pH-Dependent Bilayer Destabilization by an Amphipathic Peptide†

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ABSTRACT: A 30-residue amphipathic peptide was designed to interact with uncharged bilayers in a pH-dependent fashion. This was achieved by a pH-induced random coil–α-helical transition, exposing a hydrophobic face in the peptide. The repeat unit of the peptide, glutamic acid-alanine-leucine-alanine (GALA), positioned glutamic acid residues on the same face of the helix, and at pH 7.5, charge repulsion between aligned Glu destabilized the helix. A tryptophan was included at the N-terminal as a fluorescence probe. The rate and extent of peptide-induced leakage of contents from large, unilamellar vesicles composed of egg phosphatidylcholine were dependent on pH. At pH 5.0 with a lipid/peptide mole ratio of 500/1, 100% leakage of vesicle contents occurred within 1 min. However, no leakage of vesicle contents occurred at pH 7.5. Circular dichroism measurements indicated that the molar ellipticity at 222 nm changed from about -4000 deg cm^2 dmol^{-1} at pH 7.6 to $-11\,500$ deg cm^2 dmol^{-1} at pH 5.1, indicating a substantial increase in helical content as the pH was reduced. Changes in molar ellipticity were most significant over the same pH range where a maximum change in the extent and rate of leakage occurred. The tryptophan fluorescence emission spectra and the circular dichroism spectra of the peptide, in the presence of lipid, suggest that GALA did not associate with the bilayer at neutral pH. A change in the circular dichroism spectrum and a blue shift of the maximum of the tryptophan fluorescence emission spectra at pH 5.0, in the presence of lipid, indicated an association of GALA with the bilayer. Fragments of GALA (1, 2, 3, and 4 repeat units) were studied to determine the relative importance of conformation and hydrophobicity in inducing lytic activity of GALA. These short peptides did not change the conformation or induce leakage when protonated. Thus, the lytic activity of GALA can be correlated more closely to a conformational change rather than a change in hydrophobicity. Calcium, magnesium, or zinc cations at 20 mM induced a greater helical content than protons at pH 5.0. However, the divalent cation–peptide complexes were much less efficient at inducing leakage. This suggests that the hydrophilic face also plays a role in the lytic action of GALA on membranes.

For some time, proteins have been implicated in the control of certain biological membrane fusions (Lucy, 1984; Duzgunes, 1985); yet, an understanding of how fusion protein structure relates to their function is just beginning to emerge. Examples of proteins that regulate bilayer adhesion (Hong et al., 1981; Duzgunes, 1985) or bilayer fusion (Blumenthal et al., 1983; Lucy, 1984) have been described. The best studied are the membrane proteins of enveloped viruses (White et al., 1983). These viruses are internalized via endocytosis; subsequent to a pH drop in the endosome, the viral protein catalyzes a fusion of the viral and endosomal membranes.

In the case of the influenza virus, the hemagglutinin (HA)[1] is essential for entry of the virus into the cell, and the isolated HA mediates liposome fusion under low-pH conditions (Huang et al., 1980; Skehel et al., 1982). The low pH also results in a change of conformation of HA resulting in the exposure of the amino terminus (Skehel et al., 1982), which consists of an amphipathic helix ending in a short hydrophobic sequence of about 10 residues. This feature is highly conserved in different strains of influenza virus (White et al., 1983). Furthermore, site-specific mutagenesis at the amino terminus has demonstrated that binding is retained but membrane fusion is eliminated when the length of the hydrophobic sequence is shortened. These studies have led to the concept that fusion is triggered by a pH-induced conformational change that orients an amino-terminal amphipathic helix at the interface between the viral and cell membranes (Gething et al., 1986). The hydrophobic residues are hypothesized to then destabilize the apposed bilayers in a fashion that results in fusion of the membranes.

Early studies of peptide–liposome interactions, which have been proposed as models to study the mechanism of acid-dependent fusion, used polyhistidine (Wang & Huang, 1984) or polylysine (Gad et al., 1982; Carrier et al., 1985, 1986) to fuse liposomes composed of acidic phospholipids. In these cases, liposome fusion is primarily due to the interaction of the positively charged peptide with the negatively charged lipid. These systems may not be the most relevant to study the mechanism of virus-induced fusion since the monolayer of the endosome, that is exposed to the action of the viral fusion protein, is primarily composed of phosphatidylcholine (Alstiel & Branton, 1983). Hence, the mechanism of viral fusion is not likely to resemble that of the polycationic peptide–acidic phospholipid system.

Proteins or peptides that can induce fusion of phosphatidylcholine vesicles include alamethicin (Lau & Chan, 1975),

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; CD, circular dichroism; DPX, *p*-xylylenebis(pyridinium bromide); EPC, egg phosphatidylcholine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; REV, reverse-phase evaporation vesicles; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; HA, hemagglutinin; HPLC, high-performance liquid chromatography; Boc, *tert*-butoxycarbonyl; SDS, sodium dodecyl sulfate.

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clathrin (Blumenthal et al., 1983; Hong et al., 1985), and albumin (Garcia et al., 1984). Albumin and its pepsin cleavage fragments can induce fusion of sonicated egg phosphatidylcholine liposomes at about pH 3.5. This fusogenic activity has been related to the increase in the helical content associated with albumin as the pH is reduced from 7 to 3.5 (Garcia et al., 1984). However, the role of conformation, amino acid sequence, and amino acid hydrophobicity in mediating the effects in the above examples is not understood.

We have attempted to study the mechanism of action of viral fusion proteins by designing and synthesizing peptides that can interact with bilayers in a pH-dependent fashion. The ultimate goal is to obtain a peptide which can be attached to the surface of a liposome in a nonperturbing fashion at neutral pH, yet will fuse adjacent bilayers when the pH is lowered to 5. Studies with synthetic peptides have some advantages over those with peptides/proteins isolated from biological sources. Synthetic peptides can be obtained in comparatively large quantities. They allow a choice of hydrophobicity and amino acid sequence and peptide length. Moreover, the sequence can be chosen so that the secondary structure of the peptide is regular and to some extent predictable by means such as the Chou and Fasman method (1974). These advantages have been used extensively in the past (Sparrow & Gotto, 1980; Kaiser & Kezdy, 1984; Briggs & Gierasch, 1986) in the study of peptides which interact with lipids in one manner or the other.

As a first step, we designed a water-soluble peptide which will interact with liposomes specifically at low pH. This peptide (GALA) contains 30 amino acids with a repeat sequence of glutamic acid-alanine-leucine-alanine and an N-terminal tryptophan (Figure 1). The helical content of GALA increases, as measured by circular dichroism, when the pH is reduced from 7.6 to 5.0. Simultaneously, its ability to bring about leakage from egg phosphatidylcholine (EPC) vesicles increases. Fluorescence and circular dichroism (CD) studies indicate that the peptide associates with the bilayer as the pH is lowered. Our results indicate that the lytic activity of GALA is correlated with its helical content. Moreover, using relatively simple predictive schemes, one can design peptide sequences that change conformation and show concomitant dramatic changes in lytic activity.

