Orientation of the Pore-Forming Peptide GALA in POPC Vesicles Determined by a BODIPY-Avidin/Biotin Binding Assay

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ABSTRACT We determined the orientation of a biotinylated version of the pore-forming peptide GALA (WEAALAEALAE-ALAEHLAEALAEALEALAA) at pH 5.0 in large unilamellar phosphatidylcholine vesicles, using the enhancement of BODIPY-avidin fluorescence subsequent to its irreversible binding to a biotin moiety. GALA and its variants were biotinylated at the N- or C-terminus. BODIPY-avidin was either added externally or was pre-encapsulated in vesicles to assess the fraction of liposome-bound biotinylated GALA that exposed its labeled terminus to the external or internal side of the bilayer, respectively. Under conditions where most of the membrane-bound peptides were involved in transmembrane aggregates and formed aqueous pores (at a lipid/bound peptide molar ratio of 2500/1), the head-to-tail (N- to C-terminus) orientation of the membrane-inserted peptides was such that ¾ of the peptides exposed their N-terminus on the inside of the vesicle and their C-terminus on the outside. Under conditions resulting in reduced pore formation (at higher lipid/peptide molar ratios), we observed an increase in the fraction of GALA termini exposed to the outside of the vesicle. These results are consistent with a model (Parente et al., Biochemistry, 29:8720, 1990) that requires a critical number of peptides (M) in an aggregate to form a transbilayer structure. When the peptides form an aggregate of size i, with $i < M = 4$ to 6, the orientation of the peptides is mostly parallel to the membrane surface, such that both termini of the biotinylated peptide are exposed to external BODIPY-avidin. This BODIPY-avidin/biotin binding assay should be useful to determine the orientation of other membrane-interacting molecules.

INTRODUCTION

Many organisms, including fungi, insects, amphibians and humans, secrete peptides that exhibit antibacterial, hemolytic, and tumoricidal activities by virtue of their capacity to adopt an amphipathic α-helical conformation when they bind to lipid membranes. These peptides kill a target cell by inducing permeabilization, disruption, or disintegration of its cytoplasmic membrane (Cornut et al., 1993). It is generally accepted that they exert their cytotoxic activities directly on the lipid matrix of the cell membranes (Boman et al., 1994).

For most of these natural toxins, including magainins, pardaxins, δ-toxin, melittin, and alamethicin, it has been proposed that they permeabilize model lipid membranes by forming transient or permanent multimeric pores (Schwarz and Arbuzova, 1995; Matsuzaki et al., 1995, 1996, 1997a; He et al., 1995, 1996; Ludtke et al., 1996; Rapaport et al., 1996; Rex and Schwarz, 1998). However, particularly in the case of melittin and cecropins, other processes have also been suggested to explain bilayer permeabilization, such as the perturbation of the lipid packing induced by a high density of surface-adsorbed peptides (Vogel and Jähnig, 1986; Benachir and Lafleur, 1995; Terwilliger et al., 1982; Gazit et al., 1995). In that case, bilayer destabilization can arise from the induction of a negative curvature strain that increases the lipid tendency to adopt inverted phases, or from a positive curvature strain that causes an expansion of the bilayer interface (Terwilliger et al., 1982; Batenburg and De Kriujff, 1988; Tytler et al., 1993; Epand et al., 1995).

Ion pores formed by natural amphipathic α-helical peptides are generally modeled as bundles of aligned α-helices that span the membrane with their hydrophilic sides lining the pore lumen (Sansom, 1991; Cafiso, 1994; Shai, 1994; He et al., 1996). Investigations with analogs of cytotoxic peptides clarify the importance of peptide charge and the role of particular amino acids or arrays of amino acids in the

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Abbreviations used: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; Biotin-X/SE, 6-((biotinoyl)amino)caproic acid succinimidyl ester; BODIPY, dipyrromethene boron difluoride; $C_{12}E_{8,}$ dodecyloctaethylene glycol monoether; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DPX, p-xylene-bis-pyridinium bromide; GALA $29C_C$, WEAALAEALAEALA-EHLAEALAEALEACA; GALA 30, peptide with the sequence WEAAL-AEALAEALAEHLAEALAEALEALAA; GALA $31C_C$, WEAALAEAL-AEALAEHLAEALAEALEALACG; GALA $31C_N$, CWEAALAEALAE-ALAEHLAEALAEALEALAA; GALA (N)1, peptide GALA 30 labeled with 6-((biotinoyl)amino) caproic acid, succinimidyl ester (Biotin-X/SE) on the primary amine of the N-terminus; GALA(N)2, GALA(C)2, and GALA(C)3 are peptide GALA $31C_N$, GALA $29C_C$ and GALA $31C_C$, respectively, labeled with *N*-(biotinoyl)*N′*-(iodoacetyl)ethylenediamine on the cysteine; LUV, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; REV, reverse-phase evaporation vesicles; TES, N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid.

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conformation, membrane-binding affinity, and pore-forming abilities of these toxins (Shai, 1994; Brachais et al., 1995; Matsuzaki et al., 1997b, Kaduk et al., 1997). The importance of the helix length, hydrophobicity, and hydrophobic moment on the formation, structure, and function of ion channels has also been investigated with model peptides such as GALA (Subbarao et al., 1987; Parente et al., 1990a,b; Nicol et al., 1996), LA-E-δM2 peptides (Tomich, 1993), LS and LSU peptides (Lear et al., 1988, De Grado et al., 1989), or peptide P (Spach et al., 1985).

The synthetic peptide GALA (WEAALAEALAEALAE-HLAEALAEALEALAA), named GALA 30 in this study, has been designed to undergo a pH-sensitive conformational change (Subbarao et al., 1987) that results in about a 100-fold increase in the apparent egg yolk lecithin (egg PC) bilayer/water partition coefficient of the peptide at acidic pH (Parente et al., 1990a). The membrane-interacting properties of GALA have been extensively documented (Subbarao et al., 1987, 1988; Parente et al., 1988, 1990a,b; Goormaghtigh et al., 1991; Fattal et al., 1994; Nicol et al., 1996). In aqueous solution at neutral pH, the formation of an α-helix is not favored because of the electrostatic repulsions between the glutamic acid residues. At pH 5.0, the neutralization of these residues promotes the formation of an amphipathic α-helix and GALA binding to egg PC bilayers (Subbarao et al., 1987; Parente et al., 1990a). In the optimal pH range of 5.0 and below, GALA induces the leakage of egg PC liposome-encapsulated ANTS/DPX and the rapid flip-flop of membrane phospholipids at very low ratios of membrane-bound peptides per vesicle (Subbarao et al., 1987; Parente et al., 1990b; Fattal et al., 1994). In the case of neutral egg PC or POPC liposomes at pH 5.0, analysis by a mathematical model of the percent ANTS/DPX leakage (Parente et al., 1990b; Nicol et al., 1996) and the percent flip-flop of phospholipids (Fattal et al., 1994) as a function of lipid/peptide molar ratio predicted that the peptide aggregates in a quasi-irreversible fashion in the bilayer. When an aggregate reaches a critical size of 10 ± 2 monomers, it forms a pore that induces ANTS/DPX leakage and rapid phospholipid flip-flop (Parente et al., 1990b; Fattal et al., 1994; Nicol et al., 1996). The addition of the anionic phospholipid POPG (up to 66.6 mol%) to POPC in the vesicles composition does not modify the peptide efficiency to induce ANTS/DPX leakage at pH 5.0 (Nicol et al., 1996). However, the presence of membrane cholesterol (Nicol et al., 1996) and modifications in the structure of the phospholipids alter pore formation by GALA. For all liposome compositions tested, we have shown that no leakage of a vesicle-encapsulated fluorescent dextran 3000 MW occurs at peptide concentrations inferior or equal to the minimum concentration necessary for complete ANTS/DPX leakage. Given the Stokes radius of the dextran and the evolution of the pore size with the number of peptides forming it, this result means that the pores are restrained to less than 14 peptides in the range of lipid/peptide ratios where ANTS/DPX leakage is monitored. However, to obtain a more thorough description of the GALA pore, structural information is required.

The position of individual peptides in a membrane at equilibrium can be detected by spectroscopic techniques such as solid-state NMR (Bechinger et al., 1991; Smith et al., 1994), oriented circular dichroism (Wu et al., 1990; Ludtke et al., 1994), attenuated total reflection/Fourier transform infrared (Goormaghtigh et al., 1991; Lüneberg et al., 1995), fluorescence (Matsuzaki et al., 1994) or EPR (Barranger-Mathys and Cafiso, 1996). These methods can reveal the degree of peptide insertion in the membrane, depending on peptide sequence, lipid/peptide molar ratio, membrane composition, ionic strength, pH, and level of lipid hydration. Although the position of membrane-bound peptides relative to the bilayer plane has been extensively studied, the head-to-tail (N- to C-terminus) orientation of the membrane-inserted peptides has not been as carefully defined. The most widely assumed model, which is in accord with the model for channel-forming proteins, has all of the helices in a pore arranged with the helix dipoles pointing in the same direction (parallel) (Sansom, 1991). For voltage-dependent ion channels, the alignment of the dipoles, in response to the presence of a transmembrane potential, might stabilize the pore structure. For some peptide sequences, the parallel assembly might also stabilize the high energy packing of peptides by increasing the favorable intermolecular interactions. However, the rationale for a parallel organization is less evident in the case of synthetic peptides with a simplified sequence such as GALA.

Fourier transform infrared studies on oriented egg PC multibilayers showed that, at relatively low lipid/peptide molar ratios (under conditions of extensive pore formation), the principal axis of GALA amphipathic helix is oriented perpendicular to the bilayer surface at pH 5.0 (Goormaghtigh et al., 1991). In the present study, we determined the surface-adsorbed or transbilayer orientation of the peptide as a function of the lipid/peptide molar ratio, and we elucidated the head-to-tail (N- to C-terminus) orientation of the transbilayer peptides in POPC vesicles at pH 5.0, by using a modification of a BODIPY-avidin/biotin binding assay (Emans et al., 1995).

The avidin/biotin system was used by Qiu et al. (1994, 1996) and Slatin et al. (1994) to show that the gating of colicin Ia, a bacteriocidal protein that forms a voltage-sensitive channel, is associated with the translocation across the membrane of a segment of the protein of at least 68 residues. In these studies, site-directed mutagenesis was followed by biotinylation of a series of unique sites on colicin Ia. Biotinylated residues that translocated or inserted upon channel opening in planar lipid membranes were identified by streptavidin binding to them on the *cis* side (side of colicin Ia addition) in the closed state and streptavidin binding impeding the channel opening. Residues that translocated upon channel closing were identified by streptavidin binding to them on the *trans* side in the open state. In this case, streptavidin binding resulted in interference with channel closing.

Recently, Emans et al. (1995) applied the enhanced fluorescence of a BODIPY-modified avidin upon its binding to biotin to develop an in vivo endosome fusion assay. We modified this BODIPY-avidin/biotin binding assay to resolve the orientation of GALA in POPC liposomes at pH 5.0. We attached biotin to either termini of GALA and used its high affinity binding to a BODIPY-avidin to determine the location of the labeled terminus of the membrane-bound peptide, inside or outside the vesicle. Under conditions where all membrane-bound GALA are involved in transmembrane aggregates (at a lipid/peptide molar ratio of 2500/1), ¾ of the peptides expose their N-termini to the inside of the vesicle and their C-termini to the outside, whereas the rest of the peptides are oriented in the opposite direction. Under conditions of reduced pore formation (at high lipid/peptide molar ratios), there is an increase of the external exposure of both ends of the peptide. Based upon model calculations, we conclude that the surface aggregate formed by GALA must attain a critical number of between 4 to 6 peptides before it inserts into the bilayer.

MATERIALS AND METHODS

Reagents

POPC in chloroform was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). ANTS, DPX, Biotin-X/SE) and *N*-(biotinoyl)*N*′-(iodoacetyl) ethylenediamine were purchased from Molecular Probes (Eugene, OR). Neutralite BODIPY-avidin (Biwersi et al., 1996) was a generous gift from N. Emans, J. Biwersi, and A. S. Verkman (University of California, San Francisco). D-Biotin was obtained from SIGMA (St. Louis, MO).

Vesicle preparation and sizing

Reverse-phase evaporation vesicles (REV) were prepared as described previously (Szoka and Papahadjopoulos, 1978) in (i) 5 mM TES, 100 mM KCl (pH 7.0) or in (ii) 5 mM TES, 12.5 mM ANTS, 45 mM DPX, 20 mM KCl (pH 7.0). When BODIPY-avidin was encapsulated, the liposomes were prepared in a 5 mM TES, 100 mM KCl (pH 7.0) buffer containing 41.5 μM BODIPY-avidin, and the sonication time of the REV preparation was 20 s. The vesicles were extruded five times through a 0.1-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) with a hand-held extrusion device (Avestin, Ottawa, Canada). When ANTS/DPX or BODIPY-avidin were encapsulated in liposomes, a Sepharose 4-B column (1 × 16 cm) was used to separate vesicles from unencapsulated material with the elution buffer 5 mM TES, 100 mM KCl (pH 7.0). Lipid phosphorus was determined by a modification of the Bartlett (1959) method. The size distribution of the POPC liposomes was determined by quasi-elastic light scattering with a Coulter apparatus N4 and was typically characterized by a number average of 115 nm in diameter and a standard deviation of 20 nm.

