	lipid	
	7	α	8	δ
	10	α	11	δ
DOPC[a]	1	C7	3	C4
PS	3	C5	4	δ
	4	γ	6	γ
	7	α	8	δ
	10	α	11	δ

[a] Spectrum overwhelmed by lipid.

correlation times led to a dearth of crosspeaks in aqueous solution NOESY spectra, whereas the rotating frame ROESY spectra, which have a different dependece on τ_c and are more sensitive to faster motions, contained significant crosspeaks, indicating the β-turn structure (Jelicks et al., 1988).

Discussion

Effect of α-Factor on Lipid. Examination of ^{31}P spectra for various lipids with or without α-factor reveals that the pheromone affects both the line width and the ^{31}P chemical shift. As expected, the line widths of pure lipids are greatest below T_c (hydrocarbon chains in gel phase) and decrease significantly as the lipid phase becomes more fluid (Table II). For all lipids examined, the ^{31}P line widths are greater for lipid plus α-factor than for the lipid alone. The line width of a ^{31}P resonance of a phospholipid is a function of the overall mobility of the lipid headgroup and the heterogeneity of the environments of the phosphorus nuclei (Cullis & de Kruijff, 1979; Gabriel & Roberts, 1986; Smith & Ekiel, 1984; Thayer & Kohler, 1981). The QLS measurements (Table I) show that α-factor causes significant increases in the sizes of the phospholipid vesicles, which would lead to an increase in overall τ_c and hence to ^{31}P resonance broadening (in the absence of rapid local motions). Experiments with $PrCl_3$ and DPPC vesicles show that α-factor interacts with the outer leaflet of the phospholipid vesicle but not with the inner leaflet phospholipid (Naider et al., 1989). The resulting heterogeneity of ^{31}P chemical shifts may add to the line width. Finally, additional line broadening could result from chemical shift anisotropy.

The ^{31}P resonances of all neutral lipids shift upfield (0.02–0.19 ppm) upon addition of peptide, indicating some change in magnetic environment. The upfield shifts in the ^{31}P spectra observed in the presence of α-factor are similar to shifts observed by others upon addition of drug molecules to liquid-crystalline lipids (Fung et al., 1979; Kuroda & Fujiwara, 1987; Srivastava et al., 1984). Such shifts are thought to indicate either electrostatic interaction or binding to the phosphate headgroups (Fung et al., 1979). The small additional phosphorus peak found in the spectrum of certain phospholipid–α-factor samples was only observed for those systems wherein addition of the pheromone resulted in a significant increase in polydispersity (DPPC, DSPC, DPPA; Table I). Such small isotropic peaks have been observed by others upon interaction of egg phosphatidylethanolamine with methemoglobin (Deslauriers et al., 1986).

To summarize, QLS and ^{31}P investigations support an interaction between α-factor and lipid vesicles. These phosphorus results are clearly preliminary. The effect of the peptide on lipid structure is quite complex, and further studies are currently under way to better characterize the details of this interaction. Additional information concerning the pheromone and phospholipid interactions comes from the ^1H NMR studies.

^1H NMR Studies: Effect of Lipid on α-Factor. Affinity constants were calculated from data obtained on resonances at different positions along the peptide chain. Examination of Table III shows that the apparent affinity of α-factor for negatively charged PS vesicles is significantly greater than that observed for the neutral lipids. In their studies of α-factor binding to mixed DPPC–DLPS (dilauroylphosphatidylserine) vesicles, Wakamatsu et al. (1987) also found enhanced binding when the negatively charged headgroup was present. This strong binding of α-factor to PS is consistent with the extensive broadening of peptide resonances observed even at low peptide to PS ratios. The K_a's determined for α-factor in the presence of PS and the phosphatidylcholines (DPPC, DSPC, and DOPC) are similar to those reported for enkephalin–lipid binding. Deber and Benham (1985) observed a greater affinity of enkephalin for PS than for PC and have attributed this enhanced binding to an electrostatic interaction of enkephalin with the charged PS headgroup in addition to the hydrophobic interaction with the membrane.

Our 2D NOE studies (NOESY and ROESY) on α-factor in aqueous and organic solvents indicate that the peptide is quite flexible but that the central portion of the molecule (residues 7–10) forms a type II β-turn (Jelicks et al., 1988). On the basis of our studies of biological activity of substituted peptides and on conformational studies of these peptide in solution, all results to date support the importance of this structural feature for biological activity.

There are several NOEs that identify the presence of a β-turn for residues 7–10 of α-factor in the presence of DPPC (Table IV). Of these, the Pro^8 α-CH–Gln^9 NH NOE observed in solution and in the presence of lipid is particularly diagnostic of the type II β-turn. This NOE and that between the Gly^9 NH and the Gly^9 α-CH are very intense in the lipid–peptide system. The NOEs for Gly^9 NH–Gln^{10} NH, Pro^8 α-CH–Gln^{10} NH, and Gly^9 α-CH–Gln^{10} NH were not observed in aqueous solution and were observed in DMSO with weak intensity. The NOE between the Pro^8 β-protons and the Gly^9 NH was not observed in any solution spectra. These results clearly demonstrate that the type II β-turn observed in solution is both present and much more stable when α-factor interacts with lipid.

