Interaction of the HIV-1 Fusion Peptide with Phospholipid Vesicles: Different Structural Requirements for Fusion and Leakage†

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ABSTRACT: This paper presents a study on the membrane fusion activity of a 23-residue synthetic peptide, representing the N-terminus of gp41 of the human immunodeficiency virus type I (HIV-1; LAV_{1a} strain), in a model system involving large unilamellar vesicles (LUV) composed of the negatively charged 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG). The peptide (HIVarg) induced fusion of POPG LUV as evidenced by (i) mixing of membrane lipids, (ii) mixing of aqueous vesicle contents, and (iii) an irreversible increase in vesicle size. Fusion could be induced only in the presence of millimolar concentrations of Ca^{2+} or Mg^{2+}, needed for induction of vesicle aggregation; the divalent cations by themselves did not induce any fusion. The rate constant of the fusion reaction, as determined by simulation of the process according to a kinetic model, increased dramatically with the peptide-to-lipid molar ratio, indicating that the peptide was the mediator of the process. In the absence of divalent cations, the HIVarg peptide induced leakage of small molecules due to formation of pores in the membrane of single vesicles. Final extents and kinetics of this leakage process could be simulated adequately by model calculations for peptide-to-lipid ratios ranging from 1:25 to 1:750. Experiments, in which the order of peptide and Ca^{2+} addition to the vesicles was varied, indicated that the peptide is likely to adopt two different structures, one in the absence of Ca^{2+}, primarily supporting leakage by formation of pores in separate vesicles, and one in the presence of Ca^{2+}, primarily supporting fusion. Once a final structure had been established, it persisted even upon addition or removal of Ca^{2+}. From the infrared spectroscopy of the peptide at equilibrium with POPG vesicles it is concluded that the structure formed in the absence of Ca^{2+}, supporting leakage, represents predominantly an α-helix, whereas in the presence of Ca^{2+}, i.e., under conditions supporting fusion, the peptide adopts mostly an extended antiparallel β-structure.

The envelope glycoprotein gp160 (gp120/41) mediates the membrane fusion activity of the human immunodeficiency virus type 1 (HIV-1), a multistep process which is essential for the infectious entry of the virus into the host cell (Larsen et al., 1992; Moore et al., 1993). It is established that the endoproteolytic cleavage of the gp160 precursor is required for virus infectivity (McCune et al., 1988; Hallenberger et al., 1992). Proteolytically generated gp41 and gp120 subunits remain associated through noncovalent interactions (Kowalski et al., 1987). Conserved regions in the gp120 subunit are responsible for the binding of the virus to the CD4 receptor prior to fusion (Lasky et al., 1987; Ho et al., 1991). Soluble CD4 has been shown to be able to induce conformational changes in gp120/41 after binding (Moore et al., 1990), which result in the dissociation of the subunits with the subsequent exposure of previously cryptic epitopes in the ectodomain of gp41 (Sattentau & Moore, 1991). The exposure of the putative fusion sequence, a conserved segment with hydrophobic character of about 25 amino acids located at the N-terminus of gp41, has been proposed to constitute an integral part of the fusion mechanism of HIV (Moore et al., 1993).

The fusion peptide of gp41 shares homology with those of other enveloped viruses, such as ortho- and paramyxoviruses (Gallaher, 1987). Syncytium formation in cell cultures transfected with HIV or SIV[1] envelope glycoprotein has been demonstrated to be enhanced by mutations within the fusion sequence which increase its hydrophobicity (Bosch et al., 1989), while substitutions that increase the charge inhibit or block the process (Freed et al., 1990). Recently, it has been reported that oligopeptides homologous to the gp41 N-terminal peptide inhibit fusion of HIV-1 infected cells (Owens et al., 1990) and cellular entry of the virus (Slepushkin et al., 1993).

Studies on membrane interactions of viral fusion sequences have first employed peptides derived from the influenza hemagglutinin (Murata et al., 1987; Lear & DeGrado, 1987; Wharton et al., 1988; Takahashi, 1990; Rafalski et al., 1991). With respect to the HIV fusion sequence, Rafalski et al. (1990)

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CL, cardiolipin; CHOL, cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylenebis(pyridinium)bromide; EDTA, ethylenediaminetetraacetic acid; FITC-dextran, fluorescein isothiocyanate dextran; FTIR, Fourier transform infrared spectroscopy; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HIVarg, N-terminal sequence (23 aa) of HIV-1 (LAV_{1a}) gp41; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phophatidylethanolamine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; SIV, simian immunodeficiency virus.

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have reported on destabilization of POPG liposomes induced by the peptide HIVarg, representing the N-terminal 23 residues of the gp41 (LAV_{1a} strain). They also showed that limited membrane lipid mixing occurs between small sonicated POPG vesicles. Slepushkin et al. (1992) showed that gp41 peptides can induce an increase in conductance of planar lipid bilayers, lysis of liposomes, and mixing of liposomal lipids. It should be noted, however, that the latter studies were performed at high peptide-to-lipid ratios and peptide-induced nonspecific effects are likely to occur under those conditions. HIVarg has been shown to induce lysis of human erythrocytes and CD4+ lymphocytes (Mobley et al., 1992), probably after insertion of the amino-terminal alanine into the hydrophobic core of the membrane (Gordon et al., 1992). The latter finding is in apparent contradiction with proposed theoretical models which imply that related gp41 peptides interact with membranes by inserting the C-terminus into the hydrophobic core in an oblique orientation (Brasseur et al., 1988). A shorter 16-residue peptide was recently reported to induce, at high doses, lipid mixing and leakage of contents in PE-containing vesicles (Martin et al., 1993).