Materials and Methods

Materials. Solvents for HPLC were obtained from Fisher Scientific (Springfield, NJ). EPC was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were obtained from Molecular Probes (Eugene, OR). The protected amino acids and Merrifield resin for the synthesis of peptides were obtained from Bachem (Torrance, CA) or Peninsula Laboratories (Belmont, CA). All other reagents were analytical grade or better.

Synthesis and Purification of Peptides. GALA was synthesized on a Biosearch automatic synthesizer (San Raphael, CA) using Merrifield resin. *N*-Boc-protected amino acids and formyl-protected tryptophan were coupled by using the symmetrical anhydride method. The peptide was cleaved from the resin by using hydrogen fluoride in the presence of *p*-cresol as a scavenger. The free peptide was extracted from the resin with 0.2 M ammonium acetate at pH 8, chromatographed on a Sephadex G-10 column in 0.1 M ammonium acetate at pH 8, and purified by reverse-phase HPLC on a Rainin Semiprep C_{18} column using the following conditions: solvent system A, 0.1% trifluoroacetic acid in water; solvent system B, 0.1% trifluoroacetic acid in acetonitrile; gradient, 40–70% B in 25 min followed by isocratic elution with 70% B, flow rate 3 mL/min. The solution for injection was prepared by suspending 25 mg of crude peptide in 0.5 mL of 6 M urea and adjusting the pH to 11 by using a few microliters of 30% ammonium hydroxide. The solution was incubated at room temperature for 30 min to deprotect the tryptophan before adjusting its pH to 5 with acetic acid. When the eluant was monitored at 260 nm, the crude material gave two major peaks, which eluted at 59% B (peak I) and 69% B (peak II). The 69% B peak (peak II) was collected and lyophilized. The purified peptide was suspended in water at a concentration of 1 mg/mL and dissolved by using ammonium hydroxide, and its pH was adjusted to 5 by using 0.2 N acetic acid. This solution gave a single peak (greater than 99% purity) at 69% B on a Vydac C_{18} analytical column under the following conditions: solvent systems A and B as before, gradient 50–70% B in 10 min followed by isocratic elution at 70% B, flow rate 1.5 mL/min. The eluant was monitored at 220 nm. Liquid secondary ion mass spectrometry of the purified GALA on a Kratos MS-50S double-focusing mass spectrometer equipped with a pulsed acceleration detection and Cs^+ ion source yielded the molecular ion of the peptide at mass unit 3033 ± 1 which corresponds to the protonated deformylated peptide ($C_{136}H_{217}N_{33}O_{45}$, mass unit = 3032.4).

Truncated peptides of GALA were prepared on a Peninsula manual peptide synthesizer (Belmont, CA) using Merrifield resin, *N*-Boc-protected L-amino acids, and diisopropylcarbodiimide as the coupling agent. They were cleaved from the resin by using hydrogen fluoride in the presence of *p*-cresol as a scavenger, extracted with 0.1 M ammonium acetate at pH 8, and purified on a Rainin Semiprep column using a gradient of 0–50% B in 30 min. Each of these peptides had a purity of greater than 99% when analyzed on a Vydac C_{18} reverse-phase column using a UV monitor at 220 nm. The amino acid analysis using a Waters Picotag system is given in Table I for all the peptides.

Polyacrylamide gel electrophoresis in 20% acrylamide in 1.0-mm-thick slab gels was carried out by using the continuous buffer system of Weber and Osborn (1969) (0.1 M sodium phosphate and 0.1% SDS, pH 7.2). Protein was visualized by staining with Coomassie blue or electroblotting onto nitrocellulose and staining with Ponceau S Red.

Gel filtration to determine whether GALA existed in the monomeric or an aggregated state at neutral pH was conducted on a Bio-Gel P-10 column (Bio-Rad, Richmond, CA) (0.7 × 35 cm). The column was equilibrated with 5 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes/NaOH) and 100 mM KCl, pH 7.5, and calibrated by using potassium chromate, cytochrome *c*, egg white lysozyme, and ovalbumin (Sigma molecular weight markers, MW-SDS-70).

Circular Dichroism Studies. The CD spectra of the peptides were scanned by using a Jasco 5000A spectropolarimeter attached to an IBM PC. All spectra were obtained in a sample chamber flushed with nitrogen and, unless otherwise mentioned, at 23 °C. The spectra were scanned in capped quartz optical cells with 1-mm path length. GALA was taken as a 0.5 mg/mL stock solution in 50 mM Tes/NaOH at pH 7.5 and was diluted into the appropriate degassed buffer to obtain a 0.05 mg/mL solution to scan the spectrum, unless otherwise mentioned. All θ values are expressed as degrees centimeter squared per decimole.

To obtain the CD spectra at varying pH, the stock peptide solution was diluted into 100 mM KCl, and the pH was adjusted to the required value by using a few microliters of 0.2

M sodium hydroxide or 0.2 M hydrochloric acid. The CD spectra in solutions with varying concentrations of KCl in 5 mM Tes/NaOH were scanned at pH 6.5. For CD spectra in the presence of divalent cations, the stock peptide solution was diluted into 50 mM Tes/NaOH at pH 7.5 or 50 mM sodium acetate at pH 5 containing calcium chloride, magnesium chloride, or zinc chloride, giving an effective buffer concentration of 35 mM. For the experiments in the presence of lipid, EPC films were hydrated with 5 mM Tes/NaOH, 100 mM KCl at pH 7.5, vortexing for a total of 5 min at room temperature. The lipid suspensions were added to the peptide in 50 mM Tes/NaOH at pH 7.5 or 50 mM sodium acetate at pH 5 to obtain the lipid/peptide ratio of 50/1 with a final buffer concentration of 35 mM. The solutions were incubated at room temperature for 30 min before the samples were scanned. The CD spectra of the lipid suspensions at the same concentrations in the respective buffers were subtracted in an attempt to correct for the CD spectra of the lipid. This correction was less than 5% of the intensity at 222 nm. The CD spectra at varying temperatures were scanned in 5 mM sodium acetate, 100 mM KCl at pH 5. The CD spectra of $GALA_3$ and $GALA_4$ were scanned by using the above conditions except the peptide concentration was 0.1 mg/mL.

Vesicle Preparation. Reverse-phase evaporation vesicles (REV) composed of EPC were prepared in 12.5 mM ANTS, 45 mM DPX, 20 mM KCl, 5 mM Tes/NaOH at pH 7.5 or in 5 mM Tes/NaOH, 100 mM KCl at pH 7.5 as described previously (Szoka & Papahadjopoulos, 1978). Vesicles were extruded under approximately 80 psi nitrogen pressure through 0.1-μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Samples containing encapsulated ANTS and DPX were chromatographed on a Sephadex G-75 column (0.7 × 19 cm), eluted with 5 mM Tes/NaOH, 100 mM KCl, pH 7.5, to remove unencapsulated material from the vesicle dispersion. Lipid phosphorus was measured by the method of Bartlett (1959).

