VSV Transmembrane Domain (TMD) Peptide Promotes PEG-Mediated Fusion of Liposomes in a Conformationally Sensitive Fashion†

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Received April 30, 2002; Revised Manuscript Received August 20, 2002

ABSTRACT: Helical instability induced by gly residues in the transmembrane domain (TMD) of G protein, the fusion protein of vesicular stomatitis virus (VSV), was speculated to aid in the later steps of the fusion process, because G protein with ala's substituted for the two TMD gly's was inactive (Cleverley, D. Z., and Lenard, J. (1998) *Proc. Natl. Acad. Sci. U. S. A. 95*, 3425−30). Here we examine the conformations of synthetic peptides corresponding to fusion-active (GGpep) and inactive (AApep; G's replaced by A's) TMDs by CD spectroscopy, and then their effects on the kinetics of poly (ethyleneglycol) (PEG)-mediated fusion of small unilamellar vesicles. GGpep and AApep both assumed history-dependent, non-interconvertible ordered structures. Both peptides were largely helical under all conditions if derived from trifluoroethanol solutions, and aggregated in a β-sheet form if derived from acetonitrile solutions. In solvent, detergents or lipid bilayers, GGpep showed a greater range of secondary structural features than did AApep. The two peptides had large but different effects on PEG-mediated fusion. Both enhanced the rate but not the extent of lipid mixing. AApep significantly inhibited the extent of fusion pore formation while GGpep had no effect. The initial rate of fusion was enhanced 6-fold by GGpep and less than 2-fold by AApep. Addition of 5 mol % hexadecane overrode all peptide-induced effects. We suggest that both GGpep and hexadecane promote pore formation by stabilizing the nonlamellar structures in fusion intermediates or initial small pores. AApep, which had fewer nonhelical features in its CD spectrum than GGpep, actually inhibited fusion pore formation.

The mechanisms by which viral and cellular proteins mediate membrane fusion have been intensively studied in recent years. Structural and functional studies of the fusion proteins of several enveloped viruses, especially HIV (gp41) and influenza (HA), and of the cellular SNARE proteins, have generated the suggestion that many protein-mediated fusion reactions proceed by similar mechanisms (*47*, *51*). Further, studies of protein-free bilayer fusion mediated by poly(ethylene glycol) (PEG[1]) have shown that this reaction proceeds via a series of steps that closely resemble those occurring during protein-mediated fusion (*23*). A unified picture of membrane fusion may thus be emerging (*26*).

It is now well-established that the transmembrane domains (TMDs) of fusion proteins are essential for efficient fusion. A role for the TMD in the later stages of fusion was demonstrated for several viral fusion proteins by substituting glycerylphosphatidylinositol (GPI) moieties for TMDs. A GPI anchor uses a lipid to attach the proteins in a fairly nonperturbing way only to the outer leaflet of the membrane bilayer, in contrast to a TMD, which traverses and perturbs lipid packing in both leaflets. Fusion proteins engineered so that the external domains were anchored to the membrane by a GPI linkage could mediate hemifusion (lipid mixing between outer leaflets of the donor and acceptor membranes), but not aqueous contents mixing (*19*, *35*, *37*, *41*, *52*), which requires lipid mixing between the inner leaflets and requires formation and/or enlargement of a fusion pore (*9*). The mechanism(s) by which TMDs of fusion proteins facilitate pore formation remains unknown.

Efforts to elucidate the role of TMDs in protein-mediated fusion have included studies that address the importance of specific TMD residues or amino acid sequences. Chimeric

† Supported by USPHS GM32707 (BRL) & GM59204 (JL).

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[1] Abbreviations: AApep, 24-residue peptide identified in Materials; AcCN, acetonitrile; AcCN-H_2O-TFA, 50% acetonitrile-49.9% H_2O-0.1% trifluoroacetic acid; β-BODIPY500-PC, 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a, 4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; β-BODIPY530-PE, 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a, 4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine; C8E5, pentaoxyethylene octyl ether; CD, circular dichroism; CH, cholesterol; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DOPE, 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine; DPA, dipicolinic acid; DPC, dodecylphosphocholine; GG peptide, 24-residue peptide identified in Materials; GPI, glycerylphosphatidylinositol; HIV, human immunodeficiency virus; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; SDS, sodium dodecyl sulfate; SM, sphingomyelin; SUVs, small unilamellar vesicles; TES, *N*-[tris(hydroxymethyl)methyl]2−2-aminoethane sulphonic acid; TFE-H_2O, 90% trifluoroethanol-10% H_2O; TMD(s), transmembrane domain(s); TN buffer, 0.112 M; NaCl, 0.055 M; tris, pH 6.7; VSV, vesicular stomatitis virus; PEG, poly (ethylene glycol); SUVs, small unilamellar vesicles.

10.1021/bi0203233 CCC: $22.00
Published on Web 11/14/2002

proteins have been constructed in which TMDs from various nonfusion proteins have been substituted for those of the fusion proteins. Many different sequences were found to support fusion activity, implying that specific TMD sequences were not required (*34*, *37*). At the same time, other findings indicate that not all TMD sequences support membrane fusion and that certain residues are critical. We have shown that, in the VSV fusion protein, G protein, substitution of the two TMD gly residues in the native structure with ala or leu abolished fusion without affecting insertion, processing, or transport of the protein to the plasma membrane (*10*). Substitution of only one of the two gly residues reduced fusion activity but did not abolish it. The inactive mutant proteins retained the ability to mediate hemifusion (*10*), again indicating that the TMD promotes a step in the fusion process beyond formation of the initial or hemifusion intermediate.

It was reported recently that a similar AA substitution in a shortened, synthetic peptide based on the VSV TMD also reduced lipid mixing relative to the corresponding GG-containing peptide (*21*). While this report did not establish whether incorporation of the TMD peptide into vesicles promoted fusion or some other form of lipid reorganization (*21*), it still suggested a correlation between a bilayer perturbation induced by a TMD-like peptide and the fusion role of the corresponding TMD sequence in the intact protein. A similar correlation has been noted for gly residues of the HA fusion peptide, the N-terminal sequence of HA2 that is also essential for HA-induced membrane fusion. Thus, substitutions for certain gly residues in a synthetic HA fusion peptide interfered with vesicle lysis (*33*), while the corresponding substitutions in the intact HA fusion protein limited viral fusion activity (*40*). These results lead us to ask: *May the specific (and perhaps related) roles of the TMD and fusion peptide regions of viral fusion proteins be modeled by studying the effects of corresponding synthetic peptides on liposome fusion?*