Synthesis and biotinylation of GALA peptides

Peptides GALA 30, GALA 29C_C, and GALA 31C_C were synthesized with an Applied Biosystems 430A peptide synthesizer using standard scale F-moc chemistry and a procedure with double coupling of amino acids. The synthesis of GALA 31C_N was carried out by coupling a cysteine to the resin-attached GALA 30 using small scale F-moc chemistry. Peptides were cleaved from the resin (200 mg) with neat TFA (5 mL) in the presence of water (250 μL), phenol (375 mg), ethanedithiol (125 μL), and thioanisole (250 μL). The free peptides were separated from the resin by filtration and then precipitated from solution by addition of ether. The dried precipitates were dissolved in a 5 mM TES, pH 8.0 solution containing urea (6 M), chromatographed on a Sephadex G-10 column (1 × 20 cm) with the elution buffer 5 mM TES, pH 8.0, and the peptide-containing fractions were lyophilized. The resulting lyophilized powders were dissolved in a 5 mM TES, pH 8.0 solution containing urea (6 M) and purified by reverse-phase high performance liquid chromatography (HPLC) on a Perkin-Elmer instrument equipped with a Rainin Dynamax preparative C_{18} column, using the following conditions: solvent system A, 0.1% TFA in water; solvent system B, 0.1% TFA in acetonitrile; gradient, 0–48% B in 5 min, followed by 48–72% B in 120 min, and 72–100% in 5 min, flow rate 1 mL/min. GALA 30, GALA 31C_C and GALA 31C_N eluted at 63–64% B. In the case of GALA 29C_C, the slowest part of the gradient was 43–67% B instead of 48–72% B, and the peptide eluted at 58% B. Mass spectrometry was used in the positive mode to characterize the molecular mass of the purified peptides. Mass spectra were obtained on a PerSeptive Biosystems (Framingham, MA) Voyager linear time-of-flight mass spectrometer. Sample desorption/ionization was accomplished by matrix-assisted laser desorption ionization (MALDI) (Hillenkamp et al., 1991) with a 337-nm nitrogen laser, and data were recorded with a 500-MHz digitizer. Samples were prepared by mixing an aqueous solution of peptide (1:4) with a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 60% ethanol/40% acetonitrile and allowing a one μL droplet of the mixture to air-dry on the sample plate. The instrument was externally calibrated using standard peptides (bradykinin and ACTH 18–39), and the maximum error of mass measurement of the analyte ions was estimated to be <2 Da. Based on the singly-charged molecular ion $(M + H)^+$ and the sodium ion $(M + Na)^+$, the molecular masses of GALA 30, GALA 29C_C, GALA 31C_C, and GALA 31C_N are 3032.4, 2950.3, 3120.5, and 3134.3 Da, respectively. Based on the peptide sequences, the expected masses are 3032, 2951, 3121, and 3135 Da, respectively.

We labeled the peptide GALA 30 and its variants with a biotin reagent at the C- or N-terminus to determine the peptide orientation in POPC liposomes at pH 5.0. For biotinylation of GALA 30 (yielding GALA(N)1), the peptide was dissolved in a mixture of 0.1 M sodium bicarbonate pH 8.5 solution/ethyl alcohol 1/1 (v/v). Biotin-X/SE in DMF was added to the stirred peptide solution at a biotin/peptide molar ratio of 6. After 3 h, glycine was added to the solution at a glycine/biotin molar ratio of 8/1 to stop the reaction. For biotinylation of GALA 29C_C, GALA 31C_C, and GALA 31C_N with *N*-(biotinoyl)*N*′-(iodoacetyl)ethylenediamine (yielding GALA(C)2, GALA(C)3, and GALA(N)2, respectively), the peptides were dissolved in a 50 mM TES, pH 8.3 solution. *N*-(biotinoyl)*N*′-(iodoacetyl) ethylenediamine in a mixture of DMF/DMSO 1/1 (v/v) was added to the stirred peptide solutions at a biotin/peptide molar ratio of 4.5. After 90 min of continuous stirring in the dark, β-mercaptoethanol was added to the solutions at a β-mercaptoethanol/biotin molar ratio of 8/1 to stop the reactions. After biotinylation, the reaction mixtures were chromatographed on a Sephadex G-10 column (1 × 20 cm) with the elution buffer 5 mM TES, pH 8.0 to separate biotin-labeled and -unlabeled GALA from the low molecular weight byproducts of the coupling reaction. Separation of the biotin-labeled GALA from the unlabeled peptide was carried out by reverse phase HPLC on a Perkin-Elmer instrument equipped with a Dynamax C_{18} column, using the following conditions: solvent system A, 0.1% TFA in water; solvent system B, 0.1% TFA in acetonitrile; gradient, 0–54% B in 5 min, followed by 54–61% B in 40 min, 61–75% in 105 min, and 75–100% in 5 min, flow rate 1 mL/min. GALA(N)1 eluted at 68% B, whereas GALA(C)3 and GALA(N)2 eluted at 66% B. In the case of GALA(C)2 the slowest part of the gradient was 56–70% B, instead of 61–75% B, and the peptide eluted at 62% B. Similar to the unlabeled peptides, mass spectrometry was used in the positive mode to characterize the molecular mass of the purified biotinylated peptides. Based on the singly-charged molecular ion $(M + H)^+$ and the sodium ion $(M + Na)^+$, the molecular masses of GALA(N)1, GALA(N)2, GALA(C)2, and GALA(C)3 are 3371.3, 3462.7, 3277.6, and 3448.8 Da, respectively. Based on the peptide sequences, the expected masses are 3372 (3032 + 340), 3462 (3135 + 327), 3278 (2951 + 327), and 3448 (3121 + 327), respectively.

Fluorescence measurements

Measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) under the control of an IBM PC equipped with DM3000 software, using a 150-W xenon light source, as previously described (Parente et al., 1990a).

Measurements of ANTS/DPX leakage

The ANTS/DPX assay (Ellens et al., 1984) was used to measure the ability of the GALA peptides to induce leakage of ANTS/DPX pre-encapsulated in POPC liposomes. Details of this assay can be found in Nicol et al. (1996). To initiate a leakage experiment, a few microliters of a 5 mM TES, 50 mM KCl, pH 8.0 solution containing a GALA peptide was added to a stirred POPC vesicles suspension (0.1 mM lipid) at 20°C in a buffer at pH 5.0 containing 5 mM sodium acetate and 100 mM KCl, to achieve the stated lipid/peptide molar ratio.

BODIPY-avidin/biotin binding assays

The BODIPY-avidin/biotin binding assay developed by Emans et al. (1995) is based on the enhancement of the green fluorescence of a BODIPY-modified avidin upon its quasi-irreversible binding to d-biotin. To determine GALA surface-adsorbed or transbilayer orientation as well as the head-to-tail (N- to C-terminus) orientation of the transbilayer peptides in POPC liposomes at pH 5.0, we used the differential enhancement of BODIPY-avidin fluorescence upon its binding to d-biotin or to a biotinylated GALA peptide. In all measurements, we monitored the fluorescence of BODIPY-avidin at $\lambda = 510$ nm (2.5 mm slits), with the sample being irradiated at $\lambda = 480$ nm (1.25 mm slits).

Determination of external accessibility of biotinylated peptide by external addition of BODIPY-avidin

The purpose of the assay was to estimate *R*, the fraction of POPC liposome-bound biotinylated GALA that expose their labeled termini (N or C) on the external side of the vesicles at pH 5.0. The assay is illustrated in Fig. 1 and required the measurement of three fluorescence intensities at the wavelength corresponding to maximum emission of BODIPY ($\lambda = 510$ nm). Based on these intensities and a refinement procedure, we could determine the fraction of POPC liposome-bound biotinylated GALA that bound to BODIPY-avidin after addition of the protein to the liposome/peptide solution at pH 5.0, which corresponded to *R*.

The measurement of the first fluorescence intensity required for the computation of *R*, denoted "external layer exposure," started with the

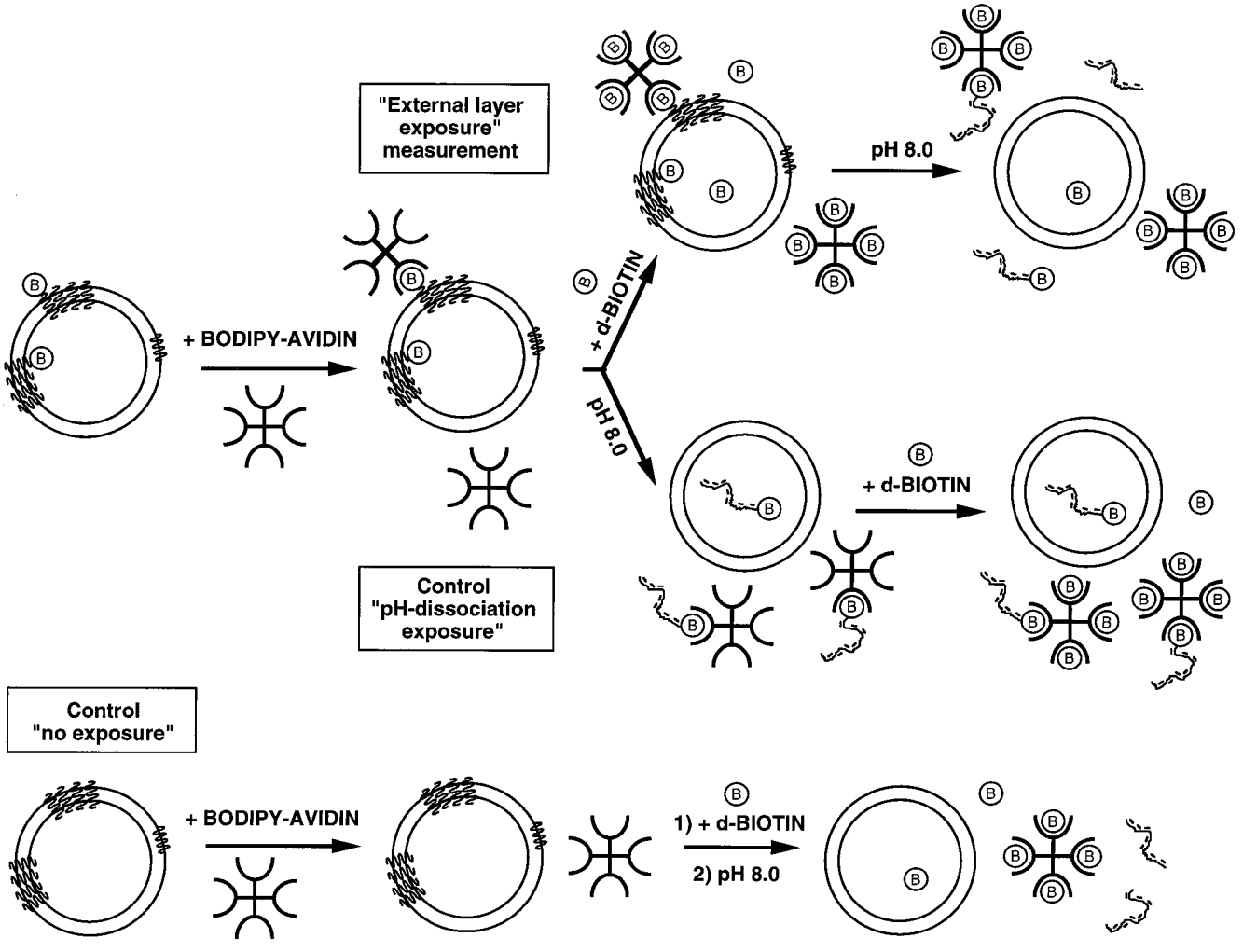


FIGURE 1 Illustration of the three independent measurements performed to obtain the three fluorescence intensities F_0, F, and $F_{<100}$ required to calculate r ($= R/Z$).

addition of an aliquot of a 3 mM TES, 50 mM KCl (pH 8.0) buffer containing a mixture of GALA 30 and a biotinylated GALA (typically at a molar ratio of 19/1) to a stirred 2.5 mL vesicle suspension (2.5 mM POPC) at 20°C in a 25 mM TES, 80 mM KCl (pH 5.0) buffer. With the lipid concentration used (2.5 mM), all the added peptide bound to the vesicles (Nicol et al., 1996). Peptides aggregated in the liposome bilayer to form pores. After 15 min, an aliquot of a BODIPY-avidin solution containing 3 mM TES and 100 mM KCl (pH 7.5) was added to the peptide/liposome solution, typically at a BODIPY-avidin/biotinylated peptide molar ratio of 2, unless otherwise specified. At this point of the experiment, the pH of the solution remained at 5.0. Every biotinylated GALA that exposed its labeled terminus to the external side of the vesicle bound to a BODIPY-avidin. After 15 min, d-biotin was added to the solution at a d-biotin/BODIPY-avidin molar ratio of 16/1. Consequently, d-biotin saturated the BODIPY-avidin binding sites that were unoccupied by the biotinylated GALA. Finally, the pH of the solution was elevated to 8.0 by adding 200 μL of a 0.5 M TES pH 8.5 solution, which resulted in dissociation of GALA from the liposomes (Parente et al., 1990b). The fluorescence intensity, F, was recorded 3 min after the pH increase.

For the measurement of the second fluorescence intensity required to compute the percent exposure, denoted control "pH-dissociation exposure," the addition of the peptide mixture to the liposome suspension and the subsequent addition of BODIPY-avidin were identical to the first measurement. Fifteen minutes after the addition of BODIPY-avidin, the pH of the solution was elevated to 8.0 by adding 200 μL of a 0.5 M TES pH 8.5 solution, which dissociated the peptides from the vesicles. At this point, the biotinylated peptides that were dissociated to the external solution (as opposed to the ones that were released on the inside of the vesicles) bound to a BODIPY-avidin binding site. After ~3 min, d-biotin was added to the solution to establish a d-biotin/BODIPY-avidin molar ratio of 16/1. Again, d-biotin saturated the unoccupied BODIPY-avidin binding sites. The fluorescence intensity, $F_{<100}$, was recorded 3 min after the addition of d-biotin.

For the measurement of the third fluorescence intensity needed for the computation, denoted control "no exposure," the protocol was identical to that of the external layer exposure measurement, except that the peptide mixture consisted exclusively of nonbiotinylated GALA 30 at the same total peptide concentration. The fluorescence intensity, F_0, was recorded 3 min after the pH elevation.

In Eq. 1, let F_d be the BODIPY-avidin fluorescence at pH 8.0 when all the BODIPY-avidin molecules have their binding sites saturated with d-biotin.

$$F_0 = F_d + A, \tag{1}$$

in which A is a constant term arising essentially from the contribution of the liposomes and the buffer. Let F_{Gd} denote the BODIPY-avidin fluorescence at pH 8.0 that would arise if all BODIPY-avidin molecules had three of their four binding sites occupied by d-biotin and the last one occupied by a biotinylated GALA. Let X be the fraction of BODIPY-avidin at the end of the control pH-dissociation exposure that had its binding sites occupied in such a pattern (3 d-biotins/1 biotinylated GALA). If we assume that, because of the excess of BODIPY-avidin, each BODIPY-avidin molecule bound, at most, one biotinylated GALA in the control pH-dissociation exposure, then

$$F_{<100} = XF_{Gd} + (1 - X)F_d + A, \tag{2}$$

where F denotes the measured fluorescence intensity at the end of the control pH-dissociation exposure.

Let r denote the fraction of biotinylated GALA bound to BODIPY-avidin in the external layer exposure measurement (R) divided by the fraction of biotinylated GALA bound to BODIPY-avidin in the control pH-dissociation exposure (Z, the labeled peptides that were dissociated on the outside of the liposomes after the pH increase to 8.0). If we assume that each BODIPY-avidin molecule bound, at most, one biotinylated GALA in the external layer exposure measurement, then

$$F = rXF_{Gd} + (1 - rX)F_d + A, \tag{3}$$

where F denotes the measured fluorescence intensity at the end of the external layer exposure measurement. The combination of Eqs. 1–3 yields

$$r = (F_0 - F)/(F_0 - F_{<100}). \tag{4}$$

Ultimately, we wished to determine R, the fraction of membrane-bound N- (or C-) terminus biotinylated GALA that exposed its N- (or C-) terminus on the outside of the POPC liposome at pH 5.0 during the external layer exposure measurement and bound via its biotin moiety to BODIPY-avidin added to the liposome/peptide solution. To determine R, we needed to determine Z, the fraction of biotinylated peptides released on the outside of the liposomes after the pH elevation to 8.0 in the control pH-dissociation exposure.