Fluorescence. Fluorescence intensity and 90° light-scattering measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ). Membrane destabilization was followed by leakage of vesicle contents induced by interaction with the peptide by using the ANTS/DPX assay (Ellens et al., 1984). Leakage was followed by the dequenching of ANTS released into solution. Samples were irradiated at 360 nm; emission was measured through a Schott GG-435 cutoff filter (50% transmittance at 435 nm). Data were collected at 0.5-s intervals for 10 min. After 10 min, the vesicles were lysed with the detergent dodecyloctaethylene glycol monoether (Calbiochem, San Diego, CA). This maximum level of fluorescence was set to 100% leakage. Zero percent leakage was set with intact vesicles in the appropriate buffer. Results were normalized by using this scale. Initial rates were calculated as the slope of the tangent line to the region of maximum change in fluorescence. Vesicles were added at a concentration of 0.1 μmol/mL to 5 mM Tes/NaOH, 100 mM KCl, pH 7.5; 5 mM sodium acetate, 100 mM KCl, pH 5 or 4.5; 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), 100 mM KCl, pH 6.0; 5 mM Mes, 100 mM KCl titrated to pH 5.5 or 5.7 with 0.2 M acetic acid. Peptide in 5 mM Tes/NaOH, 100 mM KCl, pH 7.5, was added as small aliquots to the stirred vesicle suspension at 20 °C to begin the experiment.

The tryptophan moiety of GALA was excited at 280 nm. Samples containing a constant peptide concentration of 0.005 mg/mL were scanned in the presence and absence of EPC REV at 0.5-nm intervals from 300 to 500 nm. In each case, a corrected tryptophan spectrum was obtained after subtraction of an appropriate vesicle blank.

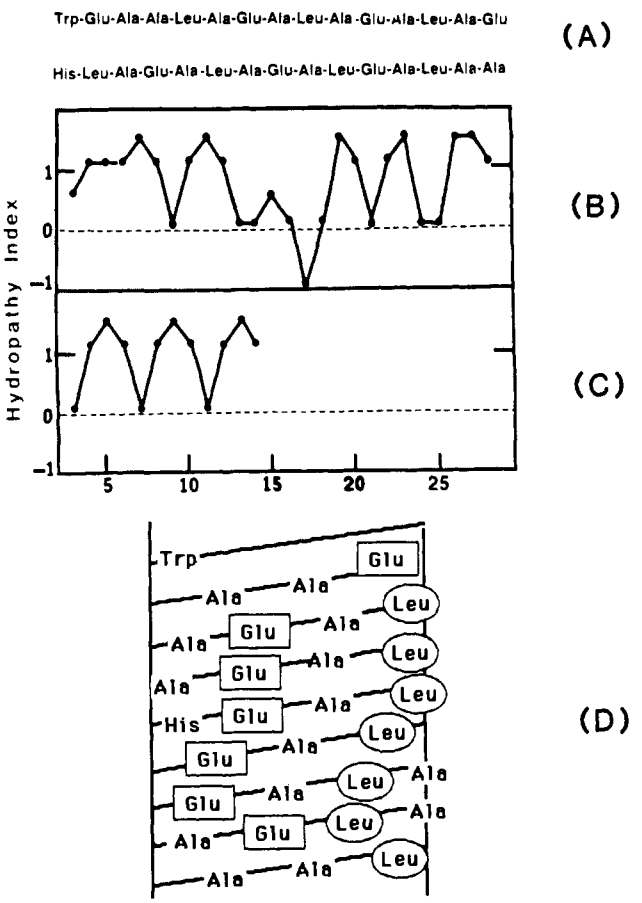

FIGURE 1: (A) Sequence of GALA. (B) Hydropathy indexes plot of GALA. (C) Hydropathy indexes plot of $GALA_4$. (D) Helical grid representation of GALA demonstrating the stacking of the glutamic acid residues.

RESULTS

The sequence of GALA is given in Figure 1A (see Discussion for details of design). It has Glu-Ala-Leu-Ala as the repeat unit in most of its structure and a molecular weight of 3032. The five-point averaging of the hydropathic indexes (Kyte & Dolittle, 1983) is given below the sequence (Figure 1B). The hydropathy attains a maximal value of +1.54 at a number of residues. The helical amphipathic moment (Eisenberg, 1982, 1986) is +0.258 kcal/residue and is exerted at an angle of −1.60° with respect to tryptophan (Figure 1D). The mean residue hydrophobicity is −0.25 kcal/residue. GALA therefore falls between the cluster of "surface-seeking" and "membrane-seeking" proteins as described by Pownall et al. (1983). By these criteria, it would not be expected to associate with lipid at neutral pH.

The average P_α value (Chou & Fasman, 1974) is 1.39, indicating that the peptide has a strong α-helical tendency.

Synthesis and Purification of Peptides. GALA was synthesized by using the Merrifield resin. After the peptide was cleaved from the resin, the purification on Sephadex G-10 had to be conducted with 0.1 M ammonium acetate at pH 8 as the peptide had a tendency to adhere to the column at low ionic strength and pH. Two major peaks were observed on reverse-phase HPLC on a C_{18} semi-prep column, one at 59% B (peak I) and the other at 69% B (peak II) under the conditions used. When collected and injected back on the semi-prep column, under the same conditions, peak I gave a single peak at 59% B, but peak II gave two peaks, one at 59%

Table I: Amino Acid Analysis of the Peptides

	Ala	Glu	Leu	His
GALA (peak I)	13.53 (14)[a]	6.84 (7)	7.14 (7)	1.03 (1)
GALA (peak II)	13.03 (14)	6.59 (7)	6.87 (7)	1
$GALA_1$	2.02 (2)	1.0 (1)	1.09 (1)	
$GALA_2$	4.1 (4)	2.0 (2)	2.3 (2)	
$GALA_3$	6.17 (6)	2.9 (3)	3.38 (3)	
$GALA_4$	8.0 (8)	4.17 (4)	3.64 (4)	

[a] Obtained (expected). The differences between peak I and peak II are described in the text. Tryptophan is not quantitated by the method used.

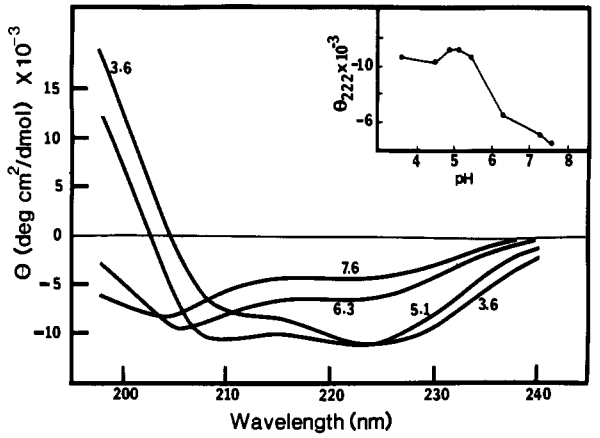

FIGURE 2: Circular dichroism spectra of GALA in 5 mM Tes, 100 mM KCl at varying pHs.

