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Association of a pH-Sensitive Peptide with Membrane Vesicles: Role of Amino Acid Sequence†

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ABSTRACT: The solution properties and bilayer association of two synthetic 30 amino acid peptides, GALA and LAGA, have been investigated at pH 5 and 7.5. These peptides have the same amino acid composition and differ only in the positioning of glutamic acid and leucine residues which together compose 47% of each peptide. Both peptides undergo a similar coil to helix transition as the pH is lowered from 7.5 to 5.0. However, GALA forms an amphipathic α-helix whereas LAGA does not. As a result, GALA partitions into membranes to a greater extent than LAGA and can initiate leakage of vesicle contents and membrane fusion which LAGA cannot (Subbarao et al., 1987; Parente et al., 1988). Membrane association of the peptides has been studied in detail with large phosphatidylcholine vesicles. Direct binding measurements show a strong association of the peptide GALA to vesicles at pH 5 with an apparent K_a around 10^6. The single tryptophan residue in each peptide can be exploited to probe peptide motion and positioning within lipid bilayers. Anisotropy changes and the quenching of tryptophan fluorescence by brominated lipids in the presence of vesicles also indicate that GALA can interact with uncharged vesicles in a pH-dependent manner. By comparison to the peptide LAGA, the membrane association of GALA is shown to be due to the amphipathic nature of its α-helical conformation at pH 5.

Low molecular weight synthetic peptides have been employed to unravel the role of amino acid sequence in the function of membrane-interactive protein segments. These synthetic peptides are being used as simplified models of lipoproteins, membrane channels, or fusogenic proteins in an attempt to relate peptide conformation, orientation in the lipid bilayer, and supramolecular structure to their function (Spach et al., 1989; Lear et al., 1988).

One of our main objectives was to investigate the role of hydrophobicity and secondary structure, especially the amphipathic α-helix, in defining the interaction of proteins with membranes. We designed a peptide using predictive algorithms governing hydrophobicity (Kyte & Doolittle, 1982;

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Eisenberg, 1984), helicity (Chou & Fasman, 1974), and membrane interaction potential (Pownall, 1983). It was also clear to us that many active membrane segments of larger proteins are not water soluble when isolated, leading to difficulties in experimental manipulations. Therefore, a peptide that was water soluble at physiological pH and would partition into membranes in a controlled manner was desired. The resulting peptide, GALA, has 30 amino acids and a sequence of W-E-A-A-L-A-E-A-L-A-E-A-L-A-E-H-L-A-E-A-L-A-E-A-L-E-A-L-A-A. It undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5 (Subbarao et al., 1987). Deviations in the major repeat sequence of Glu-Ala-Leu-Ala were made to give better alignment of hydrophilic (Glu) and hydrophobic (Leu) residues on apposing helical faces. Glutamic acid residues were chosen due to their high tendency to be in helices and to lend pH specificity to the peptide (Urness & Doty, 1961). Protonation of the Glu residues was associated with a conformational change from a random coil to an α-helix. The structural characterization of GALA has been detailed previously (Subbarao et al., 1987) and shown in preliminary experiments to interact with membranes most when in a helix at pH 5. At this pH and at a low lipid/peptide ratio, GALA also induces fusion of small unilamellar vesicles composed of egg PC[1] (Parente et al., 1988). Short analogues with the amino acid composition (Glu-Ala-Leu-Ala)$_n$ (n = 1–4) did not have well-defined secondary structure or membrane lytic or fusion properties. In this paper we have investigated the interaction of GALA in the membrane specifically at pH 5 and 7.5. We have also made use of a modified peptide, LAGA, with identical amino acid composition with and similar structural properties to GALA to examine the importance of amino acid topology in membrane activity. In the accompanying paper (Parente et al., 1990) we have focused on the ability of GALA to induce membrane destabilization, investigated the mechanism responsible for this leakage activity, and proposed a model to explain our findings.

Materials and Methods

Materials. Egg phosphatidylcholine (egg PC) and 1,2-bis(9,10-dibromooleoyl)-*sn*-glycero-3-phosphocholine in chloroform (BrPC) (East & Lee, 1982) were purchased from Avanti Polar Lipids Inc. (Pelham, AL). 5,6-Dibromocholestan-3β-ol was prepared as described (Simmonds et al., 1982) with cholesterol obtained from Sigma (St. Louis, MO). Ficoll was purchased from Pharmacia (Piscataway, NJ).

Vesicle Preparation. Reverse-phase evaporation vesicles (REV) were prepared as described (Szoka & Papahadjopoulos, 1978). Vesicles were made at pH 7.5 in 5 mM TES and 100 mM KCl or in 12.5 mM ANTS, 45 mM DPX, 5 mM TES, and 20 mM KCl and extruded three times through 0.1-μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA) at 100 psi N_2 pressure. When appropriate, a Sephadex G-75 column (20 × 0.7 cm) was used to separate vesicles from unencapsulated material with 5 mM TES–100 mM KCl, pH 7.5, as the elution buffer. Lipid phosphorus was determined by a modification of the Bartlett (1959) method.