Since the TMD is only one small part of a viral fusion protein, it clearly cannot perform the necessary docking or membrane-aggregating functions of the intact protein. We therefore examined the effect of the VSV TMD peptide on fusion of vesicles aggregated by PEG. Previous studies have shown that PEG promotes vesicle fusion by aggregating and pressing bilayers close to one another without interacting directly with them (*24*), and by providing a compressive osmotic stress that makes vesicles more fusogenic (*30*). The latter effect is not necessary for fusion, while PEG-mediated aggregation is not sufficient for fusion (*25*). In the work reported here, we have used PEG to aggregate vesicles so that we could determine the effect of VSV TMD on the ability of aggregated vesicles to fuse. We have used vesicles composed of a lipid mixture that is optimized for fusion (*16*) so that our studies could be carried out at a low concentration of PEG. We have focused on highly curved vesicles both because virus-induced fusion seems to occur at highly curved local contact points (*18*) and because curvature has been demonstrated to promote efficient, nonleaky fusion (*27*, *30*, *48*). Using this model system, we ask: *Can the VSV TMD alone affect fusion of these model membranes? If so, which steps in the process are affected?* Since certain mutations in the TMD of VSV G protein block complete membrane fusion (*10*), we also ask: *How does one of these mutations (GG → AA) alter the structure of a synthetic TMD peptide? How does this alteration affect the ability of synthetic TMD peptides to influence vesicle fusion mediated by PEG?*

We report here that the TMD-like peptides GGpep and AApep, both have large but different effects on fusion of PEG-aggregated phospholipid vesicles. The effects are consistent with the previously reported lack of fusion activity of VSV G protein containing the corresponding TMD AA mutation (*10*). Further, AApep was found to be more helical than GGpep, suggesting that a TMD sequence with the ability to explore conformations involving significant deviations from complete helicity may be required for a TMD sequence to promote fusion pore formation. Finally, we showed that the peptide-mediated effects are largely overridden by addition of hexadecane, suggesting that the influences of TMDs and hexadecane on the fusion process might be similar, reflecting their ability to compensate for hydrophobic mismatch within nonlamellar regions associated with fusion intermediates.

MATERIALS AND METHODS

Materials. The two peptides used in this study are shown in the diagram below:

	462	483
GG peptide	(K)KSSIASFFFII**GL**II**G**LFLVLR(R)	
AA peptide	(K)KSSIASFFFII**A**LII**A**LFLVLR(R)	

The GG peptide (GGpep) has the sequence of the 20-residue TMD of wild-type VSV G protein plus a lys and arg residue that flank it on the N- and C- terminal sides in the G protein sequence, respectively. One additional N-terminal lys and one additional C-terminal arg, not present in the G protein sequence, were introduced to improve solubility. The AA peptide (AApep) represents the corresponding sequence in which the two boldface gly residues within the TMD sequence have been replaced by ala residues. GGpep and AApep were synthesized and purified using reverse-phase HPLC by GenoSys (The Woodlands, TX). Chromatographic profiles and mass spectrometric analysis indicated that both peptides were >90% pure. Additional purification by reverse-phase HPLC (C4 or $C_1$8 columns) increased the purity slightly. Repurified samples behaved similarly to those used without further purification. Dodecylphosphocholine (DPC) and chloroform stock solutions of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), 1,2-dioleoyl-3-*sn*-phosphatidylcholine (DOPC), 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine (DOPE), bovine brain sphingomyelin (SM), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All except cholesterol were used without further purification. The concentrations of all the stock lipids were determined by phosphate assay (*7*). Cholesterol was purified as previously reported (*45*). 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine (BODIPY530-PE), 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY500-PC) were the products of Molecular Probes (Eugene, OR). Terbium Chloride was obtained from Johnson Matthey Co. (Ward Hill, MA). Dipicolinic acid (DPA), pentaoxyethylene octyl ether (C_8E_5), and *N*-[tris(hydroxymethyl)methyl]-2,2-aminoethane sulfonic

acid (TES) were purchased from Sigma Chemical Co. (St. Louis, MO). Poly(ethyleneglycol) of molecular weight 7000−9000 (PEG 8000) was purchased from Fisher Scientific (Fairlane, NJ) and further purified as reported previously (*27*). Dodecyloctaethylene glycol monoether ($C_{12}E_8$) was purchased from Calbiochem (La Jolla, CA). All other reagents were of the highest purity grade available.

MATERIALS AND METHODS

Sample Preparation for CD Spectroscopic Studies. GGpep and AApep were dissolved either in TFE-H_2O (9:1 v/v) or in AcCN−H_2O-TFA (50:49:1 v/v) at ca. 1 mg/mL, with sonication to give optically clear solutions as necessary. Aliquots of these stock solutions were mixed with aliquots of amphiphiles dissolved in the same solvent as the peptide, and the mixture was dried under a stream of N_2 and lyophilized to remove any remaining solvent, generally overnight. The dried film was resuspended in TN buffer (0.112 M NaCl, 0.055 M tris, pH 6.7) and sonicated to optimize dispersion. Final peptide concentrations were determined using the Biuret reaction, and ranged from 120 to 155 μM, although similar results were obtained from preparations containing as little as ca. one-tenth as much, with corresponding increases in final amphiphile:peptide ratios. Final concentrations of amphiphiles were 20 mM DPC, 100 mM C8E5, and 2.56 mM POPC.

CD Spectroscopy. CD spectra were measured on an Aviv model 620S spectropolarimeter (Lakewood, NJ) using a 1 mm quartz cell. Spectra were obtained at 25 °C at 0.25−0.5 nm intervals and time averaged from 1 to 2 s. The raw spectra were smoothed using the variation of the method of Savitsky and Golay (*43*) supplied by Aviv using a moving window of 10−25 points and a polynomial order of 3.

Vesicle Preparation for Fusion Studies. Small unilamellar vesicles (SUVs) composed of DOPC/DOPE/SM/CH (35:30:15:20 molar ratio) were prepared as reported earlier (*16*). We have shown previously that this particular lipid composition, which mimics the composition of naturally fusogenic synaptic vesicles, is optimized for providing stable vesicles that nonetheless fuse readily with minimal loss of trapped contents (*16*). Mixtures of different lipids at this molar ratio in chloroform were dried under a stream of nitrogen and dried in vacuo for an hour to remove any remaining solvent. Appropriate amounts of stock solution (1 mg/mL) of GGpep or AApep in TFE-H_2O (9/1 v/v) were added to the lipids dried on the wall of a glass vial, and the lipid was dissolved in this solvent, which was again removed under nitrogen. The residues were dissolved in cyclohexane with addition of a small amount of ethanol, frozen in a dry ice/acetone bath, and then lyophilized overnight. The dried lipids with and without peptides were suspended by rapid agitation in an appropriate buffer at 23 °C. The lipid suspensions were sonicated for about 15 min at 50% duty cycle using a Heat Systems Model 350 Sonicator (Plainview, NY) equipped with a Titanium probe tip. Vesicles were fractionated (*3*) by centrifugation at 70000 rpm for 25 min at 4°C using a Beckman TL-100 ultracentrifuge (Palo Alto, CA). For some preparations, controls were performed in which the concentration of phospholipid in the vesicle samples was measured using the activity of the radioactive lipid added in trace quantity to the phospholipid mixture. A noninterfering protein assay (Geno Technology, Inc., St. Louis, MO) was used to estimate the concentration of peptides in the vesicle samples. These controls showed that the lipid/peptide (*L*/*P*) ratio was, within experimental error, the same before and after fractionation of vesicles and matched the expected *L*/*P* ratio in the dried lipid-peptide sample before addition of buffer. This demonstrates that all peptide associated with vesicles.