B and the other at 69% B. Both I and II gave the expected amino acid composition for GALA (Table I). It seems unlikely that I was a chemical decomposition product of II. It is also unlikely that GALA can form covalent dimers under the conditions used for the purification. Thus, the two peaks may be due to aggregated forms of the same peptide. On SDS gel electrophoresis, the HPLC pure material gave a band at a molecular weight less than insulin and a barely detectable band with a greater molecular weight. On a Bio-Gel P-10 column, when the peptide was loaded at comparatively high concentrations (10 mg/mL) and eluted with 5 mM Tes/NaOH, 100 mM KCl, pH 7.5, it gave only a small peak at the region where a 3000 molecular weight peptide was expected to appear. The major portion of the peptide eluted with an apparent mass of ≈20 000 daltons with a low molecular weight shoulder (results not given here). This indicated that the peptide showed a strong tendency to aggregate.

GALA at Varying pH. GALA was designed to change conformation with pH. The variation of helical content was followed by scanning the CD spectra of the solution. The CD spectra of GALA at pH 3.6, 5.1, 6.3, and 7.6 are given in Figure 2. The value of θ_{222} changed from −4000 deg cm^2 dmol^{-1} at pH 7.6 to −11 500 deg cm^2 dmol^{-1} at pH 5.1, showing that the helical content of the peptide increased as the pH decreased. These experiments were conducted in Tes buffer; in acetate buffer, the θ_{222} was −13 600 deg cm^2 dmol^{-1}. The half-maximal change in helical content occurs at about pH 6.0 (see inset of Figure 2). The θ_{222} remained the same below pH 4.9, but the pattern of the CD was altered and showed no minimum at 208 nm. The spectral changes in GALA observed by CD upon reducing the pH to 5.0 were completely reversed when the pH was increased to 7.5.

CD Spectra of GALA in Solutions of Varying Ionic Strength. The helical content of GALA is expected to increase with increasing ionic strength due to more effective shielding of the glutamic carboxylates. The CD spectra of GALA were therefore scanned in solution with 50–400 mM KCl at pH 6.5,

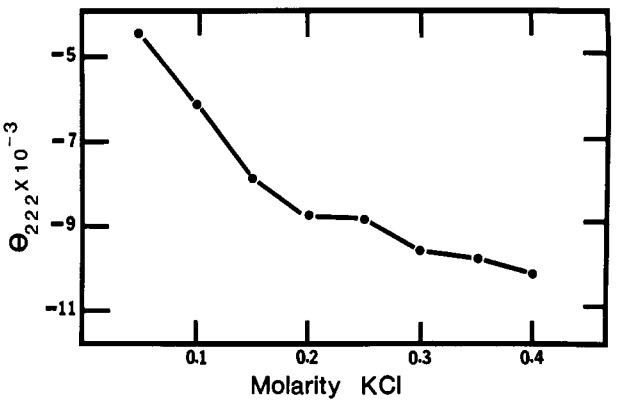

FIGURE 3: Molar ellipticities of GALA in 5 mM Tes at pH 6.5 with varying molar concentrations of KCl.

Table II: θ_{222} of GALA in the Presence of Divalent Cations in 35 mM Tes at pH 7.5 and 35 mM Acetate at pH 5.0[a]

cation	pH	molarity × 10^{-3} (divalent cations)	θ_{222} (deg cm^2 dmol^{-1})
35 mM Tes/NaOH	7.5		−4 000
35 mM sodium acetate	5		−13 600
Ca^{2+}	7.5	5	−12 500
Ca^{2+}	7.5	15	−13 600
Ca^{2+}	7.5	100	−15 300
Ca^{2+}	5	5	−14 600
Ca^{2+}	5	15	−14 200
Ca^{2+}	5	100	−15 300
Mg^{2+}	7.5	15	−14 800
Mg^{2+}	5	15	−14 600
Zn^{2+}	7.5	15	−13 400
Zn^{2+}	5	15	−13 300

[a] The molar ellipticities of GALA in the same buffers in the absence of divalent cations are also quoted for comparison.

and the conformation of GALA was found to be quite sensitive to ionic strength. The ellipticity becomes more negative at 222 nm as the ionic strength is increased (Figure 3). An almost linear change in θ_{222} is observed from 50 to 200 mM KCl.

CD Spectra in the Presence of Divalent Cations. The peptide was designed so that in the helical form, all the carboxylic acid groups would reside on the same face. These carboxylic acid groups are close enough to allow formation of divalent cation bridges connecting adjacent groups. Interaction of GALA with divalent cations should result in charge neutralization and might promote helix formation. The CD spectra of GALA in the presence of calcium, magnesium, and zinc cations were scanned. At pH 7.5, the θ_{222} value becomes more negative with increasing concentrations of Ca^{2+} (Table II). The maximum ellipticity observed was around −15 000 deg cm^2 dmol^{-1} at 100 mM Ca^{2+}. This is more negative than the ellipticity at pH 5.0 in the absence of divalent cations. In the presence of 5 mM Ca^{2+}, the θ_{222} value changes from −12 500 to −14 500 deg cm^2 dmol^{-1} by lowering the pH of the solution to 5. The θ_{222} value in the presence of 100 mM Ca^{2+} cannot be decreased further at lower pH values; 15 mM Mg^{2+} induces the same ellipticity as 15 mM Ca^{2+} both at neutral and at low pH. In the presence of Zn^{2+}, the shape of the CD spectrum changes, and the minimum at 208 nm becomes relatively small. The θ_{222} value with 15 mM zinc is −13 300 deg cm^2 dmol^{-1} at pH 7.5 and remains the same when the pH is reduced to 5.

CD Spectra at Varying Temperature. The helical structure of GALA at pH 5 should be destabilized when the temperature is increased above the denaturing point. The CD spectra of the solution at pH 5 was scanned at temperatures varying from

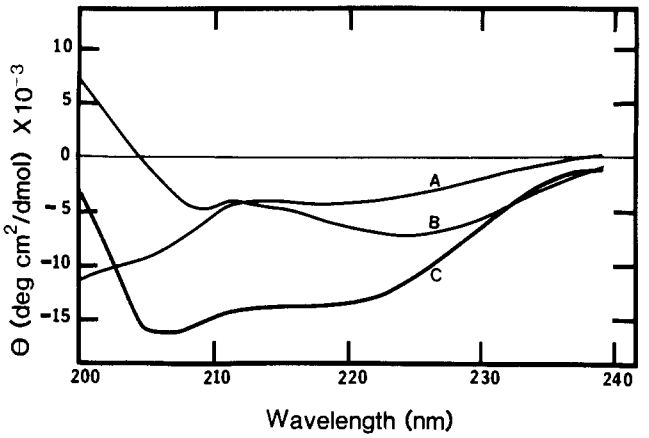

FIGURE 4: Circular dichroism spectra of GALA in the presence of EPC at a lipid/peptide ratio of 50/1. (A) In 35 mM Tes at pH 7.5 in the presence and absence of EPC. (B) In 35 mM acetate at pH 5 in the presence of EPC. (C) In 35 mM acetate at pH 5 in the absence of EPC.