To determine Z, we performed two additional controls similar to the pH-dissociation exposure and no exposure mentioned above, except that the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) was added at the end of both protocols, to lyse the vesicles. The final fluorescence intensities were $F_{Z<100}$ and F_{Z0}, respectively.

In a third measurement, denoted control "total exposure," the procedure was identical to that of the control pH-dissociation exposure, except that the addition of $C_{12}E_8$ preceded that of d-biotin. This treatment generated the complete release of the biotinylated peptides in solution before the addition of d-biotin. Thus, all the biotinylated peptides bound to BODIPY-avidin. When d-biotin was added, it saturated the remaining BODIPY-avidin binding sites not occupied by the biotinylated GALA. The fluorescence intensity at the end of the control total exposure was F_{Z100}. The fraction of biotinylated peptides released on the outside of the POPC vesicles after the pH elevation to 8.0 in the control pH-dissociation exposure, Z, could be determined by

$$Z = (F_{Z0} - F_{Z<100})/(F_{Z0} - F_{Z100}). \tag{5}$$

Because $r = R/Z$ by definition, R could be determined directly from r and Z, using

$$R = r * Z. \tag{6}$$

When the C-terminus-labeled GALAs (GALA(C)s) were studied, the difference ($F_{Z0} - F_{Z100}$) at pH 8.0 was too small. To accurately determine Z in this case, the pH was decreased to 5.0 at the end of the three measurements. This increased the difference between F_{Z100} and F_{Z0} and consequently enhanced the accuracy of the determination of Z.

Determination of internal accessibility of biotinylated peptide by pre-encapsulation of BODIPY-avidin

In this assay, BODIPY-avidin was pre-encapsulated in POPC liposomes. The assay required the measurement of three fluorescence intensities at the wavelength corresponding to maximum emission of BODIPY (λ = 510 nm). From these intensities, we could determine R', the fraction of liposome-bound N- (or C-) terminus biotinylated GALA that bound at pH 5.0 to pre-encapsulated BODIPY-avidin. R' corresponded to the fraction of bilayer-bound peptides exposing their N- (or C-) termini on the internal side of the vesicles.

The procedure with the N-terminus biotinylated peptides GALA(N), (GALA(N)1, and GALA(N)2) was as follows. To begin the measurement leading to the first fluorescence intensity, denoted "internal layer exposure," an aliquot of a mixture of GALA 30 and GALA(N) (at a molar ratio of 14/1) in a 3 mM TES, 50 mM KCl (pH 8.0) buffer was added to the stirred 2.5 mL suspension of vesicles containing BODIPY-avidin (0.65 mM POPC) at 20°C, in a 25 mM TES, 80 mM KCl (pH 5.0) buffer. A lipid/total membrane-bound peptide molar ratio of 2500/1 was used, which corresponded to a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8/1. At the lipid concentration used (0.65 mM), 80% of the peptide bound to the vesicles (Nicol et al., 1996). Every membrane-bound biotinylated GALA that exposed its biotinylated terminus on the internal side of the vesicle bound to a BODIPY-avidin. After 15 min, d-biotin was added

to the solution at a d-biotin/BODIPY-avidin molar ratio of 64/1. A consequence of the formation of pores by GALA in POPC vesicles at pH 5.0 is that d-biotin (244.3 MW) could easily translocate into the liposome and saturate the BODIPY-avidin binding sites not occupied by the biotinylated GALA. The pH of the solution was then elevated to 8.0 by adding 200 μL of a 0.5 M TES pH = 8.5 solution, and the vesicles were lysed with the detergent $C_{12}E_8$. The final fluorescence intensity, F', was recorded 3 min after lysing the vesicles.

For the fluorescence measurement required to obtain the second intensity, denoted control total exposure, the step of addition of the peptide mixture to the liposome suspension was identical to the first fluorescence measurement. Fifteen minutes after the peptide addition, the pH of the solution was elevated to 8.0 by adding 200 μL of a 0.5 M TES, pH = 8.5 solution, and the vesicles were lysed. At this point, each biotinylated GALA bound to a BODIPY-avidin binding site. After ~3 min, d-biotin was added to the solution at a d-biotin/BODIPY-avidin molar ratio of 64/1. Again, d-biotin bound to the BODIPY-avidin binding sites that were not occupied by the biotinylated GALA. The final fluorescence intensity, F'_{100}, was recorded 3 min after the addition of d-biotin.

For the third fluorescence measurement required for the computation of R', denoted control no exposure, the protocol was identical to that used to obtain the first intensity, except that the peptide mixture consisted exclusively of nonbiotinylated GALA 30 at the same total peptide concentration. The final fluorescence intensity, F'_0, was recorded 3 min after the pH elevation.

Let R'' be the fraction of N-terminus biotinylated GALA(N) that exposed its N-terminus on the internal side of the POPC liposomes in the internal layer exposure experiment at pH 5.0 and bound, via its biotin tag, to pre-encapsulated BODIPY-avidin. As in the determination of r, i.e., addition of BODIPY-avidin on the outside of the liposomes, R'' could be determined by using an equation similar to Eq. 4,

$$R'' = (F'_0 - F')/(F'_0 - F'_{100}). \tag{7}$$

The refinement used to determine R from r was not needed in this assay, because the addition of $C_{12}E_8$ and the pH elevation released all the peptides in solution in the control total exposure before addition of d-biotin. Let R' be the fraction of membrane-bound GALA(N) that displayed its N-terminus on the internal side of the liposomes. R' could be determined from R'', because the fraction of membrane-bound peptide, Y (= 0.8), is known (Nicol et al., 1996):

$$R' = R''/Y. \tag{8}$$

When the C-terminus labeled GALA(C)3 was studied, the pH was not elevated to 8.0 in the three experiments, because $(F'_0 - F'_{100})$ was too small to accurately determine R''. Instead, the pH was kept at 5.0 where $(F'_0 - F'_{100})$ was higher. However, the equations to determine R'' and R' were identical to those used for the N-terminus biotinylated peptides GALA(N).

Simultaneous determination of internal and external accessibility of biotinylated peptide by pre-encapsulation and external addition of BODIPY-avidin

In this assay, we determined the fraction of biotinylated peptide that bound at pH 5.0 to BODIPY-avidin present on both sides of the POPC bilayer. The protocol described above to determine R'' was modified by the addition of BODIPY-avidin in solution in the three measurements, 15 min after the addition of the peptide mixture, at a BODIPY-avidin binding site/biotinylated GALA molar ratio of 6/1. Fifteen minutes later, d-biotin was added to the solution in the control no exposure and the "internal + external sides exposure" measurement (that replaces the internal exposure measurement), and the pH was elevated to 8.0 for the control total exposure. The rest of the three measurements were identical to that mentioned above for the determination of R''. The three final fluorescence intensities were denoted F'_0, F', and F'_{100}, in a similar fashion to that used for the determination of R''. Using Eq. 7, we could determine R_{total} (instead of R''), the fraction of biotinylated GALA that bound to BODIPY-avidin at pH 5.0 either on the internal or on the external side of the vesicles during the internal + external sides exposure experiment. The fraction of labeled peptide whose biotin moiety was inaccessible to BODIPY-avidin binding, in the time-frame of the experiment, was given by $(1 - R_{total})$.

Theoretical analysis of the formation of transmembrane aggregates and pores

Our model of bilayer permeabilization by GALA assumes that 1) in the presence of liposomes the peptides bind and become incorporated into the bilayer of the vesicles, and 2) peptide aggregation occurs within the membrane. When an aggregate has reached a critical size within a bilayer, i.e., it consists of M peptides, a pore is created and complete leakage of liposome-encapsulated molecules occurs. The size of the pore depends on the number of peptides forming it and dictates the upper bound on the size and shape of molecules that can leak.

Here, we present a few selected equations derived from the model. A more complete description can be found in Rapaport et al. (1996) and in Nicol et al. (1996).

Adapting for surface aggregation of the membrane-bound GALA, the general scheme, as treated in Bentz and Nir (1981) and Nir et al. (1983), gives

$$X_1 + X_1 \underset{D_{11}}{\overset{C_{11}}{\longleftrightarrow}} X_2 \qquad X_i + X_j \underset{D_{ij}}{\overset{C_{ij}}{\longleftrightarrow}} X_{i+j} \tag{9}$$

where X_i are molar (or surface) concentrations of aggregates of order i. We have used the same simplifications made by Smoluchowski (1917) and by Blatz and Tobolsky (1945), i.e., that $C_{ij} = C$ and $D_{ij} = D$ for all i and j. The fraction of liposome-encapsulated molecules that has leaked at the plateau level is denoted by L. Because of the dependence of the encapsulated volume on the third power of the inner diameter, a small fraction of large vesicles can contribute significantly to L. As in Fattal et al. (1994), we considered the vesicles to consist of $j = 1, 2, \ldots, S$ size classes. The distribution of vesicles according to their diameters was determined by dynamic light scattering. We set $S = 10$ and we denote the fraction of encapsulated volume in vesicles of type j by f_j, $1 \le j \le 10$. Let A_{ij} be the normalized fraction of vesicles of size class j that contain i bound peptides, i.e.,

$$\sum_{i=0}^{N_j} A_{ij} = 1, \tag{10}$$

in which N_j is the largest number of peptides that can bind to a vesicle of size class j. The procedure for calculating the quantities A_{ij} from the peptide and lipid concentrations and the fraction of peptides that is bound to the vesicles at these concentrations was as in Nir et al., 1994. In the general case of reversible aggregation of the peptide, the expression for L is

$$L = \sum_{j=1}^{s} \sum_{i=M}^{N_j} Z(M, i, j, K_s) A_{i,j} f_j, \tag{11}$$

in which $Z(M, i, j, K_s)$ is the probability that a vesicle of size class j that contains i bound peptides will include an aggregate consisting of M or more peptides (only aggregates that induce leakage of encapsulated molecules), and K_s measures the reversibility of peptide aggregation in the membrane and is given by

$$K_s = C/D \tag{12}$$

in which C and D are the rate constants of peptide association and dissociation, respectively.

In previous studies, we have used this model to analyze the evolution of percent ANTS/DPX leakage from POPC or egg PC liposomes at pH 5.0 as a function of lipid/membrane-bound peptide molar ratio. We demonstrated that only GALA aggregates composed of i peptides, with $i \geq M = 10 \pm 2$ peptides, act as pores that induce the leakage of pre-encapsulated ANTS/DPX (Parente et al., 1990a; Nicol et al., 1996). As pointed out in Nicol et al. (1996), in these cases, Z approaches unity and K_s is large, reflecting the quasi-irreversibility of GALA aggregation in POPC and egg PC membranes. This reduces Eq. 11 to that derived in Parente et al. (1990a).

In the current work, we also performed calculations for a situation in which GALA aggregates formed on the membrane surface become transmembrane when they reach a critical size of M peptides, a size at which ANTS/DPX cannot leak ($M < 10 \pm 2$). The model permits calculation of the fraction of membrane-bound peptides that would be involved in transmembrane aggregates. Indeed, in analogy to Eq. 11, the fraction P of peptides participating in aggregates of size i, with $i \geq M$, can be calculated using

$$P = \sum_{j=1}^{s} \sum_{i=M}^{N_j} Z(M, i, j, K_s) A_{i,j} Q_j, \qquad (13)$$

in which Q_j is the fraction of total surface area in vesicles of type j. The model assumes that, in a vesicle where an aggregate of size i, with $i \geq M$, is formed (this situation occurs in almost all vesicles that contain M or more bound peptides because Z is close to 1), each peptide bound to this vesicle is involved in such an aggregate. We believe that this assumption results in a negligible overestimation of the peptide fraction involved in an aggregate of size i, with $i \geq M$, when $M = 4$ or 6.

RESULTS AND DISCUSSION

ANTS/DPX leakage induced by the GALA peptides

We compared the potency of the GALA peptides to induce ANTS/DPX leakage from neutral POPC LUV at pH 5.0. The efficiency with which the unlabeled peptides GALA $29C_C$, GALA $31C_C$, and GALA $31C_N$ induced leakage was almost identical to that of GALA 30 (Fig. 2 *A*). Thus, their ability to form pores in the POPC bilayer was unaltered by the slight modification made from the original GALA 30 sequence (Subbarao et al., 1987; Parente et al., 1990a,b; Nicol et al., 1996). In addition, biotinylation of the GALA peptides modified their leakage activity only slightly; this was dependent on the labeling reagent and the biotinylation site on the peptide (Fig. 2 *B*).

