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Properties of lipoamino acids incorporated into membrane bilayers

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Abstract

Several lipoamino acids were synthesized in which palmitic acid was coupled with the α-amino group of an amino acid. These lipoamino acids were tested for their inhibitory action against Sendai virus fusion to liposomes composed of egg phosphatidylethanolamine and 5 mol% of the ganglioside G_{D1a}. A commonly employed viral fusion assay based on the dilution of the fluorescent probe octadecylrhodamine (R18) exhibited an additional complication in the presence of N^{α}-palmitoyl tryptophan (palm-Trp). At higher mol fraction of palm-Trp it was observed that there was an increase in R18 quenching. Studies on the dependence of the emission wavelength of palm-Trp on excitation wavelength demonstrated that the presence of R18 alters the environment of the indole. The results illustrate one of the complexities of viral fusion assays using the R18 probe. Despite this complication it was possible to demonstrate that several of the lipoamino acids are effective at inhibiting the fusion of Sendai virus to liposomes as measured by the R18 assay. One of the most effective inhibitors of this process is palm-Trp which, at a concentration of 4 mol% in liposomes, markedly reduces the apparent rate of fusion. At pH 5.0 this amphiphile is also an inhibitor of Sendai virus fusion, indicating that the ionization of the carboxyl group of this amphiphile is not required for its antiviral activity. The inhibitory action of palm-Trp against Sendai virus was confirmed by demonstrating inhibition of Sendai-mediated cytopathic effects studied in tissue culture. A property associated with antiviral activity is the ability of amphiphiles to raise the bilayer to hexagonal phase transition temperature of dielaidoyl phosphatidylethanolamine. All of these lipoamino acids were found to possess this property, but a quantitative relationship with inhibition of viral fusion was not found.

Keywords: Membrane fusion; Octadecylrhodamine assay; Sendai virus; Antiviral agent; Lipoamino acid

1. Introduction

A number of N^{α}-palmitoylated amino acids and peptides have been synthesized in which palmitic acid is linked by an amide bond to the terminal α-amino group of an amino acid. Several of these lipoamino acids decrease the surface tension of water at lower concentrations than do more commonly used amphiphiles [1]. These compounds also exhibit

Abbreviations: CPE, cytopathic effects; DEPE, dielaidoyl phosphatidylethanolamine; DMF, dimethylformamide; DSC, differential scanning calorimetry; *E/M*, excimer to monomer fluorescence emission ratio; LUV, large unilamellar vesicle; Palm-amino acid (three letter code used for all amino acids), N^{α}-palmitoyl-amino acid sodium salt; PalmOSu, palmitoyl *N*-hydroxysuccinamide ester; PDTAB, pyrene butyl trimethylammonium bromide; PE, phosphatidylethanolamine; R18, octadecylrhodamine; T_H, bilayer to hexagonal phase transition temperature; TLC, thin layer chromatography

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strong emulsifying properties. A number of amphiphilic substances including cholesterol phosphorylcholine [2] and lipophosphoglycan [3] can markedly inhibit the rate of viral fusion. There has been particular interest recently in lysophosphatidylcholine as an amphiphile which can inhibit viral fusion processes [4–7]. Lipoamino acids are particularly attractive as amphiphiles with potential antiviral activity because of their potent surfactant activity and the possibility of preparing a series of homologous compounds. In addition, earlier studies with other lipopeptides, the a-factor from *Saccharomyces cerevisiae* and a number of its analogs [8], showed that this type of compounds raised the bilayer to hexagonal transition temperature (T_H), a property associated with antiviral activity [9]. Certain acyl amino acid derivatives have been proposed as potential antiviral agents, because of their inhibition of influenza neuraminidase [10].

In this study we have prepared a number of palmitoylated amino acids and incorporated them into model membranes to study their properties in membranes as well as their ability to affect the fusion of Sendai virus. These palmitoylated amino acids have a fatty acyl chain which will cause them to partition into membranes and hence to be at the site of membrane fusion. Palmitoylated amino acids would be degraded in vivo to the non-toxic products of a fatty acid and an amino acid.

2. Experimental procedures

2.1. Chemicals

The following N^{α}-palmitoylated amino acids as sodium salts were synthesized as described below: palm-Val, palm-Gly, palm-Ala, palm-Leu, palm-Phe, palm-His, palm-Tyr and palm-Trp. All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Gangliosides were purified according to Reed et al. [11]. All lipids showed one spot by thin layer chromatography (TLC) at a load of 50 μg. Fluorescent probes were purchased from Molecular Probes (Eugene, OR). All other chemicals and solvents were of reagent grade. Unless stated otherwise, all reagents and solvents used for synthesis were obtained from Merck (Darmstadt, Germany) of the synthetic grade and used without further purification. Amino acid methyl ester derivatives of synthetic grade were purchased from NovaBiochem (Switzerland).