Peptides. GALA (3032 MW) was synthesized by the Biomolecular Resource Center (UCSF) with an automatic synthesizer and Merrifield resin. Purification was carried out by reverse-phase HPLC on a Waters Associates instrument equipped with a Dynamax-300A (12 μm) C_{18} semipreparative column. A linear gradient of acetonitrile and water containing 0.1% trifluoroacetic acid comprised the mobile phase. Details of the synthesis and purification have been given elsewhere (Subbarao et al., 1987). An analogous peptide, LAGA (3032 MW), composed of the same amino acids but with a sequence altered from that of GALA was also synthesized on a Merrifield resin. The sequence of this peptide is W-E-A-A-L-A-E-A-*E*-A-*L*-A-L-A-E-H-*E*-A-*L*-A-L-A-E-A-*E*-*L*-A-L-A-A. The amino acids underlined represent positions altered from those of the GALA sequence. A similar HPLC purification scheme using an acetonitrile/water gradient was employed. LAGA eluted at 51% acetonitrile and was stored at 0 °C as a lyophilized powder. Analysis of purified GALA and LAGA on a Vydac 218TP10415 (10 μm) C_{18} analytical column using a UV detector at 220 nm indicated that the final peptide fractions were greater than 99% pure. Conformation of purity was also obtained from the molecular ion peak of each peptide observed by liquid secondary ion mass spectrometry on a Kratos MS-50S double-focusing mass spectrometer equipped with pulsed acceleration detection and a Cs^+ ion source. Peptide concentrations were adjusted by use of a molar extinction coefficient for tryptophan of ϵ = 5570 M^{-1} at pH 7.5 and 280 nm.

Fluorescence. Measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source. Anisotropy measurements of GALA and LAGA in solution or in the presence of vesicles made use of the N-terminal tryptophan moiety of the peptides, which was irradiated at 280 nm and observed at 350 nm. The fluorometer was equipped with Glan–Thompson polarizers, and measurements were corrected for instrument response. For measurements in the presence of lipid the peptide concentration was kept constant at 1.7 μM.

Tryptophan Fluorescence and Quenching by Brominated Lipids. GALA or LAGA was irradiated at 280 nm (tryptophan excitation), and samples were scanned from 300 to 500 nm at 0.5-nm invervals. When the wavelength of maximum fluorescence intensity as a function of GALA concentration was studied, samples were serially diluted from 100 μM to 1.5 μM final concentration at pH 7.5. At pH 5, experiments were performed by sequential addition of aliquots of a 500 μM or 2 mM stock solution of GALA to buffer such that the final concentration of GALA was affected less than 3% at the highest concentration. For bromine quenching experiments, vesicles were made with 0, 5, 12, or 25 mol % BrPC or 5,6-dibromocholesterol. GALA or LAGA was added to vesicles in the appropriate buffer, and samples were scanned from 1 to 60 min after peptide addition. The percent quenching was determined at the tryptophan maximum from the difference between the intensity in the absence and that in the presence of the brominated lipid containing vesicles. The fluorescence intensity in the absence of brominated lipid containing vesicles was set to 0% quenching. Appropriate vesicle blanks were subtracted from each scan, and spectra were corrected for instrument response. Samples were prepared at 50/1 or 500/1 lipid/peptide mole ratios with a constant amount of peptide (1.7 μM).

Peptide Association with Vesicles. Discontinuous Ficoll gradients were used to separate vesicles from unbound peptide by flotation of vesicles according to a modification of the method by Heath (1987). Starting with the least dense layer, gradients were formed in 13 × 51 mm Ultraclear centrifuge

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; BrPC, 1,2-bis(9,10-dibromooleoyl)-*sn*-glycero-3-phosphocholine; CD, circular dichroism; DPX, *p*-xylylenebis[pyridinium bromide]; egg PC, egg phosphatidylcholine; HPLC, high-pressure liquid chromatography; REV, reverse-phase evaporation vesicles; TES, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid.