0 to 70 °C. The θ_{222} value remained almost constant at about −13 200 deg cm^2 dmol^{-1} in the range 10–40 °C (data not shown). Above this value, the θ_{222} began to increase gradually to above −10 000 deg cm^2 dmol^{-1} at 70 °C.

CD Spectra of GALA–EPC Mixtures. The helical content of many peptides increases when they interact with lipid (Kaiser & Kezdy, 1984). When incubated for 30 min in the presence of EPC multilamellar vesicles at pH 7.5, the CD spectra of GALA do not change (Figure 4). At pH 5, however, the θ_{222} changed from −13 600 to −6900 deg cm^2 dmol^{-1}. Given the potential for light-scattering artifacts due to the presence of lipid vesicles, the interpretation of the spectra below 222 nm is ambiguous. However, the change in θ_{222} at low pH in the presence of EPC vesicles suggests that GALA is interacting with the bilayer.

Tryptophan Fluorescence. The sensitivity of tryptophan to the polarity of its environment was used to demonstrate an interaction between GALA and EPC REV under conditions of high and low pH over a range of lipid/peptide ratios. The fluorescence emission spectrum of GALA at pH 7.5 showed a maximum at 360 nm (Figure 5A). This was equivalent to a solution of L-tryptophan in water in our instrument. At pH 5.0, this maximum shifted to 350 nm (Figure 5A), indicating that the tryptophan, located at the amino terminus, is now in a more hydrophobic environment. This can be due to the altered conformation of the peptide at this pH or due to a different aggregation state of the peptide. The maximum of tryptophan fluorescence at pH 7.5 remained constant (360 ± 2 nm) when the peptide was added to EPC REV at a lipid/peptide ratio varying from 50/1 to 500/1. At pH 5.0, the fluorescence maximum in the presence of lipid showed a blue shift to 335 ± 1 nm over the same range (Figure 5A), indicating that the peptide has interacted with the more hydrophobic lipid environment. The fluorescence spectral changes observed with GALA upon lowering the pH were completely reversible when the pH was increased to 7.5.

The influence of the divalent cations calcium, magnesium, and zinc on the tryptophan fluorescence maximum was also investigated at pH 7.5. When GALA was in solution containing 20 mM $CaCl_2$ or 20 mM $MgCl_2$, the maximum was observed at 353 ± 1 nm vs. 360 nm in the absence of ions (Figure 5B). Varying amounts of lipid had no further affect on the position of the maximum although the shape of the curves in the presence of $CaCl_2$ and $ZnCl_2$ was slightly altered. The presence of 20 mM $ZnCl_2$ caused a further blue shift in the tryptophan maximum to 344 nm which again remained

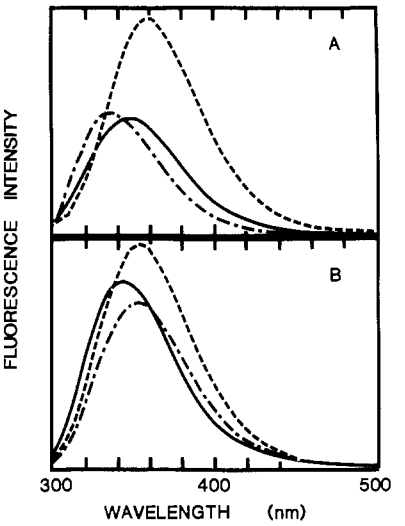

FIGURE 5: Tryptophan fluorescence spectra of GALA. (A) GALA in 5 mM Tes, 100 mM KCl at pH 7.5 (- - -), in 5 mM acetate, 100 mM KCl at pH 5 (—), and in 5 mM acetate and 100 mM KCl at pH 5 in the presence of EPC with a lipid/peptide ratio of 500/1 (-·-). No change in the tryptophan maximum occurred when vesicles were present at pH 7.5. (B) GALA in solution containing 20 mM $CaCl_2$ (-·-), $MgCl_2$ (- - -), and $ZnCl_2$ (—) at pH 7.5 shows a blue shift in tryptophan fluorescence when compared to the spectrum at pH 7.5, given in (A). The fluorescence maxima of these spectra did not change in the presence of EPC vesicles. The fluorescence intensity is not adjusted to the same scale in (A) and (B).

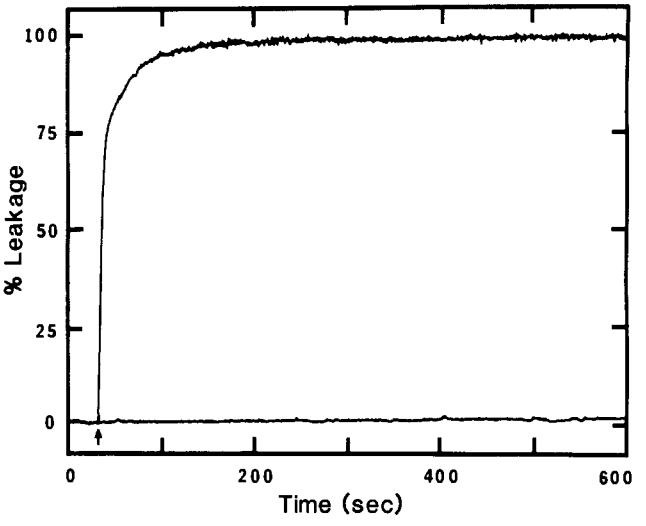

FIGURE 6: Percent leakage of EPC liposomes induced by GALA at a lipid/peptide ratio of 500/1 in (upper trace) 5 mM acetate, 100 mM KCl at pH 5 and in (lower trace) 5 mM Tes, 100 mM KCl at pH 7.5. The arrow indicates the time of injection of GALA into a solution of liposomes in the given buffer.

unchanged when lipid was present at ratios between 50/1 and 500/1 (Figure 5B).

