Structure and Topology of the Influenza Virus Fusion Peptide in Lipid Bilayers

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The secondary structure of a 20-amino acid length synthetic peptide corresponding to the N terminus of the second subunit of hemagglutinin (HA2) of influenza virus A/PR8/34 and its interaction with phospholipid bilayers are investigated using ESR, Fourier transform infrared (FTIR), and CD spectroscopy. N-terminal spin labeling of the peptide did not affect the secondary structure of the peptide either in solution or when bound to liposomes as revealed by FTIR and CD spectroscopy. ESR spectra show that the mobility of the labeled peptide is dramatically restricted in the presence of phosphatidylcholine liposomes, suggesting a strong binding to the lipid membranes. The N terminus of the peptide penetrates into the membrane and is located within the hydrophobic core. We find an oblique insertion of the peptide into the lipid bilayer with an angle of about 45° between helix axis and membrane plane using FTIR spectroscopy. No gross changes of the peptide's orientation, motion, and secondary structure were observed between pH 7.4 and pH 5.0. A model of the insertion of the fusion sequence of HA2 into a lipid bilayer is presented taking into account recent investigations on the low pH conformation of HA2 (Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Nature 371, 37–43).

Fusion of influenza viruses with target membranes is mediated by the major membrane protein of influenza, hemagglutinin (HA). HA consists of two disulfide-linked subunits, HA1 and HA2, which are generated from a precursor form, HA0, by post-translational cleavage. In the viral membrane, HA is organized as a homotrimer. The first 20 residues of the N terminus of HA2 are highly conserved within the influenza virus family (White, 1990) and appear to be crucial for virus-cell fusion as shown by site-directed mutagenesis (Gething et al., 1986; Schoch and Blumenthal, 1993). X-ray studies of the crystallized ectodomain of HA have shown that at neutral pH this region, the so-called “fusion sequence,” is buried within the stem of the trimer 100 Å away from the top of the HA spike (Wilson et al., 1981; Wiley and Skehel, 1987). The induction of fusion upon lowering the pH has been ascribed to a structural change of HA converting it into a fusogenic conformation. Although the low pH structure of HA is unknown, one consequence of this rearrangement is the exposure of the fusion sequence (White and Wilson, 1987). However, very recently, a fragment of the HA2 subunit lacking the fusion peptide has been crystallized in its low pH conformation (Bullough et al., 1994). The x-ray crystal structure of this fragment suggests that the fusion sequence can be delivered at least 100 Å away from the viral membrane surface enabling its interaction with the target membrane. This reorientation of the HA2 fragment at low pH is in line with previous observations on the pH-dependent conformation of a synthetic peptide sequence comprising 56 residues of this fragment (Carr and Kim, 1993). The hydrophobic interaction of the HA ectodomain and its fusion sequence with lipid membranes at low pH have been demonstrated by hydrophobic photolabeling (Harter et al., 1988, 1989; Brunner 1989). These studies suggested that the N terminus interacts with membranes and subsequently adopts an α-helical structure (Lear and DeGrado, 1987; Wharton et al., 1988; Rafalski et al., 1991).

In order to elucidate the particular role of the HA2 N terminus in virus fusion, several studies have been undertaken to investigate the interaction of synthetic peptides, corresponding to the fusion sequence, with lipid membranes. Several features of the peptides which are important for their bilayer destabilizing and, eventually, fusogenic activity have emerged: (a) the critical pressure of insertion into the lipid phase (Rafalski et al., 1991), (b) to adopt an α-helix upon binding to the lipid phase (Lear and DeGrado, 1987; Wharton et al., 1988; Rafalski et al., 1991; Takahashi, 1990), and (c) an asymmetric distribution of hydrophobic and hydrophilic residues which is also common for fusogenic sequences of proteins of other viruses (Brasseur et al., 1990). Indeed, the fusion sequence of the HA2 subunit is characterized by the regular abundance of glycine and hydrophobic residues (Korte et al., 1992). Provided the peptide adopts an α-helical conformation, all glycines are located on one side of the helix, whereas the bulky hydrophobic residues occupy the opposite side of the helix with respect to the helix axis (see the Edmundson wheel diagram in Fig. 1A).

Despite numerous efforts, it is still unclear how the fusion peptide corresponding to the HA2 N-terminal segment destabilizes lipid membranes. To elucidate this mechanism, information is required not only on the structure of the peptide bound to a membrane, but also on its orientation within the membrane. Very recently it has been suggested from FTIR measurements on the fusion sequence of the transmembrane protein gp32 of simian immunodeficiency virus, that a prerequisite of the fusogenicity of fusion sequences is their oblique insertion into the membrane increasing the negative curvature.
of the bilayer (Martin et al., 1994; Epand et al., 1994). However, the orientation of the fusion segment of the HA2 N terminus within the membrane is unknown. Ishiguro et al. (1993) found for water-soluble peptides, which share typical features of the sequence of the influenza fusion peptide, a rather parallel orientation of the peptide with respect to the membrane plane of lipid multibilayers by using attenuated total reflection infrared (ATR-FTIR) spectroscopy. Recently, we have studied the tryptophan fluorescence from the 20-residue synthetic peptide corresponding to the HA2 N terminus of the “B” strain of influenza virus by stationary and time-resolved fluorescence. We calculated the distance of the tryptophan residue from the bilayer center, suggesting that the tryptophan residues are at or near the hydrocarbon-polar interface (Clague et al., 1991). No significant change in position was observed between two pH values (pH 5.0 and 7.4). However, some studies do not allow judgment of the membrane orientation of the entire peptide.