Biotinylation of GALA 30 on the primary amine of the N-terminus yielded a peptide (GALA(N)1) with a slightly increased ability to induce ANTS/DPX leakage. Indeed, higher leakage extents were obtained with GALA(N)1 for the same lipid-to-peptide molar ratios. This difference might arise from the higher hydrophobicity of GALA(N)1, because of the removal of the positive charge at the biotinylation site. Extension of GALA 30 by addition of a cysteine at the N-terminus (GALA $31C_N$) and subsequent biotinylation at the free thiol group of this cysteine yielded a peptide, GALA(N)2, with a similar efficiency to permeabilize POPC vesicles as the starting sequence. The comparable activity of GALA 30 and its two biotinylated analogs GALA(N)1 and GALA(N)2 implies that the peptides labeled at the N-terminus were suitable for studying GALA

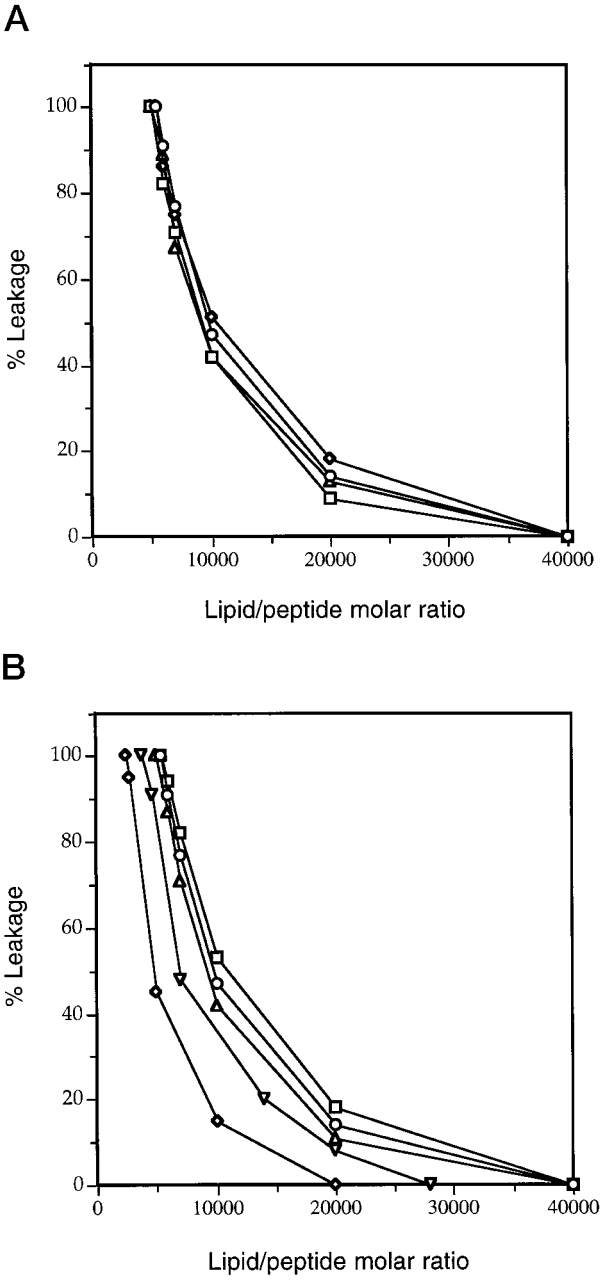


FIGURE 2 Final extent of ANTS/DPX leakage from POPC liposomes as a function of lipid/peptide molar ratio at pH 5.0. (A) Leakage induced by the nonbiotinylated GALAs (GALA 30 (○), GALA $29C_C$ (□), GALA $31C_C$ (△) and GALA $31C_N$ (◇)). (B) Leakage induced by GALA 30 (○) and the biotinylated GALAs (GALA(N)1 (□), GALA(N)2 (△), GALA(C)2 (◇) and GALA(C)3 (▽). The lipid concentration remained constant at 0.1 mM. Each data point is an average of three experiments. For every peptide tested, the final extent of leakage at a given lipid/peptide molar ratio varied no more than ±4%

30 surface-adsorbed or transbilayer orientation as well as the head-to-tail (N- to C-terminus) orientation of the transbilayer peptides in POPC liposomes.

Regarding the C-terminus labeling, biotinylation of GALA $29C_C$ at the cysteine (second amino acid from the C-terminus) yielded a peptide, GALA(C)2, with a reduced

activity in comparison to that of GALA $29C_C$ (Fig. 2 *B*). At the same lipid concentration used (0.1 mM), a two-fold increase in GALA(C)2 concentrations above those of GALA $29C_C$ was required to obtain the same extent of ANTS/DPX leakage. An improvement in the efficiency of a C-terminus-labeled GALA was achieved with the biotinylation of GALA $31C_C$, that led to the peptide GALA(C)3. In this case, only 30 mol% more biotinylated peptide than GALA $31C_C$ was needed to obtain the same extent of leakage (Fig. 2 *B*).

In GALA $31C_C$, the cysteine is located on the hydrophilic side of the membrane-bound amphipathic helix. Conversely, the cysteine of GALA $29C_C$ is positioned on the hydrophobic side of the helix. Biotinylation on the apolar side may interfere with peptide–peptide and lipid–peptide hydrophobic interactions involved in the formation of the GALA pore. This effect would explain the lower efficiency of GALA(C)2 in comparison to that of GALA(C)3. The similar activity of GALA(C)3, its nonbiotinylated version GALA $31C_C$, and GALA 30 made GALA(C)3 a suitable C-terminus-labeled peptide for studying GALA 30 surface-adsorbed or transbilayer orientation as well as the head-to-tail (N- to C-terminus) orientation of the transbilayer peptides in POPC liposomes. Overall, the similar leakage efficiency of GALA 30 and the biotinylated peptides emphasizes that the size of the label is not big enough to interfere with pore formation and dye leakage through the pore.

In the following experiments, we used a mixture of GALA 30 and a biotinylated GALA at molar ratios of 19/1, 9/1, 85/15, or 3/1 to determine GALA orientation in POPC bilayers. These mixtures exhibited the same efficiency to induce ANTS/DPX leakage as GALA 30.

Enhanced fluorescence of BODIPY-avidin upon its binding to d-biotin or to a biotinylated GALA peptide at pH 8.0

It has been observed that the binding of a biotin moiety to a BODIPY-modified avidin enhances the fluorescence of the BODIPY chromophore (Emans et al., 1995). We have monitored the enhancement of BODIPY fluorescence at its emission maximum (λ = 510 nm) resulting from the binding of d-biotin or a biotinylated GALA peptide to BODIPY-avidin. Avidin is a tetramer in which each monomer consists of an eight-stranded antiparallel β-barrel (Pugliese et al., 1993). The biotin binding site is located in a deep pocket near the center of the barrel in which two of the four tryptophan residues reside. It has been suggested that a static interaction between tryptophan and BODIPY chromophores results in quenching of their fluorescence. Biotin binding to BODIPY-avidin yields a conformational change of the protein that may weaken the interaction and consequently dequenches both BODIPY and tryptophan fluorescence (Emans et al., 1995).

In Fig. 3, the fluorescence intensity of the BODIPY-avidin/biotin complex in solution at pH 8.0 (Ex = 480 nm, Em = 510 nm) is displayed as a function of the biotin moiety-to-BODIPY-avidin molar ratio, for a constant concentration of BODIPY-avidin (0.1 μM). In comparison to the fluorescence intensity obtained without biotin binding (intensity of 1 in arbitrary units), a maximum enhancement of 4.6 was obtained when d-biotin was added to saturation of the four binding sites of the BODIPY-avidin molecules. C-terminus biotinylated peptides were slightly less efficient in dequenching BODIPY fluorescence; at saturation of the BODIPY-avidin binding sites, the intensity was 3.6 and 3.2 when using GALA(C)2 and GALA(C)3, respectively. The lowest fluorescence enhancement (2.3) was obtained with the N-terminus-labeled peptides GALA(N)1 and GALA(N)2.

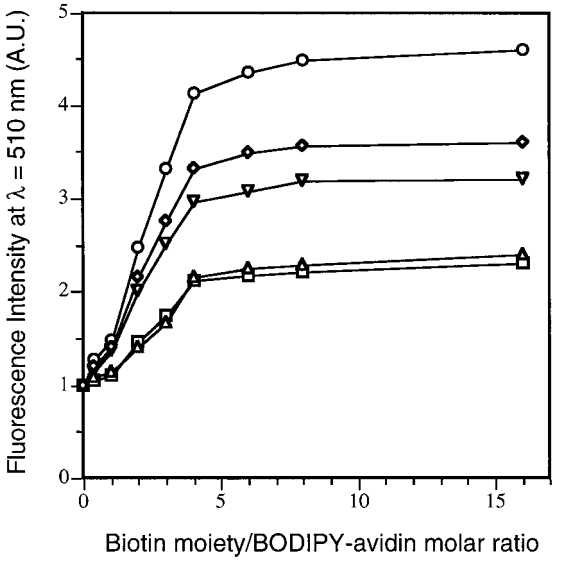


FIGURE 3 Differential enhancement of BODIPY-avidin fluorescence upon its binding to d-biotin or to a biotinylated GALA peptide at pH 8.0 as a function of biotin/avidin molar ratio. Biotinylated molecules used were d-biotin (○), GALA(N)1 (□), GALA(N)2 (△), GALA(C)2 (◇) and GALA(C)3 (▽). We monitored the fluorescence of BODIPY-avidin at λ = 510 nm (2.5 mm slits), when the samples were irradiated at λ = 480 nm (1.25 mm slits). Duplicate experiments resulted in values that differed by up to 5%.

In comparison to d-biotin, we suggest that the reduced enhancement of BODIPY-avidin fluorescence observed with GALA(C)s, and to a greater extent with GALA(N)s, is due to the presence of a tryptophan at the N-terminus of GALA. The dequenching of BODIPY-avidin fluorescence resulting from its binding to a biotin moiety is partially offset by a quenching effect caused by the peptide's tryptophan. This explanation is consistent with the observed sensitivity of BODIPY fluorescence to the aromatic amino acids tyrosine and tryptophan (Karolin et al., 1994). The tryptophan-induced quenching of BODIPY-avidin fluorescence depends on the average distance between the biotin moiety of the peptide and its tryptophan, because it was more pronounced with GALA(N)s than with GALA(C)s.

For each biotinylated peptide as well as for d-biotin, the plateau of fluorescence enhancement was obtained at a

biotin moiety-to-BODIPY-avidin molar ratio of 4, which corresponds to the number of binding sites per BODIPY-avidin molecule. Under the conditions tested, the enhancement of BODIPY-avidin fluorescence was not altered by the presence of POPC liposomes in the solution at pH 8.0 (data not shown).

In the time-frame of our experiments (less than 1 h), the binding of a biotinylated GALA or d-biotin to BODIPY-avidin at pH 8.0 was irreversible (data not shown). Indeed, when BODIPY-avidin binding sites were presaturated with GALA(N)1 or GALA(C)3 (using a biotinylated-GALA-to-BODIPY-avidin binding site molar ratio of 2), the fluorescence of the complex was not altered by the addition of a 4/1 excess of d-biotin over the BODIPY-avidin binding sites. Furthermore, when the BODIPY-avidin binding sites were presaturated with d-biotin and a biotinylated GALA was added in excess, no decrease of BODIPY-avidin fluorescence was observed.

Determination of GALA head-to-tail (N- to C-terminus) orientation in POPC bilayers at pH 5.0 by the external addition of BODIPY-avidin

To determine the fraction of membrane-bound biotinylated GALA that exposes its labeled terminus on the external side of the POPC vesicles at pH 5.0, we used the differential enhancement of BODIPY-avidin fluorescence upon its irreversible binding to d-biotin or to a biotinylated GALA. The assay was based on the following elements: 1) The lipid concentration was such (2.5 mM) that all added GALA bound to the liposomes at pH 5.0 (Nicol et al., 1996), but none remained bound when the pH was elevated to 8.0. (Parente et al., 1990b). 2) BODIPY-avidin (~60,000 MW) added to the peptides/vesicle solution did not permeate through the liposome membrane. 3) A biotinylated GALA or a d-biotin bound to BODIPY-avidin could not be exchanged by a d-biotin, or a biotinylated GALA, respectively. We believe that the quasi-irreversibility of biotin/BODIPY-avidin binding demonstrated at pH 8.0 in the presence or absence of liposomes can be extended to pH 5.0.

The assay, described in detail in Materials and Methods and illustrated in Fig. 1, comprised three independent fluorescence measurements. Each measurement yielded a fluorescence intensity required to determine the external exposure of GALA's labeled terminus. During each measurement, the sample was irradiated at $\lambda = 480$ nm, and the light emitted was monitored at $\lambda = 510$ nm (wavelength where BODIPY-avidin fluorescence emission was maximal). Measurements carried out with the biotinylated peptides GALA(N)1, GALA(C)2, and GALA(C)3 yielded traces like those displayed in Fig. 4 *A*, *B*, and *C*, respectively. In these measurements, a lipid/total peptide molar ratio of 2500/1, a lipid concentration of 2.5 mM, and a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8 were used. Measurements performed with GALA(N)2 resulted in similar traces as those obtained with GALA(N)1.

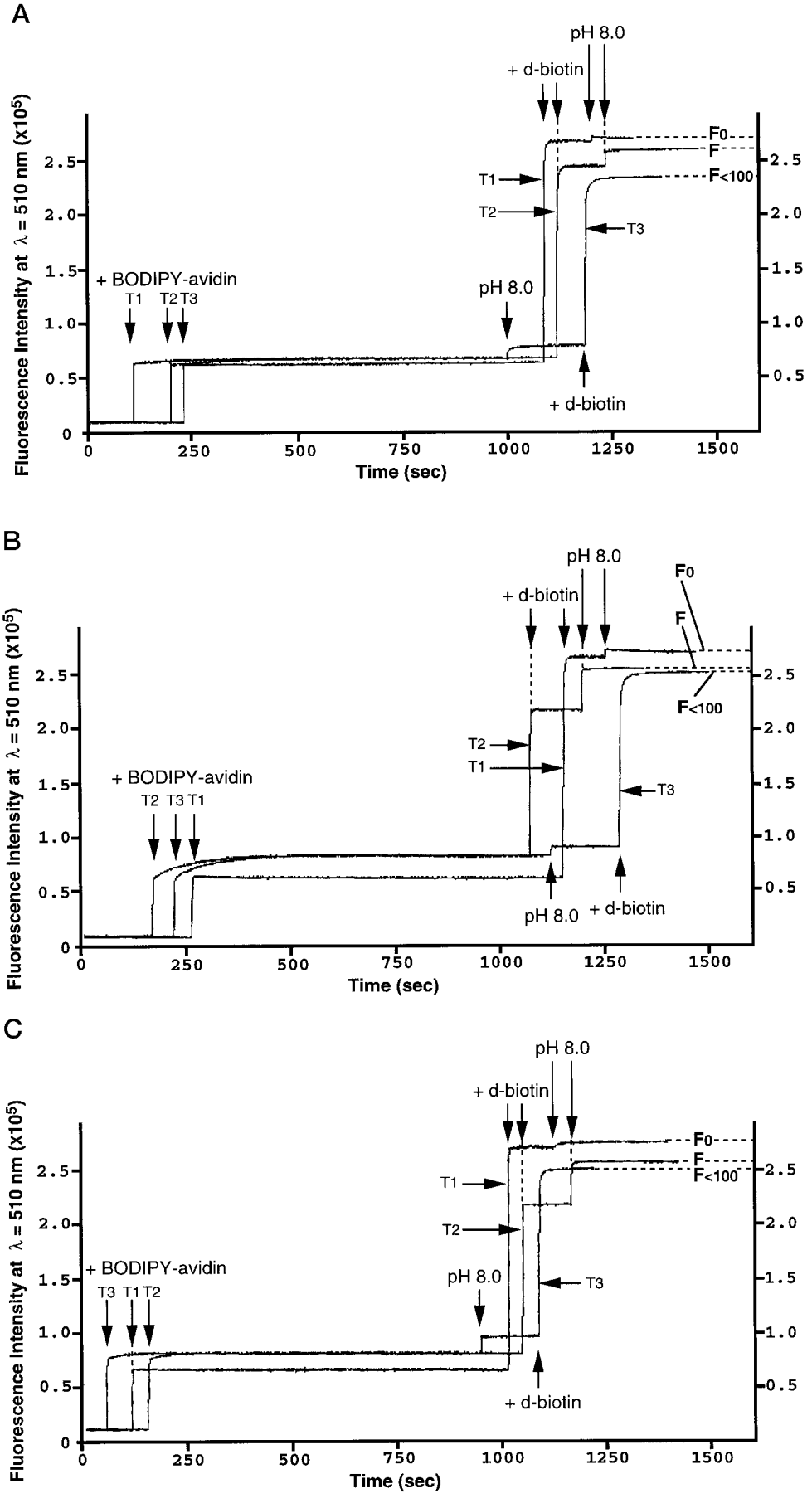
Typical traces obtained from the three measurements with GALA(N)1 are presented in Fig. 4 *A*. In the control no exposure (*trace 1*), the addition of BODIPY-avidin to the nonbiotinylated GALA 30/POPC liposome solution at pH 5.0 generated an increase in fluorescence, as expected. When d-biotin was added to the solution 15 min later, the saturation of the BODIPY-avidin binding sites by d-biotin produced an appreciable dequenching of BODIPY-avidin fluorescence. The subsequent pH elevation to 8.0 led to a small increase of the fluorescence intensity. At the end of the control no exposure, the BODIPY-avidin binding sites were all occupied by a d-biotin. A modification in the order of the addition steps did not affect the final fluorescence intensity F_0 (data not shown).