Leakage of Egg Phosphatidylcholine REV. Peptide-induced leakage of vesicle contents was monitored by dequenching of the fluorophore ANTS. The fluorescence intensity of liposomes suspended in buffer at various pHs was constant over 0.5 h, indicating that the vesicles were stable under these conditions. Typical leakage curves are presented in Figure 6 contrasting the results for a lipid/peptide ratio of 500/1 at pH 5.0 and 7.5. In most cases, one observes an initial burst of leakage followed by a region of slow change after which the extent of leakage appears to reach a plateau. Figure 7A shows the calculated initial rates and Figure 7B, the extent of leakage as a function of pH when the lipid/peptide ratio is held constant at 500/1. Below pH 5.5, leakage was maximal

Table III: Initial Rate and Extent of Leakage Induced by GALA in the Presence of Divalent Cations at pH 7.5[a]

conditions	rate (% leakage/s) at lipid/peptide ratio			extent (% leakage) at lipid/peptide ratio		
	50/1	100/1	500/1	50/1	100/1	500/1
5 mM sodium acetate, 100 mM KCl, pH 5		11	11		100	100
20 mM $CaCl_2$, 70 mM KCl, 5 mM Tes/NaOH, pH 7.5	0.56	0.35	0.02	40	30	10
20 mM $MgCl_2$, 70 mM KCl, 5 mM Tes/NaOH, pH 7.5	0.15	0.05	0.01	20	13	5
20 mM $ZnCl_2$, 70 mM KCl, 5 mM Tes/NaOH, pH 7.5	0.14	0.07	0.03	35	25	11

[a] The rate and leakage induced by pH 5 are included for comparison. Leakage was negligible at pH 7.5 at these lipid/peptide ratios in the absence of divalent cations. Also, no leakage was observed when vesicles were added to ion-containing buffers at pH 7.5 in the absence of peptide.

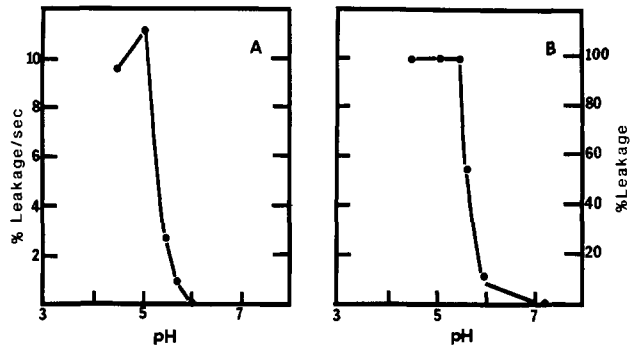

FIGURE 7: Leakage of EPC liposomes induced by GALA at a lipid/peptide ratio of 500/1. (A) Initial rate of leakage as a function of pH. (B) Extent of leakage after 10 min as a function of pH. Percent leakage at pH 4.5 became lower than the value at pH 5 at higher lipid/peptide ratios.

while the initial rate reached a peak at pH 5.2 and dropped dramatically as the pH increased. At a lipid/peptide ratio of 2500/1, the extent of leakage also decreased below pH 5.0. Leakage at other lipid/peptide ratios was studied in the range of 25/1 to 30000/1 (data not shown). In general, leakage was significant at lipid/peptide ratios greater than 500/1 only below pH 5.7.

Incubation of the peptide at pH 5.0 in the absence of lipid vesicles brought about a time-dependent loss of the lytic activity with an apparent first-order rate constant of 0.73 min^{-1}. The loss of lytic activity can be reversed by increasing the pH to 7.5 followed by decreasing the pH to 5.0. At 1000 times the concentration of GALA used in the leakage experiments, the tryptophan absorption sepctrum recorded at pH 7.5 was not changed by lowering the pH to 5.0. This indicates that at the concentration of peptide used in these experiments the loss of lytic activity is not due to the formation of an insoluble aggregate.

Simultaneous 90° light-scattering measurements did not show any significant change in turbidity when the peptide was added to the liposomes at any pH studied. This suggested that the peptide did not induce fusion or formation of stable aggregates between the liposomes.

Leakage in the Presence of Divalent Cations. Since results from CD indicated that divalent cations can promote α-helical character in the conformation of GALA at pH 7.5, the ability of GALA to promote leakage under these conditions was investigated. Calculated initial rates and extents of leakage after 10 min are recorded in Table III and are compared to values in the absence of divalent cations at pH 5. In the absence of peptide, divalent cations did not induce any leakage of EPC vesicles. Divalent cations increased the rate and extent of leakage at pH 7.5 in an ion-dependent manner; i.e., $Ca^{2+} > Zn^{2+} > Mg^{2+} >$ no ion. While the extent of leakage in the presence of Ca^{2+} and Zn^{2+} was similar over 10 min (although rates differ), it should be noted that in the presence of Zn^{2+}, the leakage did not reach a plateau characteristic of all other leakage curves observed with this peptide in a similar time frame. The most potent ion induced significantly less leakage than protons at pH 5.0.

Although not investigated in detail, leakage was also found to increase as the concentration of divalent cations increased. For example, with 5 mM $CaCl_2$, the extent of leakage at a lipid/peptide ratio of 100/1 was 10% compared to 30% for 20 mM $CaCl_2$. It was also observed that peptide suspended in 5 mM Tes/NaOH, 100 mM KCl, pH 7.5, and added to solutions of vesicles in the appropriate ion-containing buffer gave the same rates and extents of leakage as peptide preincubated with the cations.

Studies with GALA Fragments. $GALA_1$, $GALA_2$, $GALA_3$, and $GALA_4$ are shorter analogues of GALA. The sequences are $(Glu\text{-}Ala\text{-}Leu\text{-}Ala)_n$, $n = 1$–4, for $GALA_{1-4}$, respectively. A hydropathy plot of $GALA_4$ is given in Figure 1C. In these peptides, the end effects are expected to dominate the conformation, allowing little stable secondary structure. They undergo, however, a change in hydrophobicity when the side chain carboxylic acid groups are protonated at low pH. The lytic activity of these peptides was therefore studied. The CD spectra of $GALA_3$ and $GALA_4$ did not change when the pH of the solution was decreased from 7.5 to 5, showing that they (and also $GALA_2$ and $GALA_1$) were too short to have any stable secondary structure. The lipid/peptide ratio of 100/1 for GALA corresponds to 100 lipid molecules per 7 glutamic acid residues. At lipid/peptide ratios of $GALA_{1-3}$ (15/1, 30/1, and 45/1, respectively), corresponding to the same lipid to glutamic acid residue ratio given above, leakage was negligible at pH 7.5 and 4.5. In addition, no leakage was observed at higher lipid/peptide ratios. Only $GALA_4$ showed some ability to induce leakage (9%/10 min at a 60/1 ratio).

Discussion

Design of GALA. Our purpose was to design a peptide which would interact with neutral bilayers preferentially at low pH. The factors taken into consideration for choosing the sequence were (a) hydrophobicity of the residues, (b) conformational preference of the amino acids, (c) length of the peptide, (d) topology of the residues on the peptide, and (e) type of titratable amino acids. We were interested in a peptide which would increase in hydrophobicity when the pH is decreased from neutral to pH 5, thereby promoting peptide–lipid interactions at low pH. Such a peptide would mimic the action of viral fusion proteins.