In order to gain information on the membrane orientation of the whole fusion peptide, we examine here the interaction of a synthetic peptide of the influenza fusion sequence with liposomes by using both electron spin resonance (ESR) spectroscopy and ATR-FTIR spectroscopy. The primary structure of the peptide (Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Gly-Gly-Trp-Thr-Gly-Met-Ile-Asp-Gly) represents the first 20 residues of the HA2 subunit of the strain A/PR8/34 from Knauer, 16 × 250 mm). Solvent A was purified water containing 0.2% trifluoroacetic acid, and solvent B was 83% acetonitrile in purified water containing 0.2% trifluoroacetic acid. Separation was achieved using a gradient from 25% to 100% solvent B in 18 min at a flow rate of 1 mL/min. Elution was monitored with a Beckman 166 UV detector (absorption at 220 nm) and a Shimadzu fluorescence detector (excitation 290 nm, emission 340 nm) taking advantage of the peptide's tryptophan fluorescence. Labeled peptide fractions were pooled, lyophilized after removal of acetonitrile by evaporation, and subsequently stored in an evacuated desiccator at 4°C.

Preparation of Unilamellar Vesicles

Large unilamellar vesicles (LUV) about 100 nm in diameter were prepared by an extrusion procedure according to Clague et al. (1991). The buffers we used to hydrate the dried lipids could be derived from the figure legends (PBS: 5.8 mmol/liter NaH2PO4 and Na2HPO4, 156 mmol/liter NaCl, and sodium acetate: 20 mmol/liter sodium acetate, 126 mmol/liter NaCl). The buffers were adjusted with NaOH to the appropriate pH. After 6 freeze and thaw cycles, the lipid suspension (10 mg/ml lipid) was extruded 10 times through two stacked polycarbonate filters (0.1-μm pore size, Nucleopore). Small unilamellar vesicles (SUV) were prepared by sonication of the hydrated lipids (suspended in buffer) with a Branson sonifier for at least 3 min on ice or until the suspension became opalescent (Woodle and Papahadjopoulos, 1989). In some preparations, spin-labeled fatty acids were added at a concentration of 1 mol% of phospholipids prior to sonification.

ESR Measurements

ESR measurements were performed using an X-band spectrometer ECS 106 equipped with an ER 4102 ST resonator (Bruker). The samples were measured in 50-μl quartz capillaries (Corning). The ESR spectra were recorded at room temperature (~20°C) using a microwave power of 20 milliwatts and were analyzed with the software provided by Bruker.

The N-terminal labeled fusion peptide was dissolved in Me2SO to obtain a stock solution of 1 mmol/liter. One μl of this stock solution was added to a 100-μl vesicle suspension under rapid vortexing. All samples contained less than 1% Me2SO to avoid any disturbance of the membrane bilayer. The molal lipid to peptide ratio (L/P, mol/mol) was varied by diluting the vesicle suspension before adding the peptide to ensure a constant spin label concentration in all samples. Depending on L/P, the measured spectra exhibit a superposition of two components arising from membrane-associated peptide as well as from free tumbling peptide in buffer. To obtain the membrane spectrum, the spectrum of the labeled peptide in buffer obtained by separate experiments was subtracted. The intensity was calculated by double-integrating the spectra. The relative amount of membrane-bound peptide was determined by the ratio of the membrane signal intensity to the intensity of the highest L/P, when all peptide is bound to the membrane. In some experiments, the vesicle-peptide suspension was applied to a Sephadex G-50 gel filtration column (Pharmaica Biotech Inc., 10 × 50 mm) equilibrated with buffer pH 7.4 and 5.0, respectively, in order to remove unbound peptide. Turbid, lipid-containing fractions were collected and tested for tryptophan fluorescence. The fractions with the highest tryptophan fluorescence were pooled and concentrated with Centricron 30 microcentrifugation units (Amicon) prior to use in further experiments. To characterize the insertion of the spin-labeled peptide, the accessibility of the aqueous quencher K3[Fe(CN)6] to the Ω group was investigated. For that purpose, aliquots of freshly prepared K3[Fe(CN)6] stock solution were added to the liposome-peptide suspension to a final concentration of 80 mmol/liter.

Membrane Interaction of Virus Fusion Sequence

27607

Fig. 1. Schematic structure of the influenza fusion peptide and the spin label. A, schematic wheel diagram of the 20-residue fusion peptide illustrating the amphipathic structure of the helix. The hydrophobic residues (highlighted) are located on the lower left of the wheel opposite the glycine-rich region (hatched). The N and C terminus are indicated by N and C, respectively. B, spin label 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylate attached to the peptide.
Mobility of the Fusion Peptide