2.2. General procedure for preparation of N^{α}-palmitoylated amino acids as sodium salts

The condensation of palmitic acid to the α-amino group of the amino acid was carried out in all cases using the *N*-hydroxysuccinimide ester method as previously described [1].

2.3. Synthesis of palmitoyl N-hydroxysuccinamide ester (PalmOSu)

To a solution of palmitic acid (0.0125 M) and *N*-hydroxysuccinimide (0.0125 M) in 50 ml of dimethylformamide (DMF), a solution of *N*,*N*′-dicyclohexylcarbodiimide (0.0125 M) in 10 ml of DMF was added. The mixture was stirred at room temperature for 24 h and allowed to stand overnight in a refrigerator. *N*,*N*′-Dicyclohexylurea was removed by filtration and washed with DMF. The combined filtrate and washings were extracted three times with hexane. Water was then added to the DMF solution to yield a white crystalline precipitate. After several recrystallizations from ethyl acetate with petroleum ether, the desired PalmOSu was satisfactorily obtained. Yield 80%; m.p.: 89–90°C.

2.4. Synthesis of N^{α}-palm-amino acid methyl ester derivatives

To a solution of the corresponding hydrochloride salt of the amino acid methyl ester (0.002 M) in 5 ml of DMF, triethylamine (0.004 M) was added at 0°C followed by a solution of PalmOSu (0.002 M) in 5 ml of DMF. The mixture was held at room temperature for 24 h. The solvent was then removed under reduced pressure and the residue dissolved in ethyl acetate. The organic layer was washed with 10% aqueous citric acid, 5% aqueous $NaHCO_3$ and water, and then dried. The resulting oil was precipitated from ethanol/hexane or chloroform/light petroleum mixtures.

Table 1
Chemical characteristics of the compounds synthesized

Lipoamino acid	MW	M.p. (°C)	% yield	$[\alpha]_D^a$	Analysis (%; calc. (found))		
					C	H	N
PalmGlyOMe	327	78–81	80		69.72 (70.1)	11.3 (11.28)	4.28 (4.37)
PalmGlyONa	335	198–200	90		64.47 (64.50)	10.15 (10.32)	4.18 (3.98)
PalmValOMe	341	70–72	75	−16.6	70.38 (70.63)	11.44 (11.42)	4.10 (4.10)
PalmValONa	349	120–121	95		65.33 (65.50)	10.31 (10.57)	4.01 (3.92)
PalmAlaOMe	341	77–80	75	−29	70.38 (70.50)	11.44 (11.70)	4.11 (4.18)
PalmAlaONa	349	124–125	95		65.33 (65.24)	10.31 (10.55)	4.01 (3.86)
PalmLeuOMe	383	52–54	80	−23	72.06 (72.10)	11.75 (11.60)	3.65 (3.50)
PalmLeuONa	391	77–78	95	−13	67.52 (77.32)	10.74 (10.56)	3.58 (3.18)
PalmPheOMe	417	72–74	50	13	74.82 (74.79)	10.31 (10.52	3.36 (3.40)
PalmPheONa	425	109–110	90		70.60 (70.48)	9.41 (9.26)	3.29 (2.98)
PalmHisOMe	408	124–126	40	−6.5	67.40 (67.37)	10.37 (10.46)	10.37 (10.26)
PalmHisONa	416	210–211	95		63.46 (63.11)	9.37 (9.56)	10.10 (9.89)
PalmTyrOMe	433	97–100	70	5.5	72.20 (72.05)	10.15 (9.93)	3.23 (3.26)
PalmTyrONa	441	198–200	97		68.02 (67.79)	9.07 (9.42)	3.17 (2.98)
PalmTrpOMe	457	65–68	65	1.7	73.50 (73.26)	9.84 (9.90)	6.12 (5.98)
PalmTrpONa	465	123–125	98		69.67 (69.33)	9.03 (9.44)	6.02 (5.8)

[a]0.2% methanol at 25°C. Units in deg/cm/mol.