Contents Mixing and Leakage Assay. The Tb^{3+}/DPA contents mixing and leakage assays were based on those initially proposed by Wilschut et al. (*53*) and adapted to monitor PEG induced fusion (*48*). The buffer contained 100 mM NaCl, 10 mM TES, 1 mM EDTA, and 1 mM $CaCl_2$. In the contents mixing assay, the lipids were suspended in either 80 mM DPA or 8 mM $TbCl_3$ plus 60 mM sodium citrate and 10 mM TES, pH 7.4. For the contents leakage assay, lipids were suspended in 4 mM $TbCl_3$, 40 mM DPA and 10mM TES, pH 7.4. Untrapped probe-containing buffer was removed from the vesicles using a Sephadex G-75 column equilibrated with probe-free buffer (100 mM NaCl, 10 mM TES, 1 mM EDTA, and 1 mM $CaCl_2$). To measure contents mixing due to fusion, stock PEG solutions (10 wt %) were added rapidly and with mixing to a 1:1 mixture of Tb^{3+}- and DPA-containing vesicles equilibrated at 23 °C (final PEG concentration 5 wt % and total lipid concentration 0.2 mM), and the increase in Tb^{3+} fluorescence was recorded with time, after which $C_{12}E_8$ was added to obtain the fluorescence of Tb^{3+}/DPA released from the vesicles as a reference. The percentage of content mixing was obtained by comparing the signal from the vesicle mixture with that of co-encapsulated Tb/DPA vesicles, which was taken as indicative of 100% content mixing (*27*). Leakage of contents was monitored with time by following the fluorescence of vesicles containing coencapsulated Tb^{3+}/DPA, with treatment with $C_{12}E_8$ used to mark 100% leakage of trapped contents (*27*). Contents mixing was corrected for contents leakage as described (*48*).

Lipid-Mixing Assay. The mixing of lipids was measured using the fluorescent lipid probes BODIPY500-PC and BODIPY530-PE (*31*). Vesicles containing both probes in a 1:1 molar ratio were mixed with probe-free vesicles at a 1:4 ratio, diluted with buffer, equilibrated in a fluorescence cuvette at 23 °C, and an appropriate amount of stock PEG solution (10 wt %) added to produce a final concentration of 5 wt % PEG and 0.2 mM total lipid. The details of these measurements and the calculation of percent lipid mixing are given elsewhere (*31*). Both the lipid mixing and content-mixing assays assume that the mix of vesicles within a fusing aggregate is the same as the bulk mix. When aggregates contain very few vesicles, one can correct for the probability that any aggregate contains this mix (*48*, *55*). However, this correction is small for SUVs, which form fairly large aggregates in the presence of PEG (roughly 16 SUVs per aggregate; Evans, Haque, and Lentz, unpublished results), increasing the probability that each aggregate contains a random mix of vesicles. Thus, the lipid- and content-mixing assays have comparable scales.

RESULTS

Secondary Structure of GGpep and AApep. The CD spectra of these two peptides in two different organic solvents are shown in the top frames of Figure 1. In TFE-H_2O, both

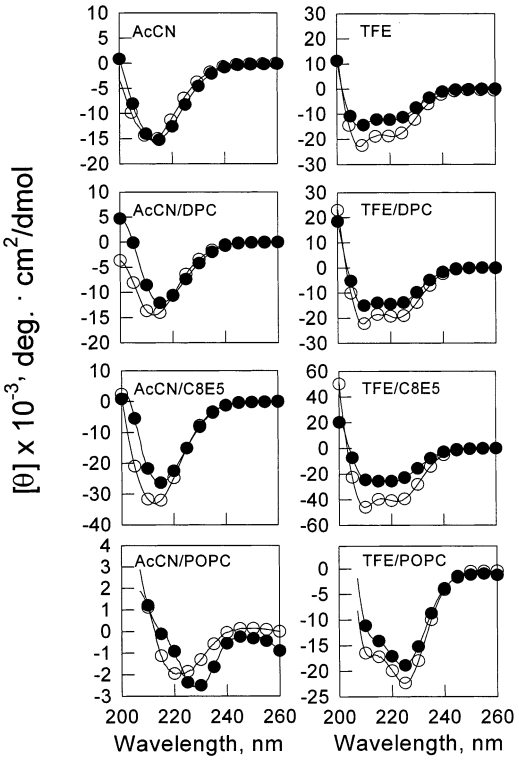

FIGURE 1: CD spectra of GGpep (filled circles) and AApep (open circles) in different environments. Row 1: In AcCN−H_2O-TFA (left), or inTFE-H_2O (right). Rows 2−4: Peptides were dried down from AcCN−H_2O-TFA (left panel) or TFE-H_2O (right panel) together with the indicated amphiphile dissolved in the same solvent. Residues were resuspended in TN buffer to final concentrations of 20 mM DPC (row 2), 100 mM C8E5 (row 3), or 2.67 mM POPC (row 4).

peptides had spectra characteristic of largely α-helical structures but the negative molar ellipticity at 222 nm (θ_{222}) was ca. 35% greater for AApep than for GGpep. In contrast, peptides dissolved in AcCN−H_2O-TFA exhibited CD spectra characteristic of largely β-sheet structures. However, structural differences between the two peptides were evident also in this solvent system, as the spectral minimum for GGpep was red-shifted compared with that for AApep.

Neither peptide could be solubilized in the aqueous buffer alone, i.e., in the absence of any amphiphile. To study the conformation of these peptides in membrane-like environments, each peptide that had been dissolved in either TFE-H_2O or AcCN−H_2O-TFA was mixed with an amphiphile (a nonionic detergent, C8E5; an ionic detergent, DPC; or a liposome-forming phospholipid, POPC) dissolved in the same solvent. After exhaustive removal of the solvent under high vacuum, the residue was resuspended with sonication in TN buffer. The CD spectra obtained from the two peptides resuspended in the presence of each amphiphile are arranged in Figure 1 such that samples derived from the TFE solvent are shown on the right and samples derived from the AcCN solvent are on the left. Figure 1 shows that those peptides that were initially dissolved in TFE-H_2O remained helical, while those that were initially dissolved in AcCN−H_2O-TFA retained the β-sheet form, despite being brought into identical aqueous amphiphile environments.