Rotational correlation times ($\tau_\text{R}$) for the predominantly isotropic motion of the fusion peptide dissolved in buffer or in 100% Me$_2$SO, respectively, were calculated according to the formula given by Keith et al. (1974):

$$\tau_\text{R} = 6.5 \times 10^{-12} \Delta H (\frac{h_0}{h_1} - 1)$$  \hspace{0.5cm} \text{(Eq. 1)}$$

where $\Delta H$ is equal to the central line width (in gauss) and $h_0$ and $h_1$ are the amplitudes of the central and the high field line, respectively. For comparison, we have calculated the correlation time of the spectra of the membrane-bound peptide from Equation 1 also, but, due to the restricted motion of the peptide, these values represent an apparent parameter of 3 gauss (Freed, 1976). Thus, the formula given by Freed (1976) assuming a Brownian rotational diffusion model:

$$\tau_\text{R}' = a \left(1 - \frac{A_0}{A_{\text{max}}} \right)^b$$  \hspace{0.5cm} \text{(Eq. 2)}$$

The outer hyperfine splitting $A_0$ was obtained from the membrane spectra of the peptide. The values a and b, 5.4 $\times$ 10$^{-10}$ s and -1.36, respectively, and $A_{\text{max}} = 33.6$ gauss were derived from spectral simulations for slow isotropic rotational motion, assuming a line width parameter of 3 gauss (Freed, 1976).

The isotropic hyperfine splitting constant $A_0$ was calculated according to Marsh (1981):

$$a_0 = \frac{1}{3} A_0 (1 + 2A_0)$$  \hspace{0.5cm} \text{(Eq. 3)}$$

The outer ($A_0$) and inner ($A_1$) hyperfine splittings were determined (in gauss) from the measured spectra after smoothing the noisy signals using the software provided by Bruker (Fourier transformation).

ATR-FTIR Spectroscopy

The secondary structure of the peptide was investigated by attenuated total reflection Fourier transform IR (ATR-FTIR) spectroscopy. A general overview of this method is given by Fringeli and Günthard (1981) and by Harrick (1967). Spectra were recorded with a Perkin Elmer FTIR 1720X spectrophotometer equipped with a gold wire grid polarizer (Perkin Elmer) at room temperature. The internal reflection element was a germanium plate (Harrick, EJ2121) with an aperture angle of 45°. For more details see, Martin et al. (1994), Caibaux et al. (1989), and Gomaraghgizh et al. (1994). For each spectrum, 64 scans were averaged with a nominal resolution of 4 cm$^{-1}$ encoded every 1 cm$^{-1}$.

Sample Preparation—The peptide, dissolved either in Me$_2$SO (4.4 mg/ml peptide) or in trifluoroethanol/PBS (0.4 mg/ml peptide) were applied to the germanium plate (10-50 μl) and dried under a stream of nitrogen. The ATR plate was then sealed in a universal sample holder and hydrated by flushing D$_2$O-saturated N$_2$ for at least 60 min. Samples with oriented multilayers were formed by adding the peptide from the Me$_2$SO stock solution (4.4 mg/ml peptide) to egg PC or DPPC LUV (10 mg/ml lipid) under immediate vortexing. Subsequently, 20 μl of the suspension were evaporated and deuterated on the ATR plate as described above. In some experiments, the peptide-containing vesicle suspension was applied to a gel filtration column in order to remove unbound peptide as described for ESR measurements. The lipid-containing fraction with the highest fluorescence activity was used without further concentration.

Secondary Structure and Orientation Determination—The region from 1700 cm$^{-1}$ to 1600 cm$^{-1}$ refers to the amide I band and is sensitive to the secondary structure (Fringeli and Günthard, 1981). An analysis of this broad band using deconvolution and band-fitting procedures leading to an estimation of the secondary structure was performed as described elsewhere (Kapunin et al., 1981; Byler and Susi, 1986). To study the orientation of the lipid and the incorporated peptide, spectra were recorded with incident light polarized parallel (0°) and perpendicular (90°) with respect to the ATR plate. For a detailed description, see Caibaux et al. (1989).

CD Measurements

CD spectra were recorded with a Jasco J-720 spectrometer using 1-mm cuvettes. The peptide was dissolved in a mixture of trifluoroethanol/H$_2$O, 4:3 (v/v). Insoluble particles were separated by slow speed centrifugation. The final peptide concentration was determined according to Lowry et al. (1951) using a calibration curve made with the peptide dissolved in PBS containing 1% SDS.

RESULTS

Interaction of the Spin-labeled Fusion Peptide with Membranes—In Fig. 2, ESR spectra of the spin label succinimidyld-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylate free in solvent (Fig. 2A) and covalently bound to the fusion peptide (Fig. 2B) are shown. Both spectra were recorded in Me$_2$SO at room temperature. In both cases, the narrow lines of the spectra indicate isotropic mobility of the spin label. The correlation time $\tau_\text{R}$ of the free tumbling label and of the peptide-bound label calculated according to Equation 1 were 250 ± 15 ps and 350 ± 20 ps, respectively. Spectra in Me$_2$SO were recorded in a peptide concentration range from 5 mg/ml to 10 μg/ml to investigate the formation of peptide oligomers. However, $\tau_\text{R}$ was independent of the peptide concentration indicating that the peptide exists predominantly as a monomer in Me$_2$SO even at high peptide concentration. Fig. 2C shows the spectrum of the labeled peptide in sodium acetate buffer at room temperature. The signal to noise ratio was extremely low due to the low solubility of the peptide in water. However, we ascribe the isotropic signal to a small portion of monomeric peptide. The low signal intensity could be explained by peptide aggregates causing an ESR spectrum with a very broad line shape due to spin-spin interaction. We mixed the labeled peptide with a 10-fold excess of unlabeled peptide (both dissolved in Me$_2$SO) in order to lower spin-spin interaction in peptide aggregates. The resulting spectrum is shown in Fig. 2D (Me$_2$SO/buffer, 1:100, v/v). Comparison with the spectrum above reveals that the sharp signal is superimposed on a second broader spectrum (see arrows). In conclusion, we ascribe the broad signal to peptide aggregates which are hardly detectable when solely labeled peptide is used, due to strong spin-spin interaction and poor signal to noise ratio. Note that spectra in Fig. 2 (C and D) were recorded with an enhanced modulation amplitude (4 gauss instead of 0.5 gauss) but otherwise at similar conditions as the spectra measured in Me$_2$SO (Fig. 2, A and B).