There are several ways of predicting lipid–protein interaction on the basis of the hydrophobicity of the residues (Kyte & Dolittle, 1982; Eisenberg et al., 1982, 1986; Pownall et al., 1983; Engelman et al., 1986). However, these methods are based on hydrophobicity scales which are determined by using data obtained without keeping the pH constant or at neutral pH. The latter are less useful for predicting changes in hydrophobicities as the pH is altered. We decided to use a sequence which fell just short of being a membrane-seeking peptide according to the criteria of Kyte and Dolittle (1982) and Pownall et al. (1983) so that the increase in hydrophobicity

at low pH would considerably enhance peptide–bilayer interactions. In addition to hydrophobicity, interaction of peptides with lipid is modified by the secondary structure of the peptide. Studies with numerous other short peptides have indicated that those which interact with bilayers have a strong helical tendency (Kaiser & Kezdy, 1984). We therefore chose only those amino acids which are strong helix formers. The helical content of peptides increases with molecular weight, and for hydrophobic peptides, the increase in length results in a concomitant increase in lipid perturbing/associating ability. For these reasons, it is advantageous to synthesize peptides with greater than 20 residues (Sparrow & Gotto, 1980). For GALA, we selected a 30-residue length so that it would have a 16–20-residue center section whose secondary structure is not disturbed by end effects.

The residues were positioned so that when it assumes a helical conformation the peptide would have one hydrophobic face and a hydrophilic face with stacked acidic residues. Glutamic acid was selected as the titratable residue which when stacked at neutral pH would destabilize the helix (Gratzer & Doty, 1963). However, when titrated to pH 5, it would promote helix formation. Upon helix formation, the hydrophobic face, capable of interacting with the bilayer, would be present. Tryptophan and histidine were introduced as fluorescence and NMR probes and also to provide a position for radiolabeling the peptide with ^{125}I. The sequence obtained is given in Figure 1A.

Conformation of GALA. GALA was designed to be aperiodic at neutral pH and α-helical at approximately pH 5. Circular dichroism measurements demonstrated the induction of helical content in GALA as the pH was decreased from 7.5 to 5.0 (Figure 2). The expression $F = \theta_{222} + 2340/-30300$ (Chen et al., 1972), without correcting for the end effect, was used to determine the helical content. The helical content changes from 7% at pH 7.5 to 30% at pH 5. The pH at the midpoint of maximum change in helical content corresponds to the pK_a of glutamic acid in polyglutamic acid (Tiffany & Krimm, 1969), indicating that the change in helical content is correlated with the protonation of the glutamic acid side chains. The aligned negative charges on the peptide at neutral pH prevent stable secondary structure. Glutamic acid protonation below its pK_a relieves repulsion, resulting in increased helical content. The helix is quite stable at pH 5 and is denatured only above 40 °C. Below pH 5, the shape of the CD curve begins to change; the 208-nm minimum becomes smaller relative to the 222-nm minimum, and the crossover point moves to a value greater than 200 nm. At high ionic strengths, the CD spectra indicated an increase in ellipticity indicating an increase in helical content to about 26% in 400 mM KCl. This can be attributed to the shielding of the carboxylates by the cations.

When the peptide is in an α-helical conformation, the side chains are close enough to allow formation of divalent cation bridges between stacked carboxylic acid groups, which might stabilize the helix. It was seen that Ca^{2+} and Mg^{2+} induce much larger helical content than low-pH conditions. In the presence of Zn^{2+}, the θ_{222} value is comparable to the value in the presence of Ca^{2+} and Mg^{2+}, however, the CD pattern is different and resembles the CD spectra of GALA at pH 4.5.

The conformation of GALA does not change in the presence of EPC at neutral pH as indicated by the CD spectra. This, in conjunction with the tryptophan fluorescence results and the absence of leakage, indicates that GALA does not interact with the bilayer at pH 7.5. At pH 5.0 when GALA is incubated with EPC, the CD spectrum changed, and θ_{222} changed from $-13\,600$ deg cm^2 dmol^{-1} in the absence of lipid to -6900 deg cm^2 dmol^{-1} in the presence of lipid. The shape of the spectrum changed and became similar to the spectrum at pH 7.5 in the presence of Zn^{2+} and also to the spectrum at pH less than 4.9, in the absence of lipid vesicles. Due to the differential light scattering that predominates below 210 nm, we are not sure of the significance of this result (Simons, 1981). This decrease in the helical content in the presence of lipid vesicles is different from that reported for melittin and other membrane-active peptides which acquire helical character when they interact with bilayers (Kaiser & Kezdy, 1984).

Tryptophan Fluorescence Studies. The maximum of tryptophan fluorescence of GALA undergoes a blue shift when the pH of the solution is decreased. CD indicates that the conformation of the peptide in this buffer is a mixture of α-helical and random coil. It is quite likely that the aggregation state of the peptide also changes with pH. Either or both of these changes could place the tryptophan residue in a region of lower polarity. When EPC liposomes are added to GALA in buffer at pH 7.5, the fluorescence maximum does not change, indicating that the lipid does not associate with the peptide at neutral pH. However, in pH 5 buffer, the emission maximum shifts as soon as the peptide is added to the lipid, implying a rapid association of the peptide with the lipid. In the presence of calcium or magnesium ions, at pH 7.5, the fluorescence again indicates movement of the tryptophan into a less polar environment. The tryptophan fluorescence blue shift in the presence of zinc at neutral pH is even greater than in pH 5 buffer. The tryptophan fluorescence spectra of GALA undergo only a slight change in shape when lipid, peptide, and divalent cations are incubated together at neutral pH. This observation suggests that helix formation under these conditions is not sufficient to induce a strong peptide–lipid interaction.

Leakage Induced by GALA. GALA interacts with neutral EPC unilamellar vesicles specifically at low pH as indicated by the leakage of aqueous contents of the vesicles, the CD of the peptide in the presence of EPC, and the change in the tryptophan fluorescence. The region where the leakage is most sensitive to pH falls in the same range as the region where maximum change in helical content occurs, indicating a correlation between the helical content of the peptide and its activity.

However, the connection between the change in conformation and the induction of leakage is complex and not yet understood. The simplest scheme that can be envisaged is that the peptide changes conformation and partitions into the membrane. The process of partitioning may be sufficient to induce leakage. Alternatively, membrane destabilization may be indirect; the peptide may have to undergo additional rearrangements in the bilayer, perhaps forming a pore or channel before the contents can leak. Finally, a conformational change in the peptide could result in a change in its state of aggregation in solution, which in turn would destabilize the bilayer.

The fact that the lytic activity of GALA is lost upon prolonged incubation at low pH in the absence of lipid suggests that the lytic species is a transient intermediate. If a bilayer is present when the pH is reduced, GALA can insert into the membrane and cause leakage. If lipid is not present, the peptide forms a nonlytic complex. The CD spectra support the idea of two different conformations of peptide at low pH. The membrane-bound form has a lower helical content than the species formed in the absence of lipid vesicles.