In this study we demonstrate that the HIVarg peptide has the capacity to induce fusion of large unilamellar phospholipid vesicles. Independent evidence is presented for peptide-induced mixing of bilayer lipids, for mixing of aqueous vesicle contents, and for the occurrence of an irreversible increase in vesicle size. We selected the negatively charged phospholipid POPG, since it allows control over the aggregation state of the vesicles, i.e., in the presence of millimolar concentrations of Ca^{2+} or Mg^{2+} the vesicles aggregate in such a way that the bilayer integrity is not affected, fusion does not occur, and the aqueous vesicular contents are completely retained (Papahadjopoulos et al., 1977, 1980). In addition, POPG vesicles have been used in a previous study on the lipid interactions of the HIVarg peptide (Rafalski et al., 1990). It is demonstrated that, in the absence of divalent cations, the HIVarg peptide forms pores in the bilayer of separate vesicles, similar to the pore formation mediated by the amphipathic peptide GALA (Parente et al., 1990). However, this pore formation does not represent the destabilization required for induction of membrane fusion, since, in the presence of Ca^{2+}, pore formation is inhibited while at the same time fusion of the vesicles is promoted. Infrared spectroscopy of the peptide in equilibrium with POPG vesicles suggests that, under conditions supporting pore formation in single vesicles, the peptide adopts an α-helix structure, whereas, under conditions promoting fusion, an antiparallel β-conformation is preferred.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylglycerol (POPG) and the fluorescent probes, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylenebis(pyridinium)bromide (DPX) were from Molecular Probes (Junction City, OR). Fluorescein isothiocyanate dextran (MW_{av} 19 600), cholesterol (CHOL), and Triton X-100 were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Peptide Synthesis. The sequence AVGIGALFLGFLGAAGSTMGARS representing the N-terminus of the HIV gp41 (LAV_{1a} strain) was synthesized as its C-terminal carboxamide by Dr. T. Saermark (EC Concerted Action Programme) as previously described (Martin et al., 1991). Purification was carried out by reversed-phase HPLC (estimated homogeneity >90%). Final amino acid analysis confirmed the correct composition. Peptide stock solutions were prepared in DMSO (spectroscopy grade).

Vesicle Preparation. Large unilamellar vesicles (LUV) were prepared according to the extrusion method of Hope et al. (1985). Briefly, dried lipid films were dispersed in 5 mM Hepes, 100 mM NaCl (pH 7.4) buffer and subjected to 10 freeze–thaw cycles prior to extrusion 10 times through two stacked polycarbonate membranes (Nuclepore, Inc., Pleasanton, CA). Vesicle size distributions were determined with a laser particle sizer NICOMP 370 (Santa Barbara, CA). Extrusion through membranes of a nominal pore size of 0.1 μm produces POPG vesicles with a mean diameter of 95 nm. Extrusion through 0.4-μm pore membranes results in the formation of vesicles with an average diameter of 202 nm. When solutes were encapsulated in the liposomes, internal and external osmolarities were adjusted with NaCl. The osmolarities of all solutions were measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Lipid concentrations of liposome suspensions were determined by phosphate analysis (Böttcher et al., 1961).

Fluorimetric Assays. Measurements were conducted at 37 °C in thermostated cuvettes with constant stirring, in a SLM-8000 spectrofluorimeter (SLM/Aminco, Urbana, IL). In all cases the kinetics of fluorescence changes were recorded continuously. In the figures, showing time courses of fluorescence development, data symbols are given solely for the purpose of a facile identification of the curves.

Membrane Mixing. Fusion of POPG vesicles detected as the mixing of their membrane components was monitored using a resonance energy transfer assay [RET assay (Struck et al., 1981)]. The assay is based on the dilution of NBD-PE and Rh-PE. Dilution due to membrane lipid mixing results in an increase in NBD-PE fluorescence. Vesicles containing 0.6 mol % of each probe were mixed with unlabeled vesicles at a 1:1 ratio. The NBD emission was monitored at 530 nm with the excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interferences. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labeled vesicles and the 100% value to complete mixing of all the lipids in the system. The latter value was set by the fluorescence intensity of vesicles, labeled with 0.3 mol % each of the fluorophores, at the same total lipid concentration as that in the fusion assay. It should be noted that, in the concentration range of the fluorophores used, the NBD-PE fluorescence intensity increases linearly with the dilution of the probes (Struck et al., 1981).

Leakage of Contents. Release of vesicular contents to the medium was monitored by the ANTS/DPX assay (Ellens et al., 1985). LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 5 mM Hepes were obtained by separating the unencapsulated material by gel-filtration in a Sephadex G-75 column eluted with 5 mM Hepes and 100 mM NaCl (pH 7.4). Osmolarities were adjusted as described above. Fluorescence measurements were performed by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The 0% leakage corresponded to the fluorescence of the vesicles at time zero; 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5% v/v). In order to rule out the possible inactivation of the peptide in solution due to adsorption to the cuvette

walls, control experiments were carried out in peptide-coated cuvettes. Essentially the same kinetic behavior was observed when these cuvettes were used.

Mixing of Aqueous Contents. We followed the FITC-dextran assay developed by Stutzin (1986) with the modifications introduced by Tournois et al. (1990). Aqueous contents mixing is monitored as dequenching of FITC fluorescence following the dilution of vesicle contents into acceptor vesicles. FITC-dextran (MW_{av} 19 600) was encapsulated in POPG vesicles extruded through 0.4-μm pore-size membranes at the self-quenching concentration of 4 mM in 5 mM Hepes and 40 mM NaCl at pH 7.4. Nonentrapped material was removed by gel filtration on a Sephacryl S-300 HR column eluted with 5 mM Hepes and 65 mM NaCl, pH 7.4. External and internal media were isosmotic. FITC-dextran-loaded vesicles were mixed in a 1:19 ratio with unloaded acceptor vesicles. HIVarg-induced fluorescence dequenching was monitored by setting the excitation at 465 nm and the emission at 530 nm. A cutoff filter at 495 nm was used in emission. The 100% dequenching values were determined by addition of Triton X-100 (0.5%, v/v).

Analysis of Fusion Kinetics. The analysis of the kinetics of fusion of POPG vesicles is relatively simple, since it is assumed that the vesicles are essentially preaggregated by Ca^{2+} or Mg^{2+}, and that the binding of the added peptides to the vesicles is fast. This procedure directly yields the fusion rate constant, f, from a simulation of the initial stages of NBD-PE fluorescence increase according to

$$I = 50[1 - \exp -(ft)] \quad (1)$$

in which t is the time and I is the percent of fluorescence increase relative to 100%, which is the level corresponding to complete merging of the membranes in a 1:1 population. The factor 50 corresponds to an initial probability of 0.5 for a fusion between a labeled and a blank vesicle.