To investigate peptide-membrane interactions, appropriate aliquots of a peptide stock solution in Me$_2$SO were added to the vesicle suspension (for details see "Materials and Methods"). The spectrum of the labeled peptide becomes anisotropic in the presence of vesicles indicating a strong interaction with the
membrane. In Fig. 3, spectra of the spin-labeled peptide in the presence of egg PC LUV at various L/P are shown for pH 7.4 and pH 5.0 (room temperature). Nearly all peptide is bound to liposomal membranes at L/P greater than 300. Lowering L/P, two phenomena could be observed: (i) a sharp component appears in the ESR spectrum which is best seen in the high field line and (ii) the total intensity of the spectra decreases.

The narrow signal arises most likely from unbound peptide. Indeed, by subtracting the spectrum of the free tumbling peptide in buffer (Fig. 2C) from the original spectrum (Fig. 3), an anisotropic spectrum without any narrow lines was obtained. Therefore, we contribute this spectrum to a peptide population which does not insert into the bilayer. Excess free spin label would cause a similar narrow spectrum. Since the free spin label and the labeled peptide can be well separated by HPLC, it is very unlikely that samples contain free spin label.

The decreasing signal intensity upon lowering L/P at constant concentration of labeled peptide certainly is a consequence of peptide aggregation in buffer accompanied by spin-spin interaction as described above.

After subtracting the narrow signal from the measured spectrum, the ratio of bound to unbound peptide could be estimated by double integration of the spectra (see "Materials and Methods"). Above L/P of approximately 300, more than 90% of the peptide is bound to the membranes. Below this L/P, the relative amount of peptide bound to vesicles decreases, indicating a saturation of binding to the bilayer. The data can be described approximately by a Langmuir binding isotherm with a limiting stoichiometry of 32 lipids/peptide for pH 7.4 at room temperature. At L/P = 60, the ratio used for FTIR measurements (see below), approximately 60% (pH 7.4) and 50% (pH 5.0) of the peptide is bound to vesicles. Only marginal differences of the bound fraction were observed upon variation of the lipid composition (egg PC or DPPC) or size of the vesicles (data not shown).

In order to separate the unbound peptide at low L/P from the vesicles, we apply the LUV suspension to a gel filtration column as described under "Materials and Methods." The lipid-containing fractions were collected and tested for ESR activity. We obtain a nearly pure membrane spectrum overlapped with a negligible narrow signal contributing to less than 2% of the total measured intensity (data not shown) ensuring that almost all unbound peptide was removed by gel filtration.

To compare the restricted motion of labeled peptide upon binding to the membrane with the free tumbling of the peptide in solvent on a quantitative basis, we calculated the apparent correlation time \( \tau_a \) according to Equation 1. We found for egg PC LUV at both pH 7.4 and pH 5.0 nearly the same value of 5.4 ± 0.2 ns and 5.3 ± 0.2 ns, respectively. The rotational correlation time \( \tau'_{a} \) estimated according to the approach of Freed (1976) (Equation 2) was in the same order: \( \tau'_{a} = 3.0 \pm 0.2 \) ns and 3.0 ± 0.3 ns for pH 7.4 and pH 5.0, respectively. Similarly, we did not observe a significant difference of the outer hyperfine splitting \( A_{h} \) between pH 7.4 and 5.0.

Localization of the Spin Label within the Membrane—The isotropic hyperfine splitting \( A_{h} \) is sensitive to the polarity of the environment of the spin label and, therefore, can be used to determine the penetration depth of the spin label probe into the bilayer (Marsh, 1981; Fretten et al., 1980). In Table I, the isotropic hyperfine splitting \( A_{h} \) of the labeled peptide calculated according to Equation 3 is presented. Upon changing the solvent from Me2SO to aqueous buffer, the value of \( A_{h} \) increases from 14.7 to 16.0 gauss due to the increasing polarity of the solvent. When the peptide binds to egg PC vesicles, \( A_{h} \) decreases to 15.1 ± 0.1 gauss and 15.2 ± 0.1 gauss at pH 7.4 and 5.0, respectively, indicating an insertion of the peptide into the hydrophobic core of the lipid phase. The insertion depth of the peptide reflected by the spin label appeared to be nearly independent of the pH in the range investigated.

In order to give an estimate of the insertion depth of the spin label, we have measured \( A_{h} \) of the spin-labeled fatty acids I(m,n) in relation to the position of the NO moiety. For that purpose, 1 mol % of I(m,n) was incorporated into egg PC and DPPC vesicles. The isotropic hyperfine splitting \( A_{h} \) as a function of the immersion depth of the NO moiety is shown in Fig. 4. The position of the NO group was taken from our previous work (Clague et al., 1991). The carboxyl group of the fatty acids corresponds to the depth 0 (membrane surface). By comparing \( A_{h} \) of the labeled peptide with this plot, we conclude that the spin label is located near the hydrocarbon-polar interface of the bilayer.