The addition of BODIPY-avidin to the [GALA 30/GALA(N)1]/POPC liposome solution in the external layer exposure measurement and in the control pH-dissociation exposure (*traces 2* and *3*, respectively) led to a fluorescence intensity that reached a plateau after a few minutes to a level slightly higher than that observed in the control no exposure. This level was higher because of BODIPY-avidin binding to the GALA(N)1 peptides that exposed their N-termini on the external side of the liposomes.

The external layer exposure measurement required the addition of an excess of d-biotin (*trace 2*). D-biotin bound to the unoccupied BODIPY-avidin binding sites. The fluorescence increase that followed this addition was lower than that observed for the control no exposure (*trace 1*), because of the partial binding of the biotinylated peptides to BODIPY-avidin. Indeed, the binding of a N-terminus biotinylated GALA to BODIPY-avidin dequenches BODIPY fluorescence less than the binding of a d-biotin. The subsequent pH elevation to 8.0 released all the peptides from the POPC bilayer, including the biotinylated ones that were exposed on the outside of the liposomes and that bound to a BODIPY-avidin molecule. $(Z * 100)\%$ of the biotinylated peptides were released on the outside of the vesicles (including those bound to BODIPY-avidin), and a small fraction of peptide was released on the inside of the liposomes (see Materials and Methods). This pH elevation induced a further dequenching of BODIPY-avidin fluorescence, as observed in the control no exposure.

At the end of the external layer exposure protocol, only the fraction of membrane-bound GALA(N)1 that exposed its biotin moiety on the external side of the POPC liposomes at pH 5.0 was bound to a BODIPY-avidin molecule. Peptides that displayed their biotin tags on the internal side of the liposomes did not bind to BODIPY-avidin. BODIPY-avidin binding sites that were not occupied by a labeled GALA were associated with a d-biotin. The partial binding of GALA(N)1 to BODIPY-avidin explains the reduced final fluorescence intensity F (*trace 2*) in comparison to that of the control no exposure, F_0 (*trace 1*).

After the addition of BODIPY-avidin in the control pH-dissociation exposure (*trace 3*), the pH was increased to 8.0,


A
pH 8.0
+ d-biotin
F0
F
F<100
T1
T2
T3
+ BODIPY-avidin
T1 T2 T3
pH 8.0
+ d-biotin
Fluorescence Intensity at λ = 510 nm (x10^5)
Time (sec)
B
pH 8.0
+ d-biotin
F0
F
F<100
T2
T1
T3
+ BODIPY-avidin
T2 T3 T1
pH 8.0
+ d-biotin
Fluorescence Intensity at λ = 510 nm (x10^5)
Time (sec)
C
pH 8.0
+ d-biotin
F0
F
F<100
T1
T2
T3
+ BODIPY-avidin
T3 T1 T2
pH 8.0
+ d-biotin
Fluorescence Intensity at λ = 510 nm (x10^5)
Time (sec)

and then d-biotin was added. As mentioned above, the pH elevation released all the GALA peptides from the membranes, $Z * 100\%$ of the biotinylated peptides being released on the outside of the vesicles. Thus, $Z * 100\%$ of the biotinylated GALA molecules bound to BODIPY-avidin. This additional binding was partially responsible for the increase in BODIPY-avidin fluorescence subsequent to increasing the pH. When d-biotin was added to the solution ~3 min later, the binding of d-biotin to the remaining BODIPY-avidin binding sites induced a further dequenching of BODIPY-avidin fluorescence. At the end of the control pH-dissociation exposure, every biotinylated GALA released on the outside of the vesicles was bound to a BODIPY-avidin molecule. The sites not occupied by a labeled GALA were associated with a d-biotin. The almost complete binding of GALA(N)1 ($Z * 100\%$ of the peptides) to BODIPY-avidin explains why the final fluorescence of the control pH-dissociation exposure, $F_{<100}$, (*trace 3*) was lower than that of the external layer exposure experiment, F, (*trace 2*).

From the three fluorescence intensities F, $F_{<100}$, and F_0, and the separately determined Z (see Materials and Methods), the fraction of membrane-bound GALA(N)1 that exposed its N-terminus on the external side of the POPC vesicles at pH 5.0, R, could be calculated (Eqs. 1–6).

Molar ratios of BODIPY-avidin binding sites to biotinylated GALA of 8 and above were used in this assay to ensure that, at most, one biotinylated GALA bound to a BODIPY-avidin. We found a similar R value when this ratio was varied from 8 to 20 (data not shown), which implies that a ratio of 8 was sufficient to meet this criterion.

At pH 5.0 and a lipid/peptide molar ratio of 2500/1, GALA 30 forms pores of 8 to 13 peptides in POPC vesicles (Subbarao et al., 1987; Parente et al., 1990a; Nicol et al., 1996). If two biotinylated GALAs participate in the same pore, we anticipate a steric hindrance for the binding of two BODIPY-avidin molecules to these two biotin moieties. This situation could prevent BODIPY-avidin binding of one of these biotin tags. We used a molar ratio of unlabeled GALA 30 to biotinylated GALA of 19/1 to minimize this possibility. This ratio also significantly reduced the chances of binding of one BODIPY-avidin molecule to two membrane-bound biotinylated GALAs.

There were remarkable differences between traces obtained with the GALA(N) peptides (Fig. 4 *A*) and that acquired with the GALA(C) analogs (Fig. 4 *B* and *C*). In the external layer exposure (*trace 2*) and the control pH-dissociation exposure (*trace 3*), the fluorescence increase following BODIPY-avidin addition was higher with the GALA(C) peptides than with the GALA(N) peptides. This difference was the result of two factors: first, GALA predominantly exposed its C-terminus to the external side of the vesicles. Second, the tryptophan of a GALA(C) bound to BODIPY-avidin was a less effective quencher of BODIPY-avidin fluorescence than the tryptophan of a bound GALA(N). This is due to a variation in the distance between the biotin moiety of the peptide and its tryptophan. This structural difference also explains why the differential intensity ($F_0 - F_{<100}$) was lower when GALA(C) peptides were used.

The assay is sensitive to a number of factors, and we found it necessary to use an excess of BODIPY-avidin over the biotinylated GALA (in *traces 2* (T2) and *3* (T3) of Fig. 4 *A–C*) and to perform a pH elevation in the three measurements (in *traces 1* (T1), *2* and *3*). This results in two populations of BODIPY-avidin molecules at the end of the measurements, one in solution at pH 8.0 completely filled with d-biotin, the other also in solution at pH 8.0 being filled ¾ with d-biotin and ¼ with a biotinylated GALA (this population does not exist in T1 because we do not use biotinylated GALA). At the end of the measurements, the only difference between them is in the proportions of the two populations of BODIPY-avidin, which makes the determination of R possible. Before the measurements are finished, we cannot quantitate R, because the BODIPY-avidin molecules are in different states in the three measurements. These differences in the state of BODIPY-avidin molecules explain, for example, the difference in fluorescence increase following pH elevation among T1, T2, and T3. This can be illustrated with the help of Fig. 5.

In T1 (Fig. 5), all the BODIPY-avidin molecules are free in solution, with their binding sites filled with d-biotin, before and after the pH increase to 8.0. In that case, the increase in BODIPY fluorescence following pH elevation arises only from the influence of pH on the fluorescence intensity of BODIPY-avidin molecules filled with d-biotin.

The effect of pH elevation is similar in T2 for the fraction $(1 - A)$ of BODIPY-avidin molecules filled with d-biotin. However, for the BODIPY-avidin molecules (fraction A) that contain a biotinylated GALA (3 of their sites are filled with d-biotin and one with biotinylated GALA), the pH

FIGURE 4 Determination of R, the fraction of biotinylated GALA that exposes its labeled terminus to the external side of POPC liposomes at pH 5.0. T1, T2, and T3 are traces that refer to the control no exposure (T1), the external layer exposure measurement (T2), and the control pH-dissociation exposure (T3). Traces were obtained by monitoring the light emitted at $\lambda = 510$ nm and following the protocol described in Materials and Methods. $r = R/Z$ was calculated from the fluorescence intensities F_0, F, and $F_{<100}$ obtained at the end of the three independent measurements, by using Eq. 4. From r and a separate determination of Z (see Materials and Methods), R could be calculated. At $t = 0$, T2 and T3 refer to solutions at pH 5.0 that contain POPC vesicles (2.5 mM lipid) and a mixture of GALA 30/biotinylated GALA 19/1 (1 μM total peptide). The peptide mixture was replaced by 1 μM nonbiotinylated GALA 30 in T1. *Arrows* indicate additions performed during the three measurements. A lipid/total peptide molar ratio of 2500/1 and a d-biotin/BODIPY-avidin binding site molar ratio of 4/1 were used in these measurements. In T2 and T3, a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8/1 was used. The biotinylated GALAs tested are (A) GALA(N)1, (B) GALA(C)2, and (C) GALA(C)3 F_0, F, $F_{<100}$, and r ($= R/Z$) values of these particular sets of measurements are listed in Table 1.

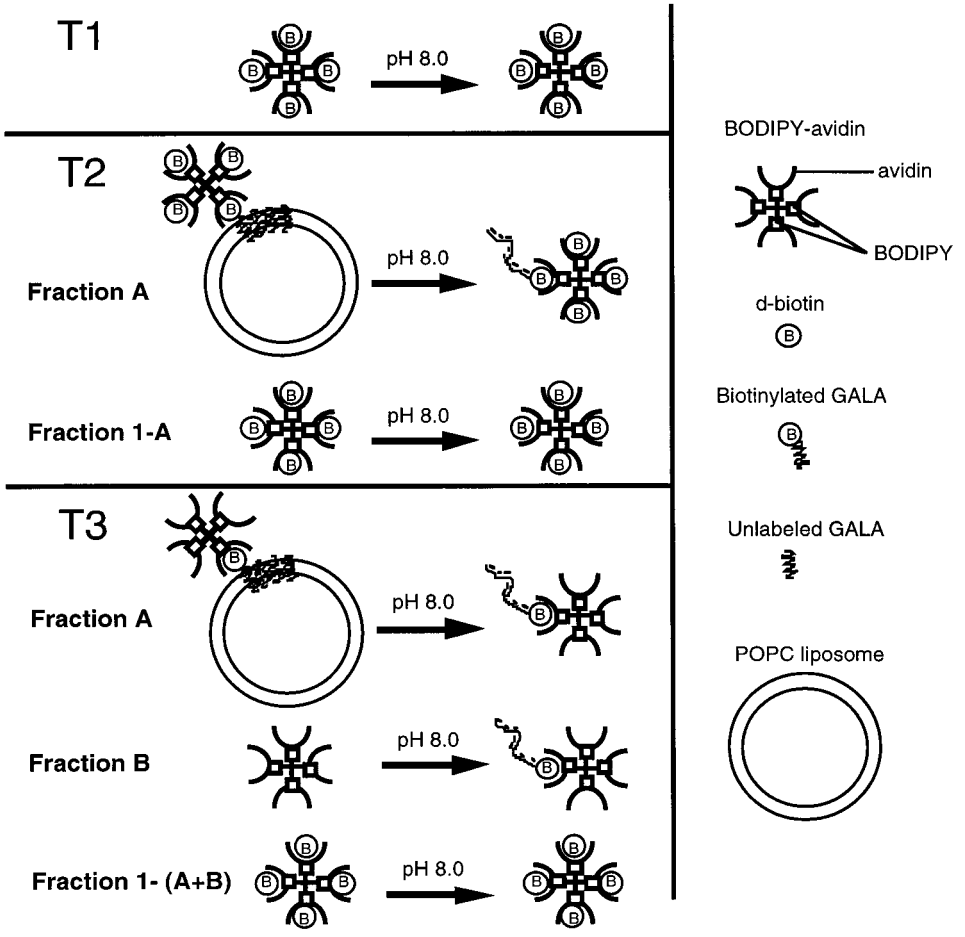


FIGURE 5 Illustration of the effect of pH on BODIPY-avidin in traces 1, 2, and 3 of Fig. 4

increase induces a change in their environment, as illustrated in T2 of Fig. 5. These BODIPY-avidin molecules evolve from a state in which they are close to the liposome membrane at pH 5.0 (the biotinylated GALA that is bound to them is inserted in the membrane) to a situation in which they are free in solution at pH 8.0, and the biotinylated GALA that is bound to them is an anionic random coil. This modification of the environment influences the change in fluorescence following pH elevation.

In T3, the pH increase induces the same environmental modifications for a similar fraction of the BODIPY-avidin molecules (fraction *A*), except that the 3 binding sites that are not occupied by a biotinylated GALA are free instead of being filled with d-biotin. Figure 5 illustrates that, in addition to this fraction of BODIPY-avidin molecules, there is another fraction (*B*) that remains in solution and binds to a biotinylated GALA following the pH increase. The fluorescence intensity of these latter BODIPY-avidin molecules should increase not only because of the pH increase per se {as in *trace 1* or with fraction $(1 - A)$ of T2 and fraction $[1 - (A + B)]$ of T3}, but also because of the binding of a biotinylated GALA.