Theoretical Analysis of Leakage Kinetics. The model employed has been described in Parente et al. (1990) for leakage induced from PC liposomes upon addition of the synthetic peptide GALA. The computational procedure is essentially as described in the above work except for several refinements as explained below. The model assumes that the HIVarg peptide binds and becomes incorporated into the bilayer, while, once within the membranes, peptide aggregation occurs. When an aggregate within a membrane has reached a critical size, a channel or a pore is created within the membrane, and leakage of encapsulated molecules can occur. The size of the pore dictates the upper bound on the size (and shape) of molecules that can leak. The analysis proceeds in two stages. First, the minimal number of peptides required to form a pore, M, is determined from a fit of the final extent of leakage. This step requires a determination of the binding constant, K, of peptides to vesicles of a given size. According to the model, the final extent of leakage is due to the leakage of contents from all the vesicles containing M or more peptides, where M is the critical number of peptides in the aggregate. For a completely homogeneous population of vesicles, the final extent of leakage $L \equiv L(\infty)$ is given by

$$L = \sum_{i=M}^{N} A_i/G_0 \quad (2)$$

in which N is the largest number of peptides that can be bound to a single vesicle, G_0 is the molar concentration of vesicles, and A_i is the concentration of vesicles containing i incorporated peptides. The dimensionless quantities $A_i = A_i/G_0$ represent the distribution function, satisfying the relation

$$\sum_{i=0}^{N} A_i/G_0 = \sum_{i=0}^{N} \bar{A}_i = 1 \quad (3)$$

The procedure for calculating the functions A_i was derived by Nir et al. (1986) and is also described in Bentz et al. (1988) and Parente et al. (1990). A refinement in the current procedure includes a determination of the distribution of vesicle sizes as already described before. This information is crucial in any attempt to employ simulations and predictions for the final extents and kinetics of leakage, since the encapsulated volume depends on the third power of the internal radius. The modified expression of eq 2 is

$$L = \sum_{j=1}^{m} \sum_{i=M}^{N} \bar{A}_{ij} f_j \quad (4)$$

in which f_j is the fraction of encapsulated volume in vesicles of size j. We have used $m = 10$ for the number of vesicle sizes considered. It has to be noted that for each size the value of K was separately calculated by the program assuming that the intrinsic affinity for peptide binding is independent of vesicle size. The results are essentially independent of the value of N used in the calculations, provided it is sufficiently large. We have used $N = 2000$ for the vesicles of the largest size.

After determining the value of M, the number of peptides required for pore formation, we determine the forward rate of peptide aggregation, $\bar{C}$, which in the program has units of s^{-1}. The relevant parameter in the calculation of the kinetics of leakage is the dimensionless quantity $\tau = \bar{C}it$, where t is the time (s) and i is proportional to the surface concentration of the peptides (units = 1/area). For the same value of i, the number of peptides per vesicle, the surface density is larger for the smaller vesicles. This was taken into account by the program. As in Parente et al. (1990) we report a value of $\bar{C}$ that corresponds to the outer surface area of a 100-nm radius vesicle. The same values of M and $\bar{C}$ are used for all peptide-to-lipid ratios.

Fourier Transform Infrared (FTIR) Spectroscopy. Prior to infrared measurements, peptide dissolved in DMSO was added to POPG LUV prepared in D_2O buffer, so that the desired lipid-to-peptide molar ratio was reached under conditions similar to those described for the fusion and leakage assays. After a 30-min incubation at room temperature, centrifugation of the peptide–lipid complexes (160 000g, 90 min) gave rise to a homogeneous band of vesicles floating on top of the D_2O. Infrared spectra were recorded in a Nicolet 520 spectrometer equipped with a DTGS detector. Samples, containing $\approx$4 mg peptide/mL, were placed between two CaF_2 windows separated by 50-μm spacers. A total of 200 scans (sample) and 200 scans (reference) were taken for each spectrum, using a shuttle device. Spectra were transferred to a personal computer where solvent subtraction and band-position determinations were performed as previously reported (Arrondo et al., 1989).

RESULTS

The HIVarg Peptide Induces Fusion of POPG LUV. Fusion was monitored by mixing of membrane components, increase in vesicle size, and mixing of aqueous contents. The HIVarg peptide alone was not able to induce fusion even in POPG vesicle suspensions as concentrated as 1 mM, due to its inability

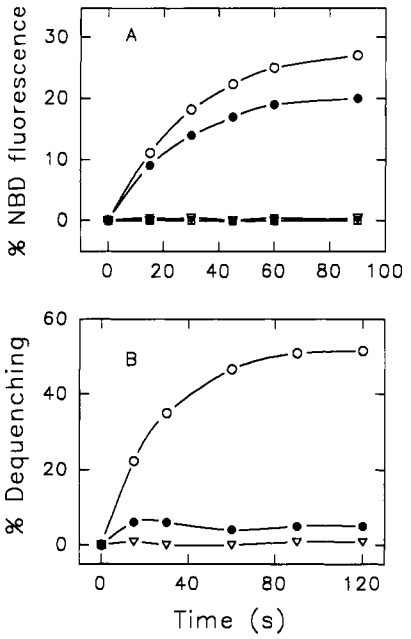

FIGURE 1: HIVarg-induced membrane lipid mixing in the presence of divalent cations. (Panel A) Lipid mixing detected by the RET assay as a function of time. The peptide (1:25 peptide-to-lipid mole ratio) was added to the POPG LUV (50 μM) in presence of the cations: (○) 5 mM Ca^{2+}; (●) 10 mM Mg^{2+}. No peptide was present in (▽) 5 mM Ca^{2+} or (▼) 10 mM Mg^{2+}. No cation was present in (□). NBD fluorescence was recorded continuously. (Panel B) HIVarg-induced aqueous contents mixing of POPG LUV as a function of time. Vesicles (final concentration, 50 μM) were extruded through 0.4-μm pore membranes. The peptide was added to a mixture containing FITC-dextran-loaded vesicles and empty vesicles at a 1:19 ratio (1:25 peptide-to-lipid mole ratio). (○) Peptide in 10 mM Ca^{2+}; (●) peptide without Ca^{2+}; (▽) 10 mM Ca^{2+} without peptide. Release was recorded continuously.