2.5. Synthesis of N^{α}-palm-amino acid sodium salts

Removal of the terminal methyl ester was carried out by classical treatment with a 10% excess of an ethanolic NaOH solution at room temperature. A white solid was collected by filtration corresponding to the final compound. The progress of the reaction and the homogeneity of the products were monitored by silica gel TLC on Merck, Kiesegel 60 plates with or without the ^{254}F fluorescent indicator. The running solvent was butanol/acetic acid/water (4.2:5:2.5) or methanol/chloroform (7:3). Primary amino groups were detected on the TLC plate with ninhydrin spray and amide groups with chlorine-toluidine developer solution [12]. The purity of the intermediate and final compounds was also checked by HPLC using a model Merck-Hitachi D-2500 apparatus with a lichrocart 125-4, lichrospher 100 pp 18 column and using a UV-VIS detector, L-4250, at $\lambda = 210$ nm. The mobile phase was a solvent gradient 10–100% of acetonitrile in water at 203–303°K for 45 min or an isocratic mixture of 20% water-80% acetonitrile. Elemental analysis for all intermediate and final compounds agreed with expected results. Melting points were determined with an FP81 measuring cell of the FP90 Mettler System; optical rotations were measured with a 141 Perkin-Elmer Spectropolarimeter (Norwalk, CT). The structures of intermediate and final products were checked by ^{1}H-NMR analysis using a Varian 200 MHz spectrometer. The IR spectra were recorded on an IR-FT Nicolet spectrophotometer. Table 1 shows the chemical characteristics of the lipoamino acids as methyl esters and sodium salts.

2.6. Preparation of large unilamellar vesicles (LUVs)

The phospholipid was dissolved in a mixture of chloroform/methanol, 2:1 (v/v), to which 5 mol% of ganglioside G_{D1a} was added from a methanolic solution. Palmitoyl amino acids were also added at this point from a methanolic solution. The solvent was evaporated with a stream of dry nitrogen gas, depositing the lipids as a film on the walls of a Pyrex test tube. Samples were placed in a vacuum evaporator equipped with a liquid nitrogen trap for 2–3 h to remove the last traces of solvent. The dried lipid film was suspended by vigorous vortexing with 5 mM HEPES, 5 mM MES, 5 mM sodium citrate, 150 mM NaCl, 1 mM EDTA at pH 7.4 (HEPES-MES buffer). The lipid suspensions were further processed with five cycles of freezing and thawing, followed by ten passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) using an Extruder (Lipex Biomembranes, Vancouver, B.C.) at room temperature [13,14]. Lipid

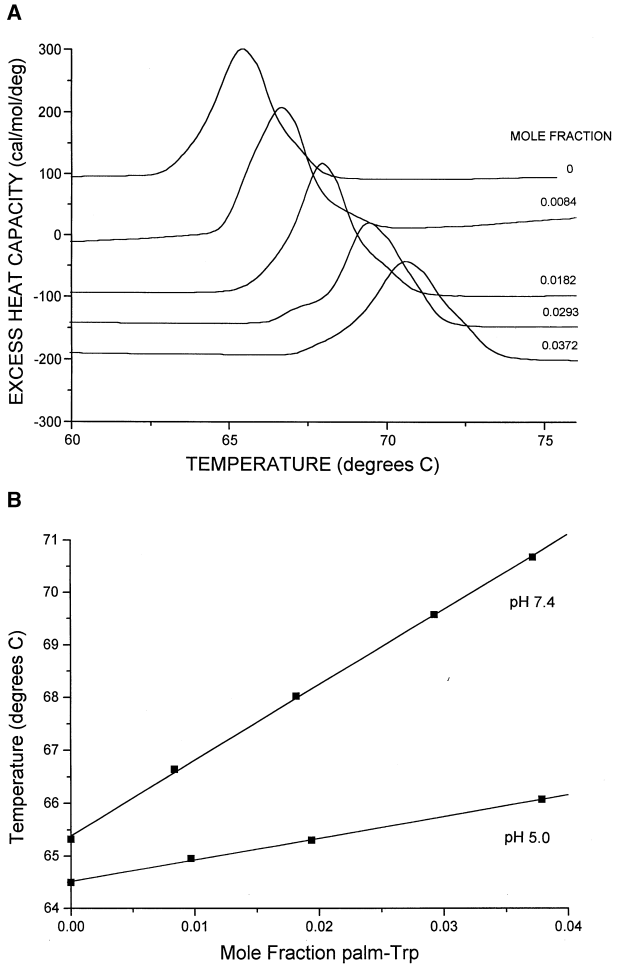

Fig. 1. (A) Thermograms of varying mole fractions of palm-Trp in DEPE. (B) Linear regression of the T_H dependence on the mole fraction of palm-Trp, at pH 7.4 and 5.0.