To sustain this observation, we have investigated the accessibility of the hydrophilic quencher \( K_{3}[Fe(CN)_{6}] \) to the nitroxy group. In the presence of 80 mmol/liter \( K_{3}[Fe(CN)_{6}] \) about 20 to 30% of the membrane spectrum is quenched, while the spin label or the labeled peptide alone in buffer is almost completely quenched. In Table I, the ratio of the signal intensity in the presence of \( K_{3}[Fe(CN)_{6}] \) \( A_{quench} \) to the intensity of the membrane spectrum \( A_{mem} \) in the absence of the quencher is given at pH 7.4 and pH 5.0. Intensities were obtained by double integration with an approximate error of ±5%.

| Nondenatured fraction of the membrane-bound peptide (A
| \( a_{h} \) (L/P = 1350) | pH 7.4 | pH 5.0 | pH 7.4 | pH 5.0 |
|-----------------|..|..|..|..|
| Egg PC | | | | |
| SUV | 0.78 | 0.75 | 15.2 | 15.1 |
| LUV | | | 15.5 | 15.2 |
| DPPC | | | 15.4 | 15.5 |
| LUV | 0.82 | 0.85 | 0.65 | 0.75 |
| Sodium acetate buffer | | | 16.0 | 16.0 |
| Me2SO | | | | |

\( ^{a} \) Nonquenchable fraction of the membrane-bound fusion peptide after adding 80 mmol/liter of the hydrophilic quencher \( K_{3}[Fe(CN)_{6}] \) to the peptide/vesicle mixture. Signal intensities are calculated by double integration with an approximate error of ±5%.

\( ^{b} \) Hyperfine coupling constant calculated according to Equation 3.
integrating the spectra. Taking into account the error of double integration, no substantial difference between pH 7.4 and pH 5.0 could be observed. The portion of the quenched signal does not change, within the experimental error, by variation of L/P in the chosen concentration range (L/P from 1350 to 135). The narrow spectrum vanishes totally as it is expected for a signal corresponding to unbound peptide in aqueous solution (data not shown). In conclusion, the spin label attached to the peptide is partly accessible to the quencher. This confirms our previous results (see above) that the label attached to the N terminus of the peptide is located in the hydrophobic part of the lipid phase close to the hydrocarbon-polar head group interface.

Secondary Structure-FTIR Measurements—FTIR spectra of the fusion peptide at room temperature dissolved in Me$_2$SO, trifluoroethanol/PBS (2:1, v/v), pH 7.4, and pH 5.0, respectively, are presented in Fig. 5. The spectrum of the peptide in Me$_2$SO (Fig. 5A) is dominated by a band with a maximum at 1637 cm$^{-1}$ which is assigned to the $\beta$-sheet conformation. A minor band at 1670 cm$^{-1}$ could be allocated to $\alpha$-turns. Table II lists the relative distribution of secondary structure elements for the peptide in various environments. In Me$_2$SO, 70% of the peptide adopts $\beta$-sheet conformation. In trifluoroethanol, an $\alpha$-helical-promoting solvent, $\beta$- and $\alpha$-structures were found to be 50% and 27%, respectively. To examine the influence of the pH, we dissolved the peptide in a mixture of trifluoroethanol and PBS (2:1, v/v) at pH 7.4 and 5.0. At both pH values, we found bands at 1637 and 1657 cm$^{-1}$ (Fig. 5, B and C) characterizing a mixture of $\beta$- and $\alpha$-structures, respectively. At pH 7.4, the portion of $\alpha$-helical structures is enhanced (40%) when compared to pH 5.0 (25%) and to pure trifluoroethanol (27%) while the $\beta$-sheet structures decrease vice versa (see Table II). The results of the secondary structure analysis of the FTIR spectrum at neutral pH in trifluoroethanol are confirmed by our CD measurements (see below).

To investigate the structure of the peptide bound to the membrane, we added the peptide from a Me$_2$SO stock solution to a suspension of egg PC LUV to a final L/P of 60 at pH 7.4 and 5.0, respectively (see the corresponding FTIR spectra in Fig. 6, A and C). The analysis of the amide I region reveals that at both pH values the peptide exhibits mainly $\beta$-sheet and random coil structures while only approximately 20% of the peptide is in an $\alpha$-helical conformation. However, the pH dependence of the ratio $\beta$-sheet/$\alpha$-helical structures is similar to that found for the peptide in trifluoroethanol/PBS: the contribution of $\beta$-sheet structures rises with decreasing pH. We applied the vesicle-peptide mixtures at pH 7.4 as well as at pH 5.0 on a gel filtration column, as described under "Material and Methods." In Fig. 6, B and D spectra of the amide I band at both pH values are given. Due to the elimination of the unbound peptide, L/P increases from 60 to 150 and 95 for pH 7.4 and 5.0, respectively. The spectra show an almost complete disappearance of $\beta$-sheet structures. The remaining peptide adopts $\alpha$-helical and random coil structures with small components of $\beta$-turns. This proves unambiguously that the membrane-bound peptide is mostly in an $\alpha$-helical and random coil conformation while the $\beta$-sheet structure corresponds to the unbound peptide.