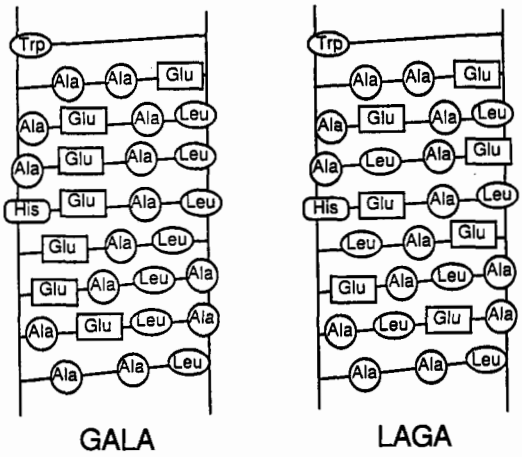

FIGURE 1: Helical grid representations of GALA and LAGA. Circular dichroism shows both peptides to be helical at pH 5. The difference between these two peptides is easily seen in this representation—Glu and Leu residues are aligned on opposite faces of the helix making GALA amphipathic, while these residues have a more uniform distribution in LAGA.

tubes (Beckman Instruments, Palo Alto, CA) by use of a peristaltic pump. In the resulting gradient, the bottom layer was composed of 1 mL of 10% Ficoll containing the lipid and/or peptide sample; the middle layer contained 3 mL of 5% Ficoll, and the top layer was 0.5 mL of the sample buffer. Gradients were centrifuged at 20 °C in a Beckman L3-50 ultracentrifuge for 30 min at 40 000 rpm in a SW 50.1 rotor. Lipid/peptide mixtures were incubated for 15–30 min prior to loading on the gradient. GALA and LAGA were iodinated at the His residue [via a modification of the chloramine-T method of Greenwood et al. (1963)] for use in these experiments. After centrifugation, 0.5-mL fractions were removed sequentially from the top of the gradient and counted in a Beckman 8000 γ-counter. Location of vesicle fractions was confirmed by phosphate analysis (Bartlett, 1959). An apparent partition coefficient was calculated from K_a = (dpm bound to lipid/dpm in solution) × [H_2O]/[lipid] (Pownall et al., 1984).

Results

Properties of Peptides in the Absence of Lipids. Structural characteristics of the peptide GALA from circular dichroism and Fourier transform infrared spectroscopy have been detailed previously (Subbarao et al., 1987; Goormaghtigh et al., personal communication). To evaluate the effect of placement of the amino acid residues in the sequence, vis-à-vis membrane interaction, we synthesized another 30 amino acid peptide. The amino acid composition was unaltered from that of the GALA sequence, but selected residues were transposed; Glu replaced Leu at positions 9, 17, and 25, and Leu replaced Glu at positions 11, 19, and 26. This results in a structure that when fit into a helical grid pattern (see Figure 1), has little amphipathic character. This peptide was designated LAGA to reflect the switched residues. By use of the normalized consensus hydrophobicity values of Eisenberg (1984) and a bilayer spanning window of 19 residues, the mean hydrophobic moment of GALA is 0.290 and the mean residue hydrophobicity is 0.337, whereas the mean hydrophobic moment of LAGA is 0.074 and the mean residue hydrophobicity is 0.389. The CD spectrum indicates LAGA to be a random coil at pH 7.5. The circular dichroism spectrum of LAGA displays a double-minimum pattern at pH 5 similar to that of GALA and indicative of an α-helix (data not shown). The θ_{222} value (at the trough of the characteristic helical pattern) for LAGA is

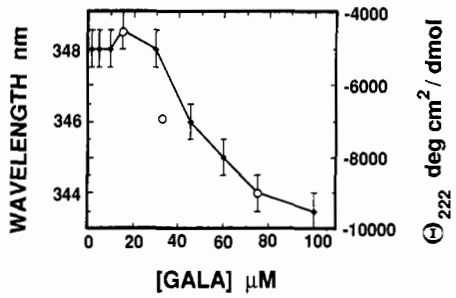

FIGURE 2: Tryptophan emission maxima (◆) and molar ellipticity at 222 nm (○) as a function of GALA concentration at pH 7.5. Errors in the emission maximum are ±0.5 nm. Both measurements indicate an altered state of self-association of GALA above 20 μM.

−9000 deg cm^2 dmol^{-1} compared to −13 600 deg cm^2 dmol^{-1} in 35 mM sodium acetate–100 mM KCl for GALA at pH 5 (Subbarao et al., 1987).

A tryptophan residue was included at the N-terminal of the peptides as a spectroscopic probe. Tryptophan is a sensitive indicator of the polarity of the peptide's environment responding with emission shifts, quantum yield changes, or polarization variations. The tryptophan fluorescence emission maximum of GALA[2] at pH 7.5 is 347 nm, which blue shifts to 335 nm at pH 5.0. When the pH was rapidly changed from 7.5 to 5.0, the change in fluorescence intensity monitored at 335 nm indicated that the tryptophan moiety and thus GALA changed its environment within the 2-s mixing time of the instrument (data not shown). The fluorescence intensity and emission wavelength changes that occur upon acidification are totally reversible upon return of the pH to 7.5. This result is in agreement with those from circular dichroism studies, which indicate that GALA can undergo a reversible helix–coil transition as the pH is cycled between 5.0 and 7.5 (Subbarao et al., 1987).