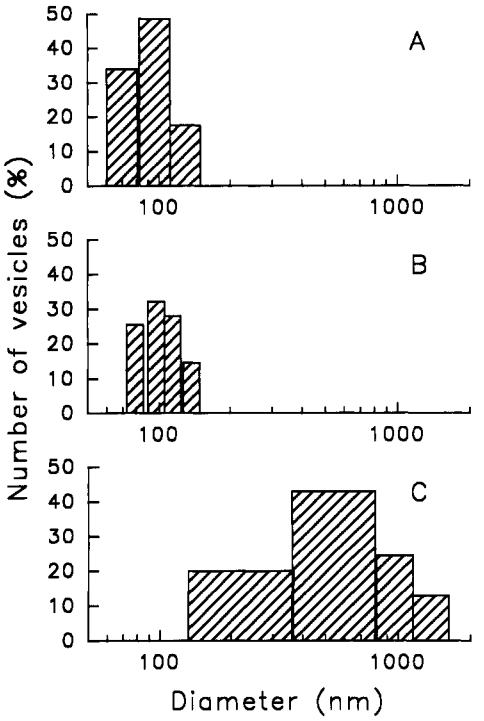

FIGURE 2: HIVarg-induced irreversible size increase of POPG LUV. The peptide was added to a final peptide-to-lipid ratio of 1:30 to a POPG LUV suspension (400 μM) in the presence of 5 mM Ca^{2+}. (Panel A) Size distribution of POPG vesicles extruded 10 times through 0.1-μm pore membranes; (panel B) size distribution after incubation with Ca^{2+} for 10 min followed by addition of 10 mM EDTA; (panel C) size distribution after treatment with Ca^{2+} and HIVarg (added 30 s after the cation) and addition of 10 mM EDTA after 10 min.

to cause aggregation of POPG LUV, as deduced from light scattering observations (data not shown). Vesicle aggregation was accomplished by adding 1–10 mM Ca^{2+} or Mg^{2+}. Importantly, the divalent cations alone did not induce any vesicle fusion in this concentration range, as shown below.

Mixing of membrane lipids was investigated by employing the RET assay (Struck et al., 1981), which monitors the increase in NBD fluorescence following probe dilution from labeled to unlabeled vesicles, which in our experiments were present in a 1:1 ratio. Figure 1 illustrates that in the presence of up to 10 mM Ca^{2+} or Mg^{2+}, but in the absence of peptide, no fusion of POPG vesicles occurred. On the other hand, addition of the HIVarg peptide induced extensive lipid mixing. Therefore, it can be concluded that, in our assay, HIVarg induces fusion of otherwise stable POPG liposomes. This is supported by the irreversible increase in size of the vesicles observed when HIVarg was added to POPG vesicles in the presence of 5 mM Ca^{2+}. Figure 2 shows the observed changes in size distribution of the vesicles. The mean diameter of the initial population was 95 nm. Large aggregates were formed by addition of 5 mM Ca^{2+}, but this aggregation was completely reversible upon addition of 10 mM EDTA after 10 min. However, addition of HIVarg, 30 s after the addition of Ca^{2+}, resulted in an increase of the mean vesicle diameter to 680 nm, as determined after the addition of EDTA.

Further evidence for fusion activity of HIVarg in this system was obtained from an assay that allowed the detection of peptide-induced mixing of vesicular aqueous contents. We employed an assay based on the dequenching of fluoresceinated dextran (Stutzin, 1986), at a 1:19 ratio of dextran-loaded to unloaded vesicles. As shown in Figure 1B, the addition of HIVarg to POPG vesicles caused dilution of fluoresceinated dextran into the lumen of the unloaded vesicles, resulting in about 50% dequenching of the fluorescence. Less than 10% dequenching was observed in the absence of unloaded vesicles, indicating that the probe did not leak out significantly to the medium after the interaction of HIVarg with the vesicles. This and the above observations taken together demonstrate that HIVarg has the capacity to induce effective mixing of aqueous contents of POPG LUV under conditions which also allow mixing of the membrane lipids.

Kinetic Analysis. The experimental results presented so far, mixing of membrane lipids, irreversible increase in vesicle size, and mixing of aqueous contents of the vesicles, provided conclusive evidence for the capacity of the HIVarg peptide to induce fusion of large POPG vesicles. In order to obtain a quantitative characterization of this fusion process, we analyzed the kinetics as obtained with the lipid-mixing assay. Figure 3 presents several kinetic curves under different experimental conditions together with calculated values obtained by simulating the experimental values with a mass-action kinetic model. The simulation yields a fair agreement between the experimental and the calculated values of fluorescence increase. An inspection of the fusion rate constants (Table 1) indicates that, at a fixed Ca^{2+} and lipid concentration, increasing the amount of peptide increases the rate constant of the fusion step in the overall process. This indicates that the peptide per se constitutes the exclusive fusogenic agent in the system.

Pore Formation by the Peptide. Figure 4 panels A and B demonstrate that the HIVarg peptide induces leakage of ANTS in the absence of divalent cations. Figure 4A illustrates the steep dependence of the kinetics of leakage on the peptide-

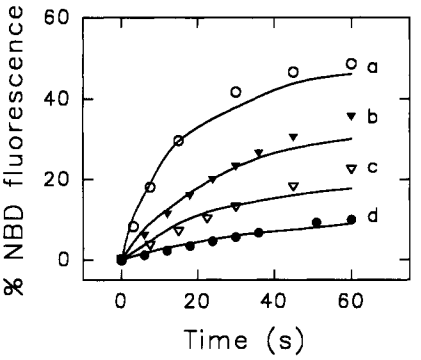

FIGURE 3: Membrane lipid mixing of POPG LUV as a function of peptide-to-lipid ratio and Ca^{2+} concentration. Vesicle concentration was 50 μM. Peptide-to-lipid ratios and Ca^{2+} concentrations were (a) 1:12.5, 5 mM; (b) 1:25, 10 mM; (c) 1:25, 5 mM; (d) 1:50, 5 mM. Experimental results recorded continuously are given by solid curves. Symbols indicate values obtained from simulation of the results using a mass-action kinetic model (see Materials and Methods).