To verify that the spin label group at the N terminus does not change the secondary structure of the peptide, we recorded IR spectra of the labeled peptide, too. The results are shown in Table II. The labeled peptide adopts the same structure as the unlabeled when it is dissolved in Me$_2$SO (70% $\beta$-sheet). Only small differences in comparison to the unlabeled peptide were found in the presence of LUV (see Table II). The secondary structure of the labeled peptide associated with the membrane was almost identical with that of the unlabeled peptide. When we applied the vesicle peptide suspension onto a G-50 column, the peptide fraction which adopted $\beta$-sheet conformation was separated from the vesicles, and the remaining bound peptide took up predominantly $\alpha$-helical and random coil structures.

Orientation Studies by FTIR—ATR-FTIR spectroscopy provides information on the orientation of phospholipid bilayers as well as on peptides inserted into oriented bilayers (Goormaghtigh et al., 1990). We characterized the orientation of the phospholipids in the bilayer in order to assess the overall membrane orientation on the germanium plate. The strong 90° polarization of the phospholipid $\gamma_{\gamma}(\mathrm{CH}_2)$ at 1200 cm$^{-1}$, due to its transition dipole moment parallel to the all-trans lipid hydrocarbon chains, and the 0° polarization of the phospholipid $\delta(\mathrm{CH}_2)$ at 1468 cm$^{-1}$, the transition dipole moment of which is perpendicular to the lipid acyl chain (Fringeli and Günthard, 1981), demonstrate that the phospholipid acyl chains are oriented almost perpendicular to the germanium plate, i.e. the
Membrane Interaction of Virus Fusion Sequence

Secondary structures of the fusion peptide in different environments

| Probe | L/P | α-Helix | β-Sheet | Random coil |
|-------|-----|---------|---------|-------------|
|       | Unlabeled | Labeled | Unlabeled | Labeled | Unlabeled | Labeled |
| Me2SO | 10 | 10 | 70 | 68 | 20 | 20 |
| Trifluoroethanol | 27 | 50 | 23 | 20 |
| Trifluoroethanol/PBS, pH 5.0 | 25 | 55 | 20 |
| Trifluoroethanol/PBS, pH 7.4 | 40 | 40 | 20 |
| LUV, pH 7.4 | 60 | 20 | 20 | 60 | 40 | 20 |
| LUV, pH 7.4/G-50 | 150 | 40 | 50 | 10 | 10 | 50 | 40 |
| LUV, pH 5.0 | 60 | 20 | 20 | 50 | 30 |
| LUV, pH 5.0/G-50 | 95 | 40 | 10 | 50 |

| a | Fusion peptide dissolved in a mixture of trifluoroethanol/PBS, 2:1 v/v at pH 7.4 and pH 5.0, respectively. |
| b | Fusion peptide dissolved in Me2SO is added to egg PC LUV suspension at pH 7.4 and pH 5.0, respectively. |
| c | Fusion peptide/egg PC LUV mixture as above but after removing unbound peptide from the vesicles using a G-50 gel filtration column (see "Materials and Methods"). |

In the present study we show that the parallel approach of ESR and FTIR measurements allows to examine both the secondary structure of membrane active peptides and their localization and orientation within the lipid bilayer. While the FTIR method can provide information on the structure and orientation of the whole peptide in the membrane, selective spin labeling of the peptide allows us to determine the insertion depth of a given sequence as well as the dynamics of peptide motion within the membrane.

Of course, the general disadvantage of the covalent (spin) labeling approach lies in the influence of the reporter group on the structure and on the membrane interaction (including the orientation) of the peptide sequence. However, we have given several lines of evidence that labeling does not affect the peptide in its structure and properties relevant for membrane interaction. (a) Investigation of the secondary structure in Me2SO by using FTIR revealed no significant differences in the relative content of α-helix, β-sheet and random coil structures between the unlabeled and labeled peptide. This finding is strongly supported by the CD spectra recorded in trifluoroethanol/H2O. (b) Similar to the nonlabeled peptide, we observed a significant enhancement of α-helical structures when labeled peptide associates with lipid membranes. (c) As shown by a lipid mixing assay, the fusogenic activity of the peptide is not modified by covalent spin labeling.2,3

2 F. Nußler, unpublished results.
3 The peptide induces fusion (lipid mixing) between SUV made of a 1:1 mixture of egg PC and DPPE only at acidic pH, regardless whether the peptide is labeled or not.
As expected, the secondary structure of the peptide is sensitive to the polarity of its environment. In Me$_2$SO, mainly $\beta$-sheet structures were found. In a mixture of trifluoroethanol and aqueous buffer, both $\alpha$- and $\beta$-structures, were present, with a higher portion of $\beta$-structure at acidic pH. A similar behavior was reported for a peptide with a related sequence (Takahashi, 1990). The $\alpha$-helical portion of the peptide increases dramatically upon binding to the membrane as shown previously for fusion sequences of the influenza strains B/Lee (Lear and DeGrado, 1987) and X31 (Rafalski et al., 1991). The membrane-bound sequence of A/PR8/34 adopts mainly $\alpha$-helical and random coil structures, at similar extents. The contribution of $\beta$-sheets was $\leq$10%. This suggests that the $\beta$-sheet is not the typical structure of membrane-bound fusion peptide. Presumably, the $\beta$-sheet peptide is not able to penetrate or even insert into the bilayer. This supports the model where the peptide upon binding to the lipid phase adopts an almost helical conformation combined with unordered structures at the ends of the helix. The last 3 residues at the C terminus of the sequence cannot adopt an $\alpha$-helical structure in principle, since hydrogen bridges cannot form. This is in line with recent observations that both ends of an $\alpha$-helical peptide exhibit typically more motional dynamics (Miick et al., 1993). An homogeneous peptide population is supported by our ESR data, since we did not find any indication for different peptide classes.