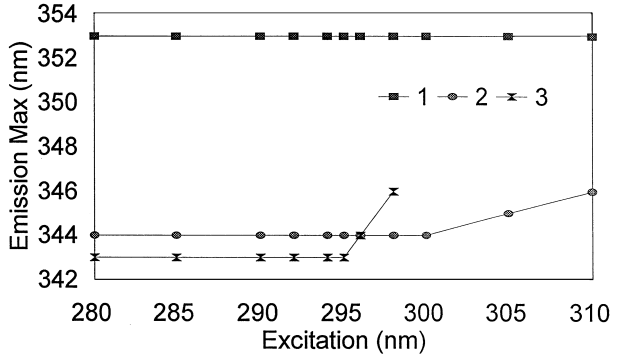

Fig. 2. Red edge effect at 37°C with 50 μM egg PE containing 5 mol% G_{D1a} and (1) 4 mol% *N*-acetyl-Trp-amide; (2) 4 mol% palm-Trp; (3) 0.1 mol% R18 and 4 mol% palm-Trp.

phosphorus was determined by the method of Ames [15].

2.7. Quasi-elastic light scattering

Particle sizing was carried out with a laser light scattering instrument from Brookhaven Instrument, equipped with a BI-200sm goniometer, version 2.0 and a BI-900AT Digital Correlator System. Size distribution analysis was calculated using a non-negatively constrained least squares method, with software provided by the instrument manufacturer. Liposomes with or without palmitoylated amino acids were found to have a mean diameter of 92 nm.

2.8. Virus preparations

The Cantell strain of Sendai virus was propagated in the allantoic sac of 10-day-old embryonated chicken eggs by incubation at 33°C for 72 h. Virus was isolated by discontinuous sucrose gradient centrifugation. The virus was washed and the final preparation resuspended in HEPES-buffered saline pH 7.4, at a viral protein concentration of 1 mg/ml. The virus was stored in the frozen state at −70°C.

2.9. Virus fusion assay

Sendai virus was labelled with octadecylrhodamine (R18) (Molecular Probes, Eugene, OR) according to the procedure of Hoekstra et al. [16]. Ten microlitres of R18 (10 nmoles) in ethanol were injected into 1 ml of a suspension of Sendai virus in HEPES-MES buffer containing approx. 1 mg of viral protein. The mixture was allowed to incubate at room temperature for 1 h. Unincorporated R18 was then removed by passing the labelled virus through a Sephadex G-

Table 2
Effect of N^{α}-palmitoyl amino acids on T_H

Amino acid	Shift of T_H (°C/mol fraction)[a]
Ala	44 ± 5
Leu	154 ± 15
Phe	100 ± 16
Trp	141 ± 7
His	232 ± 23
Tyr	225 ± 23

[a]The data above are for pH 7.4. At pH 5.0, the value for Trp reduces to 41 ± 1.

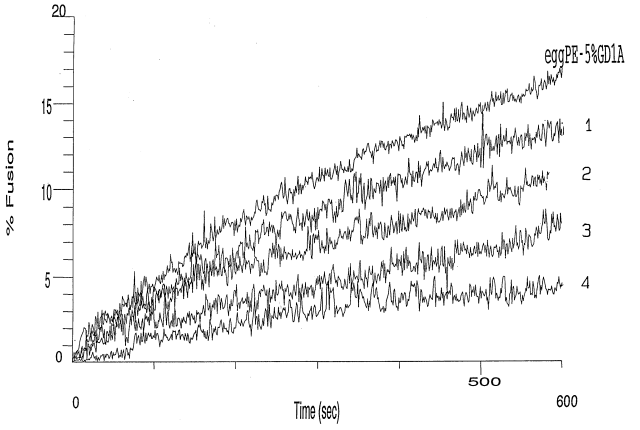

Fig. 3. Percent fusion of R18 labelled Sendai virus with 25 μM egg PE-containing 5 mol% G_{D1a} LUVs and 4 mol% of palmitoyl amino acids, at 37°C. Control: LUVs alone. 1, palm-His; 2, palm-Phe; 3, palm-Tyr; 4, palm-Trp.

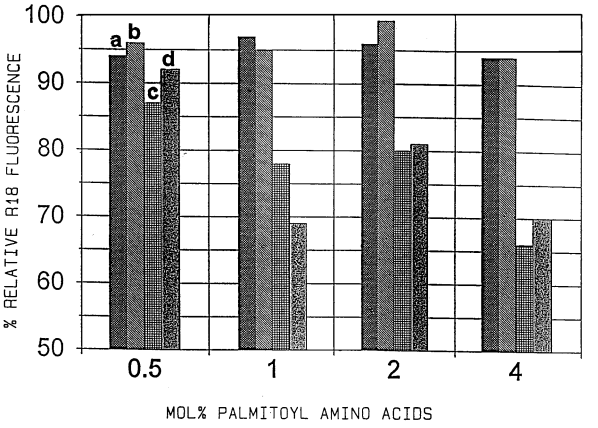

Fig. 5. Effect of concentration of palmitoyl amino acid on % relative R18 fluorescence incorporated at 0.1 mol% into LUVs of egg PE with 5 mol% G_{D1a}, at 37°C with (a) palm-Val, 25 μM LUVs; (b) palm-Val, 50 μM LUVs; (c) palm-Trp, 25 μM LUVs; (d) palm-Trp, 50 μM LUVs.