When the pH of the solution is decreased, GALA changes in both hydrophobicity and helical content due to the pro-

tonation of the side chain carboxylic acid groups. The activity of $GALA_1$, $GALA_2$, $GALA_3$, and $GALA_4$ was studied in order to distinguish between these two factors. These peptides do not show any change in CD patterns when the pH of the solution is changed, but they have the same constituent amino acids and will undergo comparable change in their hydrophobicity when protonated at low pH. $GALA_4$ shows some lytic activity, but this activity is low and only observed at low lipid/peptide (60/1) ratios. Thus, lytic activity correlates better with the change in helical content than with the change in hydrophobicity when pH is reduced. The lytic activity of GALA under conditions where helical structure is induced by means other than low pH was studied in order to understand the involvement of helical structure in the lytic process. In the presence of 20 mM Ca^{2+} or Mg^{2+} which induce a higher θ_{222} value than pH 5, the liposomes do not leak appreciably. Although Ca^{2+} and Mg^{2+} promote the same amounts of helical character in GALA, Ca^{2+} is for some reason more efficient than Mg^{2+} in inducing the lytic activity. Zinc, which gives a CD spectrum indicative of a structure different from that induced by Ca^{2+}, also shows a different leakage pattern. The rate of leakage is comparatively low, and it does not seem to decrease, as in the case of leakage-induced by Ca^{2+}, Mg^{2+}, or acid.

Leakage results suggest that divalent cation–peptide complexes interact with bilayers. Reduction in the extent of leakage compared to the low-pH form of GALA is caused either by decreased association of cation–peptide complexes with the bilayer or by intrinsically smaller bilayer perturbations induced by the cation–peptide complex. The tryptophan fluorescence spectra in the presence of $CaCl_2$ or $ZnCl_2$ were only slightly changed when lipid vesicles were added. No spectral changes were observed in the presence of $MgCl_2$. This fact suggests that these ion complexes do not associate with the bilayer as avidly as the low-pH form of GALA. However, detailed binding experiments are in progress to discriminate between the two possibilities given above.

Obviously, helical content per se is not sufficient to induce leakage. These ion complexes may be less hydrophobic than the protonated peptide due to the polarity of the carboxylate–metal bonds, or they might be interfering in the formation of a lytically active form. Further experiments are required to unravel these complexities.

The lytic action of GALA at low pH is similar to the activity of melittin and other cytolytic peptides on cells and liposomes at neutral pH (Levin, 1984; Bernheimer & Rudy, 1986). As in the case of melittin, leakage induced by GALA is initially fast; it gradually slows down, becoming almost negligibly slow. Either the lipid bilayer repairs the damage or leakage stops because the contents are depleted and the peptide cannot transfer to a "full" vesicle. GALA resembles melittin in having a high helical tendency and forming an amphipathic structure in the helical state. GALA forms aggregates, a characteristic of peptides which cause lysis of cells by physically disrupting bilayers (Bernheimer & Rudy, 1986). GALA differs from such peptides in having pH-triggered lytic activity. Structurally, it differs in not being a basic peptide, and it lacks the cationic amino end which has been proposed to be important in the activity of melittin (Degrado et al., 1982).

Our original goal was to design a pH-triggered peptide fusogen. However, GALA does not cause the aggregation or fusion of REV as indicated by light-scattering measurements. This may not be too surprising since in the examples of peptides/proteins that can bring about fusion of neutral vesicles, small unilamellar vesicles were induced to fuse (Lau et al., 1975; Blumenthal et al., 1983; Cabiaux et at., 1984; Garcia et al., 1984). Large radii vesicles were not affected. The small unilamellar vesicles have high radii of curvature and are poised to fuse, and in fact do so under appropriate conditions in the absence of peptides (Wong & Thompson, 1982). To catalyze the fusion of large structures, it appears that close apposition and bilayer destabilization are more difficult to achieve (Duzgunes, 1985). Results from studies with influenza virus fusion proteins demonstrate that the fusogenic sequences must be anchored to the bilayer surface in order to catalyze fusion (White et al., 1982; Gething et al., 1986). Whether this is due to the state of aggregation of the fusion protein or the ability of the membrane-anchored protein to simultaneously destabilize both bilayers is not known. Thus, it might be necessary to anchor GALA to the liposome surface in order to induce fusion of large unilamellar vesicles.

Conclusions

We have designed, synthesized, and studied a peptide, GALA, which induces leakage of aqueous contents of neutral liposomes preferentially at pH 5.0. By taking into account factors such as topology of the residues, peptide length, hydrophobicity, and conformation, one can design a peptide which causes pH-triggered lysis of liposomes. This capability should permit us to study the mechanism of action of viral fusogenic proteins.

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Registry No. GALA, 107658-43-5; $GALA_1$, 107658-39-9; $GALA_2$, 107658-40-2; $GALA_3$, 107658-41-3; $GALA_4$, 107658-42-4; $(Glu\text{-}Ala\text{-}Leu\text{-}Ala)_n$, 107658-44-6.

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Selective Outside–Inside Translocation of Aminophospholipids in Human Platelets†

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ABSTRACT: Spin-labeled analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin were added to human platelet suspensions. Due to the partial water solubility of these spin-labeled lipids which possess a relatively short β-chain (C_5), they incorporate rapidly in membranes. The orientation of the spin-labels within the platelet plasma membrane was assessed by following the spontaneous reduction at 37 and 4 °C due to endogenous reducing agents present in the cytosol. The rate of spontaneous reduction showed unambiguously that the labels incorporated initially in the outer leaflet of the plasma membrane and that the rate of outside–inside translocation of the aminophospholipids was faster than that of the choline derivatives. For example, at 37 °C, the half-time for the transverse diffusion of a phosphatidylcholine analogue was found to be of the order of 40 min, while it was less than 7 min for the phosphatidylserine analogue. At low temperatures, a fraction of the labels gave rise to a strongly immobilized ESR component. This fraction, which corresponded to 20–30% of the initial spin-label concentration, was found resistant to chemical reduction from the inner side of the membrane and also to externally added reducing agents such as ascorbate. Presumably these immobilized lipids are trapped in a gel phase formed in the outer leaflet at 4 °C. Cell aging, which depletes the cells of ATP, resulted in the progressive inhibition of the fast transport of the aminophospholipids from the outer to inner leaflet. Treatment of the cells with iodoacetamide completely blocked the transverse diffusion of the spin-labels. These experiments suggest the existence in platelets, as in erythrocytes, of an ATP-driven translocator of aminophospholipids, which would be responsible for maintaining the lipid asymmetry of the platelet plasma membrane, under normal physiological conditions.

Since the pioneer work of M. Bretscher in 1972, phospholipid transverse asymmetry had been well documented in erythrocytes and in platelet plasma membranes. The compositional asymmetry was assayed by chemical derivatization and enzymatic hydrolysis [see the reviews by Etemadi (1980), Van Deenen (1981), and Op den Kamp et al. (1985)]. In erythrocytes, the choline derivatives (phosphatidylcholine and sphingomyelin) are found principally on the outer monolayer, while the aminophospholipids (phosphatidylethanolamine and phosphatidylserine) are found principally on the inner monolayer (Verkleij et al., 1973). Although similar in overall lipid

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