Table 1: Rate Constants of Fusion for POPG LUV

peptide-to-lipid mole ratio	Ca^{2+} concentration (mM)	rate constant of fusion f (s^{-1})
1:12.5	5	0.06
1:25	10	0.02
1:25	5	0.01
1:50	5	0.005

to-lipid ratio. At a 1:100 peptide-to-lipid ratio, 40% of the pre-encapsulated ANTS was released within 30 s after peptide addition, while after 5 min leakage had reached its maximum of 58%. Conversely, under the same conditions dextran was mainly retained within the vesicles (maximum amount released 10%), indicating that the overall vesicle integrity was preserved during the leakage process (data not shown). Figure 4B illustrates the dependence of the extent of leakage on the peptide-to-lipid ratios. At a 1:25 ratio leakage was complete, whereas at a 1:750 ratio only 3.3% leakage was observed at equilibrium. The termination of the leakage after a few minutes and the existence of partial release are similar to the observations of leakage induced from neutral liposomes by the amphipathic peptide GALA at pH 5 (Parente et al., 1990). In the latter study the leakage data were explained and predicted by employing a pore model. According to this model the peptides bind to the vesicles and then aggregate within the membrane. When a peptide aggregate in a vesicle includes at least M peptides, a pore is formed and all the contents leak. On the other hand, vesicles that include less than M peptides do not leak at all. Thus one feature of this model is the occurrence of an all-or-none mechanism of leakage. We investigated the possibility that the HIVarg peptide forms pores in POPG vesicles. Vesicles treated with HIVarg were separated from unencapsulated material on a Sephadex G-75 column. The fluorescence in the collected vesicles was shown to be quenched to the same degree (81 ± 5%) as that in the original vesicles (78 ± 5%), consistent with an all-or-none mechanism of leakage. In the case of graded release the vesicles should exhibit a range of self-quenching values after passage through the column. For example, a final extent of 80% leakage would result in 55% quenching of ANTS remaining in the vesicles. Next we performed calculations as in Parente et al. (1990) to simulate the experimental values and determine M, the minimal number of peptides required to form a pore. Such calculations generate the normalized distribution of vesicles containing i peptides (see eqs 1 and 2) from a knowledge of the binding constant, K. In the absence of such information we assumed a range of K values or binding

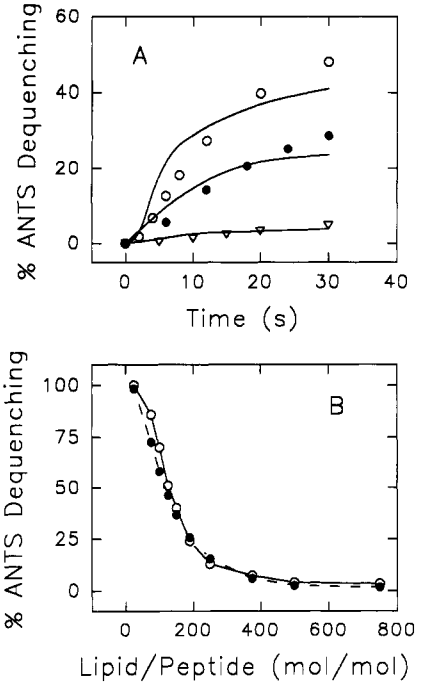

FIGURE 4: (Panel A) Kinetics of HIVarg-induced leakage of ANTS/DPX from POPG LUV in the absence of divalent cations. Vesicle concentration was 50 μM. The peptide-to-lipid ratios were (a) 1:100; (b) 1:150; (c) 1:375. The experimental data are given by solid lines, and the symbols represent the best fit to the data with $M = 6$ and $C = 0.008$ s^{-1} (see text). (Panel B) Experimental (○) and predicted (●) final extents of leakage as a function of peptide-to-lipid mole ratio. Vesicle concentration as in panel A. The predicted values were calculated using $M = 6$ (see Materials and Methods).

ratios, and for each value the calculated values of final extents and kinetics of leakage were generated as described under Materials and Methods. In these calculations the peptide-to-lipid ratios varied from 1:125 to 1:750. The range of binding values and corresponding M values, which yielded a fair simulation of the final extents and kinetics of leakage, varied from 0.0065 ($M = 4$) to 0.0175 ($M = 10$). Outside this range some of the deviations between calculated and experimental values doubled. By using $M = 6$ (which corresponds to a binding ratio of 0.01), we could simulate the experimental values of final extents of leakage fairly well (Figure 4B). Using the above pore size, we also obtained a reasonable simulation of the kinetics of leakage by employing $C = 0.008$ s^{-1} for the forward rate of surface aggregation of the peptides (Figure 4A).

Peptide Association and Exchange. Figure 5 presents another aspect of the interaction of the peptide with POPG vesicles. In panel A, curve a shows the kinetics of leakage for a 1:125 ratio of peptide to lipid, when the peptide was added to the liposomes. In curves b, c, and d the vesicles were added to a peptide suspension after 10, 30, and 60 s. The rate and final extent of leakage progressively decreased with the time of preincubation of the peptide alone. After 60 s very little leakage was observed. A simple explanation of this effect may be that the peptide in the absence of vesicles self-aggregates. Such a process has been recently observed by electron microscopy (Slepushkin et al., 1992) and is confirmed by our own FTIR spectroscopy data (see below). The results in Figure 5 imply that this self-aggregation of the peptide is essentially irreversible. The existence of leakage levels below 100% (Figure 4) rules out any significant exchange of peptide between the vesicles at later times. When a population of liposomes loaded with ANTS/DPX was added to unloaded ones that had been preincubated for 30 min with HIVarg, no