Attempts to interconvert the α-helical and the β-sheet forms of these two peptides in aqueous or solvent conditions were unsuccessful, even after heating to 90 °C. A gradual

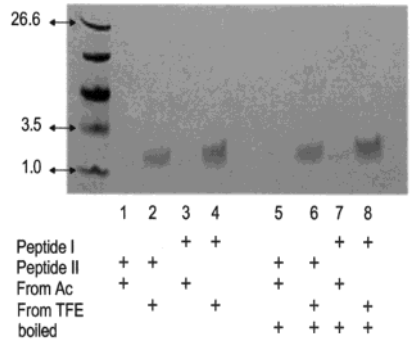

FIGURE 2: Gradient SDS gel of GG peptide (lanes 1,2,5,6) or AA peptide (3,4,7,8) dried from 90% TFE-H_2O (lanes 2, 4, 6, and 8) or from AcCN-H_2O-TFA (lanes 1,3,5,7) and redissolved in 1% SDS without (lanes 1−4) or with (lanes 5−8) boiling for 3 min. 10−20% precast gradient gels (Fisher Supragel) were run at 100 v under standard buffer conditions (*44*).

decrease in helicity of the α-helical form of both peptides was observed upon heating to 60 °C, with no evidence for a discrete thermal transition, as seen for many highly helical peptides. This decrease was reversible, since identical spectra were obtained before and after heating (not shown). Although the β-sheet forms tended to precipitate at elevated temperatures, the spectra retained their characteristic β-sheet nature, and no detectable α-helical spectral component was seen.

SDS-gel electrophoresis showed that the TFE-derived helical form migrated as a monomer, while the AcCN-derived β-sheet structure formed a high molecular weight complex that did not penetrate the gel (Figure 2). Boiling these samples in SDS solution did not change their electrophoretic behavior, providing further evidence of the thermal stability of both peptide structures. An attempt was made to interconvert the two forms by mixing them together and incubating for an extended period. The two forms of each peptide were mixed together in varying ratios and incubated at 30 °C for one month. Gel electrophoresis after this prolonged incubation period showed no change from the original proportions of helical and β-sheet structures (not shown), indicating that prion-like recruitment of the monomeric helical peptide to the β-sheet structure had not occurred.

A comparison of the conformations of GGpep and AApep that had been incorporated into amphiphiles by identical procedures showed that these were always different. The mean residue ellipticity (θ_{222}) of GGpep from TFE-H_2O was less than that of AApep in all three amphiphilic environments. Differences in θ_{222} ranged from ca. 20% to nearly 50%, being greatest for TFE and C8E5 and least for a membrane-like environment (POPC and DPC). Similarly, the spectral minimum of the β-sheet form of GGpep from AcCN−H_2O-TFA was always red-shifted relative to that of AApep.

Ideally one would like to obtain a detailed analysis of the secondary structures of the peptides incorporated into phospholipid vesicles. Unfortunately, scattering from the vesicles greatly distorts CD spectra, precluding their use for detailed structural analysis. The CD spectra of the AA and GG peptides from TFE-H_2O in DPC, however, were qualitative similar to those of TMD in POPC membranes (see above) and of sufficient quality to be analyzed in detail, since solutions of this detergent do not scatter light. Computer

Table 1. Secondary Structure Content of GGpep and AApep in DPC

program	peptide	% alpha	% beta	% other[a]
K2D	AA	68.0	5.0	26.0
	GG	52.0	16.0	32.0
CDNN	AA	71.9	5.5	25.9
	GG	51.3	10.3	38.7
Average	AA	70.0	5.3	26.0
	GG	51.7	13.2	35.4

[a] "other" structures include beta bends, which are not well-estimated by the methods employed here.

programs, namely, CDNN (*5*) and K2D (*1*), which estimate the secondary structure of peptides from CD data by neural net analysis, were used to analyze the spectra obtained with DPC solubilized peptides. These programs, which utilize a database of proteins with known conformations to train a network that is then used to analyze the spectra of protein or peptides with unknown conformations, provide a good estimate of β and other structures and a very good estimate of α-helix (*13*). In both analyses, AApep had significantly higher helical content than GGpep, which contained contributions from a broader range of local peptide bond geometries (Table 1).

We conclude that the VSV TMD peptides we have examined have very stable, history-dependent conformations corresponding to an α-helical form and an aggregated β-sheet form. Since we have been unable to interconvert these two forms, it is impossible to say which conformation corresponds to the global free energy minimum. For the purpose of these fusion studies, we have focused on the α-helical form for reasons summarized in the Discussion. The possible functional implications of the different degrees of helical content of the two TFE-derived peptides were next examined by asking how they affected fusion of model membrane vesicles.

Effects of GGpep and AApep on the Kinetics of PEG-Mediated SUV Fusion. We have studied the effect of GGpep and AApep in their helical form (peptides initially dissolved in TFE-H_2O) on the PEG-mediated fusion kinetics of DOPC/DOPE/SM/CH SUVs. Figure 3 shows representative time courses of lipid mixing (A), contents mixing (B), and contents leakage (C) for peptide-free control vesicles and for vesicles containing AApep and GGpep at a peptide/lipid ratio (*P*/*L*) of 1/200. Several distinct peptide-mediated effects are seen in these data. Both peptides enhanced the initial *rate* of lipid mixing, but the *extent* of lipid mixing at long times remained essentially unaffected by the presence of either peptide. AApep increased by less than 2-fold the initial *rate* of contents mixing but significantly impeded the extent of this process at longer times. GGpep, on the other hand, increased the initial *rate* by 6-fold and also the *extent* of contents mixing at all times up to 500 s. Contents leakage, minimal in control vesicles, was unaffected by GGpep, but was enhanced ca. 2-fold by AApep.

To make a more detailed comparison of the different effects of AApep and GGpep, we repeated these experiments at several *P*/*L* ratios. The simplest fit for the resulting kinetic data required two exponentials, which provided two rate constants (k_1 and k_2) and two preexponential factors (C_1 and C_2), which expressed the extent of the contribution of the corresponding kinetic process. The results are shown in

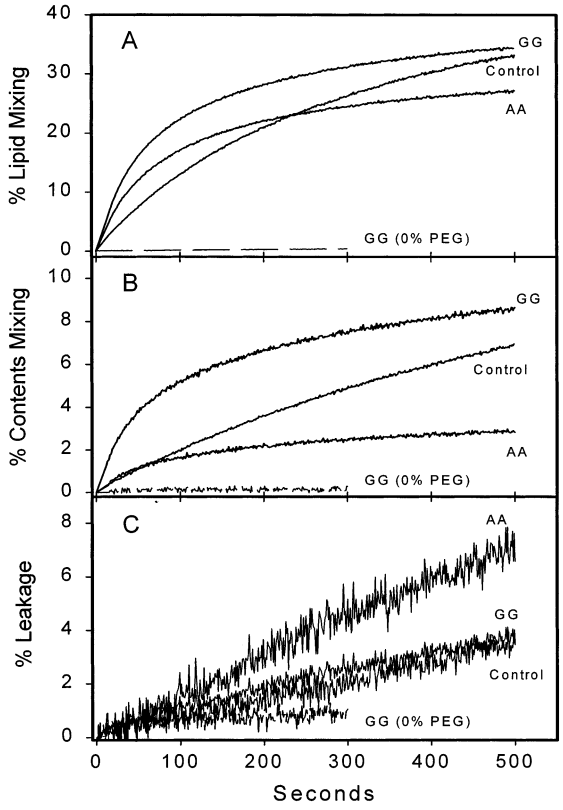

FIGURE 3: Effects of GGpep and AApep on PEG-induced fusion of DOPC/DOPE/SM/CH SUV's. Time dependence of lipid mixing (A), contents mixing (B), and contents leakage (C) are shown for peptide-free control SUVs and for SUVs containing GGpep and AApep at peptide:lipid ratio 1:200. Representative data for lipid mixing, contents mixing, and contents leakage for vesicles containing GGpep in the absence of PEG are shown as dashed lines; data for peptide-free control vesicles and vesicles containing AApep were nearly the same (not shown).