The wavelength at the tryptophan fluorescence maximum of GALA and the ellipticity values of GALA at 222 nm obtained from circular dichroism measurements are plotted as a function of GALA concentration (up to 100 μM) at pH 7.5 in Figure 2. Interestingly, there is a gradual blue shift in the tryptophan intensity maximum at concentrations greater than 20 μM at pH 7.5, while there is virtually no change at pH 5 over the same concentration range (λ_{max} = 335 nm; data not shown). The ellipticity values at pH 7.5 also increase with increasing peptide concentration, indicating formation of oligomers. The apparent half-maximal concentration for this change is 45 μM. Indeed, at millimolar concentrations of GALA oligomerization was apparent from gel permeation chromatography. The major peptide fraction eluted at approximately 20 000 daltons while only a small peak was observed at 3000 daltons (monomer) (Subbarao et al., 1987).

The tryptophan moiety of LAGA (positionally identical with that of GALA) also shows sensitivity to pH. The fluorescence maximum occurs at 347 nm at pH 7.5 and is blue shifted to 340 nm at pH 5.

The internal motions of GALA and LAGA in two pH environments were studied over a range of peptide concentrations by observing the anisotropy of the tryptophan moiety. The anisotropy of the peptides is plotted as a function of concentration at pH 5 (circles) and 7.5 (squares) in Figure 3. Over a similar concentration range, the anisotropy of GALA (0.019 ± 0.002) does not change up to 10 μM at pH 7.5. At 50 μM

[2] The wavelengths of maximal tryptophan fluorescence intensity reported in Subbarao et al. (1987) were not corrected for instrument response.

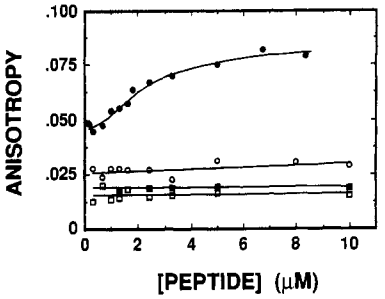

FIGURE 3: Anisotropy of GALA and LAGA as a function of peptide concentration at pH 5 (circles) and 7.5 (squares). Closed symbols are for GALA, and open symbols are for LAGA. Regression lines have been drawn through the data points for LAGA and GALA at pH 7.5. Each point represents the average of four to six measurements with errors ranging from ±0.002 to ±0.005.

the anisotropy increases to 0.142 ± 0.003, which is consistent with the data in Figure 2 and is indicative of formation of a water-soluble aggregate of GALA at pH 7.5 at this concentration. At each GALA concentration the anisotropy is greater at pH 5 as compared to pH 7.5. For instance, at pH 5, the anisotropy of 0.33 μM GALA (0.046 ± 0.005) is significantly greater than the anisotropy at pH 7.5 (0.019), and it undergoes a further increase as the GALA concentration is increased to 10 μM. In addition, at pH 5, increasing the peptide concentration results in a further increase in the anisotropy, which could reflect changes in the self-association of GALA at this pH.

The anisotropy of LAGA (0.016 ± 0.002) at pH 7.5 is similar to that of GALA (0.019 ± 0.002) at pH 7.5, and neither have a concentration dependence up to 10 μM. At pH 5, the anisotropy of LAGA (0.026 ± 0.003) is somewhat greater than that at pH 7.5 and is independent of concentration over the range measured in this study. LAGA does not exhibit a change in spectral properties indicative of self-association as GALA does at pH 5.

Peptide Association with Vesicles. The apparent association of GALA and LAGA with vesicles was measured at pH 5 and 7.5 by separation of ^{125}I-peptide–vesicle mixtures on discontinuous Ficoll gradients. The method of equilibrium dialysis was also attempted to determine an association constant of GALA for vesicles but was not satisfactory since GALA did not reach equilbrium. This was due to aggregates of GALA which formed at pH 5 and were unable to diffuse through the membrane. With the Ficoll gradient method, vesicles float to the top of the gradient while free peptide remains at the bottom. As seen in Figure 4A, when GALA was applied to the gradient and run at pH 5 or 7.5 in the absence of lipid, it remained in the bottom fractions as expected. When mixed in a 5000/1 lipid to peptide mole ratio with egg PC vesicles for 0.25 h at pH 5 before being applied to a gradient run at the same pH, GALA comigrated with the vesicles to the top fractions. The apparent distribution coefficient for this condition is 2.0×10^5 which can also be considered an apparent association constant. When incubated at pH 7.5 under similar conditions, GALA remained largely in the bottom fractions of the gradient while the vesicles had floated (apparent K_a = 1.8×10^3). If GALA was incubated at pH 5 for 0.5 h and then the pH was raised to 7.5 for 0.5 h before GALA was loaded on a gradient at pH 7.5, the result mimics the outcome at pH 7.5 (apparent $K_a = 3.2 \times 10^3$). When the top three fractions from the pH 5 gradient, which contained both lipid and GALA, were combined and reapplied to a new gradient run at pH 7.5, GALA resided in the botom fractions after centrifugation (apparent $K_a = 2.5 \times 10^3$). These results