If the fraction *A* of BODIPY-avidin was undergoing the same fluorescence increase in T2 and in T3 following the pH elevation, then the total fluorescence increase in T3 should be higher than that in T2, because of the additional binding of a biotinylated GALA to fraction *B* in T3. However, we believe that fraction *A* in T2 undergoes a fluorescence increase much higher than fraction *A* in *trace 3*. In T2 and T3, if the fluorescence increase of fraction *A* after the pH elevation involves only the BODIPY fluorophore coupled to the binding site occupied by the biotinylated GALA, this increase should be the same for both traces. However, we believe that the BODIPY fluorophore coupled to each of the 3 avidin binding sites occupied by a d-biotin (fraction *A* in T2) undergoes a higher fluorescence increase due to pH elevation than the the BODIPY fluorophore coupled to each of the 3 unoccupied binding sites (fraction *A* in T3). This effect would explain why the total fluorescence increase in *trace 2* is higher than that in *trace 3*. The reason for the higher increase in fluorescence of fraction *A* in *trace 2* than in *trace 3* could arise from a quenching of BODIPY fluorescence before pH elevation, this quenching being higher for binding sites filled with d-biotin than for unoccupied binding sites. The quenching could arise from the proximity of the BODIPY-avidin molecule to the membrane surface or from its proximity to GALA peptides that are forming a membrane aggregate with the biotinylated GALA bound to the BODIPY-avidin molecule. A similar kind of explanation can be put forward for the difference in fluorescence

increase between T2 and T3 following detergent addition in Fig. 6.

The fluorescence intensities F_0, F, and F_{100}, and the intermediate parameter r determined from the measurements displayed in Fig. 4, are listed in Table 1, as well as the R value determined from 3 to 6 sets of measurements of r and Z.

Measurements carried out with the N-terminus biotinylated peptides GALA(N)1 and GALA(N)2 yielded R_N values (R values related to N-terminus biotinylated GALAs) of 0.265 and 0.255, respectively. These R_N values correspond to the fraction of membrane-bound GALA(N) that exposed its N-terminus on the external side of the POPC liposomes at pH 5.0. The corresponding Z values were 0.87 and 0.86,

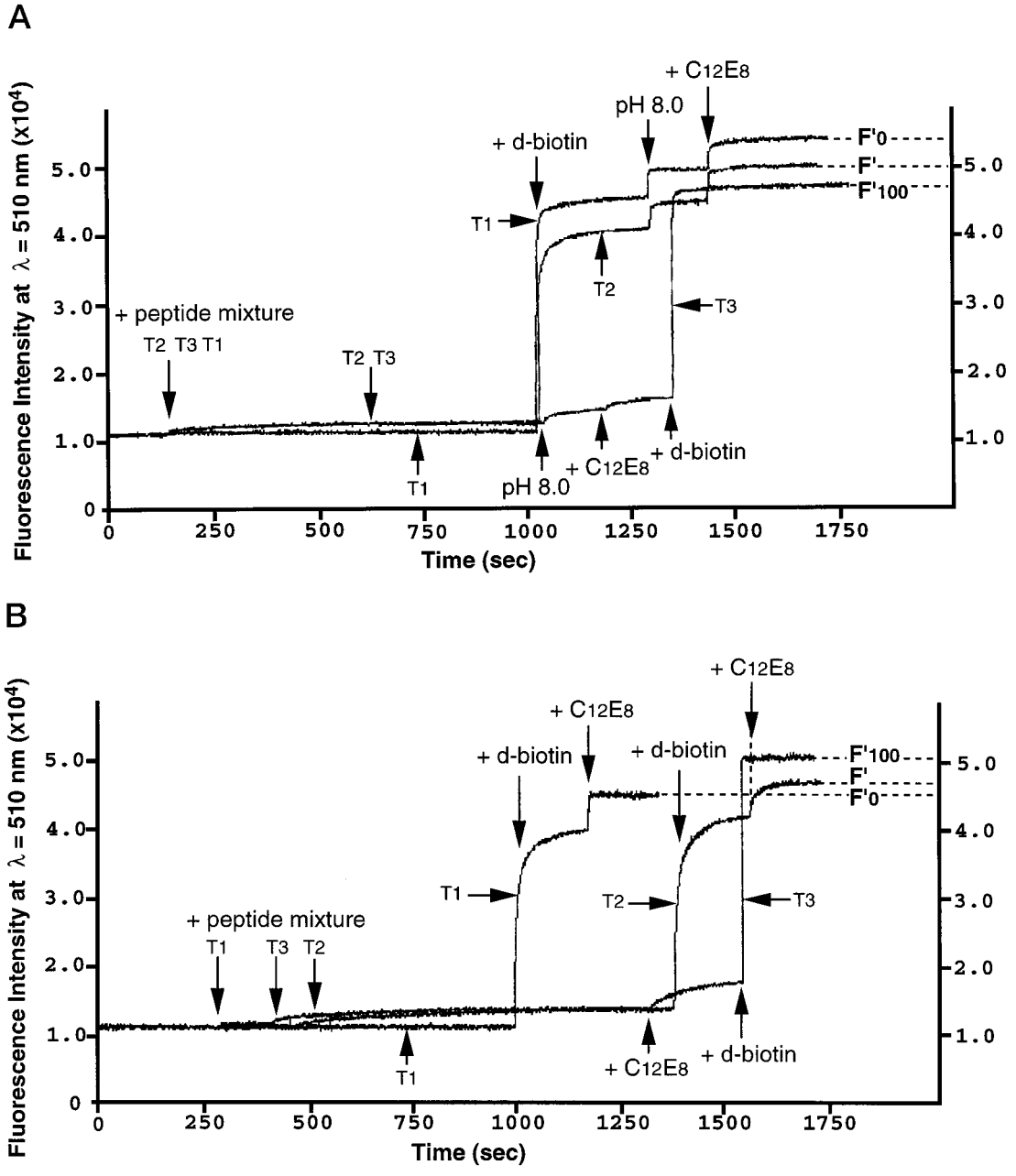


FIGURE 6 Determination of R', the fraction of biotinylated GALA that exposes its labeled terminus to the internal side of POPC liposomes at pH 5.0. T1, T2, and T3 are traces that refer to the control no exposure (T1), the internal layer exposure determination (T2), and the control total exposure (T3). Traces were obtained by monitoring the light emitted at $\lambda = 510$ nm and following the protocol described in Materials and Methods. R' could be calculated from the fluorescence intensities F'_0, F', and F'_{100} obtained at the end of the three independent measurements, by using Eq. 7 and $R' = R''/0.8$. At $t = 0$, T1, T2, and T3 refer to solutions at pH 5.0 that contain POPC vesicles (0.65 mM lipid) with pre-encapsulated BODIPY-avidin. *Arrows* indicate additions performed during the measurements. A lipid/total membrane-bound peptide molar ratio of 2500/1 and a d-biotin/BODIPY-avidin binding site molar ratio of 16/1 were used in these measurements. In T2 and T3, a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8/1 and a GALA 30/biotinylated GALA molar ratio of 14/1 were used. The biotinylated GALAs tested were (A) GALA(N)1 and (B) GALA(C)3. F'_0, F', F'_{100}, R'', and R' values of these particular sets of measurements are listed in Table 1.

TABLE 1 Percentage of GALA N-terminus and C-terminus exposed on the external and internal side of POPC liposomes at pH 5.0

	GALA(N)1	GALA(N)2	GALA(C)2	GALA(C)3
$100 * R$ (external)	26.5 ± 3*	25.5 ± 4	81 ± 7	73 ± 6
$100 * R'$ (internal)	74 ± 5	71 ± 5	N.D.[#]	33 ± 7
Determination of r from Measurements Displayed in Fig. 4[§]				
F_0	2.73		2.75	2.77
F	2.37		2.59	2.58
$F_{<100}$	2.63		2.56	2.52
r	0.30		0.84	0.76
Determination of R′ from Measurements Displayed in Fig. 6[¶]				
F'_0	5.35			4.51
F'	4.95			4.65
F'_{100}	4.66			5.00
R''	0.58			0.29
R'	0.72			0.36

*Percentage of membrane-bound biotinylated GALA that bound to BODIPY-avidin at pH 5.0 when the protein was either added in the external medium of the POPC liposomes/peptides solution ($100 * R$) or pre-encapsulated in POPC liposomes ($100 * R'$). Values reported are based on the average of $n = 3$ to 6 determinations of R and R'. GALA(N) peptides are biotinylated at the N-terminus and GALA(C) peptides are biotinylated at the C-terminus. Experiments to determine R and R' were performed with 2.5 mM and 0.65 mM POPC concentrations, respectively. We used a lipid/total membrane-bound peptide molar ratio of 2500, a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8, and a GALA 30/biotinylated GALA molar ratio of 19 and 14 for R and R' determinations, respectively.

[#]N.D. = not determined.

[§]Fluorescence intensities F_0, F, and $F_{<100}$ and intermediate parameter $r = (F_0 - F)/(F_0 - F_{<100}) = (R/Z)$ determined from measurements displayed in Fig. 4 and required to calculate R.

[¶]Fluorescence intensities F'_0, F', and F'_{100} and intermediate parameter $R'' = (F'_0 - F')/(F'_0 - F'_{100}) = (R' * 0.8)$ determined from measurements displayed in Fig. 6 and required to calculate R'.

respectively, meaning that most of the biotinylated peptides were released on the outside of the vesicles after the pH elevation during the control pH-dissociation exposure.

Experiments performed with the C-terminus biotinylated peptides GALA(C)2 and GALA(C)3 yielded R_C values (R values related to C-terminus biotinylated GALAs) of 0.81 and 0.73, respectively (Table 1). The corresponding Z values were 0.97 and 0.95, respectively.

The biotinylated peptides (particularly GALA(N)1, GALA(N)2, and GALA(C)3) exhibited a similar efficiency to induce ANTS/DPX leakage as the unlabeled peptide GALA 30 (Fig. 2 *B*), which demonstrates that the biotin-modified peptides have pore-forming properties comparable to GALA 30. Thus, we believe that the orientation of GALA 30 in the bilayer [surface-adsorbed or transmembrane orientation as well as the head-to-tail (N- to C-terminus) orientation of the transbilayer peptide] and that of the biotinylated peptides are similar. Therefore, the R_N and R_C values determined with the biotinylated peptides characterize the exposure of GALA 30 termini and the orientation of the unlabeled peptide in the bilayer. We cannot rule out the possibility that biotinylation influences peptide orientation to some extent. However, GALA(N)1 and GALA(N)2 are labeled on different sites on the N-terminus with a different biotinylation reagent but yield similar R_N values. In addition, GALA(N)1 and GALA(C)3 are labeled with the same biotinylation reagent, both on a cysteine on the hydrophilic side of the helix, but the R value associated with GALA(N)1 N-terminus (R_N) and the R value associated with GALA(C)3 C-terminus (R_C) are significantly different and complementary ($R_N + R_C \approx 1$, see next paragraph). These results emphasize that GALA orientation is not significantly affected by biotinylation.

We attribute more significance to the results obtained with GALA(C)3 than that generated with GALA(C)2, because the efficiency of GALA(C)3 to induce ANTS/DPX leakage was closer to that of GALA 30. Therefore, we expect GALA(C)3 to better mimic GALA 30 behavior. The importance of selecting the right biotinylation reagent can be illustrated by our first attempt to label GALA $29C_C$ at the C-terminus with N-(3-maleimidylpropionyl) biocytin. This labeling resulted in a peptide (GALA(C)1) with a highly reduced efficiency to induce ANTS/DPX leakage in comparison to that of GALA $29C_C$. Determination of the orientation of this peptide at a lipid/peptide ratio of 2500/1 yielded an external exposure of its labeled C-terminus close to 100% (data not shown). This almost complete external exposure was most probably arising from a reduced insertion of the peptide in the membrane, leading to reduced pore formation and ANTS/DPX leakage. We believe this effect of the labeling reagent was caused by its negatively charged carboxylic acid that prevented peptide aggregation in the membrane and/or aggregate insertion.

The sum of the R value obtained with GALA(C)3 ($R_C = 0.73$) and that obtained with the GALA(N) peptides ($R_N = 0.265$ or 0.255) is close to 1, which supports the notion that, at a lipid/peptide molar ratio of 2500/1, only one terminus per membrane-bound GALA (GALA(N), GALA(C)3, or GALA 30) is exposed to the external side of the POPC bilayer. This result implies that a vast majority of the GALA peptides are involved in transmembrane aggregates, as opposed to being adsorbed on the membrane surface. Fourier transform infrared spectroscopy studies previously indicated that the orientation of membrane-bound GALA 30 is parallel to the POPC bilayer normal, at pH 5.0, and at low lipid/peptide molar ratios (Goormaghtigh et al., 1991).

From the present experiments, we can conclude that the head-to-tail (N- to C-terminus) orientation of transmembrane GALA 30 is such that 26 ± 3.5% of the peptide has its N-terminus facing the outside of the liposome and 73 ± 6% of the peptide has its C-terminus facing the outside of the liposome. As indicated earlier, the coherence of our data implies that biotinylation has little influence on the head-to-tail (N- to C-terminus) orientation of the peptide in the bilayer. It is the amino-acids sequence that determines this orientation and explains the higher tendency of the N-terminus to insert into the bilayer.

Determination of GALA head-to-tail (N- to C-terminus) orientation in POPC vesicles at pH 5.0 by the pre-encapsulation of BODIPY-avidin

If all the GALA peptides are oriented perpendicularly to the membrane surface, an appreciable fraction of the N-termini ($1 - R_N \approx 0.74$) and the C-termini ($1 - R_N \approx 0.27$) should be exposed to the internal side of the liposomes. To verify that the biotinylated peptides are transmembrane at a lipid/membrane-bound peptide molar ratio of 2500/1, we determined the accessibility of the peptides biotin to BODIPY-avidin pre-encapsulated in POPC liposomes. We used again the differential enhancement of BODIPY-avidin fluorescence upon its irreversible binding to d-biotin or to a biotinylated GALA to quantify the exposure of GALA's biotin to the internal side of the vesicle.

The assay comprised three independent measurements. Each measurement yielded a fluorescence intensity required to determine the internal exposure of GALA's labeled terminus. Measurements performed with the labeled peptides GALA(N)1 and GALA(C)3 yielded traces like those shown in Fig. 6 *A* and *B*, respectively, when a lipid/membrane-bound peptide molar ratio of 2500/1, a lipid concentration of 0.65 mM [concentration at which ~80 mol% of the peptides are membrane-bound (Nicol et al., 1996)], a GALA 30-to-biotinylated GALA molar ratio of 14/1, and a BODIPY-avidin binding site/biotinylated GALA molar ratio of 8 were used. Measurements performed with GALA(N)2 resulted in similar traces as those obtained with GALA(N)1.