Figures 4−7, summarized in Table 2, and described below.

Lipid Mixing. Biexponential kinetics of lipid mixing were observed previously for PEG-mediated fusion of both moderately and highly curved vesicles (*12*, *22*). It was suggested that these two rates corresponded to two processes of lipid movement: first, between contacting outer leaflets, and second, between noncontacting inner leaflets (*12*, *22*). Both peptides enhanced to a similar extent the initial rate of lipid mixing (inset in Figures 4A and 5A), an effect that reflected an increase in both the rate (k_1) and the extent (C_1) of the fast process (circles in Figures 4 and 5). The major difference between the effects of the two peptides on lipid mixing was that the magnitude of the fast component (expressed as C_1) increased more dramatically with GGpep than with AApep. Since the final extent of lipid mixing was not influenced significantly by either peptide ($C_1 + C_2$ in Table 2), the contribution of the slow component necessarily decreased more markedly with increasing AApep than with GGpep.

Contents Mixing. Contents-mixing time courses were adequately described by a single exponential for peptide-free control vesicles (Figures 6 and 7). The single rate constant describing contents mixing in the peptide-free controls was nearly identical to the rate of the slow component of lipid mixing (Table 2), as has been observed previously for PEG-mediated fusion of SUVs (*12*). This is consistent with the idea that the slow lipid-mixing process corresponds

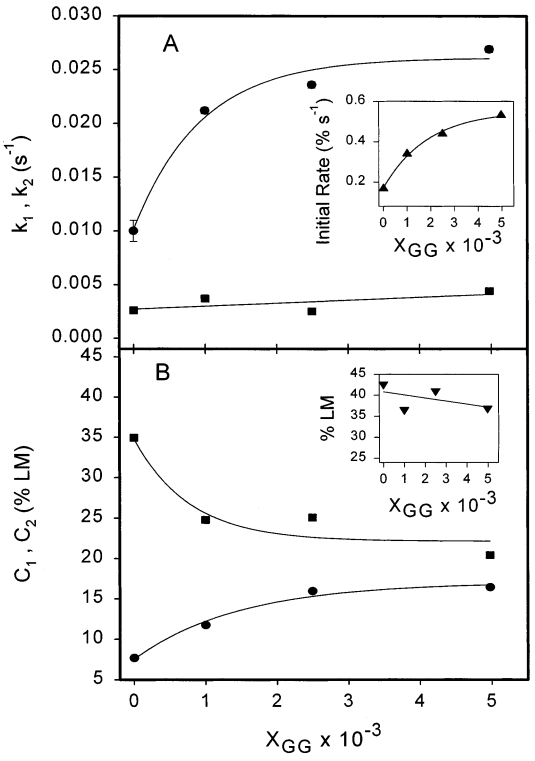

FIGURE 4: Kinetic parameters and the extent of lipid mixing as a function of mole fraction of GGpep (X_{GG}). A: The rate constants k_1 (circles) and k_2 (squares) and initial rate of lipid mixing (inset). B: The preexponential factors C_1 (circles), C_2 (squares), and extent of lipid mixing (inset).

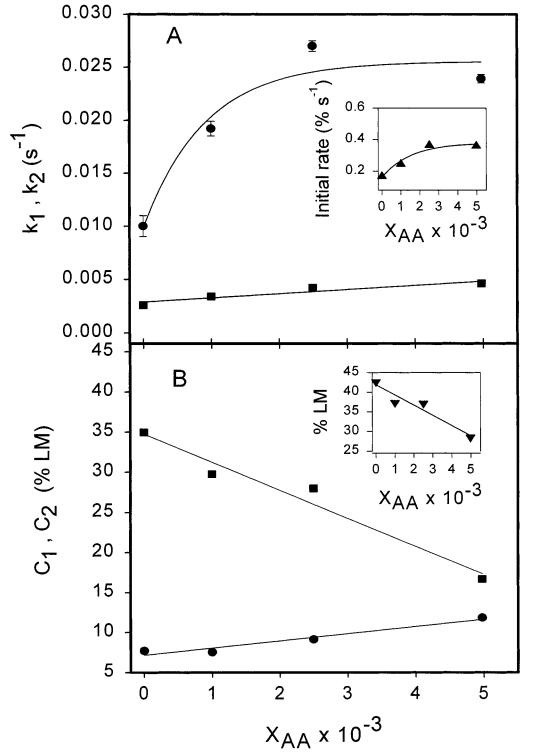

FIGURE 5: Kinetic parameters and the extent of lipid mixing as a function of mole fraction of AApep (X_{AA}). A: The rate constants k_1 (circles) and k_2 (squares) and initial rate of lipid mixing (inset). B: The preexponential factors C_1 (circles), C_2 (squares), and extent of lipid mixing (inset).

to inner leaflet lipid mixing that occurs simultaneously with pore formation in peptide-free vesicles (*12*, *22*).

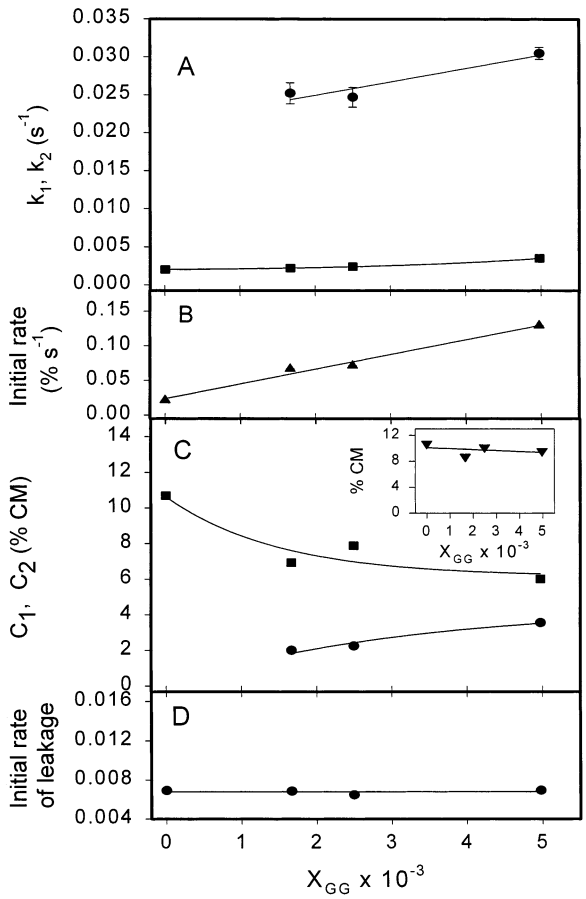

FIGURE 6: Kinetic parameters of contents mixing and contents leakage as a function of mole fraction of GGpep (X_{GG}). A: Rate constants k_1 (circles) and k_2 (squares). B: initial rate of contents mixing. C: The preexponential factors C_1 (circles) and C_2 (squares). D: Initial rate of leakage.