75 gel filtration column eluted with the HEPES-MES buffer, and collecting the virus in the void volume. The final viral protein concentration was determined using the BCA assay (Pierce Chemical, Rockford, IL). LUVs were diluted into 2 ml of HEPES-MES buffer pH 7.4, maintained in a thermostatted cuvette holder at 37°C with continual magnetic stirring. Five micrograms of R18-labelled Sendai virus was rapidly injected into the cuvette after thermal equilibration. Fluorescence was recorded using an SLM AMINCO Bowman Series 2 Luminescence Spectrometer interfaced with a 386/20 IBM compatible computer. The instrument used a xenon arc light source with a 560 nm filter between the excitation slit and sample and a 590 nm cutoff filter between the sample and the photomultiplier tube to minimize any contribution of light scattering to the fluorescence signal. The excitation and emission monochromators were set at 565 and 600 nm, respectively. The fluorescence intensity immediately after addition of the labelled virus was taken as F_0. A 20 μl aliquot of 10% Triton X-100 was added in order to measure F_{100}. The percentage of R18 dequenching was calculated at time t from:

$$\% \text{ R18 dequenching} = 100(F_t - F_0)/(F_{100} - F_0)$$

A variation of this assay was performed in order to measure the effect of pH on the rate of viral fusion. This was done in two ways; either the virus was added to liposomes in 10 mM citrate, 0.15 M NaCl, 1 mM EDTA, pH 5.0 buffer in the fluorimeter cuvette or the viral fusion assay was begun at pH 7.4 with the addition of virus as described above and then the mixture was acidified to pH 5.0 with a small volume of 1 M citric acid solution.

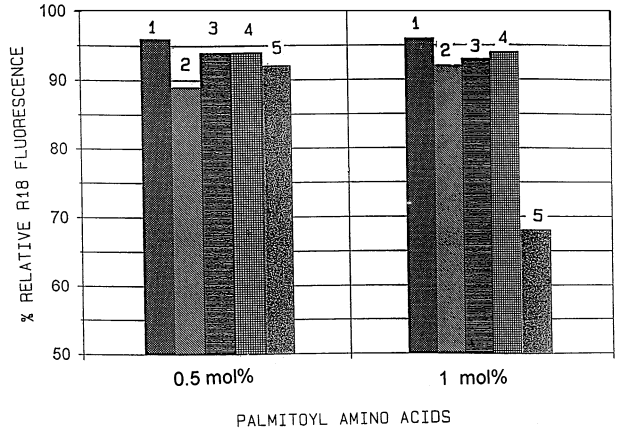

Fig. 4. Percentage of relative fluorescence of R18 incorporated at 0.1 mol% into LUVs of egg PE with 5 mol% G_{D1a}, containing 0.5 or 1 mol% of palmitoyl amino acids. 25 μM LUVs in cuvette at 37°C. 1, palm-Val; 2, palm-His; 3, palm-Phe; 4, palm-Tyr; 5, palm-Trp.

2.10. Differential scanning calorimetry (DSC)

Lipid films were made from dielaidoyl phosphatidylethanolamine (DEPE) dissolved in chloroform/methanol (2:1, v/v) and to some tubes small aliquots of a palmitoyl amino acid were added from a methanolic solution. After solvent evaporation with nitrogen, final traces of solvent were removed in a vacuum chamber for 90 min. The lipid films were suspended in either 20 mM PIPES, 1 mM EDTA,

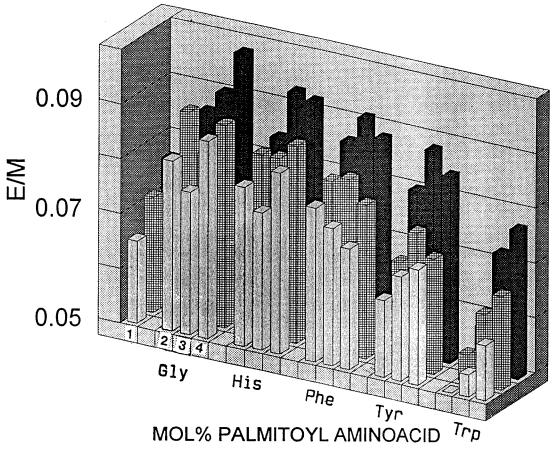

Fig. 6. Fluorescence of 8 mol% PDTAB incorporated into egg PE with 5 mol% G_{D1a}, containing varying concentrations of different palmitoyl amino acids. X-Axis: (1) LUV control (no palmitoyl amino acid present). Concentrations of palmitoyl amino acids are: (2) 0.5 mol%; (3) 2 mol%; (4) 4 mol%. Z-Axis: effect of different concentrations of LUVs: front row (grey dots), 10 μM; middle row (crossed lines), 20 μM; back row (black columns), 50 μM. Y-Axis: excimer/monomer fluorescence emission ratios.