As judged from the isotropic ESR signals in Fig. 3, at L/P > 300 nearly all the peptide present binds to the lipid bilayer. Upon lowering L/P, the bound peptide portion decreases, indicating a saturation effect of the membrane. We found a lipid to peptide stoichiometry of 32 at pH 7.4 which is similar to the value reported for the 20-residue fusion peptide of influenza B Lee (Lear and DeGrado, 1987).

The spin-labeled peptide is highly mobile in solution as deduced from ESR spectra: $\tau_e$ equals 350 ps when the peptide is dissolved in Me$_2$SO. In this solvent, we found no indication for oligomerization of the peptide in even at high concentrations. In contrast, the solubility of the peptide in aqueous buffer is so low that only a small portion of the peptide is monomeric while most of the peptide aggregates. The correlation time of the monomeric peptide in Me$_2$SO is lower than expected for a molecule with a molecular mass of approximately 2000 Da, which is known to be $>800$ ps (Miick et al., 1993). This indicates that the spin label group undergoes additional motion relative to the global mobility of the peptide.

The correlation time, $\tau_p$, of the rotational diffusion of such rather small spin labels as used here in the membrane is about 300 ps.$^4$ This value is one order of magnitude lower than the value measured for the spin-labeled peptide bound to the membrane. Although the covalent attachment of the label to the peptide reduces significantly its rotational motion, we can surmise that the membrane spectrum of the labeled peptide does not reflect solely the global motion of the peptide but, presumably, also a “local segment” motion within the peptide.$^5$

$^4$ A. Herrmann, unpublished results.

$^5$ The apparent rotational correlation time of the spin-labeled peptide associated with the membrane is significantly lower as one would expect for a value reflecting the global motion of the peptide. To estimate this value, we assume that the membrane-bound peptide can be described to a first approximation as an ellipsoid and the rotational relaxation time $\phi$ is inverse proportional to the coefficient of rotational diffusion about a short axis, $D_s$,

$$\phi = \frac{1}{D_s}$$

(Eq. 4)

with $D_s$ (Perrin, 1936; J ohn and J ähnerg, 1988).
result may also account for the absence of significant differences between spectra recorded in the presence of egg PC and DPPC, which is rather surprising, since at room temperature the DPPC membrane is in the crystalline-gel state, while the membranes consisting of egg PC are in a fluid-crystalline-like state. Most likely, the local motions within the peptide structure are not sensitive to the membrane fluidity.

As has been shown recently, the depth of the NO group covalently bound to a residue within the sequence does not necessarily reflect the average insertion depth of the peptide backbone (Yu et al., 1994). Here, we have estimated that the distance between the NO moiety of a spin label (bound to the peptide terminus) and the $\omega$-carbon of the first residue is in the order of 6 Å. Both the partial quenching by K3[Fe(CN)6] and the hyperfine coupling constant $a_\phi$ of the spin-labeled peptide bound to the membrane clearly show that the spin label inserts into the bilayer close to the hydrocarbon-polar interface independent of the pH. Thus, the N terminus of the peptide as well is localized near the interface or even deeper in the hydrophobic core. The concluded immersion depth of the N terminus is in accordance with our previous investigation on the localization of the tryptophan of the fusion sequence of influenza B/Lee. We found that this residue is at or near the hydrocarbon-polar interface without any gross positional change when switching the pH between 7.4 and 5.0 (Clague et al., 1991).

Since recent data have suggested that the hydrophobicity of the N-terminal viral fusion peptide as well as the $\omega$-helix structure are not the only parameters required for fusion to occur and that the orientation of the peptide at the lipid-water interface plays a crucial role during the fusion event (Horth et al., 1991; Martin et al., 1993a, 1994), we have investigated the orientation of influenza fusion peptide when it is inserted in the lipid bilayer.

Our polarized FTIR results indicate that influenza peptide is obliquely inserted in the vesicle membranes (see Fig. 7). As has been pointed out previously (Martin et al., 1991), FTIR spectroscopy does not discriminate between a fixed uniaxial orientation and the motional averaging of different orientations resulting in an average oblique orientation. However, as deduced from our ESR measurements, the strong motional restriction of the peptide bound to the membrane makes it very unlikely that the FTIR spectra reflect the mean of an ensemble of different orientations. The oblique mode of insertion has been predicted by computer analysis for the N-terminal fusogenic domains of a series of enveloped viral proteins (Brasseur et al., 1990) and has been experimentally demonstrated for the simian immunodeficiency virus fusion peptide (Martin et al., 1994) as well as for the human immunodeficiency virus fusion peptide (Martin et al., 1993b). We note that the angle $\phi$ between the helix long axis and the bilayer plane, which we determine to be about 45$^\circ$, differs significantly from the angle $\phi$ about 20$^\circ$ which was observed for water-soluble peptides related to this fusion peptide (Ishiguro et al., 1993). The oblique membrane insertion of viral fusogenic peptides seems to be an essential requirement for membrane fusion. It is likely that this orientation markedly alters the rather parallel alignment of the phospholipid acyl chains. This alteration of membrane organization does not occur in the case of amphipathic or transmembrane peptides and could prefigure a more dramatic change in lipid order, giving rise to new lipid phases which are thought to be associated with the initial events of membrane fusion. Moreover, the existence of a transitional nonbilayer lipidic structure has been demonstrated recently by $^{31}P$ NMR in presence of the influenza fusion peptide (Epend et al., 1994).