We observed very few NOEs involving the C-terminal residues other than those between scalar-coupled nuclei (intraresidue interactions). The only other NOEs observed involving these residues are those between the Gln^{10} α-CH and Pro^{11} δ-protons (diagnostic of a trans X–Pro peptide bond) and between the Gln^{10} NH and the Pro^{11} β-protons. This NH_i–β_{i+1} NOE cannot be correlated with any specific structural feature. Other than this apparent folding of the Pro^{11} side chain toward the Gln^{10} amide proton, the C-terminus does not appear to assume any specific ordered structure.

Several interresidue contacts are found in the NOESY spectrum for the N-terminal region of the peptide. In particular, NOEs between the side chains of residues 1 or 3 (Trp) and the side chains of residues 4 or 6 (Leu) and between residues 2 (His) and 5 (Gln) are observed. A number of NH–NH, NH–α-CH, and NH–β-CH interactions are also observed for the N-terminus (Table IV). The interactions that are known to occur in regular α-helices or 3_{10}-helices, and distances expected for such interactions, are indicated in the table. Interresidue contacts between Leu^6 and Lys^7 and between Lys^7 and Pro^8 are also seen. These NOEs suggest a compact conformation for the N-terminus of α-factor bound to DPPC; however, these data do not provide strong evidence for any regular helical structure. Similarly, the CD spectral patterns for α-factor in the presence of lipid were not those expected for a peptide in an α-helical conformation (Higashijima et al., 1983; Naider et al., 1989).

Table V lists the interresidue NOEs observed in the presence of other lipids; those in the presence of DSPC are very similar to those observed in the presence of DPPC at 25 °C, indicating interaction between the side chains of residues 1, 3, 4, and 6. Because the exchangeable protons are not observed in D_2O, those NOEs indicative of the β-turn do not appear. In the presence of DPPC at 41 °C, very few interresidue NOEs are seen, indicating that either peptide structure is lost at the elevated temperature or that while the conformation of the

peptide is similar at both temperatures, at the higher temperature motion is more rapid (shorter correlation times), leading to decreased NOE intensity. Further support for rapid motions leading to decreased NOE intensity while the peptide remains structured is provided by the results in other lipids. In the presence of DOPE, which is liquid crystalline at 25 °C, the spectrum is very similar to that obtained for the α-factor in D_2O. This suggests either that the correlation time upon interaction with DOPE vesicles is similar to that in solution or that peptide is binding very weakly or not at all to this lipid. However, the ^{31}P spectrum of DOPE is broadened beyond detectability upon addition of α-factor to the lipid, indicating interaction. The NOEs observed in the presence of PS vesicles (Table V) are also of weak intensity, whereas peptide binding to this lipid is stronger than to the other lipids (see Table III and vide infra), further suggesting that the correlation time in the liquid-crystalline lipids is such that NOEs are very small. The fact that the crosspeaks in the NOESY spectra are of positive intensity (same sign as diagonal) and decrease with temperature supports this idea. We observed a similar situation in our solution studies. Crosspeaks (of positive intensity) appeared in NOESY spectra of samples in DMSO but not in samples in H_2O–D_2O. ROESY spectra revealed that the same structure was present in both solvents, although the lifetimes were different (Jelicks et al., 1988).

The picture that emerges from our NOE data and binding studies bears some resemblance to that proposed by Wakamatsu et al. (1986), although there are notable differences. Wakamatsu et al. proposed a membrane-bound conformation for α-factor that includes a possible 3_{10}-helix at the N-terminus (residues 1–5), which inserts into the bilayer, an extended structure for residues 6–9 (in contradiction to our results), and extension of the C-terminal residues, 10–13, into the aqueous environment. In a more recent study Wakamatsu et al. (1987) propose that the Trp and Leu side chains are oriented toward the hydrophobic portion of the bilayer, while the terminal NH_3^+, His^2 ring, and Gln^5 side chain are oriented toward the hydrophilic portion. This amphiphilic behavior for the N-terminus is consistent with our NOE results (vide supra). Since the His^2 and Gln^5 side chains are too far apart in a helical structure to exhibit NOEs, our results suggest some structural flexibility for the N-terminus even when bound to lipid. In contrast to Wakamatsu et al., however, we have definitive evidence for a β-turn spanning residues 7–10. One explanation for the discrepancies between our structure and that of Wakamatsu et al. is that two-dimensional NOE experiments are intrinsically all inclusive, whereas one-dimensional NOE studies require specific irradiation of selected resonances. It is possible, therefore, that an interaction found by using NOESY spectroscopy could be overlooked in a one-dimensional NOE analysis. Furthermore, the one-dimensional transferred NOE studies of Wakamatsu et al. were performed in D_2O, and not H_2O. Thus, the important NOEs (involving the exchangeable amide protons) identifying the β-turn could not be observed. Without the NOEs determined in H_2O, only an incomplete structure can be proposed. Our results clearly demonstrate the importance of performing experiments in both D_2O and H_2O. The β-turn in the middle of the α-factor would allow the peptide chain to insert into the bilayer, to change orientation, and to lie along the vesicle surface. In this conformation the Tyr^{13} side chain could be adsorbed to the bilayer surface, which would explain the apparent similar binding affinities of the two peptide termini.