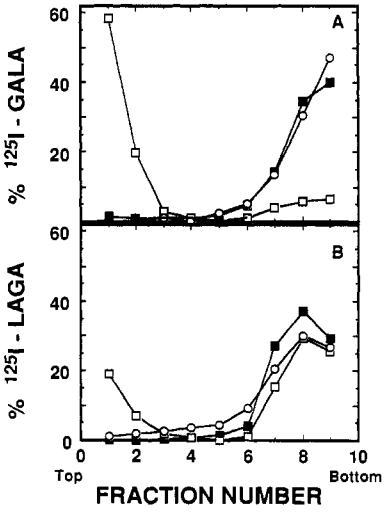

FIGURE 4: Elution profiles of GALA–lipid and LAGA–lipid mixtures on discontinuous Ficoll gradients. For these plots the lipid/peptide ratio was 5000/1 with 2 μM peptide in the initial incubation mixture. Phosphate analysis showed vesicles to be in the top three fractions after centrifugation. Data presented are from a representative experiment. (A) GALA in the absence of lipid (■) remains at the bottom of the gradient when run at pH 5 or 7.5. In the presence of lipid at pH 5 (□) GALA moves with vesicles to the top of the gradient while at pH 7.5 (○) GALA remains at the bottom. (B) LAGA in the absence of lipid at pH 5 (■) and in the presence of lipid at pH 7.5 (○) does not migrate. At pH 5 in the presence of lipid (□) a small fraction of LAGA is vesicle associated.

confirm the hypothesis that GALA is dissociated from vesicles at pH 7.5.

Keeping the peptide concentration constant at 2 μM in the initial incubation mixture, while lowering the lipid to peptide ratios, gave qualitatively similar results, showing association of GALA with vesicles at pH 5 but little interaction at pH 7.5. At pH 5 the distribution coefficient went from 2.0×10^5 at 5000/1 to 1.4×10^6 at 500/1 and 4.5×10^6 at 50/1, indicating that under these conditions partitioning is dependent on lipid concentration. This is partially attributable to the experimental method used to separate the bound from the free peptide. At high lipid concentration even a 1% contamination of radiolabeled water-soluble impurities such as free ^{125}I in the GALA solution would result in a significant underestimation of K_a. As the lipid concentration is reduced, the effect of any water-soluble impurities would decrease. Alternatively, we cannot rule out the possibility that a different type of lipid–peptide complex forms at low lipid/peptide ratios relative to the higher ratios. However, dynamic light scattering has been used to measure vesicle size before and after addition of GALA at these three ratios. No change in size occurred, indicating that there is no gross reorganization of the vesicular structure when GALA is present.

When LAGA was applied to the gradient in the absence of lipid at pH 5, it also remained at the bottom of the gradient as expected (Figure 4B). When incubated in the presence of lipid at a 5000/1 ratio at pH 5, a small fraction of the peptide floated with the lipid giving an apparent partition coefficient of 1.9×10^4, while there was almost no discernible partitioning at pH 7.5 (apparent $K_a = 1 \times 10^2$). In spite of the similar structural features of the two peptides at pH 5, the partitioning of LAGA at this concentration is at least an order of magnitude less than that of GALA under the same conditions. Thus, we would predict that LAGA would be less able to induce contents release or cause membrane fusion than GALA. In spite of evidence from these Ficoll gradient studies that there is some association of LAGA for membranes, when viewed

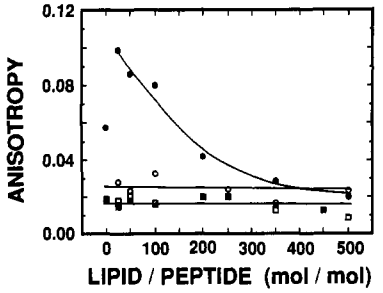

FIGURE 5: Anisotropy as a function of lipid/peptide ratio at pH 5 (circles) and 7.5 (squares). GALA (closed symbols) and LAGA (open symbols) were kept constant at 1.7 μM. Values plotted are the average from two to three experiments with different vesicle preparations and have an error of less than ±0.004.

by tryptophan fluorescence, there is no change in the tryptophan emission maximum of LAGA at pH 5 in the presence of lipid vesicles at a 50/1 mole ratio (data not shown). This is in contrast to a 6–8-nm blue shift observed between GALA in solution at pH 5 and in the presence of vesicles at this pH (Subbarao et al., 1987). Since the tryptophan environment is unchanged, this could suggest that LAGA may be associated at the surface of the membrane while GALA burys itself in the hydrophobic interior of the membrane. It is also possible that the large fraction of unassociated LAGA dominates the tryptophan spectrum, making any further blue shift undetectable. Not surprisingly, at pH 7.5, there is no change in the spectrum of LAGA upon the addition of lipid as also observed for GALA. Both the tryptophan fluorescence and the results from the Ficoll gradients support the idea of decreased membrane interaction of the peptide LAGA as compared to GALA.