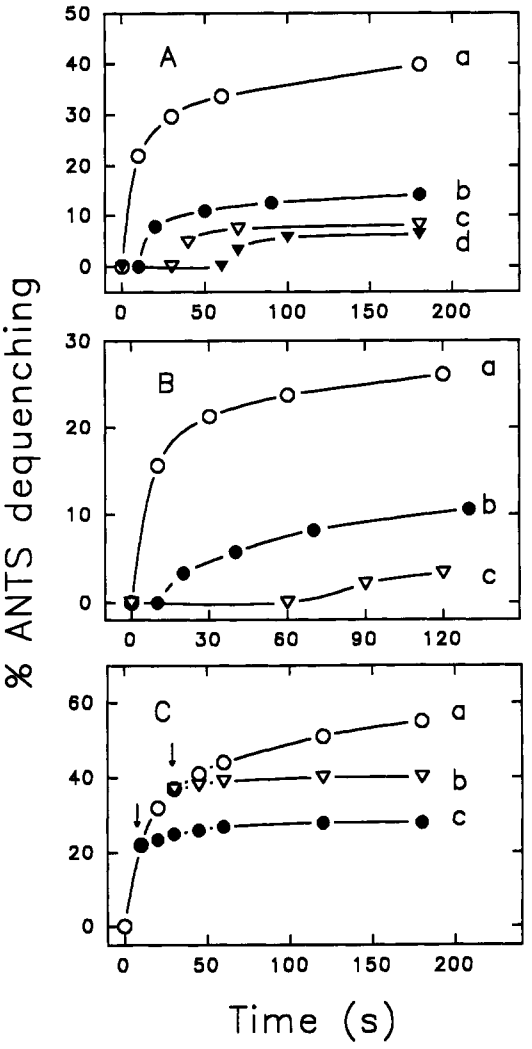

FIGURE 5: Exchange of HIVarg between peptide aggregates in solution and POPG vesicles and among POPG LUV. The figure presents ANTS/DPX leakage from vesicles recorded continuously. (Panel A) Vesicles (final concentration 50 μM) were added at different times after addition of the peptide to the solution (peptide-to-lipid ratio 1:125), (a) 0 s; (b) 10 s; (c) 30 s; (d) 60 s. (Panel B) Vesicles loaded with ANTS/DPX (50 μM) were added at different times to unloaded vesicles (50 μM), (a) 0 s; (b) 10 s; (c) 30 s. The peptide was added at time 0 (peptide-to-lipid ratio in unloaded vesicles, 1:100). (Panel C) Unloaded vesicles (50 μM) were added at different times to ANTS/DPX-loaded vesicles (50 μM), (a) no addition; (b) 30 s; (c) 10 s. The peptide was added at time 0 (peptide-to-lipid ratio in loaded vesicles, 1:100). The arrows indicate the times of empty vesicle addition.

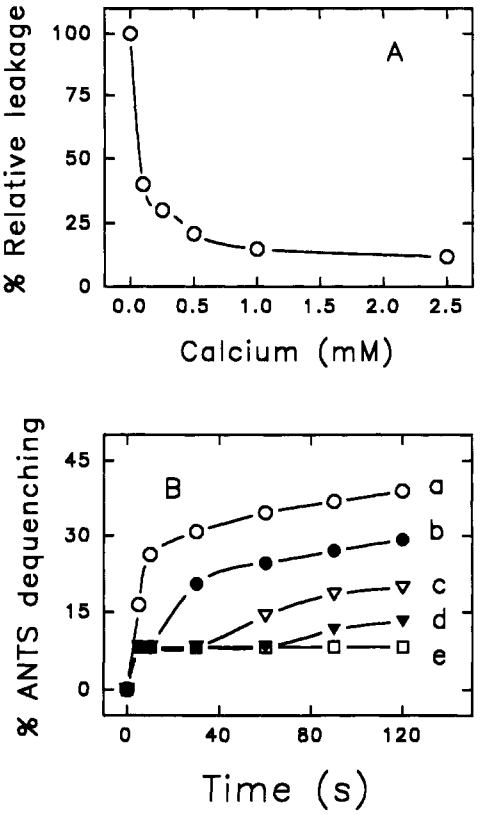

FIGURE 6: Inhibition of HIVarg-induced leakage of ANTS/DPX from POPG LUV by Ca^{2+}. (Panel A) Final extents of leakage relative to the maximal leakage at a peptide-to-lipid mole ratio of 1:125 as a function of the Ca^{2+} concentration (lipid concentration, 50 μM). (Panel B) Kinetics of leakage in the presence of 0.5 mM Ca^{2+}-EDTA (1 mM) was added at different times, (a) 0 s; (b) 10 s; (c) 30 s; (d) 60 s; (e) no EDTA. The final lipid concentration was 50 μM, and the peptide-to-lipid mole ratio was 1:125.

leakage was observed (data not shown), whereas very little leakage occurred after 60 s (Figure 5, panel B). This implies that, within 1 min, the process of peptide binding to the vesicles is completed. Furthermore, the results also indicate that, at later times, when the peptides have aggregated within the liposomal membrane, peptide dissociation from the bilayer no longer occurs. On the other hand, at earlier times exchange between vesicles does occur, since addition of unloaded liposomes to loaded ones 10 or 30 s after peptide addition resulted in a reduction of leakage (Figure 5, panel C).

Ca^{2+} Inhibits Pore Formation. While the results in Figure 1 and Table 1 demonstrate that Ca^{2+} promotes the peptide-induced fusion of POPG liposomes by supporting aggregation, the results in Figure 6 (panel A) demonstrate that Ca^{2+} inhibits leakage. At a 1:125 ratio (nonfusogenic conditions) it appears that Ca^{2+} (0.5 mM) arrests the leakage almost completely (panel B). Addition of EDTA (1 mM) to the suspension at 1:125 ratio restores the leakage when it is added at an early stage (Figure 6B). When EDTA is added 60 s after the addition of Ca^{2+} the rate of leakage is minimal, and, after 3 min (data not shown), it is not restored at all.

Effect of the Order of Peptide and Ca^{2+} Addition on Leakage and Fusion. Figure 7A shows that even if Ca^{2+} is present from time zero in the vesicle suspension, the fusion reaction is initiated only after addition of HIVarg peptide, indicating, as shown before, that Ca^{2+} alone (5 mM) does not induce fusion at this concentration. Conversely, when the peptide is added first and Ca^{2+} is added 30 s later, very little fusion occurs. Under the same conditions, leakage is maximum from the start when the peptide is added first, while it is reduced by 3-fold when Ca^{2+} is added first (Figure 7B).

Infrared Spectroscopy. Figure 8 shows the conformation-sensitive amide I region of the infrared spectrum of the peptide in D_2O buffer. The spectrum exhibited two well-defined maxima at 1625 and 1673 cm^{-1}. The former is characteristic of the peptide backbone in a β-sheet conformation while that at 1673 cm^{-1} could arise from the high-frequency component of the antiparallel β-structure. The dominance of the low wavenumber component at 1625 cm^{-1} suggests that, under our experimental conditions, the peptide alone aggregated, adopting an intermolecular β-sheet structure (Muga et al., 1990). Ca^{2+} has no effect on the conformational properties of the peptide in solution (data not shown). Curve B corresponds to HIVarg bound to POPG vesicles, at an initial peptide-to-lipid ratio of 1:65, in the presence of 5 mM Ca^{2+}, i.e., under conditions where fusion can occur (see Figure 1).