150 mM NaCl with 0.002% NaN_3, pH 7.4 or in 10 mM citrate, 1 mM EDTA, 150 mM NaCl, pH 5.0 by vortexing at 45°C for 30 s. The final lipid concentration was 5 mg/ml. The palmitoyl amino acids were incorporated at varying concentrations up to 4 mol%. The lipid suspension was degassed under vacuum before being loaded into an MC-2 high sensitivity scanning calorimeter (Microcal, Amherst, MA)

Table 3
Antiviral activity

Compound	CPE[a], EC_{50} (μg/ml)	Neutral red[b], EC_{50} (μg/ml)	50% cytotoxicity[c]
Palm-Trp	>3	3	4
Palm-Val	5	4	14
Ribavirin	4	4	164

[a]CPE is a measure of the viral cytopathic effects as determined by the number of surviving cells. The EC_{50} is the concentration of compound required to reduce the viral induced CPE by 50%.

[b]Neutral red is a microscopic assay measuring the integrity of the cell membrane and is also a manifestation of the viral CPE. The EC_{50} of the compound has the same meaning as with CPE.

[c]Cytotoxicity is a measure of the lethal effect of the compound in the absence of virus. The assay is the same as for CPE, but no virus is added.

or a Nano Differential Scanning Calorimeter CSC5100 (Calorimetry Sciences, Provo, UT). A heating scan rate of 37°C/h was generally employed. The observed phase transitions were independent of scan rate between 10 and 60°C/h. The bilayer to hexagonal phase transition was fitted using parameters to describe an equilibrium with a single van 't Hoff enthalpy [17] and the transition temperature reported as that for the fitted curve.

2.11. Tryptophan fluorescence

Fluorescence emission spectra in LUVs of egg PE with 5 mol% G_{D1a} were measured at 37°C, using an SLM Aminco Series II luminescence spectrometer. The liposomes containing palm-Trp had the same lipid composition and size as those used for the viral fusion studies. In addition, LUVs with 4 mol% palm-Trp and 0.1 mol% R18 or with 4 mol% *N*-acetyl-Trp-amide were measured. The liposomes were diluted with either the pH 7.4 or the pH 5.0 buffer used for DSC, so as to minimize inner filter effects. Excitation wavelengths from 280 to 310 nm were used in order to observe the red edge effect. LUV blanks were subtracted from the observed spectra. This had little effect on the emission spectra except for removing the Raman band of water.

2.12. Leakage studies

Aqueous content leakage from liposomes was determined using the ANTS-DPX assay [18]. Films were hydrated with 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl and 10 mM TES at pH 7.4. After passage through a 2.5×2 cm column of Sephadex G-75, the void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis. The fluorescence measurements were performed in 2 ml of buffer containing 10 mM TES, 0.15 M NaCl, 0.1 mM EDTA at pH 7.4 (TES buffer), in a cuvette equilibrated at 37°C. Aliquots of LUVs with and without palmitoyl amino acids were added to the cuvette and then fluorescence was recorded as a function of time using an excitation wavelength of 360 nm and an emission wavelength of 530 nm. The value for 100% leakage was obtained after adding 20 μl of a 10% Triton X-100 solution to the cuvette.

2.13. R18 quenching

LUVs containing egg PE with 5 mol% G_{D1a} and 0.1 mol% R18, with and without palmitoyl amino acids, were made. The R18 fluorescence was measured in 2 ml of HEPES-MES buffer pH 7.4 in a cuvette equilibrated at 37°C, with stirring, to which an amount of LUVs were added. The 100% dequenching value was obtained by adding 20 μl of 10% Triton X-100.

2.14. Pyrene butyl trimethylammonium bromide fluorescence

Pyrene butyl trimethylammonium bromide (PDTAB) was incorporated into films of egg PE with or without 5 mol% G_{D1a}, at concentrations of 5 or 8 mol%. At these concentrations the excimer to monomer ratio (*E*/*M*) was in the range of 0.06–0.09 for LUVs. Palmitoyl amino acids were incorporated into films at concentrations between 0 and 4 mol% and LUVs were made by extrusion. Fluorescence was measured at 37°C in a cuvette containing 2 ml of HEPES-MES buffer pH 7.4, to which aliquots of up to 50 μM LUVs were added. The excitation wavelength was 342 nm. The excimer and monomer fluorescence intensities were measured at wavelengths of 494 nm and 377 nm, respectively.