The anisotropy of GALA and LAGA, measured at constant peptide concentration, is plotted as a function of lipid/peptide ratio at pH 5 (circles) and 7.5 (squares) in Figure 5. The anisotropy of both peptides is indistinguishable and does not change with increasing lipid concentration at pH 7.5 (Figure 5). The anisotropy of LAGA in the absence of lipid (0.026 ± 0.003) was unchanged in the presence of lipid (0.024 ± 0.005) at pH 5. In contrast, at pH 5, the anisotropy of GALA significantly increases at a ratio of 25/1 (0.10 ± 0.003) and steadily decreases until at 500/1 it is 0.02 ± 0.005. Higher amounts of lipid caused the samples to become too turbid to accurately measure anisotropy values. No change in the anisotropy of GALA occurred over an 8-fold dilution range at each lipid to peptide ratio, indicating that the observed results are not influenced by the depolarization of tryptophan in the presence of lipid. The anisotropy differences with pH further illustrate the altered association of GALA with lipid in these two environments, while also indicating that LAGA has no observable lipid interaction when measured by this technique.

Tryptophan Quenching. In an attempt to resolve whether the N-terminal of GALA is predominantly interacting with the bilayer surface or whether it is embedded further in the bilayer, bromine-labeled lipids were employed as quenchers of the tryptophan fluorescence. A decrease in fluorescence intensity from the tryptophan of GALA is a qualitative indicator of the extent of interaction of GALA. The depth of penetration of the N-terminal tryptophan within the bilayer can be probed by including brominated compounds that position the bromine at different locations in the bilayer. 5,6-Dibromocholesterol, which would position the bromine near the headgroup region of the bilayer, and BrPC, which would position the bromine at the 9,10-position of the lipid acyl chains, were each incorporated into vesicles at varying mole percents. Spectra were recorded from 1 min to 1 h after peptide addition to the brominated vesicles and compared to spectra obtained in the presence of nonbrominated vesicles. Virtually no changes occurred after 5 min. Table I shows the percent quenching at the tryptophan fluorescence maximum at pH 5 for two lipid/peptide ratios. At pH 7.5, the tryptophan spectrum of GALA in the presence of vesicles containing brominated lipids was not changed from the spectrum of GALA in the absence of lipids. Both brominated cholesterol and brominated phosphatidylcholine were able to quench tryptophan at pH 5. For both quenchers, the extent of quenching is a linear function of quencher concentration in the bilayer (Table I). These results indicate that the N-terminal of GALA has penetrated into the hydrophobic portion of the bilayer and reached equilibrium within a few minutes.

Table I: Quenching of Tryptophan in GALA by Brominated Lipids

	lipid/GALA (mol/mol)	% quenching of tryptophan[a]
EPC/BrChol (mol/mol)		
90/10	50/1	24
90/10	500/1	31
75/25	50/1	49
75/25	500/1	51
EPC/BrPC (mol/mol)		
95/5	50/1	19
95/5	500/1	19
88/12	50/1	31
88/12	500/1	33

[a] Quenching was measured from the tryptophan fluorescence maximum, which occurs at 335 nm at pH 5. At pH 7.5, no quenching was observed at any ratio for either brominated lipid. The error associated with the quenching measurements in replicate experiments was ±2% of the stated values.

The fluorescence of LAGA was unaffected by the presence of brominated lipids in the vesicles. This is also consistent with the idea that LAGA does not interact with the bilayer to any appreciable extent under the conditions employed in the spectroscopic studies.

Discussion

The peptide GALA is unique in being a completely synthetic peptide having the ability to alter its conformation in response to discrete changes in pH. The fact that it was designed from simple principles makes it a good model to study the interplay of components in the progression from choosing an amino acid sequence to secondary structure to function. In this paper we have concentrated on a more detailed picture of the solution properties of GALA at pH 5 and 7.5 and the changes which occur in the presence of lipid vesicles. Using a modified peptide, LAGA, we have also shown how making defined changes in primary sequence can have a profound effect on activity even though secondary structure was largely retained.