Fusion of peptide-containing vesicles showed a very different behavior. When peptides were present, a fast component of contents mixing appeared in addition to the slow component that was present in the absence of peptide (Table 2). Both components were comparable in rate to the two components of lipid mixing. Although small in magnitude, this fast component increased the initial rate of contents mixing by a factor of 6 or 7 for GGpep but only slightly for AApep. Since the rate constants of the slow and fast components of contents mixing were about the same for either peptide, the principal difference between the two peptides was the greater extent of rapid contents mixing induced by GGpep (C_1 in Table 2), and the significantly reduced magnitude of the slow component (C_2 in Table 2) in the presence of AApep. In summary, GGpep significantly increased the rate of contents mixing, although it had no effect on the extent of the process ($C_1 + C_2$ in Table 2), while AApep caused a small increase in the initial rate of contents mixing and actually significantly inhibited the extent of this process ($C_1 + C_2$ in Table 2).

GGpep did not increase the rate of leakage up to a mole fraction of 1/200, in contrast to the enhanced leakage caused by AApep. However, extensive vesicle leakage (>70% by 500 s at $P/L = 1/100$) was observed for vesicles containing either peptide at higher P/L ratios. Thus, fusion experiments could not be performed at higher peptide concentrations.

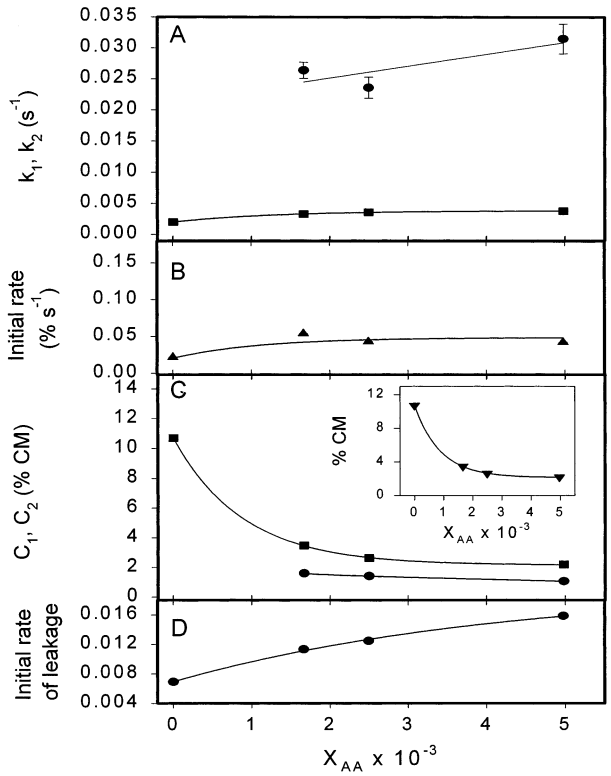

FIGURE 7: Kinetic parameters of contents mixing and contents leakage as a function of mole fraction of AApep (X_{AA}). A: Rate constants k_1 (circles) and k_2 (squares). B: initial rate of contents mixing. C: The preexponential factors C_1 (circles) and C_2 (squares). D: Initial rate of leakage.

Effects of Hexadecane. We have reported elsewhere that 5 mol % hexadecane enhances contents mixing with little effect on lipid mixing, presumably due to its ability to help fill and stabilize regions of hydrophobic mismatch associated with the nonlamellar intermediate structures of the fusion process (*30*). To test whether TMD peptides perform a similar function, we included 5 mol % hexadecane in the peptide-lipid mixture from which SUVs were prepared and examined the effects on PEG-mediated fusion in the presence and absence of TMD peptides, as reported in Figure 8. As summarized in Table 2, 5 mol % hexadecane had little effect on either the rate or extent of PEG-mediated lipid mixing in the absence of peptides. However, both peptides enhanced the rate of the rapid component of lipid mixing in the presence of hexadecane to the same extent as they did in the absence of hexadecane (Table 2). Thus, the effects of TMD peptides and hexadecane on the initial step of the fusion process seem to be independent and not related. However, there were significant similarities between the effects of hexadecane and those of TMD peptides on contents mixing. First, hexadecane induced a second, rapid component of contents mixing, similar to the fast component induced by both TMD peptides. Second, hexadecane, like both peptides, also caused a small increase in the rate of the slow component of contents mixing (Table 2). The only difference between TM peptides and hexadecane was in the specific effects of TM peptides on the extent of contents mixing: hexadecane and GGpep both had little effect on the extent of content mixing, while AApep inhibited it (Table 2). Finally, neither peptide had a significant effect on contents mixing in the presence of hexadecane, suggesting that the effects of TMD peptide and hexadecane have a common mechanistic origin and that the influence of hexadecane dominated the specific effects of the two peptides.

DISCUSSION

Effects of GGpep and AApep on PEG-Mediated Fusion. Figure 3 shows that GGpep or AApep had striking, sequence-specific effects on PEG-mediated fusion behavior of SUVs. Analysis of these effects reveals a complex picture. GGpep induced a 6-fold increase and AApep only a 2-fold increase in the initial rate of contents mixing (Figures 6 and 7). Both peptides promoted a 2- to 3-fold increase in the initial rate of lipid mixing (Figures 4 and 5). Two aspects of these results are surprising: (1) the enhancement of the initial *rate* of contents mixing occurred without a significant increase in the *extent* of contents mixing, and even a decrease in the case of AApep, and (2), in the case of GGpep, the increase in the rate of contents mixing was greater than the increase in the rate of lipid mixing. Thus, although both peptides did enhance somewhat the rate of initial intermediate formation (measured by lipid mixing), the main effect of GGpep on fusion is on a later step leading to formation of a functional fusion pore.

The Differential Effects of GGpep and AApep Arise from Both the Fast and Slow Components of the Fusion Process. For all experiments, with the single exception of contents mixing between lipid vesicles in the absence of added peptide, the time courses for lipid mixing and contents mixing were best described by a biexponential equation (Table 2). This analysis provided, for each process, two first-order rate constants and two preexponential factors that expressed the relative contribution of the two first-order processes. We have argued previously that the slow and fast components of contents mixing between 22 nm SUVs likely correspond to the two types of pores that form during PEG-mediated fusion (*12*). In 45 nm vesicles, the first type of pore is transient, is associated with the initial hemifused intermediate, and conducts only protons (*22*). The second passes larger solutes and seems to constitute the final fusion pore (*22*). An analogy has been drawn between these two types of pores and the "flickering" pores and "fusion" pores observed by patch clamping exocytotic cells (*23*). The times of appearance of these two types of pores were separated in 45 nm vesicles by a lag during which an initial intermediate slowly converted to a final, committed intermediate (*22*). In more highly curved, 22 nm vesicles, such as those examined here, no lag phase was detected between formation of an initial intermediate and the fusion pore, although fusion pore formation at minimal PEG concentrations still followed a single, slow exponential time course that had the same rate constant as the slow component of lipid mixing (*12*). At higher PEG concentrations, a fast component of contents mixing accompanied lipid mixing and formation of the initial fusion intermediate (*12*).