In the three traces in which GALA(N)1 was used (Fig. 6 *A*), the initial intensity arose from the fluorescence of the liposome-encapsulated BODIPY-avidin. The addition of GALA 30 to the POPC liposome solution at pH 5.0 in the control no exposure (Fig. 6 *A*, *trace 1*) did not alter BODIPY-avidin fluorescence. The addition of GALA 30/GALA(N)1 mixture in the internal layer exposure measurement (*trace 2*) and the control total exposure (traces 3) induced a partial dequenching of BODIPY-avidin fluorescence. This dequenching indicates that a significant fraction of the membrane-bound biotinylated GALA peptides bound to encapsulated BODIPY-avidin.

The addition of an excess of d-biotin, 15 min later, in the control no exposure (Fig. 6 *A*, *trace 1*) and in the internal layer exposure determination (*trace 2*), yielded a rapid increase in BODIPY-avidin fluorescence. The extent of this increase indicates that d-biotin translocated into the liposomes and saturated the unoccupied BODIPY-avidin binding sites. D-biotin (244.3 MW) could translocate rapidly through the membrane pores formed by GALA 30 or by the mixture GALA 30/GALA(N)1. In the absence of peptide, d-biotin permeated through the liposome bilayer at a much slower rate (data not shown). The rapid translocation of d-biotin was observed 15 min after the peptide had been added to the POPC liposomes, which indicates that the pores persisted for a long time.

The addition of d-biotin induced a larger dequenching of BODIPY-avidin fluorescence in the control no exposure (Fig. 6 *A*, *trace 1*) than in the internal layer exposure determination (*trace 2*). This difference was due to the partial binding of GALA(N)1 to BODIPY-avidin in the internal layer exposure (*trace 2*). The subsequent pH elevation to 8.0 and addition of the detergent $C_{12}E_8$ both enhanced BODIPY-avidin fluorescence.

At the end of the control no exposure (Fig. 6 *A*, *trace 1*), BODIPY-avidin binding sites were all occupied by a d-biotin. A modification in the order of the addition steps did not affect the final fluorescence intensity F'_0 (data not shown). At the end of the internal layer exposure (*trace 2*), only the fraction of membrane-bound biotinylated GALA(N)1 that exposed its biotin tag on the internal side of the liposomes at pH 5.0 was bound to a BODIPY-avidin binding site. Peptides that exposed their biotin tags on the external side of the liposomes did not bind to BODIPY-avidin. The BODIPY-avidin binding sites not occupied by a labeled GALA were associated with a d-biotin. The final fluorescence intensity was F'.

For the control total exposure the steps following the addition of the peptide mixture to the liposomes, i.e., the pH increase and the subsequent addition of $C_{12}E_8$, led to the complete binding of GALA(N)1 to BODIPY-avidin, which explains the observed dequenching in BODIPY-avidin fluorescence (Fig. 6 *A*, *trace 3*). When d-biotin was added to the solution ~3 min later, the binding of d-biotin to the unoccupied BODIPY-avidin binding sites induced a further dequenching of BODIPY-avidin fluorescence. At the end of the control total exposure (*trace 3*), all biotinylated GALA(N)1 were bound to a BODIPY-avidin binding site. The sites not occupied by a labeled GALA(N)1 were associated with a d-biotin. We verified that a modification in the order of the addition steps did not affect the final fluorescence intensity, F'_{100}, as long as d-biotin was added after all biotinylated GALA bound to a BODIPY-avidin (data not shown).

From the three fluorescence intensities F', F'_{100}, and F'_0, the fraction of membrane-bound GALA(N)1 peptide that exposed its N-terminus on the internal side of the POPC liposomes at pH 5.0, R', could be calculated (Eqs. 7–8).

We modified the protocol when the C-terminus-labeled peptide GALA(C)3 was investigated and did not increase the pH to 8.0 because of lack of sensitivity with this peptide (Fig. 6 *B*). However, we believe that the determination of R' was still accurate at pH 5.0. Particularly, the presence of $C_{12}E_8$ ensured a complete binding of the biotinylated peptides to BODIPY-avidin in the control total exposure (*trace 3*). With GALA(N)1, the lack of pH elevation did not affect R' significantly (data not shown). We think this result is extendible to the C-terminus-labeled GALA.

For the determination of R (external addition of BODIPY-avidin), where F_{100}, F and F_0 were obtained at pH 8.0 without $C_{12}E_8$, F_0 was higher than F_{100} with both GALA(N)1 and GALA(C)3 (Fig. 4 *A* and *C*). Similarly, for the determination of R', where F'_{100}, F', and F'_0 were acquired in the presence of $C_{12}E_8$ at pH 8.0, F'_0 was higher than F'_{100} with GALA(N)1 (Fig. 6 *A*). However, in the case of GALA(C)3, where F'_{100}, F', and F'_0 were obtained in the

presence of $C_{12}E_8$ at pH 5.0, F'_{100} was higher than F'_0 (Fig. 6 *B*). Thus, in this latter case, the binding of the C-terminus-labeled GALA to BODIPY-avidin dequenched the fluorescence of the protein more than the binding of a d-biotin. We attribute this difference to the location of the tryptophan of the labeled GALA bound to BODIPY-avidin.

The fluorescence intensities F'_0, F', F'_{100}, the intermediate parameter R'', and R' determined from the measurements displayed in Fig. 6 are listed in Table 1, as well as the R' value determined from 3 to 6 sets of measurements with GALA(N)1, GALA(N)2, or GALA(C)3.

The fact that we obtained R_N (0.265) + R'_N (0.74) $\approx$ 1 for GALA(N)1 and R_C (0.73) + R'_C (0.33) $\approx$ 1 for GALA(C)3 is compatible with a transmembrane orientation of all the membrane-bound GALA peptides, at the lipid/peptide molar ratio of 2500/1 used in this study. We conclude that, on average, GALA molecules are oriented in transmembrane aggregates such that 74 $\pm$ 5% of the peptide has its N-terminus facing the inside of the liposome and 33 $\pm$ 7% of the peptide has its C-terminus facing the inside of the liposome. This is compatible with the results found in the assay with the external addition of BODIPY-avidin.

The binding of the biotinylated GALAs to BODIPY-avidin in the internal side exposure experiment could partially occur on the external side of the liposomes if there was leakage of BODIPY-avidin in solution through the GALA pores formed at pH 5.0. At a lipid/peptide molar ratio of 2500/1, GALA pores are too small to allow the permeation of vesicle-entrapped molecules such as BODIPY-avidin (~60,000 MW) (Nicol et al., 1996).

We demonstrated that, indeed, there is no leakage of BODIPY-avidin from the vesicles at the 2500/1 ratio. In a variation of the control no exposure, we raised the pH to 8.0 15 min after the addition of GALA 30 to the liposome suspension at pH 5.0. This pH increase dissociated all the peptides from the POPC bilayer. When we subsequently added d-biotin, the fluorescence enhancement of liposome-encapsulated BODIPY-avidin was slow and similar to the case in which no peptide was included in the experiment. This enhancement arose from the slow translocation of d-biotin into the liposomes that occurred in the absence of GALA pores. If a fraction of BODIPY-avidin had been released in solution during the 15 min at pH 5.0, d-biotin addition would have resulted in a rapid (<1 sec) enhancement of the fluorescence of the released BODIPY-avidin. From the lack of rapid enhancement, in the presence or absence of GALA, we could estimate that the percentage of external BODIPY-avidin, with or without pores in the membrane, was lower than the limit of detection of this assay and less than 1% of the encapsulated material.

After the pH increase, the percentage of released BODIPY-avidin was also determined by separation of the vesicles from the released material on a Sepharose 4-B column (1 $\times$ 15 cm). No fluorescent material was detected in the fractions where free BODIPY-avidin would elute. Thus, the observed BODIPY-avidin binding of the biotinylated GALAs in the internal side exposure experiment occurred only on the internal side of the vesicles.

Relationship between the external exposure of GALA termini and the formation of transmembrane aggregates

We suggest that an aggregate undergoes a transition between a surface state and a transmembrane configuration when its size reaches a minimum number (M) of GALA monomers. When the lipid/total peptide molar ratio is increased, we expect GALA membrane aggregates to be smaller because of the reduction in the average number of peptides bound per liposome. Therefore, increasing the lipid/total peptide ratio should reduce the fraction of peptides that participate in transmembrane aggregates and increase the fraction of peptides positioned parallel to the bilayer surface. In the latter case, peptides expose both of their termini to the external side of the bilayer, whereas the inserted ones display only one terminus. Thus, the fraction of biotinylated GALA that bind to BODIPY-avidin added to the vesicle/peptides solution at pH 5.0 is expected to be higher when the lipid/total peptide ratio is increased.

This is indeed the trend observed with the biotinylated peptide GALA(N)1 (Table 2, first and second columns). Measurements performed with GALA(N)1 are shown in Fig. 7 *A* and *B*, for lipid/total peptide molar ratios of 20,000/1 and 40,000/1, respectively. Raising the lipid/total peptide molar ratio from 7500/1 to 80,000 (Table 2) resulted in a drastic increase of the fraction of GALA(N)1 N-termini exposed on the external side of the liposome bilayer, as indicated by an increase of R_N from 0.285 to 0.91.

This tendency was also verified for GALA(C)3 C-terminus, as evidenced by an increase of R_C. At a lipid/total peptide molar ratio of 40,000/1, we obtained $R_N + R_C = 1.6$ ($\gg$ 1), meaning that 60% of the membrane-bound peptides exposed both termini on the outside of the vesicles and 40% were inserted. These results imply that the fraction of peptides oriented parallel to the membrane surface increased when the average number of peptides per liposome decreased.

We demonstrated by the mathematical model developed in greater details in Parente et al. (1990a), Rapaport et al. (1996), and Nicol et al. (1996), that GALA 30-induced leakage of ANTS/DPX from POPC liposomes at pH 5.0 is only generated by the aggregates that comprise at least M = 10 $\pm$ 2 peptides (Nicol et al., 1996). The peptides bind to the liposomes, aggregate quasi-irreversibly, and form transmembrane pores. We used the same model to gain insight into the transition of the peptides from lying parallel to the bilayer surface to rearranging into a transmembrane orientation. We tested the hypothesis that aggregates of a smaller size (e.g., M = 4, 6) than that needed for ANTS/DPX leakage might be transmembrane. Equation 13 allows us to calculate, for a particular value of M (minimum size of a transmembrane aggregate) and a fixed lipid/peptide molar ratio, the fraction of peptides that is involved in aggregates

TABLE 2 Relationship between the percentage of GALA N- (or C-)terminus exposed on the external side of a POPC bilayer, the formation of transmembrane aggregates, and the leakage of ANTS/DPX by pore formation at pH 5.0

Lipid/peptide molar ratio	N-terminus* outside (Exp., %)	C-terminus* outside (Exp., %)	Leakage of[#] ANTS/DPX (Exp., % (Cal., %))	Peptides in[§] transmembrane aggregates (Calc., %, M = 4 (6))	N-terminus[¶] outside (Calc., %, M = 4 (6))	C-terminus[¶] outside (Calc., %, M = 4 (6))
80,000	91 ± 7	N.D.**	1 (0.3)	14.4 (3.6)	89.2 (97.3)	N.D.
40,000	71 ± 6	89 ± 8	13 (7)	47.4 (21.7)	64.5 (83.7)	88.2 (94.6)
20,000	46 ± 6	N.D.	37 (32.2)	86.9 (64)	34.8 (52)	78.3 (84)
7,500	28.5 ± 4	76 ± 7	98 (94)	99.6 (98.9)	25.3 (25.8)	75.1 (75.3)
2,500	26.5 ± 3	73 ± 6	100 (99.1)	99.9 (99.7)	25 (25.2)	75 (75.1)

*Experimental values of N-terminus and C-terminus outside correspond to the percentage of membrane-bound GALA(N)1 and GALA(C)3, respectively, that bound to BODIPY-avidin added to a POPC liposomes/peptides solution at pH 5.0 (100 ∗ R). Values reported are based on the average of $n = 3$ determinations of R. We used a peptide mixture composed of GALA 30 and a biotinylated GALA (GALA(N)1 or GALA(C)3) at molar ratios of 19/1, 19/1, 9/1, 85/15, and 3/1, when lipid/total peptide molar ratios of 2500/1, 7500/1, 20,000/1, 40,000/1, and 80,000/1 were studied, respectively. The BODIPY-avidin binding site/biotinylated GALA molar ratio was always 8.

[#]Extent of GALA 30-induced ANTS/DPX leakage measured at the plateau, which was obtained in less than 15 min after peptide addition to the POPC vesicles. Calculated values are derived from the model described in Materials and Methods, taking $M = 10$ and $Ks = 15$ (Nicol et al., 1996).

[§]The percentage of peptides involved in transmembrane aggregates is determined as explained in Materials and Methods, assuming that only the aggregates that contain $M = 4$ (or $M = 6$, value in parenthesis) or more peptides are transmembrane.

[¶]Calculated values of N-terminus and C-terminus outside are obtained from the percentage of peptides involved in transmembrane aggregates (column 5), assuming that ¼ of the transmembrane peptides exposed their N-termini on the external side of the bilayer, whereas the rest of them (¾) expose their C-termini. In surface aggregates (size $i < 4$ (6)), all the peptides expose both termini on the external side of the vesicle bilayer.

**N.D. = not determined.

of size i, with $i \geq M$ (Table 2, column 5). If we make the assumptions that 1) in transmembrane aggregates (of size $i \geq M$), the average fraction of membrane-bound GALA peptides that exposes its N-terminus on the external side of the liposome is ¼, and 2) in surface aggregates (of size $i < M$), every peptide exposes both termini to the external side, the model can predict the percentage of GALA that exposes its N-terminus (or C-terminus) on the external side of the vesicles. Calculated values for $M = 4$ and $M = 6$ are displayed in Table 2, columns 6 and 7, for various lipid/peptide molar ratios. Experimental values of the percentage of externally exposed N-termini (or C-termini) are displayed in column 2 and 3. An interpretation of these calculated and experimental results is given in the General Discussion.

GENERAL DISCUSSION

Applicability of the assay to determine the orientation of GALA and other peptides

Finkelstein and coworkers have demonstrated the application of the avidin–biotin interaction to provide information on the transbilayer location of residues in membrane proteins (Slatin et al., 1994; Qiu et al., 1994, 1996). These studies showed that biotinylated residues not positioned in the hydrophobic core of the membrane are accessible to streptavidin binding. Based upon this precedent, we have modified a fluorescent BODIPY-avidin/biotin binding assay (Emans et al., 1995) to elucidate GALA 30 surface-adsorbed or transbilayer orientation as well as the head-to-tail (N- to C-terminus) orientation of the transbilayer GALA 30 in POPC liposomes at low pH. This assay can be applied to other membrane-interacting peptides to determine their orientation as well as to discern how rapidly they move across bilayers or between membranes.