On the basis of our earlier studies (Subbarao et al., 1987), pH 5 and 7.5 were chosen for further investigation since this is where maximal differences in secondary structure were observed by circular dichroism. Also, phosphatidylcholine was selected for examining membrane interaction because its net neutral charge enabled us to avoid consideration of electrostatic effects as a major factor in lipid interaction with the highly negatively charged GALA (at neutral pH).

Tryptophan fluorescence clearly indicates the pH reversibility of GALA in solution. Cycling the pH between 5 and 7.5 causes the peptide to change conformation as observed by CD (Subbarao et al., 1987) and as indicated by a blue shift in tryptophan fluorescence. This shift occurs within the 2-s mixing time of the fluorometer, illustrating the delicate sen-

sitivity of GALA to its environment. Differences in the steady-state anisotropy of GALA in solution at pH 5 vs pH 7.5 imply an altered environment around the N-terminal tryptophan. The more restricted motion of the tryptophan at pH 5, as observed by anisotropy, is in accord with a constrained helical structure while a lower anisotropy value is what one would expect from the relatively unrestricted motion of tryptophan in a random structure as at pH 7.5. Alternatively, this could indicate a greater amount of self-association of GALA at pH 5 vs pH 7.5 for a given peptide concentration. One might imagine that the amphipathic nature of GALA in its helical state might promote association of monomers in solution in an arrangement such that leucine residues would be protected from the polar environment. Such an association could also give rise to greater anisotropy values at pH 5 as compared to pH 7.5.

Aside from gross changes in conformation, more subtle changes in the self-association of GALA at a given pH are observed as one varies the concentration of the peptide. This is observed at pH 5 by an increase in anisotropy from 0 to 10 μM GALA. The anisotropy value observed for GALA at pH 5 and the rise in anisotropy as the concentration is increased are of a magnitude similar to that observed for solutions of melittin and its analogues as the salt concentration is increased (Hermetter & Lakowicz, 1986; Weaver et al., 1989). In the case of melittin, the increase in anisotropy with increasing salt concentration is ascribed to a change from a monomer to a tetramer. On the basis of a similar line of reasoning, we think at pH 5 the self-association of GALA is enhanced as the peptide concentration increases. At pH 7.5, a blue shift in tryptophan fluorescence, a decrease in ellipticity, and an increase in anisotropy imply that GALA exists as an oligomer at concentrations greater than 20 μM. A precedent for this change occurs in the blue shift seen in the maximum emission wavelength of melittin at pH 7.4 and low ionic strength as the peptide concentration is increased (Schulze et al., 1987). The magnitude of this shift (6 nm) is comparable to that observed for GALA, although millimolar amounts of melittin are necessary. The anisotropy values of GALA at pH 7.5 increased considerably when carried out to 100 μM as has been observed for melittin solutions in the millimolar range (Faucon et al., 1979).

Membrane Association. The interaction of GALA with lipid vesicles is also strongly pH dependent. Interaction occurs at pH 5 and is drastically diminished at pH 7.5. This underscores the requirement of a helical conformation of GALA for membrane interaction, showing that structure can influence function. A blue shift in tryptophan fluorescence at pH 5 in the presence of lipid vesicles indicates that peptide has entered a more hydrophobic environment. In contrast, the wavelength maximum is unchanged with lipid present at pH 7.5 (Subbarao et al., 1987).

Direct binding measurements show an apparent bilayer/aqueous partition coefficient on the order of 10^6 at pH 5 while this association is greater than 2 orders of magnitude weaker at pH 7.5. An estimation of the partition coefficient of an α-helical peptide inserting into membranes can be made by free energy calculations (Jahnig, 1985). The free energy of protein incorporation is calculated as the sum of three individual components: (a) hydrophobicity (ΔG_W), (b) protein immobilization (ΔG_P), and (c) lipid perturbation (ΔG_L). For an α-helical peptide composed of 20 amino acids ΔG_W is -35 kcal/mol, ΔG_P is 16 kcal/mol, and ΔG_L is 2 kcal/mol. For GALA, the hydrophobic effect is reduced by a factor of $^1/_2$ due to its amphipathicity and increased by $^3/_2$ due to its 30 amino acid length, while other effects remain the same. Therefore, the total free energy of incorporation of GALA into membranes is -8.3 kcal/mol, giving a partition coefficient of 1.2×10^6. This calculation is in general agreement with our experimental findings.