Conclusions. QLS and ^{31}P NMR results indicate that α-factor increases lipid vesicle size and may interact with the phospholipid headgroup. NOESY studies demonstrate the presence of a type II β-turn spanning residues 7–10 of α-factor both in solution and in the presence of lipid. This turn, which has been correlated with biological activity, is stabilized upon interaction with lipid, suggesting that it may be necessary for proper orientation of the peptide at the membrane-bound receptor. Binding and NOE studies in lipid suggest that a compact N-terminal structure with an amphiphilic arrangement of hydrophobic and hydrophilic side chains is induced by interaction of the peptide with the lipid.

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Quantitative Fluorescence Measurement of Chloride Transport Mechanisms in Phospholipid Vesicles†

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ABSTRACT: A quantitative fluorescence assay has been developed to measure Cl flux across liposomal membranes for use in chloride transporter reconstitution studies. A Cl-sensitive fluorophore [6-methoxy-*N*-(3-sulfopropyl)quinolinium; SPQ] was entrapped into phospholipid/cholesterol liposomes formed by bath sonication, high-pressure extrusion, and detergent dialysis. Liposomes containing entrapped SPQ were separated from external SPQ by passage down a Sephadex G25 column. There was <10% leakage of SPQ from liposomes in 8 h at 4 °C and in 2 h at 23 °C. Cl influx (J_{Cl} in millimolar per second or nanomoles per second per centimeter squared) was determined from the time course of SPQ fluorescence, measured by cuvette or stopped-flow fluorometry, in response to inward Cl gradients. In 90% phosphatidylcholine/10% cholesterol liposomes at 23 °C, J_{Cl} in response to a 50 mM inward Cl gradient was 0.06 ± 0.01 mM·s^{-1} (SD, n = 3) in the absence and 0.27 ± 0.02 mM·s^{-1} in the presence of a K/valinomycin voltage clamp (0 mV), showing that the basal Cl "leak" is conductive; J_{Cl} increased (1.7 ± 0.1)-fold in the presence of a 60-mV inside-positive diffusion potential. Accuracy of chloride influx rates determined by the SPQ method was confirmed by measurement of ^{36}Cl uptake. In liposomes voltage-clamped to 0 mV, J_{Cl} was linear with external [Cl] (0-100 mM), independent of pH gradients, and strongly dependent on temperature (activation energy 18 ± 1 kcal/mol, 12-42 °C) as predicted for channel-independent Cl diffusion. To test this method for measurement of rapid Cl transport rates, liposomes were reconstituted with the Cl/OH exchanger tributyltin. Tributyltin incorporation gave rapid, pH gradient driven Cl influx (J_{Cl} = 13 mM·s^{-1}, no pH gradient; 24 mM·s^{-1}, 1.5-unit pH gradient). These results establish a rapid and accurate method for measurement of Cl influx in liposomes suitable for reconstitution studies.

Purification and physical characterization of chloride transport proteins require a quantitative functional assay for chloride permeability across membranes reconstituted with candidate chloride transporters. The transport assay should ideally (a) have good time resolution, (b) require small quantities of transport protein, (c) be sufficiently rapid for the screening of multiple samples, and (d) be applicable for measurement of neutral and conductive chloride transport. For example, the half-time for chloride equilibration for a single continuously open, 50-pS chloride channel in a 0.2-μm-diameter liposome in 50 mM Cl would be ~10 ms in the absence of a potential gradient (Fong et al, 1988). Standard ^{36}Cl uptake methods (Karniski & Aronson, 1985) or chloride-sensitive electrode methods (Dubinski & Monti, 1986) are inadequate to measure this very rapid chloride transport, particularly if a limited amount of chloride transport protein is available.

We report here a simple fluorescence assay for chloride transport in liposomes based on the use of the entrapped chloride-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ). SPQ has peak excitation and emission wavelengths of 350 and 445 nm, respectively. SPQ fluorescence is quenched by chloride by a collisional mechanism with a response time for changes in chloride concentration of under 1 ms (Illsley & Verkman, 1987). SPQ fluorescence is not altered by pH, bicarbonate, sulfate, nitrate, phosphate, or cations. The method gives a continuous record of chloride

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