On examining the fluorescence change associated with occupancy of the biotin binding sites of BODIPY-avidin, it became apparent to us that a simple assay that merely looked at the fluorescence increase associated with the binding of liposome-bound biotinylated GALA to BODIPY-avidin at pH 5.0 was unable to quantify the extent of biotinylated GALA binding to BODIPY-avidin because of a number of factors. These include the low fluorescence increase of BODIPY fluorescence associated with the binding of BODIPY-avidin to a membrane-bound biotinylated GALA at pH 5.0 and the impossibility to evaluate the fluorescence increase that would arise from complete biotinylated GALA binding to BODIPY-avidin in the exact same conditions. These factors led us to assays that require three measurements in which multiple additions of reagents are performed. Despite the complexity of the assays, the difference between the three fluorescent determinations is only due to the proportion of two populations of BODIPY-avidin that are in the same environment. This situation permits a precise determination of biotinylated GALA binding to BODIPY-avidin, and thus GALA orientation, in a POPC bilayer at pH 5.0. It could be that, for other peptides, a simpler assay based upon the fluorescence increase following binding to the BODIPY-avidin would suffice for quantification of peptide orientation. This was not the case for GALA.

BODIPY-avidin/biotinylated GALA binding assay to determine GALA orientation in POPC vesicles

To study the orientation of GALA 30 in POPC bilayers at pH 5.0 (the surface-adsorbed or transbilayer orientation as

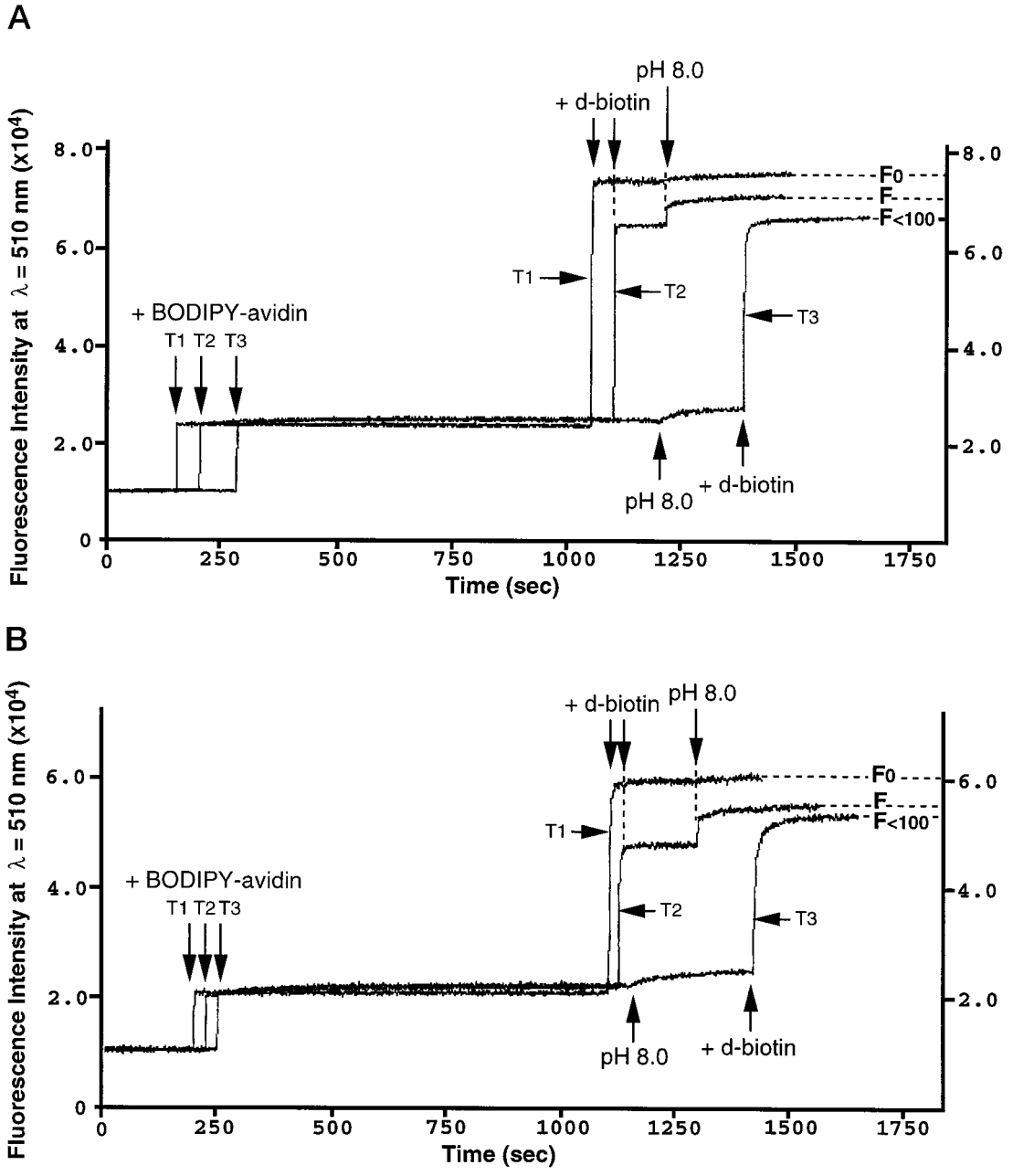


FIGURE 7 Determination of R as a function of the lipid-to-total peptide molar ratio using the biotinylated peptide GALA(N)1. Traces were obtained by monitoring the light emitted at $\lambda = 510$ nm and following the protocol described in Materials and Methods. The legend is similar to that of Fig. 4, except that the lipid-to-total peptide molar ratios used are (A) 20,000 and (B) 40,000 instead of 2500. At $t = 0$, T2 and T3 refer to solutions at pH 5.0 that contain POPC vesicles (2.5 mM lipid) and (A) a mixture of GALA 30/GALA(N)1 9/1 (total peptide concentration of 125 nM) or (B) a mixture of GALA 30/GALA(N)1 85/15 (total peptide concentration of 62.5 nM). The peptide mixture was replaced by nonbiotinylated GALA 30 at the same peptide concentration as in T1. A BODIPY-avidin binding site/GALA(N)1 molar ratio of 8/1 and a d-biotin/BODIPY-avidin binding site molar ratio of 4/1 were used in these experiments. The r ($= R/Z$) values of these particular sets of experiments were (A) 0.51 and (B) 0.73.

well as the head-to-tail (N- to C-terminus) orientation of the transbilayer peptide), we selected the biotin/avidin binding as the basis of our assays. GALA 30 and its variants were biotinylated and avidin was modified with BODIPY. These labeled components were used in fluorescence-based determinations of the percentage of biotinylated membrane-bound peptides accessible for binding to BODIPY-avidin. Biotin's interaction with avidin is among the strongest non-covalent affinities known ($Ka = 10^{15}\ M^{-1}$). Thus, when a BODIPY-avidin/biotin binding occurs, it should persist at least for the time needed to determine the orientation of the peptide in the membrane. We can also expect that an avidin-bound biotinylated molecule would not exchange with another biotin-containing molecule during this time frame.

A biotinylation reagent includes, at one end, the bicyclic ring system necessary for binding to avidin and, at the other

end, a reactive functional moiety to couple with the target group on the molecule to be labeled. A spacer arm is included in between to extend the biotin group away from the modified molecule, thus ensuring better interaction capability with avidin. In our assays, when the biotin moiety attached to a membrane-bound GALA is exposed on the external (or internal) side of the liposome bilayer, its spacer arm must be long enough to allow binding of the bicyclic ring system to BODIPY-avidin. The biotinylation reagents used in this study to label GALA were selected to satisfy this criterion. The original GALA sequence, GALA 30, was labeled directly on the primary amine of the N-terminus with Biotin-X/SE, yielding GALA(N)1, whereas the three other labeling procedures were performed by coupling *N*-(biotinoyl)*N′*-(iodoacetyl)ethylenediamine to a cysteine on a GALA 30 analog. GALA $31C_N$, which is GALA 30 elongated with a cysteine at the N-terminus, was labeled to yield GALA(N)2. GALA $29C_C$ and GALA $31C_C$, which contain a cysteine at the C-terminus, were labeled to yield GALA(C)2 and GALA(C)3, respectively. The two biotinylation reagents selected for labeling, Biotin-X/SE and *N*-(biotinoyl)*N′*-(iodoacetyl)ethylenediamine, have sufficiently long spacer arms (22.4 Å and 21.4 Å, respectively) to bind to BODIPY-avidin.

In addition, to avoid changing the pore-forming properties of the peptide, biotinylation should not change considerably its hydrophobicity. It is indeed the case, because when we separated the biotinylated peptide from the unlabeled peptide by HPLC on a C18 preparative column by the solvent system: (A) water-0.1% TFA/(B) water-0.1% TFA, the difference in elution corresponded to less than 4% B.

GALA forms a stable pore in bilayers

Unlike several natural cationic peptides for which transient pores have been advocated to explain peptide-induced dye release from liposomes [e.g., melittin, mastoparan, and magainin-2 (Rex and Schwarz, 1998; Matsuzaki et al., 1995, 1996, 1997a; Schwarz and Arbuzova, 1995)], GALA 30 forms stable pores in POPC, POPC/POPG, and egg PC liposomes at pH 5.0. This statement is supported by Fourier transform infrared studies (Goormaghtigh et al., 1991) and by the finding that ANTS/DPX leakage proceeds through an all-or-none mechanism (the permeabilized vesicles leak their entire content of ANTS/DPX), with a very slow rate of peptide transfer between membranes after insertion (Parente et al., 1990a). Our current studies confirmed that GALA forms stable pores in POPC large unilamellar vesicles at pH 5.0. When d-biotin molecules were added to the POPC vesicles in the absence of the peptide at pH 5.0, they exhibited a very slow penetration through the membrane, whereas their addition at any time after peptide addition resulted in rapid translocation. This result indicates that the pores were still present at the time of d-biotin addition. Furthermore, if the pores would undergo processes of formation and destruction, then eventually all the membrane-bound biotinylated peptides would be bound to BODIPY-avidin molecules added externally or pre-encapsulated in the vesicles. However, the fraction of labeled peptides bound irreversibly to BODIPY-avidin in the internal layer exposure and external layer exposure measurements did not vary appreciably upon changing the time of incubation of BODIPY-avidin with the liposome/peptides at pH 5.0 from 10 to 30 min, before the addition of d-biotin (data not shown).

GALA orientation (surface-adsorbed or transbilayer) in relation to pore formation

Our studies indicated that all the vesicle-bound GALAs were involved in transmembrane aggregates at a lipid/peptide molar ratio of 2500/1 and at pH 5.0, because one terminus per peptide was displayed on each side of the bilayer ($R_N + R_C \approx 1$ and $R'_N + R'_C \approx 1$ in Table 1). Moreover, the fact that all the peptides displayed their N-termini and their C-termini either on the external or the internal side of the liposomes ($R_N + R'_N \approx 1$ and $R_C + R'_C \approx 1$ in Table 1) confirmed the transbilayer orientation of the peptides. Thus, the percentage of peptide that exposed its N- or C-terminus to both sides of the bilayer, in the time frame of the measurements, was minimal. The fraction of peptides that was buried in the bilayer core such that one of their termini was inaccessible to BODIPY-avidin binding on either side of the bilayer was also minimal. The fact that almost 100% of the N-terminus or C-terminus biotinylated peptides ($R_{total} = 0.96$ for GALA(N)1 and $R_{total} = 0.98$ for GALA(C)3) bound to BODIPY-avidin when the protein was, in the same measurement, pre-encapsulated and added externally (data not shown), demonstrated the accessibility of all the termini for binding to BODIPY-avidin at a lipid/peptide molar ratio of 2500/1.

At lipid/total peptide molar ratios of 2500/1 and 7500/1, where all the peptides are transmembrane and form or are associated with pores, there was complete leakage of ANTS/DPX (Table 2) and the fraction of GALA N-termini exposed to the external side of the bilayer was similar (0.265 and 0.285, respectively). This result suggests that a fixed fraction (~0.25) of the peptide that composed the pores was oriented with its N-terminus exposed to the external side of the membrane. The N-terminus predominantly resided on the inner side of the membrane ($R'_N = 0.74$ at a ratio of 2, 500/1).

We cannot determine from these measurements whether, in all the pores, the helices are oriented in an alternating fashion at a 3/1 N-terminus inside/N-terminus outside ratio, or if ¼ of the pores have all the helices with their N-terminus outside and ¾ of the pores have all the helices with their N-terminus inside.

We demonstrated an increase in the fraction of peptide that exposed its labeled terminus to the external side of the vesicles when the lipid/peptide molar ratio was raised, i.e., under conditions at which pore formation and leakage of

ANTS/DPX were reduced (Table 2). This result indicates that the fraction of peptide that was positioned parallel to the bilayer surface increased when the number of membrane-bound peptides per vesicle decreased. At a ratio of 80,000/1, where little leakage of ANTS/DPX occurred, the fraction of external N-terminus was increased to 0.91 (Table 2). The sensitivity of the assays makes the measurements more difficult at very high lipid/total peptide molar ratios, but the trend is that the fraction of the N-terminus exposed to the external side of the membrane tends toward unity. A similar evolution was observed with the C-terminus.

Additional details of the mechanisms of pore formation by the amphipathic peptide GALA in POPC vesicles at pH 5.0 have emerged from the results obtained in this study. Previously, Parente et al. (1990a) showed that the leakage of ANTS/DPX requires pores consisting of at least $M = 10 \pm 2$ peptides. The calculations using Eq. 13 demonstrated that, if we assume that surface aggregates become transmembrane when they reach a size of $M = 4$ or 6 peptides, the experimental values for the fraction of N-terminus exposed on the external side of the membrane at various lipid-to-peptide molar ratios could be explained (Table 2). Thus, the model provided a consistent relationship between the observed exposure of the termini and a putative transmembrane aggregate.

The emerging picture of the process of pore formation by GALA in POPC vesicles at pH 5.0 is as follows. The initial rapid binding of the peptide to the vesicle does not involve a complete penetration of the peptide into the bilayer. The peptides aggregate within the membrane at what we refer to as the parallel orientation, resulting in both termini being exposed to the external medium. When an aggregate has reached a critical size ($M = 4$ to 6), a rearrangement of the peptides occurs, which leads to their rapid insertion into the membrane and the formation of a transmembrane structure. However, the aggregate apparently contains too few monomers to form a pore that would allow the passage of ANTS/DPX. If the bilayer contains sufficient peptides, the aggregate can grow in size to 10 ± 2 peptides to form a pore through which ANTS/DPX can leak.

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