Perhaps the most striking effect revealed by our results is the development, in both peptide- and hexadecane-containing vesicles, of a rapid component in the kinetics of contents mixing (k_1 and C_1 in Table 2) that is absent in the protein-free reaction (Figures 4−7). This has two possible interpretations. First, the peptide and/or hexadecane might stabilize the transient pore associated with the initial intermediate so that it can pass larger molecules. Alternatively, these agents

Table 2. Rate Constants and Preexponential Factors of Lipid Mixing and Contents Mixing of Peptide-Free Control Vesicles and Vesicles Containing GGpep and AApep (at *P*/*L* ratio 1/400) with and without 5 mol % Hexadecane.

	lipid mixing					contents mixing				
	k_1, s^{-1} ($\times 10^{-2}$)	C_1, %	k_2, s^{-1} ($\times 10^{-3}$)	C_2, %	$C_1 + C_2$, %	k_1, s^{-1} ($\times 10^{-2}$)	C_1, %	k_2, s^{-1} ($\times 10^{-3}$)	C_2, %	$C_1 + C_2$, %
Without *n*-Hexadecane										
control	1.00 ± 0.10	7.68 ± 1.37	2.60 ± 0.20	34.93 ± 0.73	42.61			2.00 ± 0.00	10.69 ± 0.04	10.69
GGpep	2.36 ± 0.03	15.95 ± 0.11	2.50 ± 0.10	25.05 ± 0.11	41.00	2.47 ± 0.01	2.25 ± 0.06	2.40 ± 0.10	7.88 ± 0.15	10.13
AApep	2.70 ± 0.05	9.12 ± 0.12	4.20 ± 0.00	27.97 ± 0.08	37.09	2.36 ± 0.17	1.41 ± 0.07	3.60 ± 0.20	2.63 ± 0.03	4.04
With 5 mol % *n*-Hexadecane										
control	1.19 ± 0.03	14.88 ± 0.71	3.00 ± 0.10	27.34 ± 0.41	42.22	2.04 ± 0.04	3.70 ± 0.07	2.30 ± 0.10	6.52 ± 0.07	10.22
GGpep	2.87 ± 0.03	18.48 ± 0.03	3.90 ± 0.10	19.74 ± 0.07	38.22	2.95 ± 0.11	2.62 ± 0.08	3.10 ± 0.10	8.08 ± 0.05	10.70
AApep	2.75 ± 0.02	16.26 ± 0.14	4.40 ± 0.00	23.45 ± 0.08	39.71	2.99 ± 0.04	3.51 ± 0.04	3.10 ± 0.02	7.07 ± 0.02	10.58

[a] Rate constants (k_1 and k_2) and preexponential factors (C_1 and C_2) were obtained by fitting the time courses of lipid mixing and contents mixing to the expression $C_1[1 - \exp(-k_1 t)] + C_2[1 - \exp(-k_2 t)]$.

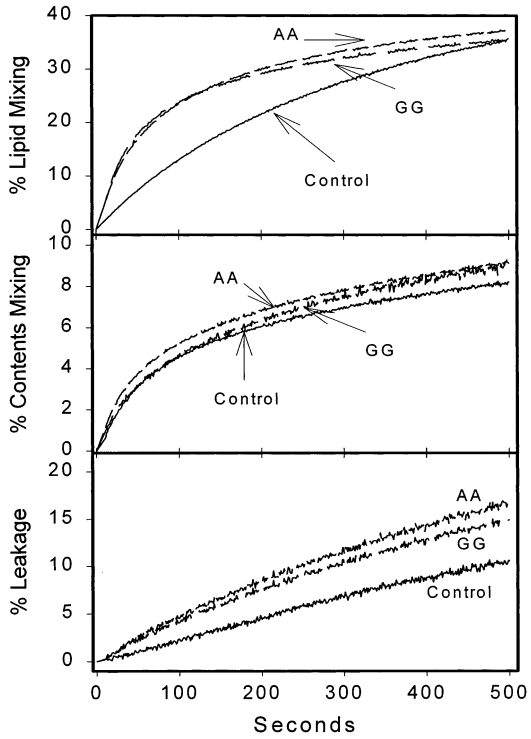

FIGURE 8: Kinetics of PEG (5 wt %)-induced fusion of DOPC/DOPE/SM/CH SUVs with 5 mol % *n*-hexadecane. Time courses of lipid mixing (A), contents mixing (B), and contents leakage (C) are shown for peptide-free control vesicles (solid line) and vesicles containing GGpep (long dashed) and AApep (short dashed) at a peptide/lipid ratio of 1/400.

might lower the free energy barrier between the initial and final intermediates, increasing the probability of conversion to a committed pore. Both interpretations seem to contribute to the effect of TMD peptide. The second possibility is supported by the fact that there was a slight increase in the rate of slow pore formation associated with the presence of both GGpep and AApep, meaning that both lowered slightly the free energy barrier between the initial intermediate and either the final intermediate or fusion pore. However, a much larger effect was that both peptides doubled the rate of formation of the initial lipid-mixed fusion intermediate and stabilized a fast pore associated with this. This supports the first possible interpretation as the major effect of TMD peptides. Only GGpep, however, was able to sustain an appreciable extent of contents mixing through this pore (Figures 5 and 7). Similarly, hexadecane had a large influence on the extent of contents mixing associated with the fast pore. However, hexadecane did not affect the rate of initial intermediate formation (Table 2). We conclude that both hexadecane and GGpep mainly act to stabilize a small, rapidly forming pore, formed as one of the unstable structures (*22*, *46*) associated with the initial intermediate, but also seem to promote the conversion of the initial intermediate to a stable fusion pore. Hexadecane is known to partition into and stabilize hydrophobic interstices (*8*) and is thought by this means to promote fusion (*4*, *50*). The similarity of the effects of GGpep and hexadecane as well as the ability of hexadecane to substitute for and override the pore-promoting effect of GGpep (Table 2) suggests that GGpep may also function by stabilizing intermediates leading to pores or structures associated with small initial pores.

Our results also showed that both TMD peptides enhanced the rate of formation of the initial fusion intermediate. However, the fact that hexadecane did not have a similar effect suggests that this does not result from peptide-induced stabilization of nonlamellar structures. It may be that the TMD peptides destabilize the bilayers of intact liposomes, thus lowering the activation energy for formation of the first intermediate. There is certainly president for such an effect of trans-membrane peptides (*20*).