Anisotropy measurements are unchanged at pH 7.5 in the presence or absence of lipid, indicating no significant interaction, while there is a definite change in anisotropy at pH 5 with lipid present. A distinct rise in anisotropy is observed at the lowest lipid/peptide ratio measured (25/1). Such an increase in anisotropy over that measured in the absence of lipid is also seen for melittin (Hermetter & Lakowicz, 1986; Faucon et al., 1979). With melittin, the anisotropy seems to reach a plateau at a 15/1 lipid/peptide ratio, but measurements were not carried out beyond 50/1. However, for GALA, the anisotropy value drops significantly at lipid/peptide ratios above 150/1, reaching a value at 500/1 approximately comparable to that observed at pH 7.5. The explanation for the decrease in anisotropy is not discernible. It may indicate an alteration in the state of association of GALA in the bilayer, a change in the interaction between oligomers of GALA in the bilayer [see Parente et al. (1990), or even a change in the bilayer location of GALA (surface versus embedded). It is interesting to note that the increased anisotropy values occur over the range of lipid/peptide ratios where GALA-induced fusion of small unilamellar vesicles is observed (Parente et al., 1988).

The depth of penetration of a protein into membranes can be estimated from the quenching of tryptophan (Markello et al., 1985). Having only one tryptophan residue, GALA is well suited for this type of study. We have used bromine incorporated into cholesterol or the 9,10-position of the acyl chains of phosphatidylcholine to localize the position of the tryptophan in the bilayer. Tryptophan quenching by brominated phospholipids is due to a static mechanism (London, 1982; East & Lee, 1982). For quenching to occur, bromine must contact or be within 5 Å of the tryptophan moiety (Everett et al., 1986). McIntosh and Holloway (1987) have shown that bromines located at the 6,7- and 11,12-positions of the *sn*-2 acyl chains lie 3.5 and 8 Å from the headgroup–hydrocarbon boundary, respectively. Since the bromines in the phospholipid used here are at the 9,10-position of both chains, they will be closer to 5.5 Å from the headgroup–hydrocarbon boundary. Assuming that the diffusional properties of the two brominated lipids in the bilayer are similar, one would expect the quenching efficiency to be due to the position of the bromine moieties in the bilayer as well as the sphere of action of the bromines (Lakowicz, 1983; Blatt & Sawyer, 1985). The phospholipid probe contains four bromine atoms per molecule and will have a larger sphere of action than the cholesterol with its two bromines per molecule. On a molar basis, the brominated cholesterol gives almost a comparable degree of quenching as the brominated phosphatidylcholine in our experiments. Therefore, we conclude that the tryptophan of GALA is closer to the headgroup–hydrocarbon interface than to the interior of the bilayer. Additional work is required with a complete series of phosphatidylcholines specifically brominated at different positions in the acyl chain to more accurately determine the tryptophan location (Markello et al., 1985). Nonetheless, a localization of the N-terminal tryptophan near the headgroup–hydrocarbon interface is consistent with a transbilayer oligomeric "channel" of GALA in unsaturated PC bilayers. We have proposed this structure on the basis of an analysis of the kinetics of leakage induced by GALA [see Parente et al. (1990)] as well as FTIR measurements of GALA in or-

iented multilayers (Goormaghtigh and colleagues, unpublished results).

Peptide Modification. The rearrangement of Glu and Leu residues between GALA and LAGA shows how function is critically linked to sequence. The secondary structure of LAGA determined by CD was similar to that of GALA at pH 5 and 7.5, with α-helical structure predominating at pH 5 and random coil structure at pH 7.5. A change in tryptophan fluorescence of LAGA in solution was observed as the pH was varied but was not as great as the shift observed for GALA. Also, there was no increase in the anisotropy of LAGA in solution.

Several lines of evidence support the idea of substantially diminished membrane interaction for LAGA: (1) there is a greater than an order of magnitude decrease in the apparent bilayer/aqueous partition coefficient of LAGA vs GALA, (2) LAGA fluorescence does not undergo a further blue shift in the tryptophan fluorescence maximum in the presence of lipid vesicles, (3) the anisotropy does not change in the presence of lipid vesicles, and (4) there is no quenching of the tryptophan fluorescence of LAGA by brominated lipids. Since both peptides are in an α-helix at pH 5, the difference in activity arises from the altered sequence. GALA is an amphipathic helix while LAGA is not. Amphipathic helices have typically been found as a common motif among membrane-spanning segments of proteins. Similar structures have been proposed for membrane proteins ranging from apolipoproteins (Segrest et al., 1974) to peptide hormones (Kaiser & Kezdy, 1984) to toxins (Brasseur et al., 1986) and ion channels (Hall et al., 1984, Cascio & Wallace, 1988).

Our data support the view that the amphipathic helical conformation of GALA is directly linked to its interaction with membranes. We have shown that GALA reversibly changes from a helix to a random coil in solution in less than 2 s and can only interact with lipid vesicles at low pH where it is helical. We believe that GALA is embedded into the hydrophobic core of the bilayer at pH 5 (Trp quenching) and can dissociate from this location when the pH is raised to 7.5 (Ficoll gradients). In the following paper we investigate pH-dependent membrane destabilization by GALA and propose a kinetic and structural model to explain its channel-like behavior.

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