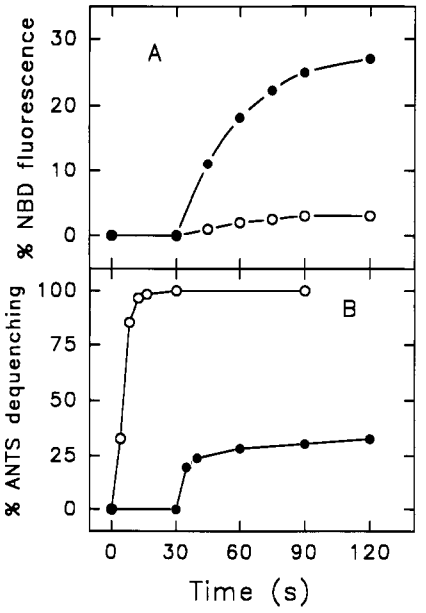

FIGURE 7: Effect of the order of Ca^{2+} (5 mM) and HIVarg addition on membrane lipid mixing (panel A) and leakage of ANTS/DPX (panel B). (●) Ca^{2+} was added to the vesicles (50 μM) at time 0 and the peptide after 30 s; (○) the peptide was added at time 0 and Ca^{2+} after 30 s. The peptide-to-lipid ratio was 1:25.

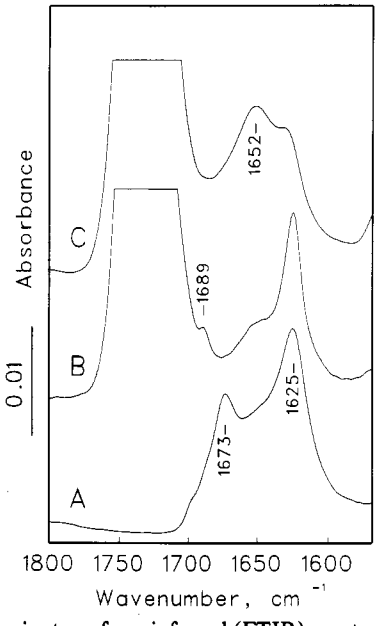

FIGURE 8: Fourier transform infrared (FTIR) spectra of HIVarg in solution and bound to POPG LUV. The peptide (peptide-to-lipid ratio, 1:65) was added to the vesicles (0.4 mM) under the experimental conditions used in the fusion and leakage assays (see Materials and Methods), in the presence of 5 mM Ca^{2+} (curve B) or in the absence of Ca^{2+} (curve C). Curve A represents the spectrum of the peptide in solution.

The spectrum displayed an intense absorption maximum at 1624 cm^{-1}. This fact, together with the presence of the high-frequency component at 1689 cm^{-1}, suggests that the peptide adopted an extended antiparallel β-structure. The spectrum of the peptide bound to POPG LUV in the absence of Ca^{2+} (curve C), i.e., under conditions where no fusion occurs, is different. In this case, the amide I band exhibited a maximum at 1652 cm^{-1} and a weaker band at 1625 cm^{-1}. While the component at 1652 cm^{-1} is representative of a peptide backbone in an α-helical conformation, the less intense band at 1625 cm^{-1} indicates residual β-structure. In both B and C, the spectra displayed an intense band centered at 1734 cm^{-1} which corresponds to the COO^--stretching vibration of the phospholipid ester bonds.

DISCUSSION

In this study we have demonstrated, for the first time, that a synthetic peptide derived from the N-terminus of the HIV-1 gp41 membrane protein has the capacity to induce fusion of large unilamellar phospholipid vesicles, as evidenced by the most stringent criterion for membrane fusion, i.e., mixing of membrane lipids (Figure 1A), accompanied by coalescence of the internal aqueous volumes of the interacting vesicles (Figure 1B) and an irreversible increase in vesicle size (Figure 2). The system we employed involved the use of negatively charged LUV consisting of POPG. This system allows the induction of vesicle aggregation by the addition of divalent cations, such as Ca^{2+} or Mg^{2+}, in the absence of any membrane fusion (Figure 1). Fusion could be induced solely by addition of the HIVarg peptide to the vesicles preaggregated by Ca^{2+} or Mg^{2+}. In this respect, the demonstration of aqueous contents mixing, on the basis of dequenching of fluoresceinated dextran encapsulated in the vesicles, was particularly important, since it indicates that the overall vesicular integrity is preserved during the fusion process. Only low levels of FITC-dextran dequenching were observed when the peptide was added to Ca^{2+}-aggregated loaded vesicles in the absence of empty vesicles. Significant dequenching of FITC-dextran was observed only when the peptide was added to a preaggregated mixture of probe-containing and empty vesicles, indicative of vesicle fusion with concomitant retention of aqueous vesicle contents. The rate constant of the fusion process increased with increasing overall peptide-to-lipid ratio, indicating that the peptide was the mediator of the fusion process observed in this system.

It is well established that, for a number of enveloped viruses including influenza virus and HIV, N-terminal sequences in the envelope glycoproteins generated by proteolytic cleavage of fusion-inactive precursors play a crucial role in the expression of the viral membrane fusion activity (White et al., 1983; Gething et al., 1986; Gallaher, 1987; Bosch et al., 1989; White, 1990). However, thus far attempts to mimic this fusion activity in lipid vesicle systems by utilizing synthetic peptides corresponding to these fusion segments have either failed or been only partially successful. In most cases, despite the observation of peptide-induced fusion of sonicated small unilamellar vesicles (SUV), fusion of large unilamellar vesicles (LUV) could not be accomplished (Lear & Degrado, 1987; Rafalski et al., 1990, 1991; Düzgünes & Shavnin, 1992). Fusion of SUV is likely to occur readily due to the built-in strain in the packing of the bilayer lipids as a result of the high degree of membrane curvature in these small vesicles (Wilschut, 1990). In addition, in many cases LUV aggregate less easily than SUV. For example, it has been shown that the synthetic pH-sensitive amphipathic peptide GALA does not induce aggregation of PC LUV (Parente et al., 1988), although it does interact with the vesicles and induces leakage of vesicle contents. In our system, the HIVarg peptide cannot by itself aggregate the negatively charged POPG LUV, and therefore it does not induce fusion, unless Ca^{2+} or Mg^{2+} are added first in order to preaggregate the vesicles. However, importantly, the peptide is capable of inducing fusion of zwitterionic LUV consisting of DOPC/DOPE/CHOL (1:1:1) in the absence of divalent cations (Nieva et al., manuscript in preparation).