In a recent investigation, a fragment of the HA2 subunit was crystallized in its low pH conformation. The x-ray structure analysis suggested that the N terminus of the HA2 subunit could be delivered approximately 100 Å toward the target membrane when a coiled coil ensemble is formed of the HA2 subunits of the HA trimer at low pH (Bullough et al., 1994). This fragment lacks the fusion peptide and two antiparallel $\beta$-strands at its N terminus. In Fig. 9A, we present a model of the membrane topology of the three fusion peptides of such a coiled coil structure. According to our results, the fusion peptides are inserted at an oblique angle with the N terminus located in the hydrophobic core. The C terminus with its two charged residues is located in the polar head group region. The arrangement of the three peptides shown in Fig. 9B (view along the long axis of the coiled coil) reflects the C3 symmetry of the coiled coil. The wedge shape of the peptide due to its asymmetric distribution of small and bulky moieties (see introduction to the text) supports a concave local bilayer curvature which may

![Fig. 9. Model of the insertion of the fusion peptides of a HA trimer in the coiled coil form at low pH into a phospholipid membrane.](http://www.jbc.org/)

\[ D_\phi = \frac{3kT}{2w_b} \left[ 2 \ln \frac{2a}{b} - 1 \right] \]  
(Eq. 5)

where $a$ and $b$ refer to the half-axes of the ellipsoid. To approximate the dimension of the peptide, we used $a = 1.5$ nm and $b = 0.6$ nm. Taking the viscosity of the membrane, $\eta$, with 0.1 pascal, we obtain $D_\phi = 1.6 \times 10^9$ s$^{-1}$ and $\phi = 6.3 \times 10^{-5}$ s. Even if we assume an uncertainty of a factor 10, the estimated rotational relaxation time is still one order of magnitude higher than the measured $\tau_A$. 
be involved in the local alteration of the lipid phase.

An important result of our study is that we did not detect any significant influence of the pH either on the secondary structure or on the orientation of the peptide and the insertion depth of the N terminus in the limit of the accuracy of our methods. Due to the presence of negatively charged carboxyl groups in the sequence of the influenza fusion peptide one would anticipate, for example, that acidification would favor a deeper insertion of the peptide into the membrane. However, assuming that the pK_a of 3.86 and 4.24 for isolated aspartic and glutamic acid (Abraham and Leo, 1987), respectively, is not changed dramatically within the fusion sequence, it is obvious that a significant amount of theses residues is still in the deprotonated form at pH 5.0. We have previously shown by a hydrophobicity plot of the N terminus of the HA2 subunit of A/PR8/34 (including pH-dependent protonation of carboxyl groups) that the hydrophobicity profile does not change dramatically when shifting the pH from 7.4 to 5.0 (Korte et al., 1992). Using the scale values of Abraham and Leo (1987), the average hydrophobicity of the first 20 residues of this sequence changes only from 0.64 (pH 7.4) to 0.90 (pH 5.0). For comparison, the average hydrophobicity values of the membrane-spanning part of glycolipid and HA2 are significantly higher, 1.51 and 1.75, respectively (Clague et al., 1991). This suggests that, even at pH 5.0, the fusion peptide is not able to penetrate through the very hydrophobic core (midplane) of the bilayer. A similar conclusion can be drawn from the average hydrophobic moment which is indicative for the formation of peptide helices with amphipathic properties (Eisenberg, 1984). Assuming an (α-helical structure, the average hydrophobic moment of the fusion sequence is 0.76 and 0.58 at pH 7.4 and 5.0, respectively. The value for the membrane-spanning part of HA2 is 0.19. Thus, even the hydrophobic moment of the fusion peptide at acidic pH suggests amphipathic properties. From this, it can be concluded that the peptide will remain near the hydrocarbon-polar interface after acidification, which is in agreement with our experimental results here and those presented recently (Clague et al., 1991).

We cannot rule out that an alteration of the oblique angle of peptide orientation occurs upon lowering the pH within the range of the experimental error (±5°). A rough estimate shows that even a peptide reorientation of about 10° may account for the observed pH dependence of tryptophan fluorescence (see Clague et al. (1991)).

In summary, our approach confirms previous results that the fusion peptide of influenza virus adopts an (α-helical structure upon binding to phospholipid bilayers and extends these studies in the respect that for the first time the orientation of the peptide within the membrane has been elucidated. We could show that the peptide inserts with an oblique angle into the bilayer with the N terminus localized within the hydrophobic core of the bilayer. This topology of the influenza fusion peptide may promote intermediate lipid structures which may resemble a major step in merging of the virus with the target membrane.

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