2.15. Cellular assay for antiviral activity

Antiviral screening assays were performed at the Institute for Antiviral Research (Utah State University, Logan, UT). These assays used the Cantell strain of Sendai virus (parainfluenza type 1) infecting African green monkey cells (cell line MA-104). Two criteria for antiviral activity were used. One is the inhibition of the viral cytopathic effect (CPE). This is done by adding the drug to the wells of microtitre plates containing a cell monolayer. Within 5 min the virus was then added and the plate incubated at 37°C for 3–7 days. Methanol was used to dissolve the palmitoyl amino acid and a solvent control was therefore also measured. The final concentration of methanol was less than 1% and showed no toxicity by itself. In addition, a positive control, rivabirin, was evaluated in parallel. The CPE is read microscopically and neutral red was subsequently added to the media. Cells not damaged by virus take up more dye. The data are expressed as the drug concentration required to observe 50% of maximal effect. Cytotoxicity of the drugs on African green monkey cells is also evaluated in the absence of virus.

3. Results

We have measured by DSC the changes that a number of lipoamino acids produce on the bilayer to hexagonal transition temperature (T_H) of DEPE. A thermogram showing changes occurring with varying mole fractions of palm-Trp is given in Fig. 1A. We find that all of the N^{α}-palmitoylated amino acids raise T_H (Table 2). A linear dependence on mole fraction is observed. At pH 5.0 the palm-Trp had a much smaller effect on T_H (Fig. 1B), raising it by only 41 °C/mol fraction.

The fluorescence emission spectrum of Trp is sensitive to its solvent environment. The palm-Trp exhibits an emission maximum at about 344 or 341 nm at pH 7.4 and pH 5.0, respectively. This indicates that the Trp moiety is within the membrane but positioned close to the interface. The Trp is slightly more buried at pH 5, as expected subsequent to the protonation of the carboxyl group, making the lipoamino acid more hydrophobic.

The dependence of the emission wavelength on the excitation wavelength is thought to be a consequence of the degree to which the solvent can relax during the lifetime of the excited singlet state [19,20]. The palm-Trp exhibits about a 2 nm red edge shift when embedded at 4 mol% in liposomes of egg PE containing 5 mol% of the ganglioside G_{D1a}, which acts as a receptor for Sendai virus in the fusion assays. In contrast, *N*-acetyl-Trp-amide has a maximal emission at 352 nm which does not shift with excitation wavelength even in the presence of the egg PE with 5 mol% G_{D1a} liposomes (Fig. 2). Addition of 0.1 mol% R18 to liposomes composed of 4 mol% palm-Trp with 5 mol% G_{D1a} in egg PE, greatly increases the red edge shift (Fig. 2). Accurate emission maxima for these samples could not be measured above an excitation wavelength of 298 nm because of the low emission intensity and the inner filter effects caused by the presence of R18. Nevertheless, it is clear that R18 markedly enhances the red edge shift effect.

The effect of these lipoamino acids on the fusion of Sendai virus was tested with liposomes composed of egg PE with 5 mol% G_{D1a} (Fig. 3). The ganglioside was incorporated to act as a receptor for Sendai virus. A lipid dilution assay with the fluorescent probe R18 was used. The *N*-palmitoylated amino acids of Ala, Val, Ile, Leu and Gly had no significant effect on viral fusion up to mole fractions as high as 0.2. Palm-His and palm-Phe showed some inhibition at mole fractions higher than 0.04. However, the lipoamino acids palm-Tyr and palm-Trp exhibited marked inhibition of fusion at mol fractions as low as 0.005. Inhibition of fusion was also seen to comparable extents at pH 5 with palm-Trp.

Quenching of R18 fluorescence was studied with both palm-Trp and R18 incorporated into LUVs and this effect was dependent on the amount of this lipoamino acid present in the vesicles. It was not observed with the other palmitoyl amino acids tested (Figs. 4 and 5). It was also observed to a comparable degree at pH 5 with palm-Trp.

E/M obtained from PDTAB fluorescence shows a biphasic behaviour with palm-Trp, first decreasing with small amounts added and then increasing progressively with increasing concentrations (Fig. 6). The other palmitoyl amino acids showed an increase in *E/M* with respect to control vesicles, particularly palm-Gly (Fig. 6); *E/M* was largely independent of concentration in the range studied here for the other palmitoyl aromatic amino acids.