It is interesting to speculate that the role of Ca^{2+} in HIVarg-mediated vesicle fusion may not be restricted entirely to the prefusion aggregation of the vesicles. First, in the DOPC/

DOPE/CHOL system noted above, Ca^{2+}, although not required, does stimulate the fusion process (Nieva et al., manuscript in preparation). Second, Ca^{2+} has also been reported to stimulate HIV-1 fusion with erythrocyte ghost membranes and with liposomes of several compositions (Larsen et al., 1993). Furthermore, in a recent study, Dimitrov et al. (1993) have shown that Ca^{2+} promotes the induction of syncytia in HIV-1-infected cells. Therefore, Ca^{2+} may play a role in the expression of the actual membrane fusion activity of the HIV gp120/gp41 in both model and biological systems.

In this regard, we also point out that Ca^{2+} reduces, or at a low peptide-to-lipid ratio even inhibits, the leakage from POPG vesicles induced by the HIVarg peptide (Figure 6). Clearly this action of Ca^{2+} does not stem from significantly reducing the binding of the peptide to the vesicles, since the peptide-induced fusion is promoted under the same conditions. It is of interest that Ca^{2+} is known to inhibit permeability changes in cell membranes caused by pore-forming agents, including Sendai and influenza viruses (Pasternak et al., 1985). On the other hand, in negatively charged vesicle systems, fusion in the presence of Ca^{2+} is generally accompanied by leakage of vesicle contents, particularly in the later stages of the process (Wilschut et al., 1980, 1985a,b; Nir et al., 1980, 1982, 1983). What we have found here, as will be discussed below, is that the addition of Ca^{2+} reduces significantly the leakage induced by the HIVarg peptide when it is due to pore formation.

One of the most intriguing results of this study relates to the effect of the order of addition of Ca^{2+} and peptide on the fusion of POPG vesicles (Figure 7). When Ca^{2+} was added 30 s after peptide addition, a very low rate and limited extent of membrane lipid mixing were observed. At that time a high degree of leakage of ANTS/DPX had occurred. On the other hand, when the peptide was added to POPG vesicles pre-aggregated by Ca^{2+}, leakage was suppressed in favor of vesicle fusion with retention of aqueous vesicular contents. Consequently, from the combination of the leakage and fusion results we conclude that the HIVarg peptide associated with POPG vesicles has the capacity to form two different structures. In the absence of Ca^{2+}, a pore structure is predominant, which mediates leakage of low-molecular-weight solutes from single vesicles, but this pore does not render the vesicles fusion-susceptible. In the pore structure, the peptides are likely to be oriented perpendicular to the plane of the bilayer, resembling the orientation of the transmembrane segments of integral proteins. Such an orientation is not *a priori* likely to play an active role in membrane fusion. In the presence of Ca^{2+}, i.e., when fusion is promoted, another type of structure appears to be formed. This structure, or an intermediate leading up to this structure, is likely to be involved in the induction of vesicle fusion, while leakage of vesicular contents is reduced. It would appear as though Ca^{2+} seals the POPG membranes by causing structural changes which are not compatible with pore formation. Once the final structures, i.e., the pore structure or the "Ca^{2+} structure", have been established, they appear to be relatively stable. Addition of Ca^{2+} to vesicles in which the peptide pores had been established did not result in an induction of the fusion process (Figure 7), while, also, once the "Ca^{2+} structure" had been established, addition of EDTA did not restore the rate of leakage to its value in the absence of Ca^{2+} (Figure 6). If EDTA was added rapidly after addition of peptide to Ca^{2+}-aggregated POPG vesicles, leakage rates did approach the level observed in the absence of Ca^{2+}, suggesting that intermediates leading up to the "Ca^{2+} structure" can revert to the pore structure.

The FTIR results (Figure 8) indicate the existence of two different conformations of the HIVarg peptide in our vesicle system. The peptide spectrum in the absence of Ca^{2+} is compatible with the peptide adopting mainly an α-helix conformation. This conformation has been proposed to be associated with pore formation by peptides in vesicle membranes (Lear et al., 1988; DeGrado & Lear, 1990; Parente et al., 1990). In contrast, in the presence of Ca^{2+} the peptide mostly adopts an extended antiparallel β-structure. It must be cautioned that part of the peptide still remains in an α-helix conformation even in the presence of Ca^{2+}. Hence, at higher peptide-to-lipid ratios (e.g., 1:25) there might be a sufficient number of peptides to form pores in the presence of Ca^{2+} (see Figure 7).

On the basis of the evidence presented in this paper it is tempting to speculate that the extended antiparallel β-structure of the peptide renders the vesicles susceptible to fusion. In this arrangement, the peptides are likely to reside primarily at the lipid–water interface. In membrane proteins, transmembrane stretches of β-structure appear to be the exception, rather than the rule (Unwin, 1993). In most studies on viral fusion peptides an α-helical arrangement of the lipid-bound peptide has been suggested to represent the fusion-active conformation (Lear & DeGrado, 1987; Harter et al., 1989; Takahashi, 1990; Rafalski et al., 1990). However, this notion has been questioned by Gallaher et al. (1992). In addition, recently, Epand et al. (1992) deduced that the measles virus fusion peptide adopts mostly a β-structure when immersed in a lipid environment. Our results also favor the involvement of peptides in a β-conformation in the actual fusion event in our system, although we cannot eliminate the possibility that the small fraction of the peptide that adopts an α-helix conformation is responsible for induction of fusion in a stage prior to the formation of a pore. In this respect, it should also be noted that the FTIR spectra are recorded under equilibrium conditions, after fusion has been completed. This implies that any intermediate structure, even those preceding the observed β-structure, may be involved in the fusion process.

Finally, we caution that results of studies with isolated synthetic viral fusion peptides in lipid vesicle systems are not to be directly extrapolated to their biological counterparts. Although studies on model systems, such as the one employed here, do provide important information with respect to the general requirements for membrane fusion, by necessity they provide, at best, only part of the answer to the central question regarding the molecular mechanism of viral membrane fusion. Therefore, in each case it remains to be established whether the specific characteristics of a fusion process, as revealed in a model system, relate directly to the properties of the corresponding fusion event in the infectious cellular entry of the particular virus involved.

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