How Do TMD Peptides Increase the Rate But Not the Extent of Contents Mixing? The extent of lipid mixing has been shown to reflect the size and geometry of vesicle aggregates formed in the presence of PEG (*54*, *55*). However, the extent of contents mixing should also reflect the probability that any intervesicle contact can proceed to a fusion pore (*6*). Since neither peptide nor hexadecane significantly altered the extent of lipid mixing, we can rule out any influence of these agents on the extent of aggregation. For this reason and because the peptides were present in our vesicles at very low copy numbers (≤1 per 200 lipids), we presume that they exerted their influence by concentrating in and altering the regions of contact between vesicles. The fact that GGpep did not appreciably change the extent of content mixing suggests that it did not affect the number of intervesicle contacts within aggregates, although the fact that it affected the rate suggests that it did change the nature of the contacts. In contrast, the fact that AApep significantly inhibited the extent of fusion suggests that it did interfere with either the quality or quantity of these contacts. Such regions of contact are expected to contain distorted or nonbilayer lipid arrangements (*46*) into which

"impurities" (i.e., TMD-like peptides or hexadecane) might partition. Apparently, AApep and GGpep are sufficiently different structurally that they have different effects on the ability of these unstable, nonbilayer structures either to form or to progress toward a fusion pore.

Possible Role of TMD Structural Differences in Fusion Differences. TMD Structural Diversity and Fusion. A remarkable result of this study is that both TMD peptides exist in very different and noninterconvertible conformations depending on the solvent used to disperse them. We assumed that the α-helical conformation would be most relevant to the conformation of the TMD in VSV G-protein. No X-ray crystal or NMR structure of the TMD of any intact fusion protein has yet been obtained, so we do not know with certainty that the TMD of intact VSV G-protein is helical. However, for several reasons, it is reasonable to presume that the TMD exists as a trans-membrane helix. First, the G-protein TMD consists of twenty mostly hydrophobic amino acid residues that are predicted by the structure predicting routine PROFsec (*42*) to form a helix that would just span the hydrophobic portion of a bilayer. Indeed, the peptides we have used are of an optimal length to form helical structures that span the bilayer roughly perpendicular to the plane of the bilayer (*11*). Second, single-pass TMDs such as the VSV G-protein TMD tend to be helical (*29*), while the common example of TMDs in the *β*-sheet conformation is a multipass protein (*36*). In fact, a synthetic peptide of the analogous fusion protein of influenza virus has been shown to exist as a helical bilayer-spanning structure (*49*). Finally, the *β*-sheet form of VSV TMD forms large aggregates that are not likely to form when this peptide is attached to the large extra-cytoplasmic portion of the VSV G-protein, making α helix the most likely conformation of the TMD in the native protein. Nonetheless, it is possible that a helix-sheet conformational change, as has been suggested to occur in the influenza virus fusion peptide upon interaction with specific lipids (*39*), might occur in the TMD and contribute to the fusion reaction. However, in light of the extreme structural stability of each form of the TMD peptides studied here, any such conformational change would probably have to be potentiated by interactions occurring elsewhere in the whole molecule.

In this context, it is clear from our results (Figure 1 and Table 1) that the presence of two gly residues in the VSV TMD increases the number of conformations that can be explored by the TMD in lipid, detergent, or solvent environments. This agrees with the report that gly residues are helix breakers in trans-membrane peptides in a membrane environment (*28*). A similar result has been reported previously in an organic solvent, using a model TMD peptide, not corresponding to any authentic TMD sequence, containing three gly residues dispersed at regular intervals (*29*), showing that incomplete helicity within the TMD could be induced by any one of several amino acid residues including gly and leu.

We have previously shown that substitution of the gly residues examined here in the VSV G-protein TMD with either ala or leu abolished VSV-mediated fusion (*10*). We noted that gly residues are present in fusion protein TMDs in greater abundance than in other, nonfusion protein TMDs and speculated that a TMD "glycine hinge" might promote virus-mediated fusion (*10*). While our results do not establish the precise structure of the VSV TMD and therefore cannot demonstrate a "glycine hinge", they do show that the presence of gly residues produces conformational diversity consistent with the "hinge" idea. The results of point mutations in the TMDs of several other lipid-sheathed virus proteins lend further credence to this linkage between TMD conformational diversity and fusion. In most cases[2] (*2*), substitutions that arguably should promote formation of a more stable helix abrogated fusion activity (*17*, *34*, *38*). Our results add considerable support to this concept by showing direct correlation between a demonstrated increase in conformational diversity and an increase in the ability of an isolated TMD peptide to promote fusion.

How Might a Less Helical Structure Promote Fusion? We argue above that the similar effects of GGpep and hexadecane on the initial rates of content mixing suggests that both might act by partitioning into and stabilizing nonlamellar structures at the point of contact between closely apposed membranes. How might the added structural diversity of GGpep allow it to do this job better than AApep? Small pores must involve highly curved lipidic structures that necessarily have nonlamellar regions that cannot be accommodated by the lamellar leaflets of hemifused membranes. We might expect that the highly helical AApep would have a difficult time adapting to such highly bent structures. GGpep, however, would be expected to adapt better to, and might even stabilize, such structures because of its helix-destabilizing gly residues that cause it to explore a larger range of conformational space (Table 1). The more helical AApep might limit the range of nonlamellar lipid structures that can be formed at these contact points, and create "dead-end intermediates" that cannot function in fusion. This would explain the decrease in extent of fusion seen with AApep. The stabilization of highly curved small pores by hexadecane would be expected to negate any thermodynamic advantage to peptide association with this region, explaining why the presence of hexadecane could largely override the influence of both peptides (Figure 8 and Table 2).

Relation to Biomembrane Fusion. The pore-promoting effect of GGpep is consistent with the results of several studies that show the importance of, but not absolute requirement for, TMDs in the timely formation of a normal fusion pore (*32*, *34*, *35*, *38*). This suggests that the TMDs of intact fusion proteins may play a role in pore formation similar to the role suggested by our results for the synthetic TMD peptides examined here. In addition, we note a similarity between the effects of TMD peptide that we have described here and the effects of an influenza virus HA fusion peptide (*15*) and a HIV gp41 fusion peptide (*14*) on PEG-mediated vesicle fusion. In all three cases, these membrane-located portions of fusion proteins had minimal effects on the initial step in the fusion process, as measured by lipid mixing, but all promote rapid pore formation. We have also shown that the gp41 fusion peptide, like the VSV TMD, promotes fusion in a way that is over-ridden by the presence of hexadecane (*14*). Thus, our results suggest a general theme in which these portions of fusion proteins insert into bilayers

[2] We note that several mutations in the TMD of HA from the X-31 strain of influenza virus are reported not to influence infectivity significantly (*2*). This makes it clear that not all amino acid substitutions in viral TMDs lead to loss of infectivity.

at the points of fusion contacts and promote pore formation by stabilizing nonbilayer structures that are part of fusion intermediates or of initial small fusion pores.

ACKNOWLEDGMENT

We thank Dr. Kervin Evans for his help in initiating the fusion studies and Dr. Md. Emdadul Haque for his suggestions and advice.

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BI0203233