As a complement to the R18 lipid dilution assays and to show the inhibitory effect of palm-Trp independently of the presence of a fluorescent probe, we measured the antiviral activity of palm-Trp by measuring inhibition of viral CPE. We used palm-Val for comparison and ribavirin as a positive control. The results are summarized in Table 3. They confirm that palm-Trp is a potent antiviral agent. However, both palmitoylated amino acids exhibit significant cytotoxicity and a low index of antiviral selectivity.

4. Discussion

Because the R18 lipid dilution assay is one of the most frequently used assays in viral fusion, the observation that palm-Trp quenches the fluorescence of R18, when incorporated into LUVs, deserved particular attention. This quenching is dependent on the concentration of palm-Trp and occurs also at pH 5. Both G_{D1a} and the palmitoyl amino acid are negatively charged, while the R18 is positively charged. One would expect the introduction of increasing amounts of the palm-Trp to increase the amount of negative charge present on the surface of vesicles. This could either (a) create a competitive effect for the interaction of R18 with G_{D1a}, making the effective local concentration of R18 increase around the palm-Trp due to greater affinity, thus increasing self-quenching, or (b) the palm-Trp could be causing defects on the surface which would attract the probe increasing its local concentration. Higher local concentrations of R18 result in lower fluorescence, largely as a result of energy transfer to non-fluorescent dimers of R18 [21].

Since the quantum yield is determined by a number of factors, we wanted to test a different cationic fluorescent membrane probe using a different spectroscopic property. We chose the probe PDTAB, whose excimer concentration is dependent on the frequency of collisions between a PDTAB ground state and excited state. Thus the ratio of excimer to monomer emission (*E/M*) is higher in conditions in which the PDTAB is clustered. A lower *E/M*, indicative of decreased probe concentration, was observed with the addition of small amounts of palm-Trp. This was not the case for the more hydrophobic lipoamino acid palm-Val or for palm-Gly, which increased the *E/M* with increasing concentration nor for the other palmitoyl aromatic amino acids which had little effect. Therefore, it is possible to conclude that the bulky Trp head group causes a disruption on the surface of the vesicles. The probes would accumulate in the defects attracted by the negative charge of the lipoamino acid. With more hydrophobic or less bulky lipoamino acids, this would not happen as they would be able to accommodate more deeply into the bilayer, causing less disruption on the surface. At higher palm-Trp concentrations there are more defects for the PDTAB to be diluted into and therefore the extent of quenching decreases.

Palmitoylated amino acids act very differently than free fatty acids with regard to lipid polymorphism. While palmitoylated amino acids significantly stabilize the bilayer towards non-bilayer phase formation at physiological as well as acidic pH, saturated fatty

acids have little or no effect at pH 7.4 and can even decrease T_H at lower pH [22]. This would be consistent with palmitoyl amino acids taking up a place in the head group region and increasing interfacial lateral pressure.

The observed increase in the red edge shift in the emission spectrum of palm-Trp by R18 is also consistent with the interaction between these two fluorescent probes. It also explains the quenching of the R18 fluorescence since the lipoamino acid could cause the R18 to be non-randomly distributed. The palm-Trp itself shows a small red edge shift, indicating a slower relaxation of dipoles surrounding the excited singlet state as would occur in a more rigid membrane environment. However, the red edge shift is small, consistent with the observation that Trp in membranes containing PE does not form hydrogen bonds between the lipid and the Trp, the latter having a large degree of motional freedom [23].

The complications of the R18 assay for viral fusion in the presence of palm-Trp indicate an additional caveat in the application of the R18 viral fusion assay in the presence of Trp containing peptides or proteins. This complication does not affect the apparent R18 dilution for the non-aromatic amino acids and the effects with palm-Tyr, palm-Phe or low concentrations of palm-Trp are small. The lipoamino acids which inhibit viral fusion most effectively are palm-Tyr and palm-Trp. But the fact that palm-Trp remains inhibitory at pH 5, where it is also much less effective in promoting a non-bilayer phase in the lipid, indicates that other factors are also of importance. In addition, palm-His and palm-Leu are effective in raising T_H, but have little or no inhibitory activity against viral fusion. Therefore, compounds which partition into the membrane and are effective in raising T_H, are not necessarily good inhibitors of viral fusion and a quantitative relationship cannot be established only on this basis.

As a verification that palm-Trp has antiviral activity, we tested this directly in a cell system by measuring the inhibition of viral-induced cytopathic effects. This lipoamino acid exhibited potent antiviral activity (Table 3). However, the palmitoyl amino acids also exhibit high toxicity to cells, limiting their therapeutic applications. This is likely related to their strong detergent like action [1] and their ability to promote positive monolayer curvature in membranes (Table 2).

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